NOVEL INSIGHTS IN THE MOLECULAR MECHANISMS OF ACTION OF RETINOIDS AND THEIR POTENTIAL REPERCUSSIONS ON BREAST CANCER CELL PROLIFERATION

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Ignacio Miro Estruch

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INVITATION

FOR ATTENDING THE PUBLIC DEFENCE
OF MY PhD THESIS ENTITLED:

NOVEL INSIGHTS IN THE MOLECULAR
MECHANISMS OF ACTION OF
RETINOIDS AND THEIR POTENTIAL
REPERCUSSIONS ON BREAST CANCER
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Propositions

1. Subtype-selective RAR agonists can be discriminated using RAR-coregulator binding studies.

(this thesis)

2. The effects of chemicals such as retinoids on any NR signaling can only be understood based on a multi-endpoint research approach.

(this thesis)

3. Ethics concerning cloning and the use of human stem cells currently blocks the further development of medical science.

4. The health and socio-economic risks of cross-contamination of food with undetectable amounts of allergens are underestimated.

5. A PhD journey is a magic mirror where you suddenly become aware of all your own strengths and limitations.

6. The expression of Muhammad Ali “impossible is nothing” encourages people to do anything in life, but also makes millions of lives impossible.

Propositions belonging to the Thesis entitled: “Novel insights in the molecular mechanisms of action of retinoids and their potential repercussions on breast cancer cell proliferation”

Ignacio Miro Estruch

Wageningen 19th June 2018

**Novel insights in the molecular mechanisms of
action of retinoids and their potential repercussions
on breast cancer cell proliferation**

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**Novel insights in the molecular mechanisms of action of retinoids and their
potential repercussions on breast cancer cell proliferation**

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Thesis

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Abbreviations List

Abbreviation	Meaning
Ab	Antibody
ADORA1	Adenosine A1 Receptor
AF-1	Activation Function Domain 1
AF-2	Activation Function Domain 2
Am80	4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-carbamoyl] benzoic acid
ANOVA	Analysis of Variance
AP-1	Activator Protein 1
AtRA	<i>all-trans</i> retinoic acid
AU	Arbitrary Units
Avg.	Average
BCA	Bicinchoninic acid
BDS	Biodetection Systems
BIN1	Bridging integrator 1
BMS961	3-fluoro-4-[[2-hydroxy-2-(5,5,8,8-tetramethyl-5,6,7,8,-tetrahydro-2-naphthalenyl) acetyl]amino]-benzoic acid
BrdU	Bromodeoxyuridine
BTG2	p53-Controlled Antiproliferative Gene B-Cell Translocation Gene, Member 2
CASP4	Caspase 4
CASP9	Caspase 9
CCD	Charge Coupled Device Camera
CCND1	Cyclin D1
CD2314	5-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-3-thiophenecarboxylic acid
CDK1	Cyclin dependent kinase 1
cDNA	Complementary DNA
CEACAM1	Carcinoembryonic antigen related cell adhesion molecule 1
CLMN	Calmin
CoA	Coactivator
CoR	Corepressor
CPDB	Consensus Path Data Base
CXCL12	Chemokine CXCL12
DAB2	DAB2, clathrin adaptor protein
DBD	DNA Binding Domain
DMBT1	Deleted in malignant brain tumors 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E₂	17 β -estradiol
EC50	Half Maximal Effective Concentration
EDN2	Endothelin 2
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
ERα/ESR1	Estrogen Receptor Alpha
ERβ/ESR2	Estrogen Receptor Beta
FBS	Foetal Bovine Serum
FC	Fold Change
FITC	Fluorescein Isothiocyanate
FL	Full length
FOXO3	Forkhead box O3
FRET	Fluorescence Resonance Energy Transfer
G418	Geneticine
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GJA1	Gap junction protein alpha 1
GO	Gene Ontology
GR	Glucocorticoid Receptor
GREB1	Growth regulation by estrogen in breast cancer 1
GST	Glutathione S-Transferase
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
His	Histidine
HRE	Hormone Response Element

IER5L	Immediate early response gene 5-like protein
IGFBP6	Insulin like growth factor binding protein 6
IL17RB	Interleukin 17 receptor B
IRF1	Interferon regulatory factor 1
JAK2	Janus kinase 2
KCNK5	Potassium two pore domain channel subfamily K member 5
LBD	Ligand Binding Domain
Log	Logarithm
MAPK	Mitogen-Activated Protein Kinase
MARCoNI	Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction
MI	Modulation Index
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYC	MYC proto-oncogene, bHLH transcription factor
nM	Nanomolar
NPY5R	Neuropeptide Y receptor Y5
NR	Nuclear Receptor
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PK4	Pyruvate dehydrogenase kinase 4
PKIB	cAMP-dependent protein kinase inhibitor- β
PLK2	Polo like kinase 2
pM	Picomolar
PPAR	Peroxisome Proliferator-Activated Receptors
PRKCA	Protein kinase C alpha
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Responsive Element
RARα/NR1B1	Retinoic Acid Receptor Alpha
RARβ/NR1B2	Retinoic Acid Receptor Beta
RARγ/NR1B3	Retinoic Acid Receptor Gamma
RASA4B	RAS p21 protein activator 4B
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
Ro415253	4-[(E)-2-(7-Heptoxy-4,4-dimethyl-1,1-dioxo-2,3-dihydrothiochromen-6-yl)prop-1-enyl]benzoic acid
RT-qPCR	Quantitative Real-Time (Reverse Transcription) Polymerase Chain Reaction
RXR	Retinoid X Receptor
RXRα	Retinoid X Receptor Alpha
RXRβ	Retinoid X Receptor Beta
RXRγ	Retinoid X Receptor Gamma
SCN5A	Sodium voltage-gated channel alpha subunit 5
SDS	Sodium Dodecylsulfate
SEM	Standard Error Mean
SERPINB1	Serpin family B member 1
SMAD3	SMAD family member 3
SOX9	SRY-box 9
STC2	Stanniocalcin 2
STON1	Stonin 1
STS	Steroid sulfatase
SULF1	Sulfatase 1
T47D-ERα-Luc	Breast cancer T47D reporter (ER α) cell line (luciferase)
TBS	Tris-Buffered Saline
TFF1	Trefoil Factor-1
TINAGL1	Tubulointerstitial nephritis antigen like 1
TLR3	Toll like receptor 3
TNFSF10/TRAIL	TNF superfamily member 10
TNS4	Tensin 4
U2OS-ERα-Luc	Osteosarcoma reporter (ER α) cell line (luciferase)
U2OS-ERβ-Luc	Osteosarcoma reporter (ER β) cell line (luciferase)
μM/μM	Micromolar
VDR	Vitamin D Receptor
WUR	Wageningen University & Research
ZSWIM6	Zinc Finger SWIM Domain-Containing Protein 6

Chapter 1

General Introduction

1 Background information (state-of-the-art)

Retinoids present pharmacological and toxicological interest because of the plethora of signaling pathways they can influence. In fact, retinoids can affect multiple cellular processes by binding to Retinoic Acid Receptors (RAR) in the classical genomic pathway [1,2] but also via crosstalk with other signaling pathways such as the estrogen signaling pathway [3–5]. In the context of breast cancer, it was shown that retinoids can reduce breast cancer cell proliferation *in vitro* [2,3,6] leading to a high number of studies evaluating these chemicals as therapeutic agents [2]. However, due to aspects such as the high rates of failure observed (e.g. resistance) or their toxicological adverse effects at therapeutic (μM) concentrations, the interest on retinoids as anticancer agents has decreased in the last years [1,2,7]. Up to now, the molecular mechanisms governing the differential sensitivity to retinoid therapy in breast cancer treatment are still not completely known. In consequence, acquiring novel insights in the retinoid-induced molecular events is required to better understand their variable effects on breast cancer cell proliferation. For this reason, the main objective of the present thesis was to assess different retinoid-driven molecular processes related to both the classical RAR genomic pathway and the potential interference between retinoid and estrogen signaling to better comprehend their differential observed effects on breast cancer growth.

2 Retinoids, promiscuous effectors

The term “retinoid”, is generally applied to define a fat-soluble compound which is functionally and/or chemically related to vitamin A. Retinoids generally contain three main regions in their chemical structure, namely, a hydrophobic part, a central polyene chain (linker), and a polar region (usually containing carboxylic acid) [7,8]. This type of chemicals can be either endogenously/naturally formed such as the general Retinoic Acid Receptor (RAR) agonist *all-trans* retinoic acid (AtRA) or synthetically produced such as the RAR subtype-selective agonists Am80, CD2314 or BMS961 (Figure 1) [7]. In addition, synthetic RAR antagonists (e.g. Ro415253) have been successfully synthesized.

Figure 1. Representation of the molecular structures of the naturally-occurring retinoid AtRA and the synthetically produced retinoids Am80, CD2314, BMS961 and Ro415253.

Retinoids have been described to play a role in a wide range of physiological processes such as development, growth, reproduction or vision, among others [7]. However, exposure to high concentrations of this kind of chemicals has been also linked to several adverse effects leading to liver or developmental toxicity [7,9]. In addition, pharmacological effects on different pathological processes have been proposed [10–12]. For instance, treatment with retinoids was found to be effective against certain types of cancers (e.g. breast cancer) due to its inherent effects on parameters like cell proliferation, differentiation or apoptosis [10].

Natural and synthetic retinoids have been shown to exert their effects via multiple different pathways [1,13]. Thus, these compounds can carry out their actions by means of both genomic and non-genomic signaling (Figure 2). Within the classical pathway, retinoids can activate Retinoic Acid Receptor alpha, beta or gamma (RAR α , β , γ) which form heterodimers with Retinoid X Receptors alpha, beta or gamma (RXR α , β , γ) and bind to specific regions within the genome called Retinoic Acid Responsive

Elements (RAREs) leading to the expression of RAR-RARE-regulated genes. However, treatment with retinoids can also elicit the transcription of a broad variety of non-RAR-RARE-driven genes which can also play an important role in their physiological, pharmacological and/or toxicological effects [14]. In addition, apart from the regulation of retinoid-responsive genes (RAR or non-RAR mediated), crosstalk of retinoid signaling with other molecular pathways such as estrogen- or kinase-related processes has been reported [1,13,15].

Figure 2. Representation of the different molecular mechanisms of action of retinoids leading to their different physiological, toxicological and pharmacological/therapeutic effects.

3 Retinoids and RARs. Role in Breast Cancer Growth and Treatment

Among their different pharmacological effects, retinoids were found to be capable of reducing breast cancer cell proliferation *in vitro* [2,3,6] which led to an increasing interest for these chemicals as therapeutic agents. Thus, in the context of breast cancer treatment, retinoids have been shown to decrease proliferation and promote differentiation, cell cycle arrest and apoptosis [1,3,10,16].

However, many clinical trials using retinoids as therapeutic agents in breast cancer treatment have failed so far [1]. It has been suggested that resistance to retinoid-treatment in different types of cancer, may appear due to aspects such as epigenetic changes, disruption of coregulators levels or increased retinoid metabolism, among others, however the molecular mechanisms governing this differential response are far from being completely unravelled [1]. In order to better understand such a discrepancy, it has to be noted that the different genomic and non-genomic RAR-mediated events [2] can lead to different outcomes in terms of breast cancer cell proliferation [1,2]. To add more complexity, non-RAR-mediated actions of retinoids such as the activation of the pro-proliferative peroxisome proliferator-activated receptor beta/delta (PPAR β/δ) pathway [2] or the up-regulation of the pro-apoptotic caspase 7 [14] have been described. In consequence, the effectiveness of retinoids in breast cancer treatment will not only depend on their intrinsic ligand properties (e.g. selectivity) and the RAR expression levels (usually high for RAR α and RAR γ and remarkably lower for RAR β) [1,3,10,16,17], but also on many other factors [2,7]. In fact, varying with the type of tumour and its stage, the sensitivity/resistance to different retinoid treatments will depend on many variables such as their specific effects on 1) the different RAR subtype dependent genomic or non-genomic signaling pathways, 2) the activity of other transcription factors such as Estrogen Receptors (ERs) [3,18–20] or 3) other pathways (e.g. kinase signaling) [2]. Thus, despite the wide knowledge acquired so far, the molecular mechanisms governing the success/failure of retinoid therapy are still not fully understood.

4 The classical genomic RAR-RARE pathway in the context of breast cancer

In the classical genomic RAR-RARE pathway, RARs associate with RXRs and bind to promoter regions along the genome called Retinoic Acid Responsive Elements (RAREs) and to coregulators [7]. The binding of RAR/RXR to the different coregulators (coactivators or corepressors) is a key intermediate regulatory step in this process since it modulates chromatin structure and accessibility for transcription (Figure 3) [8]. In the absence of ligand, the RAR/RXR heterodimer binds to RAREs and to the apo-form-bound corepressors NCOR1 and NCOR2 which lead to condensed chromatin structure

and active target gene silencing [8,21,22]. Upon exposure to an agonist, a conformational change on the RAR occurs [22] leading to the release of corepressors and the recruitment of ligand-dependent coactivators which in turn, attracts other protein complexes leading to modification of chromatin structure, increase of DNA accessibility and recruitment of the general transcription machinery eventually initiating gene transcription [8,23]. In addition, ligand-induced corepressors which can maintain transcription blocked also exist [7]. These distinct types of coregulators have special importance since it was shown that depending on the cellular levels of certain coactivators or corepressors, genomic RAR signaling and the related response to retinoids can vary [7,24,25].

Figure 3. Representation of the different types of coregulators present in a cell and their role in RAR-RARE-mediated gene expression. Apo CoR = Apo-form-bound Corepressor; Lig-Ind CoA = Ligand-Induced Coactivator; Lig-Ind CoR = Ligand-Induced Corepressor; HDAC = Histone Deacetylase; HAT = Histone Acetyltransferase.

One well accepted mechanism affecting proliferation and ultimately leading to breast cancer growth inhibition is the above explained induction of the classical genomic RAR signaling which leads to the expression of different tumour suppressor genes [7]. Via this pathway, RARs have been shown to

directly drive the transcription of genes such as *CASP9* or *BTG2* leading to apoptosis or differentiation/anti-proliferative processes, respectively [14,26]. Previous research showed that the different RAR subtypes mostly bind to coincident promoter regions in the DNA suggesting genomic functional redundancy. However, despite their structural and mechanistic similarity, several studies that assessed the role of the different RARs (RAR α , RAR β and RAR γ) on breast cancer cell proliferation have suggested that they can exert different and even opposite effects [27,28]. Concerning this issue, the lack or overexpression of the different RAR subtypes can provoke differential effects on the proliferative rates of different tumour cells. Whereas expression of RAR β and RAR γ have been related to anti-proliferative and pro-proliferative status, respectively, RAR α has been linked to both anti- and pro-proliferative events depending on the cellular and environmental context (e.g. presence/absence of estrogenic stimulation) [4,16,27]. Adding complexity to the equation, RAR α -selective ligands have been shown to decrease breast cancer cell proliferation, while both RAR β - and RAR γ -selective agonists have shown not to exert any anti-proliferative effect [16,29]. Altogether it becomes evident that a complete understanding on the underlying molecular mechanisms involved in the RAR subtype-specific responses is still lacking so that additional research is needed.

In order to explain the different observed actions of the RAR subtypes on breast cancer cell proliferation, coregulator actions may play an important role. Thus, differential interactions of the RAR subtypes with the distinct types of coregulators, namely, apo-form-bound corepressors, ligand-induced binding coactivators, or ligand-induced binding corepressors may lead to different retinoid-driven biological outcomes [7,24]. In relation to this presumed mechanism, previous research in other cell types suggested that the specific effects of the different RAR subtypes may be explained by a differential capacity to attract coregulators to promoter regions [30]. Consequently, studies assessing and comparing the specific ligand-induced coregulator interactions between the different RAR subtypes may bring new insights in the understanding of the differential retinoid-induced responses in breast cancer [21,31–33].

5 Crosstalk ER-RAR in the context of breast cancer cell proliferation

Breast cancer growth is a complex process that involves and depends on many different signaling pathways which are often interconnected. In fact, distinct nuclear receptors such as ERs or RARs can modulate, in one way or another, parameters such as breast cancer cell proliferation, differentiation and apoptosis (Table 1) [27,34]. In addition, regarding ligand response, while several retinoids (RAR ligands) have been shown to inhibit breast cancer growth [34], estrogens (ER ligands) mainly induce cell proliferation of ER+ breast cancer cells. Thus, RARs and ERs can play key and opposite roles in the context of breast cancer cell proliferation [34]. Furthermore, the inhibitory retinoid-mediated effects have been shown to be highly dependent on the simultaneous presence of ERs in the cells, suggesting crosstalk between the respective signaling pathways [3].

Table 1. Representation of the different Estrogen Receptor (ER) and Retinoic Acid Receptor (RAR) subtypes and the biological effects linked to their expression in the context of breast cancer.

Nuclear Receptor (NR)	NR Subtype	Effects Breast cancer Growth
Estrogen Receptors (ERs)	Estrogen Receptor alpha (ER α)	Pro-proliferative
	Estrogen Receptor beta (ER β)	Anti-Proliferative
Retinoic Acid Receptors (RARs)	Retinoic Acid Receptor alpha (RAR α)	Pro-proliferative Anti-Proliferative
	Retinoic Acid Receptor beta (RAR β)	Anti-Proliferative
	Retinoic Acid Receptor gamma (RAR γ)	Pro-proliferative

Backing up the observed dependence of retinoid-mediated effects on the levels of ERs, the interplay between retinoid and estrogen signaling has been found at many different molecular levels (Figure 4). Correlation between the expression of ER α and RARs has been reported which is partially explained by the fact that the *RAR α* gene is a direct target of ER α and is up-regulated upon estrogenic stimulation [35,36]. Next, estradiol (E₂)-induced ER α -mediated transcription and the concomitant proliferation have been shown to be hampered by retinoid treatment [4,19]. These antagonistic actions of RARs and their ligands on ER α activity were proposed to be driven by competition for DNA-binding since both

types of receptors were found to frequently associate with close or even overlapping regions within the genome [5]. Further, RARs and ER α have been shown to directly interact forming heterodimers [37] and a study by Ross Innes et al. (2010) showed that in the presence of E₂, RAR α can cooperate with the pro-proliferative ER α -genomic signaling by acting as a scaffold protein within the same transcriptional complex [4]. In the same study, the researchers showed that while the levels of ER α and RAR α on the promoter regions of many estrogen responsive genes were not highly changed upon co-exposure with retinoids, the presence of several coregulators was diminished [4]. Accordingly, protein-protein interactions between ER α , RARs and coregulators may play an important role in the retinoid-mediated modulation of estradiol-induced transcription and the eventual cell proliferation.

In the absence of any estrogenic stimulation, the activity of apo-ER α plays also an important role in the basal proliferative rates of breast tumours [38]. In fact, ligand-independent activity of ER α and in consequence, the supported breast cancer cell proliferation may be also affected by different molecular events such as the phosphorylation/activation of ER α via kinase pathways, ER α degradation or changes in ER α subcellular levels [39]. Little is known regarding a possible crosstalk between ERs and RARs under hormone-deprived condition. Concerning this matter, it is known that increased levels of apo-ER α reduce the expression of the *RAR β* gene while permitting it to be highly up-regulated by a pan-agonist such as AtRA [40]. Apo-RAR α however, has been shown to enhance the ligand-independent activity of ER α and in this way is able to stimulate breast cancer growth [38]. In addition, in the absence of estradiol, exposure to subtype-selective retinoids have been shown to differentially affect ER⁺ breast cancer cell proliferation [16,29]. Altogether, a valid hypothesis could be that the differential effects exerted by the distinct RAR subtypes may also rely on a differential interplay with ER α signaling apart from the classical genomic RAR-RARE pathway. However, studies evaluating the possible effects of specific retinoids (general or selective) on the basal ER α signaling pathway and their potential role in the observed effects are lacking.

Figure 4. Different molecular mechanisms involving interplay between estrogen and retinoid signaling in the context of breast cancer cell proliferation, differentiation and apoptosis.

While research on the inter-relation between ER α and RARs is more extensive, data on possible crosstalk between ER β and RAR signaling is highly scarce. ER β has been shown to have anti-proliferative effects in breast cancer cells antagonizing ER α signaling, however it presents low expression levels in most of the breast tumour tissues [41–44]. On the other hand, ER β and RARs have also been shown to directly interact and form heterodimers *in vitro* influencing ER β -responsive gene expression [37]. Furthermore, RAR-mediated transcription was also differentially affected by increased expression of ER β or modulation of ER β activity upon incubation with both agonists and antagonists [45,46]. As a consequence, crosstalk between the ER α , ER β and the different RAR subtypes may influence breast cancer proliferation rates. Therefore, to further elucidate the modes of

action underlying the influence of retinoids on estrogen signaling of breast cancer cells, studies making use of cell lines known to express their respective receptors (RARs and ERs) to a greater or lesser extent (e.g. MCF7 or T47D) are required [3,47].

6 *In vitro* assays to evaluate effects of retinoids on RAR or other NR (e.g. ER) signaling

To assess the effects of a compound on nuclear receptor activity, the current *in vitro* tests focus on receptor binding, transactivation and cell proliferation assays. However as reported by the U.S. Environmental Protection Agency (EPA) other aspects such as NR dimerization, chromatin or coregulator binding, play crucial roles in this process [48]. In addition, specific chemicals may modulate NR signaling via other multiple mechanisms of action different to the classical ligand-receptor binding (Figure 5). In consequence, for the characterization of the potential of a retinoid to modulate directly or indirectly NR (e.g. ER or RAR) signaling a broad variety of different biochemical and cell-based assays can be used (Figure 5).

Studies evaluating final endpoints such as proliferation can give initial insights in the eventual modulation of NR activity generated by a chemical, however such effects may be elicited by many possible molecular events. In order to acquire further knowledge on the effects of a certain compound on nuclear receptor activity, multiple methods zooming in into NR-related molecular events can be applied. First, knowledge of the NR levels in the specific cell model studied gives important initial insights to better understand the effects of a chemical. NRs can be located in different compartments such as the membrane, cytoplasm or nucleus [49–52]. Upon stimulation with a ligand mechanisms such as translocation to or retention in the nucleus can be elicited [49,53]. However, such intracellular redistribution may also appear due to causes other than the direct ligand-NR binding such as via kinase pathways [54] or in association (direct interactions) with other protein complexes guided by different molecular mechanisms. Binding assays can accurately assess whether a certain compound is a cognate ligand for a specific receptor and the affinity of that interaction [55,56]. However, this kind of technique cannot predict the type of action elicited by such association [55,56]. Thus, using binding

assays does not give any information in terms of the potential transcriptional agonism or antagonism of the investigated chemical [56]. In order to predict the effects of chemicals on NR activity, reporter gene cell-based assays (e.g. luciferase assays) have been traditionally used [56]. Although very useful to evaluate transactivation potency parameters (e.g. EC50s) of a ligand, reporter gene assays make use of a specific Hormone Responsive Elements (HRE) while in a real-life scenario several HREs of different nature can be present in a specific gene and, in turn, different receptors can bind to a specific HRE [55]. In addition, reporter cell lines have usually different origin than the target cell population of interest and in consequence cell-derived differences such as differential coregulator levels or crosstalk with other signaling pathways could influence the obtained results in a way that is dependent on the cellular context [56–58]. RT-qPCR studies in the cell model of interest, although more laborious, do not have such limitations and can provide additional information relative to specific genes related/associated to a specific biological process (e.g. proliferation). However, molecular mechanisms exerted by the chemical other than the direct binding to the specific nuclear receptor may generate misleading interpretations. For example, phospho-activation via kinase pathways or up-regulation of the expression of the NR itself could also be possible underlying causes of an increase in the expression of specific genes. Although such processes could be detected by determining phosphorylation status, effects on kinase activity or via other omics techniques, to certainly determine whether a chemical can directly bind to a specific nuclear receptor and the eventual response generated (agonism-antagonism) additional techniques can be used. These techniques include NR-coregulator binding assays. Within the classical genomic pathway NR-coregulator binding is a fundamental step. NR-coregulator binding is modulated by a necessary and direct ligand-receptor interaction which leads to conformational changes in the receptor leading to the recruitment/release of different coactivators or corepressors. Thus, by using coregulator binding assays we can acquire information on both binding of a compound to the nuclear receptor and the most likely eventual effects on transcription (activation/repression of gene expression).

Figure 5. Representation of the different possible pathways by which a compound can directly or indirectly modulate NR signaling. Examples of specific techniques to assess each molecular event are also depicted as white squares.

7 Coregulator Binding Assays. Heterodimers vs Monomers. Full length NR vs LBD

NR-coregulator interactions play key intermediate roles in the regulation of gene expression. Despite representing a crucial step on the modulation of transcription, research on NR-coregulator interactions is still limited. In order to study NR-coregulator interactions, several methods such as intermolecular FRET or yeast two-hybrid assays have been used. However most of these assays allow to study specific interactions between one type of NR and one type of coregulator [59]. In a real life scenario though, different coregulators can take part in this interaction and depending on factors like cell-type specific coregulator expression, coregulator-NR affinity and the specific ligand-generated conformational change of the receptor, the biological response to a chemical can vary [24,25,32,33,60–63]. The interactive region of coregulators is composed by leucine-rich motifs (NR-boxes) which are necessary and sufficient for the interactions with NRs [8,21]. Making use of this concept, methods such as the Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) can be used to study, in a high-throughput manner, ligand-dependent binding of NRs to multiple coregulator motifs. Thus, using such a microarray, 154 coregulator motifs belonging to 65 different coregulators can be studied simultaneously (Figure 6) [64].

Figure 6. Representation of the general principle of the Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) which has been widely used in this thesis.

Due to the actual existence of RAR/RXR heterodimers in a physiological scenario, the use of isolated RARs in coregulator binding assays such as MARCoNI may be initially argued. However, several studies have provided supporting evidence that the use of single RARs can contribute as a starting point to provide biologically relevant information concerning the RAR/RXR functioning. In fact, it became clear that the RAR/RXR is a so-called non-permissive heterodimer where RAR has a major role [8,21]. Thus, RAR agonists can activate transcription independently of the presence/absence of RXR ligands [8,21] while, on the contrary, the heterodimer does not respond to RXR agonists in the absence of RAR ligands [8,21]. In fact, in the RAR/RXR heterodimer, RAR is the principal element providing interaction surfaces for both corepressors and coactivators [8,65]. Furthermore, several studies have pointed out that the coregulator binding to RAR as monomer and to RAR/RXR heterodimers is very similar [66]. These findings support the use of the RAR monomer as a valid approach when assessing coregulator interactions.

NR monomers such as RARs are proteins that possess six different regions (A-F) containing three principal domains namely the AF-1 domain, the DNA Binding Domain (DBD) and the Ligand Binding Domain (LBD) [22]. The AF-1 domain is responsible for ligand independent (basal) activation and can bind to several coactivators and other transcription factors [22]. The DBD is the part of the receptor in charge of the binding to the HRE within the genome [22]. Finally, the LBD (which includes the AF-2 domain) is the region containing the surface of interaction with the cognate ligand (named ligand binding pocket) [22] and is the main player in other important processes such as dimerization and coregulator interactions [8]. This “independent” role distribution, which remains after separation of the respective domains, together with the difficulties to produce active purified full length NRs, has led to an extensive use of isolated LBDs as a main tool for the evaluation of chemicals as potential ligands of a certain NR or to evaluate ligand-driven NR-coregulator interactions, among others. However, inter-domain communication within the NRs where events occurring in one domain can provoke changes in the other domains have been described [60,67–73]. In addition, the mere presence

in the full length protein of domains other than the LBD, was shown to elicit different conformations in a NR upon stimulation with the same ligand [74]. Thus, studies showing concordance between the activity (e.g. coregulator binding) of full length NRs and their isolated LBDs are required to further support the general use of truncated receptors and to evaluate additional parameters within the area of NR-coregulator interactions.

8 Aim, approach and outline of the present thesis

Despite the fact that many previous studies have already provided multiple insights in the RAR genomic pathway and the crosstalk between RAR and ER signaling, the underlying molecular mechanisms leading to the different retinoid-mediated effects on the basal and E₂-induced proliferation of breast cancer cells are not completely understood. Thus, the objective of the present thesis was to investigate different molecular events possibly elicited after retinoid treatment to better understand the observed effects on breast cancer growth. Thus, both the classical RAR genomic pathway and the potential interference between retinoids and other signaling pathways (e.g. ER pathway) were assessed by carrying out biochemical and cell-based studies.

All along the present thesis, different basal and ligand-dependent NR-coregulator binding events were evaluated in detail. In order to support the posterior use of isolated LBDs for the coregulator binding assays, **chapter 2** analysed the suitability of such an approach by comparing the coregulator interactions between the full-length RAR α and its respective LBDs counterpart. Once shown that there were no remarkable differences between full length and truncated receptors, the coregulator binding of the different RAR subtypes upon agonistic/antagonistic actions of different ligands were further characterized and compared in **chapter 3** to acquire new insights on possible specific/selective interactions and their potential link to the differential RAR-subtype specific biological actions. Regarding the retinoid-mediated effects on the E₂-mediated (**Chapter 4**) and basal (**Chapter 5**) ER α signaling, the present thesis assessed the effects of the general RAR agonist AtRA and the subtype-selective RAR ligands Am80 (RAR α -selective agonist), CD2314 (RAR β -selective agonist) and

BMS961 (RAR γ -selective agonist) on the highly E₂-responsive MCF7/BUS breast cancer cell line and the U2OS-ER α -Luc reporter gene cell line. To this end, parameters such as cell proliferation, ER α -mediated gene expression, ER α coregulator binding and ER α nucleocytoplasmic shifts (subcellular localization) were tested. Next, **Chapter 6 and 7** went deeper in the understanding of the relation between retinoid-mediated effects on gene expression and the consequent proliferative response by testing the effects of the different general and selective retinoids in another ER⁺ breast cancer cell line (T47D). Thus, these chapters focused on the differential transcriptomic signatures generated by retinoids both in the presence (**Chapter 6**) and absence of E₂ (**Chapter 7**). Finally, **Chapter 8 and Chapter 9** presented a general discussion and a summary of the findings and conclusions generated throughout the present thesis. In addition, these chapters also included a general perspective concerning the implications and recommendations for follow-up studies to eventually obtain a more complete molecular understanding of the retinoid-mediated effects on breast cancer growth.

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Chapter 2

**Ligand-modulated coregulator binding profiles of the full length
Retinoic Acid Receptor alpha and its ligand binding domain**

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Abstract

The retinoic acid receptor alpha (RAR α) is important from both a toxicological and a pharmacological perspective. It forms a dimer with retinoid X receptors and regulates gene expression upon binding to its ligands (retinoids). Binding to coregulators (coactivators or corepressors) is a key intermediate regulatory step in this process. To study receptor-coregulator binding, studies are often performed using the isolated ligand binding domain (LBD). However, it has not been well characterized to what extent LBD-coregulator interactions reflect coregulator interactions with the full length (FL) receptor. The present study aims to characterize ligand-dependent RAR α -coregulator interactions using both the RAR α -LBD and the RAR α -FL focussing on potential similarities and/or differential outcomes. To this end, we used the Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) to assess RAR α interactions with 154 motifs belonging to more than 60 different coregulators. We investigated the coregulator motif interactions of RAR α -FL and RAR α -LBD in the presence of increasing concentrations of the RAR agonist *all-trans* retinoic acid (AtRA) and the RAR antagonist Ro415253. We showed that the coregulator motif interaction profiles are highly similar for the RAR α -FL and the RAR α -LBD, and that with both proteins, the agonist AtRA was successfully discriminated from the antagonist Ro415253. The agreement between RAR α -FL and RAR α -LBD was more prominent in the presence of the agonist AtRA than in the presence of the antagonist Ro415253. Additionally, analysis of AtRA-dependent coregulator motif binding profiles indicated several unknown RAR α -coregulator interactions. Altogether, this study demonstrates that coregulator binding profiles of RAR α -FL and RAR α -LBD are highly similar and that the data obtained may provide a starting point to further identify possible unknown RAR α -coregulator interactions.

1 Introduction

The retinoic acid receptor alpha (RAR α) is important from both a toxicological and a pharmacological point of view [1,2]. As all nuclear receptors (NRs), RAR α is a ligand modulated transcription factor that regulates gene expression. RAR α forms a dimer with retinoid X receptors (RXRs), which binds to specific DNA regions called retinoic acid responsive elements (RAREs) and modulates transcription upon binding to its ligands (retinoids). The binding of the RAR α to coregulators (coactivators or corepressors) is a key intermediate regulatory step in this process since it modulates chromatin structure and accessibility for transcription [3]. In general, these coregulators can be divided in three major categories, being apo-form-bound corepressors, ligand-induced coactivators, or ligand-induced corepressors [4]. Knowledge on ligand-dependent RAR α -coregulator interactions is needed to understand retinoid-induced effects, since the biological response to ligands has been shown to depend on the levels of coregulators present and their affinity/selectivity for the NR [5–8]. To assess such NR-coregulator interactions, studies are often performed using the isolated ligand binding domain (LBD) of the particular NR. Although ligand-directed coregulator binding indeed takes place at the LBD, this interaction may differ in the context of the full length receptor (FL). In fact, studies with different NRs have shown inter-domain allosteric communication in which processes occurring at the DNA binding domain (DBD) may propagate a signal influencing the LBD and in consequence, affect the final NR functioning [9–12]. In addition, there is still controversy concerning a possible differential behaviour of the LBD due to the mere presence of other domains in the FL protein [13,14]. It has not been well characterized to what extent LBD-coregulator interactions can accurately reflect coregulator interactions with the FL protein. Therefore, comparative studies assessing coregulator binding with the isolated LBD and the FL protein are needed.

To study ligand-dependent modulation of NR-coregulator interactions, several methods such as X-ray crystallography [15], GST-pull down assays [16], fluorescence anisotropy [15] or yeast two hybrid

assays [16] have been used. However, most of these assays only allow to study a specific interaction between the NR and one coregulator while in a real life scenario, many different coregulators are present in a cell and could take part in this interaction. Thus, only a limited number of coactivators and corepressors have been studied up till now. The interactive region of coregulators is composed by leucine-rich motifs (NR-boxes) [3] which are deemed to be necessary and sufficient for the interactions with NRs [5]. Coactivators possess one or several consensus sequences LXXLL while corepressors contain the canonical sequences LXXI/HIXXXI/L [5]. The Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) makes use of this concept in a high-throughput context offering the possibility to rapidly and simultaneously assess ligand-modulated binding of NRs to 154 motifs belonging to more than 60 different coregulators. Thus, by using this technique, a broad coregulator binding profile can be obtained.

The present study aims to characterize the ligand-dependent coregulator binding profiles of the RAR α -FL and the RAR α -LBD in the presence of the RAR agonist all-*trans*-retinoic acid (AtRA) or the RAR antagonist Ro415253 by using MARCoNI. It focusses on possible similarities and/or differential outcomes between coregulator interactions obtained using the RAR α -FL and the RAR α -LBD in order to assess the possible influence of domains other than the LBD on the ligand-dependent RAR α -coregulator binding profiles.

2 Materials and Methods

2.1 MARCoNI Coregulator Binding Assay

In this study, MARCoNI was used in order to evaluate RAR α -FL and RAR α -LBD coregulator interaction in the presence of the RAR agonist AtRA (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) and the RAR antagonist Ro415253 (Abcam, Cambridge, UK). In brief, all assay samples, containing the RAR α , the ligand of interest and the detecting antibody, were prepared in time-resolved fluorescence resonance energy transfer (TR-FRET) reaction buffer D (Life technologies, Ltd, UK,

#PV4420) supplemented with 5 mM DTT (Sigma, Zwijndrecht, the Netherlands, #43819) and kept on ice before the initiation of the assay. Three-fold dilution series of the ligands within the range of 10^{-4} - 10^{-9} M were tested with final solvent concentrations of 2% DMSO. Samples containing 2% DMSO without ligand were included as the solvent control (henceforth presented as 10^{-15} M). For the RAR α -FL studies, 50 nM histidine (His)-labeled human RAR α -FL (RAR α^{His} , Jena Bioscience GmbH, Jena, Germany, #PR-753) and 50 nM Alexa 488-conjugated anti-His (Qiagen, Germantown, MD, USA, #35310) as detecting antibody, were used. For the RAR α -LBD study, 10 nM glutathione S-transferase (GST)-tagged RAR α -LBD (RAR α -LBD^{GST}, amino acids 176-462, Life technologies, Ltd, UK, #PV4415) and 25 nM Alexa 488-conjugated anti-GST (Invitrogen, Breda, the Netherlands, #A11131) as detecting antibody, were utilized. For each ligand concentration, two technical replicates (2 arrays) were performed by using PamChip[®] plates (PamGene International B.V., 's-Hertogenbosch, the Netherlands) containing 96 peptide microarrays. Every microarray consists of a three-dimensional porous metal oxide carrier containing 154 spots with different immobilized NR-binding motifs belonging to more than 60 different coregulators. The automated platform PamStation[®] (PamGene International B.V., 's-Hertogenbosch, The Netherlands) and EvolveHT software (PamGene International B.V., 's-Hertogenbosch, The Netherlands) were used applying 2 (up-down) pumping cycles per minute at 20°C. Before adding the samples to the Pamchip[®] arrays, incubation for 20 cycles with 25 μ l blocking buffer composed of Tris buffered saline (Bio-Rad, Veenendaal, the Netherlands, #170-6435) supplemented with 1% BSA (Calbiochem, Merck, Darmstadt, Germany, #126609) and 0.01% Tween-20 (Bio-Rad, Veenendaal, the Netherlands, #170-6531) was carried out in order to avoid unspecific binding to the arrays. After removal of blocking buffer, samples were added to the Pamchip[®] arrays and incubated for 80 pumping cycles. Next, unbound receptor was washed away with 25 μ l Tris Buffered saline and TIFF images were taken by a charge coupled device camera (CCD) installed in the PamStation[®].

2.2 Data analysis

The software Bionavigator (Version 6.1, PamGene International B.V., 's-Hertogenbosch, The Netherlands) was used for the image analysis. RAR α -binding to the different coregulator peptides in the absence or presence of the ligands was evaluated by quantifying the fluorescence signals. Shortly, boundaries for every spot (peptide) were determined, and RAR α -coregulator fluorescence binding Arbitrary Units (AU) were quantified by subtracting the background signal from the median fluorescent signal. Concentration-response curve fitting was carried out utilizing the DRC package in R (version 2.15.3, www.r-project.org) by means of a sigmoidal, 4-parameter Hill (logistic) model ($response = ((A - D)/(1 + ((concentration/C)^B))) + D$, with parameters A = response minimum, B = Hill slope, C = EC50, and D = response maximum). Only concentration-response curves with goodness of fitting (R^2) higher than 0.8 were considered for analysis and discussion. In this work, effects were also expressed as Modulation Index (MI) which represents the log10 transformed ratio of the binding in the presence of the ligand over the binding in the solvent control. To assess whether RAR α -coregulator interactions observed using the MARCoNI assay have been previously described in the literature, the databases STRING v9.1 (<http://string-db.org/>) and BioGRID^{3,4} (<http://thebiogrid.org/>) were used.

3 Results

3.1 Assessment of interactions of RAR α -FL and RAR α -LBD with coregulator motifs in the presence of the RAR agonist AtRA or the RAR antagonist Ro415253

The present study utilized MARCoNI to determine interactions of the RAR α -FL and the RAR α -LBD with multiple coregulator motifs upon incubation with increasing concentrations of the RAR agonist AtRA (Figure 1) or the RAR antagonist Ro415253 (Figure 2).

Figure 1A shows an overview of the obtained AtRA concentration-response curves on RAR α -coregulator motif interactions with R^2 higher than 0.8 for RAR α -FL and RAR α -LBD. Concentration-

response data for the ATRA-driven interactions of RAR α -FL and RAR α -LBD with all coregulator motifs presents on the array including all individual data points underlying the curves are depicted in Supplementary Figures A.1-A.2. In addition, Supplementary Table A.1 present an overview of all EC50 values as derived from the concentration-response curves with R^2 higher than 0.8. A more detailed view of the concentration-response curves of 3 selected motifs, belonging to the RAR α apo-form-bound corepressor NCOR1 (Figure 1B) [17], the RAR α ligand-induced coactivator NCOA1 (Figure 1C) [18] and the RAR α ligand-induced corepressor MAPE (Figure 1D) [19] is shown in Figures 1B-D.

Figure 1. Concentration-response curves of binding of RAR α -FL (red lines) or RAR α -LBD (blue lines) to coregulator motifs in the presence of the RAR agonist AtRA (A). AtRA-modulated RAR α binding to the motifs NCOR1_2251_2273 (B), NCOA1_677_700 (C) and MAPE_356_378 (D) is depicted in detail. Concentration-response curves are presented if goodness of fit (R^2) was higher than 0.8. The X-axis represents the different concentrations of AtRA, while the Y-axis represents the binding in Arbitrary Units (AU).

Figure 1B shows the binding of RAR α -FL and RAR α -LBD to the motif NCOR1_2251_2273, which represents an apo-form-bound corepressor [17]. As the concentration of AtRA increases, the binding of this inhibitory protein motif decreases with an EC50 of 17 nM for both RAR α -FL and RAR α -LBD (Supplementary table A.1). RAR α also showed an AtRA concentration-dependent dissociation from a motif representing the other known apo-form-bound corepressor present at the array (NCOR2_2330_2352) [20] (Figure 1A) with identical EC50 values of 17 nM for RAR α -FL and RAR α -LBD (Supplementary Table A.1).

Figure 1C depicts the binding of RAR α -FL and RAR α -LBD to the motif NCOA1_677_700, which represents a ligand-induced coactivator [18]. For this coregulator motif, as the concentration of AtRA increases, the RAR α -binding increases. In this case, the EC50 values calculated for RAR α -LBD and RAR α -FL were 8 nM and 17 nM, respectively (Supplementary Table A.1). Further, clear concentration-dependent increases in binding of RAR α was also found for the motifs representing the known RAR α -coactivators NCOA2 [18], NCOA3 [21], NCOA6 [22], NRBF2 [23], PRGC2 [24], TIF1A [25], EP300 [26,27], MED1 [28], UBE3A [29] and CBP [27] (Figure 1A) with similar EC50 values within the low nanomolar range (Supplementary Table A.1).

Although less frequent, ligand-induced RAR α -corepressor binding has been previously described in the literature [4]. As an example of these non-conventional agonist-induced RAR α -corepressor interactions, the binding of RAR α to the motif MAPE_356_378 [19] is displayed in Figure 1D. The EC50 for this non-classical coregulator was 16 nM for the RAR α -LBD and 23 nM for the RAR α -FL (Supplementary Table A.1). Apart from this example, MARCoNI also showed the binding of RAR α to motifs of three other ligand-induced RAR α corepressors previously reported in literature namely NRIP1 [7,10], NR0B2 [30,31] and LCOR [32] (Figure 1A) with similar EC50 values in the low nanomolar range (Supplementary Table A.1).

Figure 2A displays an overview of the obtained Ro415253 concentration-response curves on RAR α -coregulator motif interactions with R^2 higher than 0.8 for RAR α -FL and RAR α -LBD. Concentration-response data for the Ro415253-driven interactions of RAR α -FL and RAR α -LBD with all coregulator motifs presents on the array including all individual data points underlying the curves are depicted in Supplementary Figures A.3-A.4. In addition, Supplementary Table A.1 present an overview of all EC50 values as derived from the concentration-response curves with R^2 higher than 0.8. Concentration-response curves for 3 selected coregulator motifs, belonging to the RAR α apo-form-bound corepressor NCOR2 [20] (Figure 2B), the RAR α ligand-induced coactivator NCOA2 [18] (Figure 2C) and the RAR α ligand-induced corepressor NRIP1 [7,10] (Figure 2D) are presented in detail in Figure 2B-D.

Figure 2. Overview of the binding of RAR α -FL (red lines) or RAR α -LBD (blue lines) to coregulator motifs in the presence of the antagonist Ro415253 (A). Ro415253-modulated RAR α binding to the motifs NCOR2_2123_2145 (B), NCOA2_677_700 (C) and NRIP1_701_723 (D) is depicted in detail. Concentration-response curves were generated if goodness of fit (R^2) was higher than 0.8. The X-axis represents the different concentrations of Ro415253, while the Y-axis represents the binding in Arbitrary Units (AU).

Upon incubation with increasing concentrations of the RAR antagonist Ro415253, not only coactivator binding, but also corepressor binding was reduced when comparing to the basal binding of unliganded receptors (Figure 2A). This global dissociative outcome was observed for both RAR α -FL and RAR α -LBD, leading to minimal coregulator binding levels at the highest concentration of the antagonist Ro415253. Thus, RAR α showed decreased binding to the different categories of coregulators represented by the motifs NCOR2_2123_2145 (Figure 2B), NCOA2_677_700 (Figure 2C) and NRIP1_701_723 (Figure 2D) upon incubation with increasing concentrations of Ro415253. The high capacity of Ro415253 to decrease the basal binding to these coregulator motifs is reflected by the low EC50 values within the range of 13-23 nM for both RAR α -FL and RAR α -LBD (Supplementary Table A.1).

When comparing concentration-response curves for RAR α -FL and RAR α -LBD binding to coregulator motifs upon agonist or antagonist action, similar trends were observed for each coregulator motif (Figure 1A and Figure 2A). However, the overall fluorescence signal was generally lower when using the RAR α -FL compared to the RAR α -LBD, even though the molar concentrations of RAR α -FL and its detecting antibody were 5-fold and 2-fold higher, respectively. Thus, for most of the coregulator motifs, basal, minimum and maximum binding were consistently lower when using the RAR α -FL. The RAR agonist AtRA generated a high number of concentration-response curves, being 111 for the RAR α -FL and 108 for the RAR α -LBD. However, Ro415253 treatment resulted in a lower number of concentration-response curves for RAR α -LBD (81) but especially for RAR α -FL, for which only 43 concentration-response curves of coregulator motifs showed an R^2 higher than 0.8.

3.2 RAR α -FL and RAR α -LBD coregulator binding profiles present high correlation and similar EC50s and Modulation Indexes

In order to further assess possible similarities and/or differences between RAR α -FL and RAR α -LBD binding to coregulator motifs, aspects such as EC50 values derived from binding concentration-

response curves (Figure 3), correlation of data on binding all along the different ligand concentrations (Figure 4) as well as modulation indexes at saturating ligand concentrations (Figure 5) were assessed.

Figure 3 and Supplementary Table A.1 show a comparison of the EC50 values derived from the concentration-response curves for RAR α -FL and RAR α -LBD binding to the coregulator motifs upon incubation with AtRA and Ro415253. The EC50 values for the different coregulators were highly similar when using RAR α -LBD and when using RAR α -FL. Median EC50 values for AtRA concentration-response curves were 19 nM and 16 nM for the RAR-FL and RAR-LBD, respectively (Figure 3A). On the other hand, median EC50 values for Ro415253 concentration-response curves were 17 nM for RAR α -FL and 12 nM for the RAR α -LBD (Figure 3B). Thus, similar EC50s in the low nanomolar range were found when comparing both receptors.

Figure 3. Distribution of EC50 values obtained from concentration-response curves of RAR α -FL and RAR α -LBD binding to the coregulator motifs in the presence of AtRA (A) and Ro415253 (B) showing interquartile ranges (boxes), median (lines within boxes), whiskers (10–90 percentiles), and dots (values outside the 10–90 percentiles). Only motifs with concentration-response curves showing R^2 higher than 0.8 in both RAR α -FL and RAR α -LBD were included for comparison leading to the analysis of EC50 values from 98 motifs for AtRA and EC50 values from 40 motifs for Ro415253.

The correlation between coregulator binding to RAR α -FL and RAR α -LBD was determined using data of all exposure conditions and applying a non-parametric Spearman test. Figure 4 shows an excellent correlation of binding in the presence of AtRA ($\rho = 0.95$) (Figure 4A), whereas a good but lower correlation was obtained when considering the Ro415253 data ($\rho = 0.80$) (Figure 4B).

Figure 4. Correlation of RARα-FL and RARα-LBD binding to coregulator motifs (in Arbitrary Units (AU)) upon exposure to AtRA (A) and Ro415253 (B). Binding of RARα-LBD to coregulator motifs is plotted against binding of RARα-FL to coregulator motifs at each ligand concentration. Only motifs with concentration-response curves showing an R^2 higher than 0.8 in both RARα-FL and RARα-LBD were included for comparison leading to the analysis of 98 motifs for AtRA and 40 motifs for Ro415253 conditions.

Figure 5 depicts the modulation indexes at saturating ligand concentrations, being 10^{-5} M for AtRA and 10^{-4} M for Ro415253. Using the modulation index concept, positive or negative modulation indexes imply an increase or decrease in coregulator motif binding when compared to the apo-form (solvent control, modulation index = 0), respectively. Opposite patterns for AtRA and Ro415253 can be readily noticed discriminating between agonist and antagonist actions (Figure 5). In the presence of AtRA the modulation index pattern for RARα-FL and RARα-LBD show a high overlap over the whole set of coregulator motifs. Similarly, Ro415253 gave rise to largely similar modulation index patterns, although to a lesser extent than the ones generated by ATRA.

Figure 6. Coregulators showing AtRA-dependent coregulator motif interactions with both RAR α -FL and RAR α -LBD in MARCoNI. Coactivators (green), corepressors (pink) and other proteins (blue) are classified according to their function described in literature, being agonist-dependent coactivators, agonist-dependent corepressors, apo-form corepressors, ligand-independent coregulators, and other proteins. Proteins previously described to bind to RAR α are presented by a connecting line.

Ligand-dependent coactivators described for other nuclear receptors but newly detected as interactors with RAR α include BL1S1 [33], BRD8 [34], CHD9 [35], MLL2 [36], NCOA4 [37], PELP1 [38], PR285 [39], PRGC1 [40], TGF11 [41], TIP60 [42], TREF1 [43], TRRAP [44] and DDX5 [44]. Further, MARCoNI identified RAR α interactions with known ligand-dependent corepressors such as CNOT1 [45], NELFB [46], IKBB [47], PROX1 [48] and RAD9 [49], which have not been reported to act as a corepressor for RAR α so far. Next, our study detected AtRA-concentration-dependent binding of RAR α to motifs of proteins that have only been described as ligand-independent interactors with RAR α , such as TRIP4 [50]. In addition, motifs of the ligand-independent coactivator ZNT9 [51] and the dual ligand-independent/dependent corepressor NROB1 [52,53], defined for other NRs but never pointed out as RAR α partners, interacted with RAR α in an ATRA-concentration-dependent manner in the present study. MARCoNI also harbours several peptides belonging to proteins that have been

reported to show other functions than the ones attributed to coregulators, such as motifs of transcription factors or proteins playing a role in kinase signaling pathways. Regarding these proteins, RAR α showed AtRA-induced binding with motifs of GNAQ, which has been described before [54] or WIPI1 [55] which has only been reported as ligand-independent RAR α -interactor. Additionally, RAR α showed AtRA-induced binding to motifs belonging to the Progesterone Receptor (PGR) [56] and to motifs of proteins such as KIF11 [58] or TRXR1 [59], which have not been reported before.

4 Discussion

The present study aimed to compare the coregulator binding profiles of the RAR α -FL and the RAR α -LBD upon exposure to the RAR agonist AtRA and the RAR antagonist Ro415253 by using MARCoNI. It specifically focussed on possible similarities and/or differential outcomes between the FL receptor and its isolated LBD, in order to assess whether coregulator interactions identified with the RAR α -LBD represent RAR α -FL coregulator interactions.

RAR α -FL and RAR α -LBD have been shown to bind AtRA with identical affinities [60], but their coregulator interactions have not been compared in detail. Our study showed that RAR α -FL and RAR α -LBD display highly similar responses in terms of coregulator motif binding upon incubation with the agonist AtRA. This agreement was also observed upon incubation with the antagonist Ro415253 although to a lesser extent. Technical factors, such as the low fluorescence signal obtained in the experiments with RAR α -FL, which is aggravated in the presence of an antagonist, may partially explain this difference. Thus, the incubation of RAR α -FL with Ro415253 leads to lower signal to noise ratios, which in turn, can influence the fitting of concentration-response curves. In addition, our study showed that the median EC₅₀ values obtained by MARCoNI for RAR α -FL and RAR α -LBD are in the same low nanomolar range, which for the case of AtRA is also in accordance with previous studies [61]. Overall, we can conclude that both RAR α -FL and RAR α -LBD are functional in MARCoNI and that they show high similarity in their ligand-modulated coregulator binding patterns. Since the RAR α -

LBD shows a highly similar coregulator binding profile to the one displayed by RAR α -FL and at the same time provides better concentration-response data, it represents an adequate option if MARCoNI is to be used for screening strategies to detect if chemicals present retinoid-like activity or as a starting point for the assessment of specific ligand-driven RAR-coregulator interactions.

MARCoNI revealed potential AtRA-dependent RAR α -coregulator interactions that have never been reported before. These data can be used as a starting point to further study such interactions under physiologically relevant conditions. Previous research demonstrated the potential use of MARCoNI in the identification of new NR-coregulator binding events [62]. Although RAR α -mediated gene expression is a dynamic process with several exchanges of coregulators at the promotor level [4], only one coregulator per RAR-RXR heterodimer can bind at a time [63,64]. Accordingly, the relevance of each RAR α -coregulator interaction that can be detected by MARCoNI will depend on various factors in a cellular context such as the binding affinity between the receptor and the full length coregulator protein or the coregulator expression levels [7,8,10,19,65]. Interestingly, motifs for coactivators such as NCOA4 or corepressors like CNOT1 showed a clear ligand-dependent interaction with RAR α in the MARCoNI, whereas they have been reported to display very weak or no interaction with RAR α in previous biochemical studies [37,45]. On the other hand, the coregulator PNRC2 has been described in literature as a ligand-dependent coregulator of RAR α [66], whereas it was not considered as such by our analysis in MARCoNI ($R^2 < 0.8$). Hence, interactions between the coregulator motifs and RAR α in MARCoNI requires further validation steps to confirm ligand-directed binding between the full-length proteins in a more physiological scenario. If confirmed, data of newly-detected AtRA-dependent binding of RAR α to certain motifs present in MARCoNI may provide information influencing genomic but also non-genomic events. Overexpression of ligand-activated corepressors (e.g. MAPE) in cancer, has been related to retinoid treatment resistance [19] and worse prognosis [65]. Our study showed concentration-dependent interaction of both RAR α -FL and RAR α -LBD with motifs of MAPE, but also with motifs of many other unknown RAR α ligand-induced corepressors such as

CNOT1, NELFB, IKBB and RAD9. MARCoNI also showed unknown AtRA-dependent direct RAR α interactions with the Progesterone Receptor (PGR) as well as with proteins associated with several effects of retinoids such as cell death pathways (TRXR1) [59] or differentiation-related autophagy (WIPI1) [55,67]. Thus, follow-up studies should focus on the toxicological and pharmacological relevance of such interactions.

Besides assessing the RAR α coregulator binding profile in the presence of the agonist AtRA, we also assessed the largely unknown coregulator binding profile in the presence of the antagonist Ro415253. Generally, RAR antagonists can cause down-regulation of retinoid responsive genes by strengthening the binding of corepressors (inverse agonism) or by provoking the dissociation of corepressors while not attracting coactivators (neutral antagonism) [3]. Upon incubation with Ro415253, both coactivator and corepressor peptide binding was reduced when compared to the unliganded receptors. Although previous studies already showed that the binding of NCOA1 and NCOR2 to RAR α is decreased by Ro415253 [68], we hereby show that this dissociative behaviour can be generalized to many other coactivators and corepressors. Thus, the present study demonstrated that Ro415253 behaves as a neutral antagonist leading to the so-called naked RAR α [69].

In conclusion, the present study showed that the ligand-modulated coregulator binding profiles of RAR α -FL and RAR α -LBD in the MARCoNI are highly similar. Thus, the choice of the type of receptor to be used will depend on the objectives of each study. Additionally, this work showed that MARCoNI successfully discriminated between agonist/antagonist actions and identified unknown ligand-dependent RAR α interactions with coregulators and other proteins. Thus, future studies should focus on evaluating these newly-described interactions and their possible consequences in a more physiological scenario.

Conflicts of Interests

The authors declare that part of the study was conducted at PamGene International B.V.

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Appendix. Supplementary Material

Supplementary material can be found in the end of this chapter.

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Appendix. Supplementary Figure A.1. Binding of RAR α -FL to the whole panel of MARCoNI motifs in the presence of the RAR agonist AtRA. In this figure, concentration-response curves are presented if goodness of fit (R^2) was higher than 0.5. The X-axis represents the different concentrations of AtRA, while the Y-axis represents the binding in Arbitrary Units (AU).

Appendix. Supplementary Figure A.2. Binding of RAR α -LBD to the whole panel of MARCoNI motifs in the presence of the RAR agonist AtRA. In this figure, concentration-response curves are presented if goodness of fit (R^2) was higher than 0.5. The X-axis represents the different concentrations of AtRA, while the Y-axis represents the binding in Arbitrary Units (AU).

Appendix. Supplementary Figure A.3. Binding of RAR α -FL to the whole panel of MARCoNI motifs in the presence of the RAR antagonist Ro415253. In this figure, concentration-response curves are presented if goodness of fit (R^2) was higher than 0.5. The X-axis represents the different concentrations of Ro415253, while the Y-axis represents the binding in Arbitrary Units (AU).

Appendix. Supplementary Figure A.4. Binding of RAR α -LBD to the whole panel of MARCoNI motifs in the presence of the RAR antagonist Ro415253. In this figure, concentration-response curves are presented if goodness of fit (R^2) was higher than 0.5. The X-axis represents the different concentrations of Ro415253, while the Y-axis represents the binding in Arbitrary Units (AU).

Appendix. Supplementary Table A.1. EC50 values upon exposure of RAR α -FL and RAR α -LBD to AtRA and Ro415253. Only EC50 values of motifs with R² higher than 0.8 are included for comparison. When R² was lower than 0.8, EC50 values were not determined (N.D).

Coregulator (Alternative nomenclature)	Coregulator motif	AtRA	AtRA	Ro 415253	Ro 415253
		RAR α -FL EC50 (nM)	RAR α -LBD EC50 (nM)	RAR α -FL EC50 (nM)	RAR α -LBD EC50 (nM)
ANDR: Androgen Receptor	ANDR_10_32	31	N.D	N.D	N.D
BLIS1 (BLOC-Isunit 1): Biogenesis of lysosome-related organelles complex 1 subunit1 MICoA: Metastasis-associated protein 1 (MTA1)-interacting coactivator	BLIS1_1_11	22	18	N.D	N.D
BRD8: Bromodomain-containing protein 8 p120 SMAP: Skeletal muscle abundant protein	BRD8_254_276	17	10	49	10
CBP: CREB-binding protein	CBP_2055_2077	18	N.D	N.D	14
	CBP_345_367_C367S	25	16	N.D	N.D
	CBP_345_368	27	N.D	N.D	N.D
	CBP_345_368_C367S	27	N.D	N.D	N.D
	CBP_57_80	17	15	19	13
CCND1: G1/S-specific cyclin-D1	CCND1_243_264_C243S/C247S	N.D	N.D	N.D	N.D
CENP-R: Centromere protein R	CENPR_159_177	20	17	11	11
ITGB3BP: Inte-grin beta-3-binding protein NRIF3: Nuclear receptor-interacting factor 3	CENPR_1_18	N.D	19	N.D	N.D
CHD9: Chromodomain-helicase-DNA-binding protein 9 PRIC320: PPAR-alpha-interacting complex protein 320 kDa	CHD9_1023_1045	N.D	N.D	N.D	93
	CHD9_2018_2040	19	21	N.D	N.D
	CHD9_855_877	N.D	N.D	N.D	N.D
CNOT1: CCR4-NOT transcription complex subunit 1	CNOT1_140_162	N.D	N.D	N.D	30
	CNOT1_1626_1648	21	17	N.D	11
	CNOT1_1929_1951_C1932S	N.D	N.D	15	10
	CNOT1_2083_2105	47	23	N.D	N.D
	CNOT1_2086_2108	50	35	N.D	N.D
	CNOT1_557_579	N.D	N.D	N.D	16
DDX5: Probable ATP-dependent RNA helicase	DDX5_133_155	26	18	N.D	N.D
DHX30: Putative ATP-dependent RNA helicase	DHX30_241_262	N.D	N.D	N.D	N.D
	DHX30_49_70	N.D	N.D	N.D	N.D
EP300: Histone acetyltransferase p300	EP300_2039_2061	22	18	N.D	8
	EP300_69_91	16	16	17	11
GELS: Gelsolin precursor	GELS_376_398	N.D	N.D	N.D	N.D
GNAQ: Guanine nucleotide-binding protein G(q) subunit alpha. G protein alpha Q (Gaq).	GNAQ_21_43	37	15	N.D	N.D
Hair protein	HAIR_553_575_C567S	N.D	N.D	N.D	12
	HAIR_745_767_C755S/C759S	18	9	21	12

IKBB :NF-kappa-B inhibitor beta	IKBB_244_266	N.D	N.D	N.D	16
TRIP9 :Thyroid receptor-interacting protein 9	IKBB_277_299	20	10	N.D	N.D
	IKBB_62_84	N.D	N.D	N.D	N.D
ILK : Integrin-linked protein kinase	ILK_131_153	18	N.D	36	11
JHD2C : Jumonji domain-containing protein 1C (JMJD1C)	JHD2C_2054_2076	N.D	6	14	12
TRIP8 : Thyroidreceptor-interacting protein 8					
KIF11 : Kinesin-like protein KIF11	KIF11_832_854_C854S	20	18	N.D	N.D
TRIP5 :Thyroid receptor-interacting protein 5					
L3R2A : Loss of heterozygosity 3 chromosomal region 2 gene A protein.	L3R2A_12_34	N.D	N.D	N.D	N.D
LCOR : Ligand-dependent corepressor (LCoR)	LCOR_40_62	19	9	16	10
MAPE : Melanoma antigen preferentially expressed in tumors PRAME : Preferentially expressed antigen of melanoma	MAPE_249_271	22	11	N.D	N.D
	MAPE_300_322	N.D	N.D	N.D	N.D
	MAPE_356_378	23	19	N.D	N.D
	MAPE_382_404_C388S	N.D	N.D	N.D	N.D
	MAPE_454_476_C472S	N.D	N.D	N.D	N.D
	MAPE_91_113	N.D	23	15	12
MED1 : Mediator of RNA polymerase II transcription subunit 1	MED1_591_614	18	8	14	14
PBP : Peroxisome proliferator-activated receptor-binding protein	MED1_632_655	17	8	18	17
TRAP220 :Thyroid hormone receptor-associated protein complex 220kDa component					
MEN : Menin	MEN1_255_277	N.D	N.D	N.D	N.D
MGMT : Methylated-DNA--protein-cysteine methyltransferase	MGMT_86_108	N.D	N.D	N.D	N.D
MLL2 : Myeloid/lymphoid or mixed-lineage leukemia protein 2.	MLL2_4175_4197	20	9	N.D	93
	MLL2_4702_4724	N.D	N.D	N.D	N.D
MTA1S : Metastasis-associated protein MTA1.	MTA1S_388_410_C393S/C396S	18	N.D	N.D	N.D
NCOA1 : Nuclear receptor coactivator 1	NCOA1_1421_1441	18	4	27	N.D
SRC-1 : Steroid receptor coactivator 1	NCOA1_620_643	20	17	17	9
	NCOA1_677_700	17	8	19	23
	NCOA1_737_759	16	15	N.D	20
NCOA2 : Nuclear Receptor Coactivator 2	NCOA2_628_651	18	10	14	14
TIF2 : Transcriptional intermediary factor 2	NCOA2_677_700	17	10	19	18
	NCOA2_733_755	17	9	N.D	N.D
	NCOA2_866_888	39	24	N.D	N.D
NCOA3 : Nuclear receptor coactivator 3	NCOA3_104_123_N-KKK	14	N.D	11	10
AIB-1 : Amplified in breast cancer 1 protein	NCOA3_609_631	19	16	15	10
TRAM-1 : Thyroid receptor activator molecule 1	NCOA3_609_631_C627S	19	11	15	11
	NCOA3_673_695	17	10	N.D	27
	NCOA3_725_747	18	10	N.D	N.D

	NCOA3_MOUSE_1029_1051	22	16	N.D	N.D
NCOA4: Nuclear receptor coactivator 4	NCOA4_315_337	11	16	N.D	N.D
	NCOA4_79_101_C101S	N.D	14	N.D	N.D
NCOA6: Nuclear receptor coactivator 6	NCOA6_1479_1501	18	9	13	10
ASC2: Activating signal co-integrator 2	NCOA6_875_897	18	11	N.D	25
TRBP: Thyroid hormone receptor-binding protein					
NCOR1: Nuclear receptor corepressor 1	NCOR1_1925_1946	N.D	N.D	N.D	24
	NCOR1_2039_2061	N.D	13	38	13
	NCOR1_2039_2061_C2056S	N.D	20	20	15
	NCOR1_2251_2273	17	17	N.D	30
	NCOR1_2376_2398	N.D	21	N.D	N.D
	NCOR1_662_684_C662S	N.D	N.D	N.D	N.D
NCOR2: Nuclear receptor corepressor 2	NCOR2_2123_2145	15	16	20	13
SMRT: Silencing mediator of retinoic acid and thyroid hormone receptor	NCOR2_2330_2352	17	17	N.D	N.D
	NCOR2_649_671_C649S	N.D	N.D	N.D	N.D
NELFB: Negative elongation factor B	NELFB_328_350	26	7	N.D	21
COBRA1: Cofactor of BRCA1	NELFB_428_450	23	18	N.D	N.D
	NELFB_80_102	18	N.D	N.D	N.D
NR0B1: Nuclear receptor subfamily 0 group B member 1	NR0B1_136_159	17	7	21	41
	NR0B1_1_23	N.D	N.D	N.D	N.D
	NR0B1_68_90_C69S	18	10	N.D	18
NR0B2: Nuclear receptor subfamily 0 group B member 2	NR0B2_106_128	20	9	18	10
	NR0B2_201_223_C207S	19	18	N.D	8
	NR0B2_237_257	29	20	N.D	8
SHP: Small/Short heterodimer partner	NR0B2_9_31_C9S/C11S	19	11	N.D	32
NRBF2: Nuclear receptor-binding factor 2	NRBF2_128_150	20	17	N.D	12
NRIP1: Nuclear receptor-interacting protein1	NRIP1_1055_1077	18	8	15	N.D
RIP140: Receptor-interacting protein 140	NRIP1_120_142	18	16	15	15
	NRIP1_121_143_P124R	18	15	N.D	15
	NRIP1_173_195	20	16	N.D	N.D
	NRIP1_173_195_C177S	19	17	N.D	N.D
	NRIP1_253_275_C263S	18	10	28	10
	NRIP1_368_390	22	7	N.D	N.D
	NRIP1_488_510	19	9	19	28
	NRIP1_700_722	20	15	N.D	10
	NRIP1_701_723	19	16	13	15
	NRIP1_805_831	20	12	N.D	36106
	NRIP1_8_30	N.D	N.D	N.D	N.D
	NRIP1_924_946	21	14	N.D	N.D
	NRIP1_924_946_C945S	19	9	N.D	N.D
NSD1: Nuclear receptor-binding SET domain-containing protein 1	NSD1_894_916	19	16	N.D	21
	NSD1_982_1004	20	30	N.D	N.D

PAK6: Serine/threonine-protein kinase PAK6	PAK6_248_270	N.D	N.D	N.D	N.D
PCAF: Histone acetyltransferase PCAF	PCAF_178_200	N.D	N.D	N.D	9
PELP1: Proline-, glutamic acid- and leucine-rich protein 1 MNAR: Modulator of non-genomic activity of estrogen receptor	PELP1_142_164	N.D	16	9	8
	PELP1_168_190	20	17	N.D	18
	PELP1_20_42	18	N.D	N.D	N.D
	PELP1_251_273	N.D	N.D	N.D	26
	PELP1_258_280	N.D	N.D	N.D	N.D
	PELP1_446_468	19	16	N.D	14
	PELP1_496_518_C496S	19	N.D	N.D	N.D
	PELP1_56_78_C71S	19	13	N.D	17
	PELP1_571_593_C575S/C581S	26	22	N.D	18
PIAS2: Protein inhibitor of activated STAT2	PIAS2_6_28	20	N.D	N.D	N.D
PNRC1: Proline-rich nuclear receptor coactivator 1	PNRC1_306_327	N.D	17	N.D	N.D
PNRC2: Proline-rich nuclear receptor coactivator 2	PNRC2_118_139	N.D	N.D	N.D	N.D
PPRC1: Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	PPRC1_1159_1181	20	15	N.D	N.D
	PPRC1_151_173	18	16	22	14
PR285: Peroxisomal proliferator-activated receptor A-interacting complex 285kDa protein PRIC285: PPAR-alpha-interacting complex protein 285 HELZ2 - helicase with zinc finger 2, transcriptional coactivator	PR285_1062_1084	21	18	N.D	18
	PR285_1105_1127	21	16	N.D	N.D
	PR285_1160_1182_C1163S	20	N.D	N.D	16
	PR285_2216_2238_C2219S	19	17	N.D	8
	PR285_432_454_C453S/C454S	35	20	N.D	N.D
PRDM2: PR domain zinc finger protein 2	PRDM2_948_970	N.D	25	N.D	N.D
PRGC1 (PPARGC1 A): Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PRGC1_130_155	19	5	19	25
	PRGC1_134_154	18	4	22	24
PRGC2 (PPARGC1 B): Peroxisome proliferator-activated receptor gamma coactivator 1-beta	PRGC2_146_166	19	N.D	7	14
PERC: PGC-1-related estrogen receptor alpha coactivator	PRGC2_338_358	21	16	11	11
PRGR: Progesterone receptor (PR)	PRGR_102_124	22	19	N.D	9
	PRGR_42_64_C64S	21	20	N.D	N.D
PROX1: Homeobox prospero-like protein	PROX1_57_79	19	13	N.D	3079
RAD9: Cell cycle checkpoint control protein	RAD9A_348_370	33	15	N.D	N.D
RBL2: Retinoblastoma-like protein 2	RBL2_875_897_C879S/C894S	N.D	N.D	N.D	9
TF65: Transcription factor p65 NFKB3: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 3	TF65_437_459	N.D	N.D	N.D	19
TGFH1 (TGFB11I): Transforming growth factor beta-1-induced transcript 1 protein ARA55: Androgen receptor coactivator 55 kDa protein Hic-5: Hydrogen peroxide-inducible clone 5 protein	TGFH1_325_347_C334S/C346S	N.D	N.D	N.D	N.D
	TGFH1_443_461_C452S/C455S	32	32	N.D	N.D

TIF1A : Transcription intermediary factor 1-alpha	TIF1A_373_395_C394S	35	16	N.D	N.D
TRIM24 : tripartite motif containing 24	TIF1A_747_769	20	7	18	19
TIP60 : Histone acetyltransferase HTATIP	TIP60_476_498	23	17	N.D	17
KAT5 : Histone acetyltransferase KAT5					
TREF1 : Transcriptional-regulating factor 1	TREF1_168_190	19	14	24	11
TREP-132 : Transcriptional-regulating protein 132	TREF1_850_872	11	18	N.D	N.D
TRIP4 : Thyroid receptor-interacting protein 4	TRIP4_149_171_C171S	27	23	N.D	N.D
ASC-1 : Activating signal co-integrator 1					
TRRAP : Transformation/transcription domain-associated protein	TRRAP_3535_3557_C3535S/C3555S	22	18	N.D	9
	TRRAP_770_792	20	18	N.D	N.D
	TRRAP_971_993	14	17	N.D	17
TRXR1 : Thioredoxin reductase 1, cytoplasmic	TRXR1_132_154	21	17	N.D	N.D
TXNRD1 : Thioredoxin reductase TR1					
UBE3A : Ubiquitin-protein ligase E3A	UBE3A_396_418	18	14	11	12
E6-AP : E6-associated protein	UBE3A_649_671	18	15	14	12
WIP1 : WD repeat domain phosphoinositide-interacting protein 1	WIP1_119_141	19	17	7	11
	WIP1_313_335_C318S	25	18	N.D	N.D
ZNH13 : Zinc finger HIT domain-containing protein 3	ZNH13_89_111	N.D	N.D	96	N.D
TRIP3 : Thyroid receptor-interacting protein 3					
ZNT9 : Zinc transporter 9 (GAC-63)	rZNT9_449_471	22	19	N.D	8
SLC30A9 : Solute carrier family 30 member 9					

Chapter 3

Characterization of the differential coregulator binding signatures of the Retinoic Acid Receptor subtypes upon (ant)agonist action

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Abstract

Retinoic Acid Receptor alpha (RAR α /NR1B1), Retinoic Acid Receptor beta (RAR β /NR1B2) and Retinoic Acid Receptor gamma (RAR γ /NR1B3) are transcription factors regulating gene expression in response to retinoids. Within the RAR genomic pathways, binding of RARs to coregulators is a key intermediate regulatory phase. However, ligand-dependent interactions between the wide variety of coregulators that may be present in a cell and the different RAR subtypes are largely unknown. The aim of this study is to characterize the coregulator binding profiles of RARs in the presence of the pan-agonist *all-trans*-Retinoic Acid (AtRA); the subtype-selective agonists Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ); and the antagonist Ro415253. To this end, we used a microarray assay for coregulator-nuclear receptor interactions to assess RAR binding to 154 motifs belonging to more than 60 coregulators. The results revealed a high number of ligand-dependent RAR-coregulator interactions among all RAR variants, including many binding events not yet described in literature. Next, this work confirmed a greater ligand-independent activity of RAR β compared to the other RAR subtypes based on both higher basal and lower ligand-driven coregulator binding. Further, several coregulator motifs showed selective binding to a specific RAR subtype. Next, this work showed that subtype-selective agonists can be successfully discriminated by using coregulator binding assays. Finally, this study demonstrated the possible applications of a coregulator binding assay as a tool to discriminate between agonistic/antagonistic actions of ligands. The RAR-coregulator interactions found will be of use to direct further studies to better understand the mechanisms driving the eventual actions of retinoids.

1 Introduction

Retinoic Acid Receptors (RARs) are transcription factors regulating gene expression in response to ligands known as retinoids. There are three different RAR subtypes, namely, Retinoic Acid Receptor alpha (RAR α /NR1B1), Retinoic Acid Receptor beta (RAR β /NR1B2) and Retinoic Acid Receptor gamma (RAR γ /NR1B3), which have been described to play important roles from both a therapeutic (e.g. breast cancer treatment) as well as a toxicological (e.g. liver toxicity) perspective [1]. In order to modulate gene expression, RARs form heterodimers with Retinoid X Receptors (RXRs) and bind to specific DNA regions called Retinoic Acid Responsive Elements (RAREs) and to different coregulators [2]. Within the genome, the DNA binding regions of the RAR subtypes have been shown to highly overlap suggesting functional redundancy [3]. However, despite their structural and mechanistic similarity, the respective RAR subtypes have been shown to often play different and even opposite roles in both physiological and pathological cellular contexts [4].

Within the RAR genomic pathways, binding of RARs to coregulators (coactivators or corepressors) is a key intermediate regulatory step since these coregulators modulate chromatin structure and accessibility for transcription [5]. Coregulators interacting with RARs can be divided in three major categories, being apo-form-bound corepressors, ligand-induced binding coactivators, or ligand-induced binding corepressors [1] and it has been shown that distinct cellular levels of some of these coregulators (e.g. MAPE) may lead to different biological responses [6]. In addition, previous studies suggested that the specific effects of the different RAR subtypes may arise from a differential capacity to recruit coregulators to promoter regions [7]. Thus, in order to better understand retinoid-induced biological effects, deeper knowledge on the specific ligand-dependent RAR-coregulator interactions is required [8–11].

In order to study retinoid-driven modulation of RAR-coregulator interactions, several methods such as GST-pull down assays, or yeast two hybrid assays have been used [12]. However, most of these

assays only allow to study one specific coregulator while in a biological scenario, many different coregulator proteins could participate in this interaction. Consequently, only a limited number of coactivators and corepressors have been studied so far. The interactive region of coregulators is composed by leucine-rich motifs called Nuclear Receptor boxes (NR-boxes) [5] which are considered to be necessary and sufficient for the interactions with NRs. Coactivators possess one or several consensus sequences LXXLL while corepressors contain LXXI/HIXXXI/L sequences [8] where L is Leucine, I is Isoleucine, H is Histidine and X represents any amino acid. Previous studies have shown differences between the functionality of NR-boxes [13,14] and that residues immediately adjacent to these canonical sequences can play an important role in the affinity and specificity of NR-coregulator interactions [15,16]. Therefore, technologies assessing NR binding to coregulator peptides containing such sequences could add initial insights to test whether these differences may also occur among the different RAR subtypes when activated by a variety of different ligands. The Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction makes use of this concept in a high-throughput manner offering the possibility to rapidly and simultaneously assess ligand-modulated binding of NRs to 154 motifs derived from more than 60 different coregulators. Thus, by using this technique, broad coregulator binding profiles can be obtained.

The present study aims at characterizing and comparing the coregulator binding profiles of RAR α , RAR β and RAR γ in the presence of both generic and subtype-selective agonists in order to get new insights in the possible role of specific coregulators in the eventual action of RAR subtypes. In addition, the coregulator binding signature generated by the RAR antagonist Ro415253 was also characterized. For this purpose, retinoid-driven coregulator motif interactions with purified recombinant ligand binding domains (LBDs) of the respective RAR subtypes were assessed.

2 Materials and Methods

2.1 Chemicals

All-*trans*-Retinoic acid (AtRA) was purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl] benzoic acid (Am80) and 4-[(E)-2-(7-Heptoxy-4,4-dimethyl-1,1-dioxo-2,3-dihydrothiochromen-6-yl)prop-1-enyl]benzoic acid (Ro415253) were obtained from Abcam (Cambridge, UK). 5-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-3-thiophenecarboxylic acid (CD2314) and 3-fluoro-4-[[2-hydroxy-2-(5,5,8,8-tetramethyl-5,6,7,8,-tetrahydro-2-naphthalenyl)acetyl]amino]-benzoic acid (BMS961) were purchased from Tocris Bioscience (Bristol, UK).

2.2 MARCoNI Coregulator Binding Assay

In the present study, the Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) was used in order to characterize and compare the different coregulator interactions of all RAR subtypes in the presence of the agonists AtRA, Am80, CD2314 and BMS961, and the antagonist Ro415253 at concentrations ranging from 0.1 nM to 200 μ M. All samples contained a final concentration of DMSO of 2% which was also used as a solvent control. Assay mixtures containing the respective RAR, the ligand of interest and the detecting antibody, were prepared in time-resolved fluorescence resonance energy transfer (TR-FRET) reaction buffer D (Life technologies, Ltd, UK, #PV4420) supplemented with 5 mM dithiothreitol (DTT) (Sigma, Zwijndrecht, the Netherlands, #43819) and kept on ice before starting the assay. Glutathione S-transferase (GST)-tagged RAR α -LBD (RAR α -LBD^{GST}, amino acids 176-462, Life technologies, Ltd, UK, #PV4415), glutathione S-transferase (GST)-tagged RAR β -LBD (RAR β -LBD^{GST}, amino acids 168-448, Life technologies, Ltd, UK, #PV4716) or glutathione S-transferase (GST)-tagged RAR γ -LBD (RAR γ -LBD^{GST}, amino acids 178-454, Life technologies, Ltd, UK, #PV4413) were used for the experiments at a final concentration of 10 nM. For all the subtypes, 25 nM of Alexa 488–conjugated anti-GST (Invitrogen, Breda, the

Netherlands, #A11131) was utilized as detecting antibody. Each ligand concentration, was tested in singular (unless otherwise stated) by using PamChip® plates (PamGene International B.V., 's-Hertogenbosch, the Netherlands) containing 4 or 96 peptide microarrays. Microarrays consist of a three-dimensional porous metal oxide carrier containing 154 spots with different immobilized NR-binding motifs belonging to 65 different coregulatory proteins. The automated platform PamStation® (PamGene International B.V., 's-Hertogenbosch, The Netherlands) and EvolveHT software (PamGene International B.V., 's-Hertogenbosch, The Netherlands) were used applying 2 (up-down) pumping cycles per minute at 20°C. Prior to the addition of the samples to the Pamchip® arrays, incubation for 20 cycles with 25 µl blocking buffer composed of Tris buffered saline (Bio-Rad, Veenendaal, the Netherlands, #170-6435) supplemented with 1% BSA (Calbiochem, Merck, Darmstadt, Germany, #126609) and 0.01% Tween-20 (Bio-Rad, Veenendaal, the Netherlands, #170-6531) was carried out in order to avoid unspecific binding. Immediately after blocking, samples were added to the Pamchip® arrays and incubated for 80 pumping cycles. As last step, unbound receptor was washed away with 25 µl Tris buffered saline and TIFF images were taken by a charge coupled device camera (CCD) installed in the PamStation®.

2.3 MARCoNI Data Analysis

Image analysis was performed using the software Bionavigator (Version 6.1, PamGene International B.V., 's-Hertogenbosch, The Netherlands). Basal and agonist-driven binding of the different RAR subtypes to the different coregulator peptides was assessed by quantifying the fluorescence signals. Shortly, boundaries for every spot (peptide) were determined, and fluorescence binding Arbitrary Units (AU) were quantified by subtracting the background signal from the median fluorescent signal. Concentration-response curve fitting was carried out utilizing the DRC package in R (version 2.15.3, www.r-project.org) by means of a sigmoidal, 4-parameter Hill (logistic) model ($response = ((A - D)/(1 + ((concentration/C)^B))) + D$, with parameters A = response minimum, B = Hill slope, C = EC_{50} , and D = response maximum). Unless otherwise stated, coregulators presenting complete

concentration-response curves with goodness of fitting (R^2) higher than 0.8 (cut-off value) were considered as ligand-dependent for analysis and discussion. Throughout this work, results were also expressed as a percentage of the solvent control or as Modulation Index (MI), the latter representing the log10 transformed ratio of the binding in the presence of the ligand over the binding in the solvent control. In order to evaluate whether the different RAR-coregulator interactions observed in this work have been previously described in the literature, the databases STRING v9.1 (<http://string-db.org/>) and BioGRID^{3,4} (<http://thebiogrid.org/>) were used

2.4 Pull Down assay

Ligand-dependent binding of RAR α to the full length coregulator DDX5 was evaluated by adapting and using the Pierce GST Protein Interaction Pull-Down kit (ThermoFisher Scientific, Rockford Illinois, USA). All protein mixtures and washing buffers except the elution buffer were prepared using time-resolved fluorescence resonance energy transfer (TR-FRET) reaction buffer D (Life technologies, Ltd, UK, #PV4420) supplemented with 5 mM dithiothreitol (DTT) (Sigma, Zwijndrecht, the Netherlands, #43819). Briefly, 500 ng of RAR α -LBD (RAR α -LBD^{GST}, amino acids 176-462, Life technologies, Ltd, UK, #PV4415) were incubated for 1 hour in the specific spin columns. After washing, 4000 ng of purified DDX5 (OriGene, Maryland, USA) and either DMSO or AtRA were added to the spin columns and incubated for two hours. After the respective washing steps, recovery of both proteins was carried out by adding elution buffer consisting of Tris Buffered Saline supplemented with glutathione (30 mM). Samples were prepared in Laemmli buffer (Bio-Rad, Veenendaal, The Netherlands) supplemented with dithiothreitol (DTT) (50 mM) (Sigma, Zwijndrecht, the Netherlands). After denaturation at 90°C for 5 minutes, samples were loaded in 10% Mini-PROTEAN TGX TM Precast Protein Gels (Bio-Rad, Veenendaal, the Netherlands). Electrophoresis was run at 200 V for 40 minutes in Tris/Glycine/SDS Buffer (Bio-Rad, Veenendaal, the Netherlands). Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Veenendaal, the Netherlands) in

Tris/Glycine Buffer containing 20% methanol (Biosolve, Valkenwaard, the Netherlands). Subsequently, the membrane was blocked for 1 hour with Tris Buffered Saline (Bio-Rad, Veenendaal, the Netherlands) supplemented with Tween 20 (0.1%) (Bio-Rad, Veenendaal, the Netherlands) and Blotting Grade Blocker (50 g/l) (Bio-Rad, Veenendaal, the Netherlands) to avoid unspecific binding. Next, the membrane was probed with the respective antibodies which were diluted in Tris Buffered Saline supplemented with Tween 20 (0.1%) and Blotting Grade Blocker (50 g/l) according to the manufacturer instructions. First, membranes were incubated with the primary antibodies against RAR α (sc-551) (Santa Cruz Biotechnology, CA, USA) or against DDX5 (ab-126730) (Abcam, Cambridge, UK). Membranes were washed with Tris Buffered Saline supplemented with Tween 20 (0.1%) and subsequently were incubated with the secondary antibody goat-anti-rabbit IgG-HRP (sc-2004) (Santa Cruz Biotechnology, CA, USA). After final washing, Clarity Western ECL Substrate (Bio-Rad, Veenendaal, the Netherlands) was used for chemiluminescent detection. Semi-quantitative determination of RAR α and DDX5 levels was performed by using Image Studio Lite (Li-COR) and ImageJ (National Institutes of Health; Bethesda, MD) softwares. Protein levels of DDX5 were first corrected by the amounts of RAR α protein eluted and then represented as a percentage of the solvent control (DMSO).

3 Results

3.1 Characterization of the basal coregulator binding profiles of the RAR subtypes

The present work first assessed the coregulator binding of all RAR subtypes in the absence of ligand in order to detect possible different outcomes among the respective apo-form proteins. Interestingly, basal coregulator binding to the different RARs varied among the motifs present in the array (Supplementary Figure A). Figure 1A illustrates this disparity by showing some examples of coactivator and corepressor motifs where the basal binding of the RAR variants clearly differed. As shown in these examples, the basal binding of the different RAR subtypes was remarkably different

depending on the specific coregulator motif. In addition, the global analysis of all peptides showed that in a relatively high proportion of coregulator motifs (56%), RAR β presented the highest basal binding (Figure 1B). Altogether the results obtained for coregulator interactions in the absence of ligands suggest that the LBDs of the respective RAR subtypes present differential basal coregulator binding profiles.

Figure 1. Basal coregulator motif binding to the different RAR subtypes (RAR α in red, RAR β in blue and RAR γ in green). Examples of specific peptides showing differential binding (AU) to the different subtypes are depicted (A). The proportion (percentage) of coregulator motifs where each subtype was shown to have the highest binding values are additionally shown (B). The respective data for all coregulator motifs are shown in Supplementary Figure A.1-A.3.

3.2 Analysis and comparison of the AtRA-driven coregulator binding profiles of RAR subtypes

In a next step, the present study characterized the coregulator motif binding profiles of RAR α , RAR β and RAR γ upon incubation with the pan-agonist AtRA. Figure 2A shows an overview of the different AtRA-induced RAR-coregulator interactions showing all the concentration-response curves with goodness of fitting (R^2) higher than 0.8. Individual concentration-response curves for the AtRA-driven interactions of the RARs with all motifs present on the array (including all data points underlying the curves) are depicted in Supplementary Figures B.1-B.3. In addition, Supplementary Table A presents an overview of all EC50 values as derived from the concentration-response curves with R^2 higher than 0.8. As shown in Figure 2A, all RAR subtypes showed AtRA-dependent binding to a high number of coregulator peptides. Thus, the number of motifs showing complete concentration-response curves ($R^2 > 0.8$) was 126 for RAR α , 102 for RAR β and 108 for RAR γ . As examples, a more detailed view of specific motifs belonging to the apo-form-bound corepressor NCOR2 (NCOR2_2123_2145), the ligand-induced coactivator NCOA1 (NCOA1_677_700), or the ligand-induced corepressor NRIP1 (NRIP1_488_510) is depicted in figures 2B, 2C and 2D, respectively. NCOR2_2123_2145 showed for all RAR subtypes a decrease in the initial basal binding with increasing concentrations of AtRA. On the other hand, NCOA1_677_700 and NRIP1_488_510 showed increased binding as the concentrations of AtRA increased.

Figure 2. Overview of the binding of RAR α -LBD (red), RAR β -LBD (blue) and RAR γ -LBD (green) to coregulator motifs in the presence of the pan-agonist AtRA (A). Individual concentration-response curves for all motifs present on the array (including all data points underlying the curves) are depicted in Supplementary Figures B.1-B.3. AtRA-modulated RAR α binding to the motifs NCOR2_2123_2145 (B), NCOA1_677_700 (C) and NRIP1_488_510 (D) is shown in detail. Concentration-response curves were generated if goodness of fit (R^2) was higher than 0.8. The X-axis represents increasing concentrations of AtRA, while the Y-axis represents the binding in Arbitrary Units (AU).

After revealing that all three RAR subtypes show AtRA-dependent interactions with a remarkably high number of coregulator motifs, a literature search was performed to assess and compare the ligand-dependent RAR-coregulator binding events with previous research. Figure 3A presents all coregulators showing at least one AtRA-dependent coregulator motif interaction ($R^2 > 0.8$) with RAR α -LBD,

RAR β -LBD and/or RAR γ -LBD distinguishing coregulators previously described in literature from those never reported before. A detailed view of this comparison together with the literature references used for this purpose and for classification of the proteins according to their main function is shown in the Supplementary Table B. Our results successfully reproduced RAR interactions previously observed by using different methodologies. In addition, our study revealed several unknown RAR interactors. In fact, a considerably high number of motifs belonging to so far unstudied coregulators were found to bind to RARs in an AtRA concentration-dependent manner. Finally, as a representative example to confirm that interactions of RARs with the coregulator motifs in MARCoNI can predict the binding with the full length protein, the newly found ligand-dependent binding of DDX5 to RAR α was evaluated by the Pull Down technique showing an AtRA-dependent increase in RAR α -DDX5 binding (Figure 3B).

Figure 3. Coregulators presenting AtRA-driven interactions ($R^2>0.8$) between at least one of their motifs and RAR α -LBD, RAR β -LBD or RAR γ -LBD (A). Coactivators (grey), corepressors (brown) and proteins with important functions apart from coregulatory (blue), are depicted. Proteins previously described to bind to the respective RARs are represented by dark colour while newly described RAR interactors are depicted in light colour. Empty spaces (white) express coregulator motifs showing weaker or no concentration-related binding to the respective RAR ($R^2<0.8$). In addition, confirmation by the pull down assay of the newly found AtRA-driven (0.25 mM) binding of RAR α to the full length DDX5 is also provided (B).

To determine whether the RAR subtypes may differentially respond to AtRA regarding coregulator binding, modulation indexes (see section 2.3 data analysis) and concentration-response curves were further evaluated (Figure 4). Figures 4A-B show the MIs (signature/heat map) obtained after incubation with the highest AtRA concentration tested (logM = -5.3). As shown, MI profiles were highly similar for all RARs mostly showing subtype-specific differences in terms of only magnitude of the effect. Whereas RAR γ often displayed higher MIs, RAR β generally showed to be the less responsive subtype at saturating concentrations of AtRA. When assessing complete concentration-response curves ($R^2>0.8$), all RARs displayed highly coincident ligand-dependent coregulator interactions with only a relatively low number of subtype-specific coregulator motifs presenting differential AtRA-induced binding patterns among the RAR variants. In fact, 87 coregulator motifs presented full concentration-response curves for all three RAR subtypes (Figure 4C).

Figure 4. Comparison of coregulator binding of RAR α , RAR β and RAR γ upon incubation with the pan-agonist AtRA. Results are expressed as modulation indexes (MIs) at saturating concentrations of ligand (A). MIs found are also represented based on hierarchical clustering (Euclidean distance) by a heat map (B). In addition, results are also expressed as a Venn diagram indicating the number of coregulator motifs showing complete concentration-response curves with R^2 higher than 0.8 for the different RAR subtypes (C).

As shown in figure 4, despite the fact that most of the coregulator motifs showed resembling binding patterns, some exceptions were found where ligand-driven RAR-coregulator interactions were pronouncedly different between the three subtypes. Figure 5 depicts several cases where concentration-response curves suggest the existence of subtype-specific coregulator motifs (see also Supplementary Table A). Figures 5A-D show some representative examples of the relatively higher number of coregulator motifs specifically binding to RAR α upon incubation with AtRA such as CBP_345_368, DDX5_133_155, MEN1_255_277, and NELFB_328_350. Coregulator binding specific for RAR β and RAR γ in the presence of AtRA was less evident. Figure 5E shows an example of a coregulator motif, NCOR1_2039_2061_C2056S, considered relatively specific in its AtRA-modulated binding to RAR β . Figure 5F shows an example of a coregulator motif, ZNHI3_89_111, mostly responsive to RAR γ , which also shows a concentration-related binding to RAR α albeit at a much lower intensity.

Figure 5. Coregulator motifs presenting differential binding to RAR subtypes upon incubation with AtRA. Concentration-response data points and fitted curves ($R^2 > 0.8$) belonging to subtype-specific motifs are shown. AtRA-driven binding for RAR α (red colour), RAR β (blue colour) and RAR γ (green colour) is expressed as percentage of the solvent control.

Overall, all RAR subtypes presented AtRA-dependent interactions with a remarkably high number of coregulator motifs and in many cases these interactions had not been studied before. Further, although this ligand-dependent coregulator binding was highly similar for all RAR subtypes, some coregulator motifs showed potential specificity/selectivity for a certain RAR-subtype, especially for RAR α .

3.3 Prediction of subtype-selectivity of ligands based on coregulator binding profiles

After characterizing and comparing ligand-dependent coregulator interactions with the different RAR variants upon incubation with the endogenous pan-agonist AtRA, we tested the possibility to directly discriminate subtype-selective ligands based on their RAR-coregulator interaction patterns. To do so, we evaluated the coregulator binding profiles induced by Am80, CD2314 and BMS961 which are known to be respectively RAR α -, RAR β - and RAR γ -selective agonists. Individual concentration-response curves depicting interactions of the RARs with all motifs present on the array (including all data points underlying the curves) are presented in Supplementary Figures C1-C3 (Am80), D1-D3 (CD2314) and E1-E3 (BMS961). In addition, Supplementary Table A presents an overview of the EC50 values for all coregulator peptides as derived from the concentration-response curves with R^2 higher than 0.8. Figure 6 describes the differential coregulator binding patterns generated by the distinct compounds on the different RAR subtypes in terms of EC50 distributions and the number of coregulators presenting complete concentration-response curves ($R^2 > 0.8$). Median EC50s were compared to previously described parameters (K_d and EC50s) obtained with different methodologies [17,18] (values between brackets in the table presented in Figure 6). As expected for the pan-agonist AtRA (Figure 6A and 6B), similar EC50s were generally found all along the different coregulator motifs, although in contrast to literature, slightly higher for the RAR γ subtype. Next, it was revealed that coregulator binding profiles were capable of predicting subtype-selectivity of Am80 (RAR α -selective agonist) and Bms961 (RAR γ -selective agonist) as reflected by the EC50 distributions (Figure 6C and 6G). Thus, these ligands generated remarkably lower EC50s for the subtype for which they are known to be selective [17,18]. However, for the RAR β -selective ligand CD2314, the selectivity was

less pronounced. This compound appeared to be a weak RAR β -selective agonist resulting in much closer EC50 distributions (Figure 6E) for all RAR subtypes in a similar way to those displayed by the pan-agonist AtRA. Besides the EC50 values, we also analysed the number of coregulators showing complete concentration-response curves ($R^2 > 0.8$) in more detail. As expected, Am80 and BMS961 generated on their preferential targets RAR α and RAR γ , respectively, a higher number of coregulators presenting full concentration-response curves (Figure 6D and 6H). This result is related to the fact that EC50 values were low enough to result in full concentration-response curves in the concentration range tested. Interestingly, CD2314 (Figure 6F) displayed a strikingly high number of coregulator motifs presenting complete concentration-response curves for RAR α while activation of this RAR subtype by CD2314 has not been described in literature [17,18].

Figure 6. Distribution of EC50 values obtained from concentration-response curves of RAR binding to the coregulator motifs in the presence of AtRA (A), Am80 (C), CD2314 (E) and BMS961 (G). Results are shown as box and whisker plots (whiskers: 10-90 percentiles) of EC50s obtained from those coregulator motifs showing concentration-response curves with R^2 higher than 0.8. A table specifying the 25P, Median and 75P obtained and data from literature (between brackets) is also provided. In addition, Venn diagrams representing the number of coregulator motifs presenting complete concentration-response curves ($R^2 > 0.8$) on the different RARs in the presence of AtRA (B), Am80 (D), CD2314 (F) and BMS961 (H) are depicted. NB = No Binding; NA = No Activation; ND = Not Determined.

In order to get further insights in possible additional factors explaining subtype-selectivity of the tested ligands, we assessed every specific coregulator motif concentration-response curve generated by the tested ligands on the different RAR subtypes (Supplementary figures B-E). Although at high concentrations, as shown by MIs (Supplementary Figure F and G), all compounds presented a high similarity in the coregulator peptides that bind each receptor, some clear differences were observed. For example, it was found that CD2314 elicited a relatively higher decrease of the initial binding of RAR β to NCOR1 and NCOR2 in comparison to what was observed for the other RAR subtypes. Next, we compared motifs belonging to NCOR1 and NCOR2 in the presence of both CD2314 and the pan-agonist AtRA finding an interesting difference (Figure 7). Unlike the similar dissociation for all subtypes caused by AtRA, CD2314 showed to more efficiently disrupt the basal binding of NCOR1_2251_2273 (Figure 7A) and NCOR2_2330_2352 (Figure 7B) to RAR β . Thus, CD2314 displayed a lower ability to dissociate these coregulator motifs from RAR α or RAR γ than from RAR β which may play a role in the reported RAR β -selectivity of CD2314.

Figure 7. Concentration-response curves of two RAR apo-form-bound corepressor motifs, namely, NCOR1_2251_2273 (A) and NCOR2_2330_2352 (B), which present differential binding trends between RAR subtypes upon incubation with AtRA and CD2314. Binding for RAR α (red colour), RAR β (blue colour) and RAR γ (green colour) is expressed as percentage of the solvent control.

Altogether, based on median EC50 values, our study successfully predicted subtype-selectivity of Am80 and BMS961, and to a lesser extent CD2314. In addition, despite that our work showed high similarity in the coregulator binding profiles induced by both general and selective ligands, the global microarray coregulator binding analysis showed to detect various subtle differences such as that observed for CD2314 on apo-form-bound corepressor motifs.

3.4 Prediction of antagonistic actions of ligands based on coregulator binding profiles

Once shown that the coregulator binding profiles can successfully recognize general and subtype-selective agonists of RARs, the present study additionally tested whether this methodology can discriminate potential antagonistic actions of a specific ligand. For this purpose, the coregulator binding signatures generated by the antagonist Ro415253 (10^{-4} M) were evaluated (Figure 8). As clearly shown, modulation indexes of all the RAR subtypes were decreased upon incubation with this specific antagonist. Thus, we can conclude that antagonistic actions of chemicals on the different RAR subtypes can be predicted based on the coregulator binding profiles. In addition, the results support that Ro415253 behaves as a neutral antagonist by hampering the basal binding of all kind of coregulators (both coactivators and corepressors).

Figure 8. Comparison of coregulator binding of RAR α , RAR β and RAR γ upon incubation with the antagonist Ro415253 (10^{-4} M). Average results from duplicates (2 arrays) are expressed as modulation indexes (MIs) at saturating concentrations of ligand (A). MIs found are also presented based on hierarchical clustering (Eucl. dist.) by a heatmap (B).

4 Discussion

Knowledge on the specific ligand-dependent RAR-coregulator interactions is needed to understand retinoid-induced effects, since the biological response to ligands has been shown to depend on the cellular levels of coregulators and their affinity/selectivity for NRs [8–11,19,20]. Besides subtype-selective binding of ligands, differences in signalling of each RAR-subtype [4] may also depend on other variables such as differential ligand-driven binding to specific coregulators. Therefore, the present study characterized and compared the coregulator binding profiles of RAR α , RAR β and RAR γ in the absence and presence of the endogenous pan-RAR-agonist AtRA. Furthermore, in this work we demonstrated an alternative experimental approach to directly discriminate between different subtype-selective ligands and also agonistic/antagonistic actions based on RAR-coregulator binding events.

Several studies using different methodologies have shown that the DNA-bound RARs initially tend to bind to the corepressors NCOR1 and NCOR2 in the absence of ligand [21,22]. Agonist binding elicits the release of these corepressors followed by binding to ligand-induced coactivators like NCOA1 [23] or ligand-induced corepressors such as NRIP1 [10,24]. The present study confirmed previously described ligand-dependent RAR-coregulator interactions while further revealing multiple unknown AtRA-driven RAR-coregulator binding events. Thus, a high number of coactivators and corepressors were found to interact with the different RARs in a ligand-dependent manner suggesting that a broad range of coregulator proteins can potentially intervene on RAR signalling. Previous research has shown that overexpression or lack of certain ligand-dependent coregulators could affect the physiological outcome driven by retinoids ultimately influencing aspects such as sensitivity and/or resistance [6,25]. In the present study we were able to show several ligand-dependent RAR interactions with various coactivators such as BLIS1, PIAS2 or MLL2 and with corepressors such as CNOT1, NELFB or NR0B1, which to the best of our knowledge, have not been described in literature before. It has to be noted that the interactions newly found in MARCoNI will require additional steps to confirm ligand-driven direct binding between RARs and other coregulator motifs (with slightly

different sequences) or purified full-length coregulators and also within a cellular context. As an initial step, we showed this concordance by using the pull down assay to confirm the AtRA-driven direct interactions between the RAR α -LBD and the full length DDX5. Thus, future studies should further focus on characterizing the relevance of these newly described interactions and their potential role in the eventual RAR-genomic signalling.

Under basal conditions or in the presence of the pan-RAR agonist AtRA, which is capable of activating all RAR subtypes [17], coregulator selectivity/specificity for a certain RAR variant may play an important role in the distinct observed phenotypes. To test this hypothesis, identifying possible coregulators with a higher tendency to associate with a specific RAR subtype would be the first step. Despite the fact that all RAR subtypes showed highly coincident coregulator binding trends, several subtype-specific features and differences were found. In the absence of ligand, the obtained basal coregulator binding signal (expressed in AU) may be influenced by technical factors such as the specific receptor tagging which may influence antibody detection and eventually, the signal. However, we observed differential basal coregulator binding among the different receptors and motifs supporting the concept that basal RAR-coregulator interactions may differ depending on both the receptor subtype and the coregulator involved. Based on the results of this work, RAR β generally showed higher basal binding to coregulators and lower binding efficacies and MIs upon AtRA action among the three RAR subtypes tested, suggesting it to possess a higher ligand-independent mode of action. In accordance with our findings, RAR β has been postulated as a protein with a highly autonomous functioning [26]. Regarding this ligand-independent activity in a physiological scenario, a differential binding of the corepressors NCOR1 and NCOR2 to apo-form RARs could play a predominant role on their eventual signalling. In fact, Farboud et al. (2003) showed that among all RAR subtypes, RAR α presents a lower ligand-independent activity due to a significantly higher basal binding to NCOR2 when compared to RAR β or RAR γ [27,28]. In agreement with this finding, our study showed higher basal binding of RAR α to NCOR2_649_671 and NCOR2_2123_2145 suggesting these motifs as possible main players

in the direct interaction between NCOR2 and RAR α . Concerning specific AtRA-driven RAR-coregulator interactions, only a few motifs were found to differentially bind to the respective RAR subtypes, and when specific interactions were detected, these were mainly involving RAR α . Previous research suggested that the differential biological responses elicited by RAR α , RAR β and RAR γ may arise from a differential recruitment of coregulators to the retinoic acid responsive elements [7]. In consequence, the subtype-specific ligand-induced coregulators found in this work should be further studied in order to determine their possible role in the eventual phenotype provoked by the different RAR subtypes at the cellular level.

Independently of the cell context (e.g. RAR or coregulator levels), ligand design can also lead to the modulation of a selected pathway related to a specific RAR subtype. In the last decades, extensive effort has been made to develop selective ligands capable of specifically modulating the desired RAR-subtype [29]. Consequently, selective ligands such as Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ) have been produced. In order to judge the subtype-selectivity of a certain compound, techniques like ligand binding and transactivation assays have been used to derive parameters such as K_d or EC₅₀ values. Whereas ligand binding assays can predict affinity values (K_d) to RARs, they cannot predict the eventual action on the receptors occurring as a result of such interaction. In order to define activation/inhibition potential of a chemical on RARs, reporter gene cell lines have been used. However, specific aspects among the different cell lines used such as differential coregulator levels or indirect mechanisms such as ligand-driven induction of the expression of the target receptor itself may elicit misleading interpretations. Using a coregulator binding assay, both direct ligand-protein interactions and eventual actions (agonist or antagonist) of a certain compound can be predicted. Thus, after showing its potential to characterize coregulator interactions induced by AtRA on RARs, the present study additionally confirmed that such an approach can successfully discriminate between RAR subtype-selective agonists. Thus, in terms of median EC₅₀ values, Am80 and BMS961 were unequivocally categorized as RAR α - and RAR γ -selective ligands, respectively. Nevertheless, based

on these criteria, CD2314 showed an only weak preference for RAR β (weak RAR β -selective agonist). As shown in previous studies, the structure of the ligand itself may elicit different conformational changes on NRs, which in turn, may affect their specific coregulator interactions [30]. Thus, we hypothesized that, apart from the well-known ligand-receptor variables such as ligand binding affinity, differential coregulator binding to the receptor-ligand complex induced by a selective agonist, could also play a pivotal role in the subtype-selective actions. It should be emphasized that all compounds (at the highest concentration tested) elicited highly similar coregulator binding profiles on each RAR subtype. However, some exceptions were found where the coregulator binding generated by the different compounds remarkably differed. For instance, CD2314 showed to generate differential corepressor dissociation among the RAR subtypes when comparing with the general agonist AtRA. In fact, based on our results, selectivity of CD2314 may partially occur due to the greater ability of this compound to dissociate NCOR1 and NCOR2 from RAR β while concurrently displaying lower capacity to do so for RAR α and RAR γ . As the release of these apo-form-bound corepressors is the first and necessary step in the genomic RAR pathway, we speculate that this specific factor may be the underlying reason of the RAR β -selectivity of CD2314.

As last objective, the present work tested the capacity to discriminate antagonistic actions of a specific RAR ligand by assessing its coregulator binding profiles as previously reported for other nuclear receptors [31]. To do so, coregulator binding signatures generated by the RAR antagonist Ro415253 at saturating concentrations were evaluated. Firstly, upon incubation with Ro415253, both coactivator and corepressor binding were reduced when compared to the unliganded receptors, which clearly distinguish this ligand from the agonists tested. Although previous studies already showed that the binding of NCOA1 and NCOR2 to RAR α is decreased by Ro415253 [32], we hereby show that this dissociative behaviour can be generalized to the other RAR subtypes and to many other coactivators and corepressors. Thus, the present study demonstrated that Ro415253 behaves as a neutral antagonist leading to the so-called naked RARs [26]. Once shown the capacity of this coregulator binding assay

to discriminate between agonism and neutral antagonism, future studies may additionally test other special RAR ligands such as inverse agonists (e.g. BMS493) which can cause down-regulation of retinoid responsive genes by strengthening the binding of corepressors [5] or compounds known to exert dual actions (agonism/antagonism) on the different RAR subtypes (e.g. BMS453) [29].

The present study characterized and compared RAR-coregulator binding events occurring upon incubation with general and subtype-selective agonists. A strikingly high number of coregulator motifs were found to bind to RARs in a ligand-dependent manner, which in many cases have not been previously reported as RAR interactors. Despite the relatively high similarity on ligand-induced coregulator binding profiles among all RARs, some remarkable findings such as the detection of coregulator motifs with a higher tendency to associate with a specific RAR subtype were found. The outcome of this study could be used as a basis for further research aiming at determining the role of these specific coregulators in the eventual cellular action of the RAR subtypes. This may facilitate the future search and development of novel therapeutic approaches such as peptidomimetics [33], which block specific RAR-coregulator interactions. Finally, this work additionally demonstrated the possible applications of a coregulator binding microarray as a tool to discriminate among subtype-selective ligands and between agonistic/antagonistic actions of different retinoids.

Conflicts of Interests

The authors declare that part of the study was conducted at PamGene International B.V.

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Appendix. Supplementary Material

Supplementary material can be found in the end of this chapter.

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A.1

A.2

A.3

Appendix. Supplementary Figure B: AtRA-driven coregulator binding data points and concentration-response curves for RAR α (B.1), RAR β (B.2) and RAR γ (B.3).

B.1

Appendix. Supplementary Figure C: Am80-driven coregulator binding data points and concentration-response curves for RAR α (C.1), RAR β (C.2) and RAR γ (C.3).

C.1

Appendix. Supplementary Figure D: CD2314-driven coregulator binding data points and concentration-response curves for RAR α (D.1), RAR β (D.2) and RAR γ (D.3).

D.1

E.1

Appendix. Supplementary Figure F: Comparison of the coregulator motif binding to RAR α (F.1), RAR β (F.2) or RAR γ (F.3) generated by the general and subtype selective-agonists AtRA, Am80, CD2314 and BMS961. Modulation indexes (MIs) at saturating concentrations of ligand are represented by heat maps based on hierarchical clustering (Euclidean distance).

G.1

Appendix. Supplementary Table A: EC50 values (nM) for all coregulatory motifs presenting concentration-response curves with $R^2 > 0.8$ (considered as ligand-dependent). EC50s from motifs presenting concentration-response curves with $R^2 < 0.8$ (weak or ligand-independent motifs) are indicated by N.D (not determined).

Coregulator	Coregulator motif ID	Sequence	AtRA EC50 (nM)			Am80 EC50 (nM)			CD2314 EC50 (nM)			BMS961 EC50 (nM)		
			RAR α	RAR β	RAR γ	RAR α	RAR β	RAR γ	RAR α	RAR β	RAR γ	RAR α	RAR β	RAR γ
Androgen Receptor	ANDR_10_32	VYPRPPSKTYRGAFONLFQSVRE	2883	N.D	235	2	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Biogenesis of lysosome-related organelles complex 1 subunit I	BL151_1_11	MLSRLLKEHQA	21	172	183	3	126	1208	502	N.D	601	1156	N.D	100
Bromodomain-containing protein 8	BRD8_254_276	TVAASPAASGAPTLRLLEAGPT	15	19	44	2	22	265	660	410	722	N.D	389	22
CREB-binding protein	CBP_2055_2077	SVOPPRSPSALQDLRLTKSP	21	27	61	6	40	817	471	N.D	N.D	N.D	279	62
	CBP_345_367_C367S	TADPEKRLLQQQLVLLHAKMS	16	N.D	N.D	3	N.D	N.D	499	N.D	N.D	N.D	N.D	N.D
	CBP_345_368	TADPEKRLLQQQLVLLHAKCQ	21	N.D	N.D	7	N.D	N.D	860	N.D	N.D	N.D	N.D	N.D
	CBP_345_368_C367S	TADPEKRLLQQQLVLLHAKMSQ	23	N.D	N.D	7	N.D	420	492	N.D	N.D	N.D	N.D	96
G1/S-specific cyclin-D1	CCND1_243_264_C243S/C247S	SLRASQEQIEALLSRLRQAQ	6	N.D	146	83	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	CENPR_159_177	PHKASHRDSYEFKAILN	34	9	134	3	N.D	1613	413	N.D	N.D	755	71	87
Centromere protein R	CENPR_1_18	MPVKRSKLDGLLENSF	N.D	N.D	N.D	N.D	83	80	786	N.D	N.D	N.D	N.D	46
	CHD9_1023_1045	LLGTPLQNTVEELFSLHLEP	N.D	N.D	N.D	1	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	CHD9_2018_2040	QYQVALSASPLTSPRLDAKGI	23	40	137	5	53	1723	750	118	690	1422	157	160
Chromodomain-helicase-DNA-binding protein 9	CHD9_855_877	KNGNQLREYLEGUNWLLFNWYN	N.D	N.D	39	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	CNOT1_140_162	DLRGFAAQFIQKLPDLLRSYID	9	N.D	N.D	3	N.D	N.D	N.D	965	1366	N.D	N.D	91
	CNOT1_1626_1648	IPPTLAMPNQAQALRSLEVVLL	25	N.D	25	6	N.D	769	411	N.D	N.D	N.D	N.D	33
	CNOT1_1929_1951_C1932S	RAKSYHNLDAFVRLLVVKHSG	N.D	20	N.D	N.D	N.D	N.D	N.D	527	122688	N.D	N.D	28
CCR4-NOT transcription complex subunit 1	CNOT1_2083_2105	ELTKPMQILYKGLRVLLVLLHD	17	N.D	500	9	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	CNOT1_2086_2108	KPMQILYKGLRVLLVLLHDFPE	N.D	N.D	N.D	11	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	CNOT1_557_579	LSRLDVAQDKALSMLLNGTFP	N.D	111	N.D	600	N.D	2494	N.D	417	800	N.D	N.D	25
	DDX5_133_155	DMVGVAQTGGSKTLYLPAVH	69	N.D	N.D	N.D	120	N.D	455	N.D	N.D	N.D	N.D	67
Probable ATP-dependent RNA helicase DDX5	DHX30_241_262	QFPLPKNLAKVQIATSSSTA	N.D	N.D	77	N.D	N.D	13304	N.D	N.D	N.D	N.D	N.D	N.D
Putative ATP-dependent RNA helicase DHX30	DHX30_49_70	EFPQPKNLNSVIGRALGISHA	1	16	N.D	N.D	9	N.D	N.D	183	5418	N.D	266	38
Histone acetyltransferase p300	EP300_2039_2061	SPLKPGTVSQQAQNLRLTRSP	25	22	119	4	13	748	527	N.D	N.D	412	N.D	66
	EP300_69_91	GMVQDAASKHQKSELRLSGSSP	20	27	108	3	24	742	473	251	612	N.D	470	70
Gelsolin precursor	GELS_376_398	QVSVLPEGGTFLPKQFFKNWRD	N.D	471	N.D	N.D	N.D	N.D	N.D	500	1451	N.D	210	N.D
Guanine nucleotide-binding protein G(iq) subunit alpha	GNAQ_21_43	RQLRKDRDARREKLKLLGTGE	14	22	67	2	11	35	1804	N.D	165	N.D	36	18
Hair protein	HAIR_553_575_C567S	GLAKHLLSGLDRLSRLRRERE	N.D	N.D	N.D	8	N.D	N.D	N.D	N.D	N.D	N.D	N.D	86
	HAIR_745_767_C755S/C759S	EDRAGRGLPSPSLSELASTAV	14	19	42	2	58	313	666	287	633	N.D	220	24
NF-kappa-B inhibitor beta	IKBB_244_266	PLHLAVEAAQADVLELLRAGAN	N.D	N.D	N.D	18	N.D	185	434	N.D	3763	N.D	N.D	365
	IKBB_277_299	PLGSAMLRPNPLARLRAHAGP	13	24	24	4	22	267	N.D	N.D	191	1405	276	25
	IKBB_62_84	LHLAVIQHEPFLDFLGFSAQT	N.D	31	85	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Integrin-linked protein kinase	ILK_131_153	KYGEIPVDKAKAPRELLRERAE	25	31	99	4	44	919	638	175	762	1119	2008	74
Jumonji domain-containing protein 1C	JHD2C_2054_2076	PLVSQNNQGGSTLRDLTTTAGK	14	35	23	3	35	330	6582	347	514	N.D	N.D	30
Kinesin-like protein KIF11	KIF11_832_854_C854S	QWVSSLNEREQEHLNLLEVVSQS	29	N.D	N.D	4	N.D	N.D	59	N.D	158	N.D	N.D	N.D
Loss of heterozygosity 3 chromosomal region 2 gene A protein	L3R2A_12_34	WHNNVLHHLVFLPHLLNQFSS	N.D	0.4	N.D	N.D	N.D	11	N.D	N.D	N.D	N.D	6	8
Ligand-dependent corepressor (LCoR)	LCOR_40_62	TTSPTAATTQNPVLSKLLMADQD	14	21	27	3	27	210	886	437	585	1423	165	18
Melanoma antigen preferentially expressed in tumors	MAPE_249_271	LAKFSPYLGQMINLRLLSHIH	12	19	40	4	27	247	N.D	N.D	N.D	N.D	N.D	26
	MAPE_300_322	ALYVDSFLFLGRGLDQLRHVMN	N.D	N.D	108	23	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	MAPE_356_378	LSGVMLTDVSPPEQLALRASA	28	34	71	7	223	1543	761	N.D	1054	1490	N.D	93
	MAPE_382_404_C388S	DLVFDESIGTDQLLALLPSLH	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	MAPE_454_476_C472S	TLHLRLAYMARLRELLSELGR	N.D	N.D	277	139	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Mediator of RNA polymerase II transcription subunit 1	MAPE_91_113	HLHLETKAVLDGLDVLLAQEVR	30	22	9	2	15	0	N.D	223	N.D	N.D	210	43
	MED1_591_614	HGEDFSKYSQNPILTSLLQITGNG	12	16	29	2	17	265	816	142	340	1581	314	15
	MED1_632_655	VSSMAGNTKNPMLNMLKNPAAQ	28	17	63	2	16	440	664	233	534	N.D	365	43
Menin	MEN1_255_277	HTDSLELLQQLKLLVLLYDGH	7	N.D	N.D	0	19	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Methylated-DNA--protein-cysteine methyltransferase	MGMT_86_108	HPVFQEQSFTRQVLWKLLKVVF	N.D	N.D	60	N.D	N.D	N.D	N.D	N.D	1138	N.D	N.D	N.D
Myeloid/lymphoid or mixed-lineage leukemia protein 2	MLL2_4175_4197	LLAGPRSEAGHLLQKLRAKVN	16	20	39	2	16	528	257	N.D	N.D	2745	168	15
	MLL2_4702_4724	PRLLKKNKGVWRKRLRLTLTKG	N.D	N.D	97	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Metastasis-associated protein MTA1	MTA1S_388_410_C393S/C396S	GAGRASESSYMSSRLILDIEE	82	26	121	3	2315	11245	251	N.D	N.D	N.D	N.D	62
Nuclear receptor coactivator 1	NCOA1_1421_1441	TSGPQTPOAQKSKSLQQLTE	17	24	16	3	24	185	2074	964	415	1106	N.D	22
	NCOA1_620_643	SDGDSKYSQTSKHLQLLTITAEQ	22	30	72	4	41	769	688	144	731	N.D	429	95
	NCOA1_677_700	PSHSSSLTERHKHLRLDQEGSPS	14	16	30	3	16	256	586	281	314	N.D	376	28
	NCOA1_737_759	ASKKESKDHQLRYLLDKDEKD	20	20	58	4	22	384	602	195	383	2509	240	54
Nuclear Receptor Coactivator 2	NCOA2_628_651	GQSRLLHDSKGTQLQLLTTSKSDQ	15	19	52	3	17	293	710	323	364	N.D	294	32
	NCOA2_677_700	STHGTSLKEKHILRLDQSSSP	16	19	59	3	15	368	635	189	319	1108	414	42
	NCOA2_733_755	EPVSPKKENALLRYLLDKDQTD	16	16	47	3	16	279	635	233	329	1618	327	25
	NCOA2_866_888	SQSTFNPNRPGLGRLLPNQNL	122	N.D	59	3	382	N.D	11521	N.D	N.D	0	N.D	N.D
	NCOA3_104_123_N-KKK	KKKGQGVWDKSLGPLLQALDG	20	36	21	6	0	909	478	N.D	N.D	N.D	N.D	21
Nuclear receptor coactivator 3	NCOA3_609_631	QRGPLESGHKHKLQLLTSSDD	27	30	86	4	22	400	666	161	481	1778	171	58
	NCOA3_609_631_C627S	QRGPLESGHKHKLQLLTSSDD	16	28	40	4	26	349	1532	258	392	1381	249	44
	NCOA3_673_695	MHGSLLQEKHRLHLLQNGNSP	21	20	38	3	26	653	437	174	N.D	N.D	293	38
	NCOA3_725_747	EQLSPKKENALLRYLLDRDDP	16	15	41	2	20	264	826	150	302	2651	252	20
	NCOA3_MOUSE_1029_1051	HGSQNRPLRLNSDLLGLPPSNA	37	N.D	N.D	4	N.D	N.D	342	N.D	N.D	4296	N.D	435
Nuclear receptor coactivator 4	NCOA4_315_337	RKPENSGRETSEKFLFQSVNV	12	N.D	91	N.D	N.D	N.D	N.D	N.D	N.D	N.D	1865	21
	NCOA4_79_101_C101S	QLKEETLQQAQQLVSLGGQFNS	110179	N.D	N.D	3	N.D	N.D	416	N.D	N.D	N.D	N.D	N.D
Nuclear receptor coactivator 6	NCOA6_1479_1501	LVPAMREAPTSLSQLLDNSGAP	20	34	54	3	82	686	1015	596	557	N.D	N.D	35
	NCOA6_875_897	PVNKDVLTSPVLNMLLOSISA	21	13	57	2	14	974	567	280	305	11605	392	25

Coregulator	Coregulator motif ID	Sequence	Atra EC50 (nM)			Am80 EC50 (nM)			CD2314 EC50 (nM)			BMS961 EC50 (nM)		
			RARα	RARβ	RARγ	RARα	RARβ	RARγ	RARα	RARβ	RARγ	RARα	RARβ	RARγ
Nuclear receptor corepressor 1	NCOR1_1925_1946	TTITAAFNFDVITRIQASDKD	N.D	N.D	N.D	N.D	29	N.D	N.D	173	N.D	N.D	N.D	N.D
	NCOR1_2039_2061	MGQVPRTHRLTLADHICQITQ	N.D	7	46	2	0	N.D	N.D	344	828	N.D	N.D	10
	NCOR1_2039_2061_C2056S	MGQVPRTHRLTLADHICQITQ	11	35	N.D	4	19	N.D	N.D	410	4213	392	N.D	N.D
	NCOR1_2251_2273	GHSFADPASNLGLEDIRKALMG	19	24	54	3	41	7945	N.D	541	7189		2982	45
	NCOR1_2376_2398	SSTGSTQFPYNPLTMRMLSSTPP	N.D	N.D	N.D	N.D	N.D	N.D	116	N.D	N.D	N.D	N.D	N.D
	NCOR1_662_684_C662S	SKNFYFNKRRHNLNLLQKHQK	N.D	0	N.D	889	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Nuclear receptor corepressor 2	NCOR2_2123_2145	APGVKGHRQVVTLAQHISEVITQ	18	16	44	3	11	47	N.D	153	45261	N.D	190	N.D
	NCOR2_2330_2352	QAVQEHASTNMGLAEIRKALMG	17	24	46	3	47	297	N.D	546	3481	N.D	N.D	37
	NCOR2_649_671_C649S	SKNFYFNKRRHNLNLLQKHQK	42	N.D	25	3	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Negative elongation factor B	NELFB_328_350	QETLPRDSPDLLLRLALGQG	29	N.D	N.D	3	N.D	N.D	308	N.D	697	N.D	1	25
	NELFB_428_450	VVLHITKQRNKNALLRLPLGLVE	18	24	130	5	N.D	31	471	N.D	N.D	329	N.D	31
	NELFB_80_102	IASEGKAEERYKKLEDLLEKFS	37	N.D	75	4	N.D	N.D	6867	N.D	523	N.D	N.D	N.D
Nuclear receptor subfamily 0 group B member 1	NROB1_136_159	GEDHPRQGSILVSLTSSKQTHVA	12	26	26	3	18	254	1113	339	275	N.D	N.D	21
	NROB1_1_23	MAGENHGWGSSLYNMLMSAKQT	22	16	51	4	17	444	500	N.D	928	1585	N.D	69
	NROB1_68_90_C69S	FSGKDHPRGSSILVSLTSSKQTHVA	18	20	79	3	10	493	662	198	306	1787	546	47
Nuclear receptor subfamily 0 group B member 2	NROB2_106_128	TFEVAEAPVPSILKILLEEPS	13	21	28	3	23	240	1083	190	378	N.D	N.D	20
	NROB2_201_223_C207S	EVLEPWSPPAAQGLRLVLTAST	30	30	104	6	73	2215	604	N.D	675	N.D	N.D	103
	NROB2_237_257	FRPIGQVDIAGLLRQDMILLR	N.D	N.D	17	N.D	N.D	349	N.D	N.D	N.D	N.D	N.D	2263
	NROB2_9_31_C9S/C11S	SPSQGAASRPAILYALSSSLKA	13	17	38	3	16	233	N.D	N.D	272	N.D	223	26
Nuclear receptor-binding factor 2	NRBF2_128_150	PEIQGIFDRDPDTLLYLQKQSE	26	22	N.D	5	51	1017	1128	177	657	N.D	498	99
Nuclear receptor-interacting protein 1	NRIP1_1055_1077	EKDSRLTKNPILYMLQKGGN	21	14	45	3	21	988	476	134	282	5926	422	34
	NRIP1_120_142	VDSVPKQKQDSTLLASLQSFSS	20	22	76	3	31	631	481	69	409		402	53
	NRIP1_121_143_P124R	DSVVRKQKQDSTLLASLQSFSS	19	19	69	3	30	462	361	N.D	N.D	1400	322	45
	NRIP1_173_195	KDLRCYGVASSHLTKLTKSKVK	203	29	154	17	N.D	132	312	N.D	N.D	N.D	N.D	37
	NRIP1_173_195_C177S	KDLRCYGVASSHLTKLTKSKVK	91	30	151	5	27	102	690	N.D	N.D	399	5996	44
	NRIP1_253_275_C263S	PATSPKPSVASSQLALLSSEAH	17	27	39	3	30	229	625	267	409	N.D	N.D	23
	NRIP1_368_390	RNNIKQAANNSLLHLKQSTIP	10	22	18	3	21	143	N.D	152	237	1068	214	17
	NRIP1_488_510	KNSKLSHQKQVTLQLLGHKNE	14	15	35	3	20	345	521	N.D	257	N.D	326	23
	NRIP1_700_722	GSEIENLERRTVLQLLGNPNK	19	17	N.D	2	19	482	546	126	416	1384	286	50
	NRIP1_701_723	SEIENLERRTVLQLLGNPNK	18	25	53	4	28	735	562	187	400	N.D	230	56
	NRIP1_805_831	PVSPQDFPSKNGSLRLRQNDQSYL	18	27	52	3	29	608	414	N.D	230	443196	N.D	30
	NRIP1_8_30	GSDVHDQSVLTVLEGLMHQAA	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	46	17	N.D	N.D
	NRIP1_924_946	RSWARESKSFNVKQLLSSENV	17	29	54	4	26	564	623	N.D	235	2147	N.D	37
	NRIP1_924_946_C945S	RSWARESKSFNVKQLLSSENV	17	16	42	3	18	416	486	81	241	1439	N.D	116
	NSD1_894_916	SSQNHPIEPDYKFTLLMLMKD	23	15	80	4	90	1145	715	N.D	449	N.D	N.D	127
	NSD1_982_1004	GGDSALSGELASLPLGSDKRD	29	N.D	N.D	4	N.D	N.D	1487	N.D	882	562	N.D	N.D
Serine/threonine-protein kinase PAK 6	PAK6_248_270	SPKTRESSLKRRLFRSMFLSTAA	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Histone acetyltransferase PCAF	PCAF_178_200	EEDADTKQVYFYLKLLAKSILQ	9	N.D	15	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	55
Proline-, glutamic acid- and leucine-rich protein 1	PELP1_142_164	QDPATMELAVAVLRDLRLRYAAQ	24	10	26	N.D	2	169	N.D	103	497	N.D	72	12
	PELP1_168_190	LFRDISMNLPLGLTLGLGRPE	21	35	84	4	72	1357	387	N.D	N.D	N.D	4260	97
	PELP1_20_42	GTGGLSASVSGPRLLRLLESVS	19	14	55	3	17	322	479	171	446	N.D	1314	64
													5	
	PELP1_251_273	SOGLKHTESEWEQELSHLASLHT	N.D	N.D	N.D	8	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	PELP1_258_280	ESWEQELSHLASLHTLGLAYE	N.D	N.D	N.D	42919	N.D	N.D	N.D	N.D	N.D	N.D	N.D	420
	PELP1_446_468	AGMLQGGASGEALLTHLLSDIS	21	25	62	4	59	715	717	147	555	1638	293	56
	PELP1_496_518_C496S	SPFFLQSLHGDGPRLLRLLESVS	19	34	92	6	144	1465	831	N.D	N.D	N.D	N.D	97
	PELP1_56_78_C71S	VHPPNRSAPHLGLMSLLRLHGS	16	21	43	4	16	183	301	N.D	289	N.D	476	39
	PELP1_571_593_C575S/C581S	TSRSRRREYSLLALLAPSPR	N.D	99	135	9	25	49	243	N.D	N.D	N.D	153	28
Protein inhibitor of activated STAT2	PIAS2_6_28	ELRNMVSSFRVSELQVLGFAGR	28	20	63	6	38	789	971	N.D	N.D	10009	N.D	182
Proline-rich nuclear receptor coactivator 1	PNRC1_306_327	ENSNQNRLEMAVHLKTLKVKQT	24	33	63	4	42	887	850	128	536	1673	N.D	64
Proline-rich nuclear receptor coactivator 2	PNRC2_118_139	SFNPDKIEIMTQKTLKVKVQ	25	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	91
Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	PPRC1_1159_1181	QAFISEIGIEASDLSLLEQFEK	34	N.D	N.D	4	N.D	N.D	14	N.D	N.D	N.D	2260	N.D
	PPRC1_151_173	DSELLVSPREGSSHLKLLTSLRT	19	28	57	4	37	942	609	N.D	417	N.D	N.D	55
Peroxisomal proliferator-activated receptor A-interacting complex 285kDa protein	PR285_1062_1084	WPWDGELNADDAIRRELDSEOK	33	44	74	6	294	2410	16781	83	683	10318	880	188
	PR285_1105_1127	QQAARLYNLPPAALRKLRAEPE	20	24	84	4	36	1179	1025	137	534	N.D	914	78
	PR285_1160_1182_C1163S	RLDSGMFAFAGDEVVLQSLGDKA	32	45	N.D	6	191	7727	440	N.D	602	51363	N.D	178
	PR285_2216_2238_C2219S	ILYSGPSNKSVDVLAGLLRRME	19	18	67	4	70	1162	520	N.D	N.D	1074	200	67
	PR285_432_454_C453S/C454S	EVRLERRASSGQALWLLPARSS	21	N.D	N.D	27	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
PR domain zinc finger protein 2	PRDM2_948_970	SPALQTPSLSSGQLPLPLPTDP	27	N.D	N.D	5	N.D	N.D	179	N.D	N.D	N.D	N.D	N.D
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PRGC1_130_155	DGTPPPQEAEPSSLKLLAPANTQ	16	20	12	7	20	N.D	1113	275	360	N.D	N.D	14
	PRGC1_134_154	PPQEAEPSSLKLLAPANT	16	48	22	0	11	206	2440	1213	735	N.D	270	14
Peroxisome proliferator-activated receptor gamma coactivator 1-beta	PRGC2_146_166	PAPEVDLSLLQKLLATSYP	27	22	78	3	55	1075	542	N.D	N.D	N.D	417	58
	PRGC2_338_358	AEFSILRELLAQDVLCDVSKP	27	22	50	5	25	385	500	188	503	1587	N.D	54
Progesterone receptor (PR)	PRGR_102_124	SPPEKDSGLLSDVLTLLAPSPG	24	48	N.D	5	N.D	3390	484	1007	N.D	N.D	N.D	64
	PRGR_42_64_C64S	SDTLPEVSAIPSLDGLFPFPPS	27	N.D	N.D	4	N.D	N.D	744	N.D	N.D	31330	N.D	67
Homeobox prospero-like protein PROX1	PROX1_57_79	SVVQHDAGSEKSNVLRKLKRRANS	17	18	36	N.D	12	115	379	N.D	N.D	N.D	1699	32
Cell cycle checkpoint control protein RAD9A	RAD9A_348_370	EPSTVPGTPPKKFRSLFFGSIL	12	N.D	N.D	N.D	80	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Retinoblastoma-like protein 2	RBL2_875_897_C879S/C894S	SHQSPFLMMDRHLQQLMSAIY	14	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Transcription factor p65	TF65_437_459	SEALLQLQDFDDEDGALLGNSTD	6589	N.D	N.D	3	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Transforming growth factor beta-1-induced transcript 1 protein	TGFI1_325_347_C334S/C346S	FHEREGRPYSRRDFQLFAPRSQ	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	TGFI1_443_461_C452S/C455S	FQERAGKPYQSPFKLFG	424	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Transcription intermediary factor 1-alpha	TIF1A_373_395_C394S	TALLYSKRLTYRLRLHRAASD	11	19	63	3	18	31	N.D	N.D	N.D	N.D	21	35
	TIF1A_747_769	ESRPQANYPNRSILTSLLNSQ	11	10	21	2	17	216	501	103	214	2436	N.D	11
Histone acetyltransferase HTATIP	TIP60_476_498	LSIEDIVDGERHAMUKRLRIQSK	26	44	111	5	42	986	567	179	599	3649	1834	87
Transcriptional-regulating factor 1	TREF1_168_190	TQSAVMDGAPDSALRQLLSOKPM	18	17	50	3	31	433	809	230	540	1748	296	29
	TREF1_850_872	HSLFEAKGDVMMVALEMLLRKPV	30	25	N.D	7	N.D	N.D	582	N.D	N.D	N.D	N.D	184

Coregulator	Coregulator motif ID	Sequence	Atra EC50 (nM)			Am80 EC50 (nM)			CD2314 EC50 (nM)			BMS961 EC50 (nM)		
			RAR α	RAR β	RAR γ	RAR α	RAR β	RAR γ	RAR α	RAR β	RAR γ	RAR α	RAR β	RAR γ
Thyroid receptor-interacting protein 4	TRIP4_149_171_C171S	FVNLTYRERQDRILAVLLPGRHPS	18	N.D	204	6	N.D	N.D	N.D	N.D	N.D	N.D	N.D	20
Transformation/transcription domain-associated protein	TRRAP_3535_3557_C3535S/C3555S	SLTESRREERVLQLRLNPSLE	25	14	64	5	35	1318	433	N.D	N.D	N.D	730	85
	TRRAP_770_792	GSHDLLYQEFLLPNLLQGLNM	25	N.D	N.D	4	N.D	N.D	606	N.D	N.D	N.D	N.D	N.D
	TRRAP_971_993	LVAMMSLEDNKHLYQLLAHPNF	51	46	126	5	329	5857	348	N.D	892	N.D	246	73
Thioredoxin reductase 1, cytoplasmic	TRXR1_132_154	GHGPTLKAYQEGRLQKLLKMNGP	25	56	103	4	44	647	571	N.D	570	N.D	682	90
Ubiquitin-protein ligase E3A	UBE3A_396_418	DDEEPIPESSSELTQELLGEERR	22	23	52	4	20	937	1554	202	617	N.D	229	44
	UBE3A_649_671	RDLGDSHPVLYQSLKDLLEYEGN	23	15	53	6	33	615	322	196	446	N.D	N.D	37
WD repeat domain phosphoinositide-interacting protein 1	WIPI1_119_141	ESYIHNIKDMKLLKTLDDIPAN	25	35	79	4	43	1231	565	149	660	2193	397	74
	WIPI1_313_335_C318S	GQRNISTLSIQKLPRLLVASSS	20	23	118	5	N.D	310	440	N.D	N.D	N.D	N.D	56
Zinc finger HIT domain-containing protein 3	ZNHI3_89_111	LQNLKNGESATLRSLLNPHLR	11	N.D	13	N.D	N.D	473	14986	284	249	N.D	N.D	7
Zinc transporter 9	ZNT9_449_471	LLGRSQPEQVQRLLTELENDPS	25	31	59	5	54	714	1317	85	1113	1325	345	100

Appendix. Supplementary Table B: Identification and function of coregulator motifs present in the array. Comparison of the results of this work with previous literature (if any) regarding ligand-dependent RAR-coregulator interactions is also provided. Coregulators with at least one motif presenting ATRA-driven interactions with RARs ($R^2 > 0.8$) in MARCoNI were considered as ligand-dependent.

Coregulator	Main Function	Coregulator motif ID	ATRA-dependent coregulator. MARCoNI vs Literature						References
			RAR α (MARCoNI)	RAR α (Literature)	RAR β (MARCoNI)	RAR β (Literature)	RAR γ (MARCoNI)	RAR γ (Literature)	
Androgen Receptor	Nuclear Receptor	ANDR_10_32	Yes	No	No	No	Yes	No	[34]
Biogenesis of lysosome-related organelles complex 1 subunit I	Coactivator	BL151_1_11	Yes	No	Yes	No	Yes	No	[35]
Bromodomain-containing protein 8	Coactivator	BRD8_254_276	Yes	No	Yes	No	Yes	No	[36]
CREB-binding protein	Coactivator	CBP_2055_2077 CBP_345_367_C3675 CBP_345_368 CBP_345_368_C3675 CBP_57_80	Yes	Yes	Yes	No	Yes	No	[10]
G1/S-specific cyclin-D1	Coactivator Corepressor	CCND1_243_264_C2435/C2475	Yes	No	No	No	Yes	No	[37,38]
Centromere protein R	Coactivator	CENPR_159_177 CENPR_1_18	Yes	No	Yes	No	Yes	No	[39]
Chromodomain-helicase-DNA-binding protein 9	Coactivator	CHD9_1023_1045 CHD9_2018_2040 CHD9_855_877	Yes	No	Yes	No	Yes	No	[40]
CCR4-NOT transcription complex subunit 1	Corepressor	CNOT1_140_162 CNOT1_1626_1648 CNOT1_1929_1951_C19325 CNOT1_2083_2105 CNOT1_2086_2108 CNOT1_557_579	Yes	No	Yes	No	Yes	No	[41]
Probable ATP-dependent RNA helicase DDX5	Coactivator	DDX5_133_155	Yes	No	No	No	No	No	[42,43]
Putative ATP-dependent RNA helicase DHX30	Signalling zinc-finger antiviral protein	DHX30_241_262 DHX30_49_70	Yes	No	Yes	No	Yes	No	[44]
Histone acetyltransferase p300	Coactivator	EP300_2039_2061 EP300_69_91	Yes	Yes	Yes	No	Yes	No	[45]
Gelsolin precursor	Coactivator	GELS_376_398	No	No	Yes	No	No	No	[46]
Guanine nucleotide-binding protein G(q) subunit alpha	Signalling p38MAPK	GNAQ_21_43	Yes	Yes	Yes	No	Yes	No	[47]
Hair protein	-	HAIR_553_575_C5675 HAIR_745_767_C7555/C7595	Yes	No	Yes	No	Yes	No	-
NF-kappa-B inhibitor beta	Corepressor	IKBB_244_266 IKBB_277_299 IKBB_62_84	Yes	No	Yes	No	Yes	No	[48]
Integrin-linked protein kinase	PI3K/ILK AKT kinase pathway	ILK_131_153	Yes	No	Yes	No	Yes	No	[49]
Jumonji domain-containing protein 1C	Coactivator	JHD2C_2054_2076	Yes	No	Yes	No	Yes	No	[50]
Kinesin-like protein KIF11	Mitosis	KIF11_832_854_C8545	Yes	No	No	No	No	No	[51]
Loss of heterozygosity 3 chromosomal region 2 gene A protein.	Corepressor	L3R2A_12_34	No	No	Yes	No	No	No	[52]
Ligand-dependent corepressor (LCoR)	Corepressor	LCOR_40_62	Yes	Yes	Yes	Yes	Yes	Yes	[53]
Melanoma antigen preferentially expressed in tumors	Corepressor	MAPE_249_271 MAPE_300_322 MAPE_356_378 MAPE_382_404_C3885 MAPE_454_476_C4725 MAPE_91_113	Yes	Yes	Yes	No	Yes	No	[6]
Mediator of RNA polymerase II transcription subunit 1	Coactivator	MED1_591_614 MED1_632_655	Yes	Yes	Yes	No	Yes	No	[54]
Menin	Coactivator	MEN1_255_277	Yes	No	No	No	No	No	[55]
Methylated-DNA--protein-cysteine methyltransferase	Corepressor	MGMT_86_108	No	No	No	No	Yes	No	[56]
Myeloid/lymphoid or mixed-lineage leukemia protein 2.	Coactivator	MLL2_4175_4197 MLL2_4702_4724	Yes	No	Yes	No	Yes	No	[57]
Metastasis-associated protein MTA1.	Corepressor	MTA15_388_410_C3935/C3965	Yes	No	Yes	No	Yes	No	[58]
Nuclear receptor coactivator 1	Coactivator	NCOA1_1421_1441 NCOA1_620_643 NCOA1_677_700 NCOA1_737_759	Yes	Yes	Yes	Yes	Yes	Yes	[59]
Nuclear Receptor Coactivator 2	Coactivator	NCOA2_628_651 NCOA2_677_700 NCOA2_733_755 NCOA2_866_888	Yes	Yes	Yes	No	Yes	No	[23]

Coregulator	Main Function	Coregulator motif ID	ATRA-dependent coregulator. MARCoNI vs Literature						References
			RAR α (MARCoNI)	RAR α (Literature)	RAR β (MARCoNI)	RAR β (Literature)	RAR γ (MARCoNI)	RAR γ (Literature)	
Nuclear receptor coactivator 3	Coactivator	NCOA3_104_123_N-KKK NCOA3_609_631 NCOA3_609_631_C627S NCOA3_673_695 NCOA3_725_747 NCOA3_MOUSE_1029_1051	Yes	Yes	Yes	Yes	Yes	Yes	[27,60]
Nuclear receptor coactivator 4	Coactivator	NCOA4_315_337 NCOA4_79_101_C101S	Yes	No	No	No	Yes	No	[61,62]
Nuclear receptor coactivator 6	Coactivator	NCOA6_1479_1501 NCOA6_875_897	Yes	Yes	Yes	No	Yes	No	[63,64]
Nuclear receptor corepressor 1	Corepressor	NCOR1_1925_1946 NCOR1_2039_2061 NCOR1_2039_2061_C2056S NCOR1_2251_2273 NCOR1_2376_2398 NCOR1_662_684_C662S	Yes	Yes	Yes	Yes	Yes	Yes	[65,66]
Nuclear receptor corepressor 2	Corepressor	NCOR2_2123_2145 NCOR2_2330_2352 NCOR2_649_671_C649S	Yes	Yes	Yes	Yes	Yes	Yes	[27]
Negative elongation factor B	Corepressor	NELFB_328_350 NELFB_428_450 NELFB_80_102	Yes	No	Yes	No	Yes	No	[67]
Nuclear receptor subfamily 0 group B member 1	Corepressor	NR0B1_136_159 NR0B1_1_23 NR0B1_68_90_C69S	Yes	No	Yes	No	Yes	No	[68,69]
Nuclear receptor subfamily 0 group B member 2	Corepressor	NR0B2_106_128 NR0B2_201_223_C207S NR0B2_237_257 NR0B2_9_31_C9S/C11S	Yes	Yes	Yes	No	Yes	Yes	[60,70]
Nuclear receptor-binding factor 2	Coactivator	NRBF2_128_150	Yes	Yes	Yes	No	No	No	[71]
Nuclear receptor-interacting protein 1	Corepressor	NRIP1_1055_1077 NRIP1_120_142 NRIP1_121_143_P124R NRIP1_173_195 NRIP1_173_195_C177S NRIP1_253_275_C263S NRIP1_368_390 NRIP1_488_510 NRIP1_700_722 NRIP1_701_723 NRIP1_805_831 NRIP1_8_30 NRIP1_924_946 NRIP1_924_946_C945S	Yes	Yes	Yes	No	Yes	No	[72]
Nuclear receptor-binding SET domain-containing protein 1	Coactivator Corepressor	NSD1_894_916 NSD1_982_1004	Yes	Yes	Yes	No	Yes	No	[73,74]
Serine/threonine-protein kinase PAK 6	Corepressor	PAK6_248_270	No	No	No	No	No	No	[75]
Histone acetyltransferase PCAF	Coactivator	PCAF_178_200	Yes	Yes	No	No	Yes	No	[72,76]
Proline-, glutamic acid- and leucine-rich protein 1	Coactivator	PELP1_142_164 PELP1_168_190 PELP1_20_42 PELP1_251_273 PELP1_258_280 PELP1_446_468 PELP1_496_518_C496S PELP1_56_78_C71S PELP1_571_593_C575S/C581S	Yes	No	Yes	No	Yes	No	[77]
Protein inhibitor of activated STAT2	Coactivator	PIAS2_6_28	Yes	No	Yes	No	Yes	No	[78]
Proline-rich nuclear receptor coactivator 1	Coactivator	PNRC1_306_327	Yes	Yes	Yes	Yes	Yes	Yes	[60,79]
Proline-rich nuclear receptor coactivator 2	Coactivator	PNRC2_118_139	Yes	Yes	No	No	No	Yes	[60,80]
Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	Coactivator	PPRC1_1159_1181 PPRC1_151_173	Yes	No	Yes	No	Yes	No	[81]
Peroxisomal proliferator-activated receptor A-interacting complex 285kDa protein	Coactivator	PR285_1062_1084 PR285_1105_1127 PR285_1160_1182_C1163S PR285_2216_2238_C2219S PR285_432_454_C453S/C454S	Yes	No	Yes	No	Yes	No	[82]

Coregulator	Main Function	Coregulator motif ID	AtRA-dependent coregulator. MARCoNI vs Literature						References
			RARα (MARCoNI)	RARα (Literature)	RARβ (MARCoNI)	RARβ (Literature)	RARγ (MARCoNI)	RARγ (Literature)	
PR domain zinc finger protein 2	Coactivator	PRDM2_948_970	Yes	No	No	No	No	No	[83]
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	Coactivator	PRGC1_130_155 PRGC1_134_154	Yes	No	Yes	No	Yes	No	[84]
Peroxisome proliferator-activated receptor gamma coactivator 1-beta	Coactivator	PRGC2_146_166 PRGC2_338_358	Yes	Yes	Yes	No	Yes	No	[85]
Progesterone receptor (PR)	Nuclear Receptor	PRGR_102_124 PRGR_42_64_C64 S	Yes	No	Yes	No	No	No	[34]
Homeobox prospero-like protein PROX1	Corepressor	PROX1_57_79	Yes	No	Yes	No	Yes	No	[86]
Cell cycle checkpoint control protein RAD9A	Corepressor	RAD9A_348_370	Yes	No	No	No	No	No	[87]
Retinoblastoma-like protein 2	Corepressor	RBL2_875_897_C 8795/C8945	Yes	No	No	No	No	No	[88]
Transcription factor p65	Corepressor	TF65_437_459	Yes	No	No	No	No	No	[89]
Transforming growth factor beta-1-induced transcript 1 protein	Coactivator	TGFI1_325_347_C 3345/C3465 TGFI1_443_461_C 4525/C4555	Yes	No	No	No	No	No	[90]
Transcription intermediary factor 1-alpha	Coactivator	TIF1A_373_395_C 3945 TIF1A_747_769	Yes	Yes	Yes	No	Yes	No	[91]
Histone acetyltransferase HTATIP	Coactivator	TIP60_476_498	Yes	No	Yes	No	Yes	No	[92]
Transcriptional-regulating factor 1	Coactivator	TREF1_168_190 TREF1_850_872	Yes	No	Yes	No	Yes	No	[93]
Thyroid receptor-interactingprotein 4	Coactivator	TRIP4_149_171_C 1715	Yes	Yes	No	No	Yes	No	[94]
Transformation/transcription domain-associated protein	Coactivator	TRRAP_3535_355 7_C35355/C35555 TRRAP_770_792 TRRAP_971_993	Yes	No	Yes	No	Yes	No	[42]
Thioredoxin reductase 1, cytoplasmic	Retinoid-driven tumour growth suppression	TRXR1_132_154	Yes	No	Yes	No	Yes	No	[95,96]
Ubiquitin-protein ligase E3A	Coactivator	UBE3A_396_418 UBE3A_649_671	Yes	Yes	Yes	No	Yes	No	[97,98]
WD repeat domain phosphoinositide-interacting protein 1	Autophagy Processes	WIPI1_119_141 WIPI1_313_335_C 3185	Yes	Yes	Yes	No	Yes	No	[99]
Zinc finger HIT domain-containing protein 3	Coactivator	ZNHI3_89_111	Yes	No	No	No	Yes	No	[100]
Zinc transporter 9	Coactivator	ZNT9_449_471	Yes	No	Yes	No	Yes	No	[101]

Chapter 4

The effects of *all-trans* retinoic acid on estrogen receptor signalling in the estrogen-sensitive MCF/BUS subline

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Abstract

Estrogen Receptor alpha (ER α) and Retinoic Acid Receptors (RARs) play important and opposite roles in breast cancer growth. While exposure to ER α agonists such as 17 β -estradiol (E₂) is related to proliferation, RAR agonists such as *all-trans* retinoic acid (AtRA) induce anti-proliferative effects. Although crosstalk between these pathways has been proposed, the molecular mechanisms underlying this interplay are still not completely unravelled. The aim of this study was to evaluate the effects of AtRA on ER α -mediated signaling in the ER α positive cell lines MCF7/BUS and U2OS-ER α -Luc to investigate some of the possible underlying modes of action. To do so, this study assessed the effects of AtRA on different ER α -related events such as ER α -mediated cell proliferation and gene expression, ER α -coregulator binding and ER α subcellular localization. AtRA-mediated antagonism of E₂-induced signaling was observed in the proliferation and gene expression studies. However, AtRA showed no remarkable effects on the E₂-driven coregulator binding and subcellular distribution of ER α . Interestingly, in the absence of E₂, ER α -mediated gene expression, ER α -coregulator binding and ER α subcellular mobilization were increased upon exposure to micromolar concentrations of AtRA found to inhibit cell proliferation after long-term exposure. Nevertheless, experiments using purified ER α showed that direct binding of AtRA to ER α does not occur. Altogether, our results using MCF7/BUS and U2OS-ER α -Luc cells suggest that AtRA, without being a direct ligand of ER α , can indirectly interfere on basal ER α -coregulator binding and basal ER α subcellular localization in addition to the previously described crosstalk mechanisms such as competition of ERs and RARs for DNA binding sites.

1 Introduction

Nuclear Receptors (NRs) are ligand-induced transcription factors that regulate gene expression by binding to specific regions in the genome called Hormone Responsive Elements (HREs) and to coregulators. In the context of breast cancer cell growth, specific NRs such as Estrogen Receptor alpha (ER α) and Retinoic Acid Receptors alpha, beta and gamma (RAR α,β,γ) and their respective ligands play key roles [1]. While estrogens (ER α ligands) such as 17 β -estradiol (E $_2$) induce breast cancer cell proliferation, retinoids (RAR ligands) such as *all-trans* retinoic acid (AtRA) have been demonstrated to often decrease proliferative rates [2]. Previous research showed that the inhibitory effects of retinoids on breast cancer cell growth can vary (different sensitivity) and are driven by multiple molecular mechanisms including the interplay with the ER pathway [3,4]. Thus, crosstalk between ER and RAR pathways has been proposed since the effects of retinoids seem to be highly dependent on the presence of ERs in breast cancer cells [5]. In fact, retinoids have been shown to decrease proliferation of especially ER $^+$ breast cancer cells while no remarkable effects are observed for most of the ER $^-$ breast cancer cells [5–7]. In addition, inhibition of estradiol-induced ER α activity and the related proliferation induced by RAR agonists has been previously described [8,9]. However, the underlying mechanisms leading to these specific interactive effects between ER and RAR signaling are still not completely understood.

Previous studies showed that ERs and RARs directly interact with each other through their ligand binding domains in a ligand-independent manner [10] and that the AF-2 domain (C-terminus) of the RAR α mediates AtRA-driven inhibition of ER α -induced transcription in breast cancer cells [11]. Regarding the classical genomic pathway of ER α in breast cancer cells, Hua et al. (2009) suggested a competition for transcriptional activity due to the existence of close/overlapping DNA binding regions for RARs and ERs within the genome [12]. In a later publication, it was shown that under E $_2$ -stimulation, RAR α appeared within the ER α transcriptional complex, suggesting that RAR α acts as a scaffold protein [9]. This study showed thus a double role where unliganded RAR α is actually

necessary for the expression of different E₂-responsive genes while ligand-bound RAR α decreases such transcription. In addition, it was found that whereas the presence of both ER α and RAR α on the promoter regions of several E₂-responsive genes was not affected by retinoid treatment, the recruitment of several coregulators was decreased [9]. Altogether, the capacity of ER α and RARs to form dimers, the proximity of their respective DNA binding regions and the simultaneous presence of both receptors in a transcriptional complex lead to hypothesize that protein-protein interactions between both NRs and other key regulatory elements within the transcription machinery (e.g. coregulators), may play an important role in the observed anti-proliferative effects of retinoids.

The aim of the present study was to assess the effects of retinoids on different ER α -related molecular events to acquire further insights in the possible mechanisms governing the crosstalk between ER α and RARs. Most of the research on the crosstalk between ER α and RARs has been carried out using the ER⁺ MCF7 (wild type) cell line. However, MCF7 is a highly heterogeneous polyclonal cell line and several studies have reported different levels of anti-estrogen sensitivity/resistance depending on the specific MCF7 clone [13,14]. The present study makes use of MCF7/BUS, an MCF7 subline known to be highly E₂-responsive [15] in which to our knowledge, interference between retinoid and estrogen signaling has not been investigated so far. To this end, this work thoroughly assessed the effects of the pan-RAR-agonist AtRA on ER α -mediated proliferation and gene expression followed by the measurement of ER α -coregulator binding profiles and ER α subcellular localization, using the MCF7/BUS cell line and U2OS-ER α -Luc (reporter gene) cell line as model systems.

2 Materials and Methods

2.1 Chemicals

The ER α agonist 17- β -estradiol (E₂), and the pan-RAR-agonist *all-trans* retinoic acid (AtRA) were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands. Dimethylsulphoxide (DMSO, 99.9%) was purchased from Acros (Geel, Belgium).

2.2 Cell lines and cell culture conditions

The human breast cancer cell line MCF7/BUS (provided by Rikilt Institute, Wageningen, The Netherlands) and the reporter cell line U2OS-ER α -Luc (provided by BDS, Amsterdam, The Netherlands) were used for all cell-based experiments. Cells were maintained in 150 cm² flasks (Corning, NY, USA) in Dulbecco's Modified Eagle Medium DMEM/F12 (Gibco, Bleiswijk, The Netherlands) supplemented with 10% (v/v) foetal bovine serum (PAA, Pasching, Austria), kept in a humidified atmosphere at 37°C and 5% CO₂ and subcultured when they reached 60-80% confluence. For U2OS-ER α -Luc cells, 0.2 mg/ml geneticine (G418) (Gibco, Bleiswijk, The Netherlands) was used as selection agent. In order to deprive the cells from estrogens, all experiments were performed using Dulbecco's Modified Eagle Medium (DMEM/F12) without phenol red (Gibco, Bleiswijk, The Netherlands) supplemented with 5% charcoal-dextran-treated foetal calf serum (Hyclone, Logan, USA) (hereafter exposure medium). For every assay, exposure medium containing retinoids (10⁻⁹-10⁻⁴ M) and/or estradiol (10⁻¹³-10⁻⁶ M) was freshly prepared with a maximum final concentration of 0.2% DMSO (solvent).

2.3 MTT-Proliferation studies

MCF7/BUS cells were seeded in flat-transparent 96-well plates (Corning, NY, USA) at a cell density of 2 x 10³ cells per well. 24h later, cells were exposed to the respective test compounds. After 72 hours exposure medium containing the compounds was renewed. The number of viable cells after 6 days

exposure to the solvent control and the respective chemicals was determined by performing the MTT proliferation assay. To this end, cells were incubated for the last three hours with the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Next, medium was removed and 150 μ l of DMSO was added to each well. After 15 minutes, absorbance of the dissolved formazan product was measured at the wavelength of 562 nm with a Microplate Reader SpectraMax M2 (Molecular Devices, Sunnyvale CA, USA).

2.4 ER α -reporter gene studies

U2OS-ER α -Luc cells were seeded in 96-well white opaque plates (PerkinElmer, Groningen, The Netherlands) at a cell density of 1×10^4 cells/well. On the next day, medium was refreshed. 48 h after seeding, exposure was carried out by adding the test compounds. After 24 h of exposure, cells were washed with half-diluted (water) Phosphate-Buffered Saline (PBS) (Gibco, Bleiswijk, The Netherlands) and lysed with 30 μ l of hypotonic low-salt buffer (pH 7.8) containing 10 mM Tris (Sigma-Aldrich, Zwijndrecht, The Netherlands), 2 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM 1,2-diaminocyclohexanetetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA). Plates were cooled down and later kept at -80 °C until analysis. In order to determine luciferase activity, plates were thawed and light emission was measured by using a luminometer (Luminoscan Ascent, Thermo Scientific, Waltham, USA) after addition of 100 μ l of flashmix (pH 7.8) composed of 20 mM tricine (Sigma-Aldrich, St. Louis, MO, USA), 1.07 mM (MgCO_3) $4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ (Aldrich, Milwaukee, WI, USA), 2.6 mM magnesium sulfate (MgSO_4 , Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM ethylenediaminetetraacetic acid ($\text{EDTA} \cdot 2\text{H}_2\text{O}$, Merck, Darmstadt, Germany), 2 mM dithiothreitol (DTT) (Sigma, Zwijndrecht, The Netherlands, #43819), 0.47 mM D-luciferin (Duchefa Biochemie, Haarlem, The Netherlands) and 5 mM adenosine triphosphate (ATP, Duchefa Biochemie, Haarlem, The Netherlands).

2.5 Gene expression (RT-qPCR) studies

MCF7/BUS cells were hormone-deprived for 3 days prior to exposure. Cells were seeded in 6-well plates (Corning, NY, USA) at a cell density of 6×10^5 cells/well. 24 h after seeding, exposure was carried out by adding the test compounds. Next, after 6 h of exposure, cell lysis with RLT Lysis buffer and isolation of RNA by using QIAshredder and RNeasy kits (Qiagen, Venlo, The Netherlands) was carried out according to the manufacturer's instructions. Quality of RNA was assessed spectrophotometrically by using a Nanodrop (ND-1000 Thermoscientific Wilmington, Delaware, USA). Synthesis of cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). Expression of the genes *trefoil factor-1 (TFF1)*, *cAMP-dependent protein kinase inhibitor- β (PKIB)*, *estrogen receptor alpha (ER α)* and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* (housekeeping gene) was assessed by real-time PCR using Rotor-Gene SYBR[®] Green Kit (Qiagen, Venlo, The Netherlands) and the Rotor-Gene 6000 cycler (Qiagen, Venlo, The Netherlands) according to the manufacturer's guidelines. To this end QuantiTect Primer Assay Hs_TFF1_1_SG, Hs_PKIB_1_SG, Hs_ESR1_1_SG and Hs_GAPDH_1_SG (Qiagen, Venlo, The Netherlands) were utilized.

2.6 Coregulator binding assays

The Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) was used in order to evaluate possible effects of the test compounds on ER α -coregulator binding profiles. Thus, using MARCoNI, the ligand-modulated binding of ER α to 154 binding motifs belonging to more than 60 coregulators was assessed. These assays were performed using purified GST-tagged ER α -LBD (Invitrogen) or full length ER α obtained from lysates of the cell line MCF7/BUS. In all cases, ligand concentrations with a maximum final solvent concentrations of 2% DMSO in the reaction buffer were applied. Samples containing identical percentage of DMSO without ligand were included as the solvent controls.

For the experiments with recombinant proteins, assay samples were prepared in time-resolved fluorescence resonance energy transfer (TR-FRET) reaction buffer E (Life technologies, Ltd, UK, #PV4420) supplemented with 5 mM dithiothreitol (DTT) and kept on ice before starting the assay. Mixtures containing 7 nM glutathione S-transferase (GST)-tagged human ER α -LBD, 25 nM Alexa 488-conjugated anti-GST (Molecular Probes, Life Technologies, Ltd, UK) and the different ligands were used. For the experiments with lysates, post-lysis treatment assays were carried out. In short, cells were hormone-deprived in 150 cm² flasks for three days and trypsinized. Next, a cell suspension with assay medium was prepared in order to neutralize the remaining trypsin. Cells were washed with PBS by centrifugation and removal of supernatant. Cell pellets were snap-frozen in liquid nitrogen and stored at -80°C. Next, lysis was performed in TNRL01 lysis buffer using a dismembrator as previously described [16] and samples were stored at -80°C. Before the initiation of the coregulator binding assay, lysate mixtures were prepared in 2xNR buffer containing 10 nM rat anti-ER α IgG2a (D547) (Santa Cruz Biotechnology, CA, USA), 8 nM goat anti-Rat IgG2a (FITC) (Axxora, CA, USA), 2 μ M dithiothreitol (DTT) and the test compounds.

For both recombinant protein and cell lysate, experiments were performed by using PamChip[®] platforms (PamGene International B.V., 's-Hertogenbosch, The Netherlands) containing 4 peptide microarrays. Every microarray consists of a three-dimensional porous metal oxide carrier containing 154 spots with different immobilized NR-binding motifs derived from 65 different coregulators. The automated platform PamStation[®] (PamGene International B.V., 's-Hertogenbosch, The Netherlands) and EvolveHT software (PamGene International B.V., 's-Hertogenbosch, The Netherlands) were used applying 2 (up-down) pumping cycles per minute at 20°C. Before adding the samples to the Pamchip[®] arrays, incubation for 20 cycles with 25 μ l blocking buffer composed of Tris Buffered saline (Bio-Rad, Veenendaal, The Netherlands, #170-6435) supplemented with 1% BSA (Calbiochem, Merck, Darmstadt, Germany, #126609), 0.01% Tween-20 (Bio-Rad, Veenendaal, The Netherlands, #170-6531) and 0.3% ELK (milk) blocking solution was carried out in order to avoid unspecific binding to

the arrays. After removal of blocking buffer, samples were added to the Pamchip[®] arrays and incubated for 80 pumping cycles. For the cell lysate samples, an additional incubation for 40 cycles was carried out. Finally, unbound receptor was washed away with 25 µl Tris Buffered Saline and TIFF images were obtained by a charge coupled device camera (CCD) installed in the PamStation[®]. Image analysis was performed using Bionavigator 6.1 (PamGene International B.V., 's-Hertogenbosch, The Netherlands). Fluorescence binding Arbitrary Units (AU) of every spot (coregulator motif) were quantified by subtracting the background signal from the median fluorescent signal.

2.7 Determination of cytoplasmic and nuclear ERα levels

MCF7/BUS cells were hormone deprived for 3 days prior to exposure to the test compounds for 1 hour in 150 cm² flasks. After exposure, cells were washed with PBS and trypsinized. Next, remaining trypsin was neutralized and removed by adding assay medium, spinning down the cells and discarding the supernatant. The same procedure was carried out for washing cells using PBS. Next, separation of the cell pellet into a nuclear and cytoplasmic fraction was performed by using NE-PER Nuclear and cytoplasmic extraction reagents (ThermoFisher Scientific, Rockford Illinois, USA) according to the manufacturer's instructions. Total protein of the respective fractions was quantified by the Pierce bicinchoninic acid (BCA) protein assay (ThermoFisher Scientific, Rockford Illinois, USA) and samples containing 20-35 µg of protein were prepared in Laemmli buffer (Bio-Rad, Veenendaal, The Netherlands) supplemented with dithiothreitol (DTT) (50 mM). After denaturation at 90°C for 5 minutes, samples were loaded on 10% Mini-PROTEAN TGX[™] Precast Protein Gels (Bio-Rad, Veenendaal, The Netherlands). Electrophoresis was run at 200 V for 40 minutes in Tris/glycine/SDS Buffer (Bio-Rad, Veenendaal, The Netherlands). Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Veenendaal, The Netherlands) in Tris/glycine Buffer containing 20% methanol (Biosolve, Valkenwaard, The Netherlands). A reversible Ponceau staining (Sigma, Zwijndrecht, The Netherlands) of the membrane was carried out to assess correct blotting. Subsequently, the membrane

was blocked for 1 hour with Tris Buffered Saline (Bio-Rad, Veenendaal, The Netherlands) supplemented with Tween 20 (0.1%) (Bio-Rad, Veenendaal, The Netherlands) and Blotting Grade Blocker (50 g/l) (Bio-Rad, Veenendaal, The Netherlands) to avoid unspecific binding. Next, the membrane was probed with the respective antibodies which were diluted in Tris Buffered Saline supplemented with Tween 20 (0.1%) and Blotting Grade Blocker (50 g/l) according to the manufacturer's instructions. First, membranes were incubated with the primary antibodies against ER α (sc-543), cofilin (sc-33779) and Lamin A/C (sc-20681) from Santa Cruz Biotechnologies (Santa Cruz Biotechnology, CA, USA); or against H3 Histone (ab176842) and GAPDH (ab128915) from Abcam (Abcam, Cambridge, UK). Membranes were washed with Tris Buffered Saline supplemented with Tween 20 (0.1%) and subsequently incubated with the secondary goat-anti-rabbit IgG-HRP (sc-2004) antibody (Santa Cruz Biotechnology, CA, USA). After final washing, Clarity Western ECL Substrate (Bio-Rad, Veenendaal, The Netherlands) was used for chemiluminescent detection. Semi-quantitative determination of ER α levels was performed by using the softwares Image Studio Lite (LI-COR) and ImageJ (National Institutes of Health; Bethesda, MD) by correcting for the housekeeping proteins Cofilin (cytoplasmic fraction) and H3 Histone (nuclear fraction). Assessment of the purity of the respective pooled fractions (cytoplasmic or nuclear samples) was carried out by using several cytoplasmic (Cofilin and GAPDH) and nuclear (Histone H3 and Lamin A/C) specific markers.

2.8 Data analysis and statistics

Concentration-response curves and their respective EC₅₀s (half maximal effective concentrations of the respective treatments) were obtained by non-linear regression fitting using the software GraphPad Prism 5 (San Diego, CA, USA). For all the experiments a minimum of three independent experiments (unless otherwise stated) were carried out. For comparison, statistical significances between the different groups (treated/untreated) were evaluated by one-way ANOVA with Dunnet's or Bonferroni's tests or by a Student's t-test. All along this work, data are expressed as percentage/fold

induction of the solvent control DMSO ($\text{Avg} \pm \text{SEM}$) or as Modulation Indexes (MI) (for the MARCoNI assay) which represent the $\log_{10}$ transformed ratio of the binding in the presence of the ligand over the binding in the solvent control (DMSO).

3 Results

3.1 Effects of exposure to AtRA on basal and E₂-induced MCF7/BUS cell proliferation

The effects of the pan-agonist AtRA on MCF7/BUS cell proliferation were tested in the presence or absence of E₂ (Figure 1). First, pro-proliferative effects of E₂ at concentrations ranging between 0.1 pM and 1000 pM were evaluated. As shown in Figure 1A, proliferation of MCF7/BUS cells increased as the concentration of E₂ augmented with an EC₅₀ of 2 pM. Next, effects of different concentrations of AtRA on E₂-induced proliferation were assessed. While co-exposure to 10 nM AtRA only decreased the maximum E₂-induced response to a limited extent, co-incubation with 1000 nM AtRA remarkably decreased both minimum and maximum responses induced by E₂. Further, effects of different concentrations of AtRA in the absence or presence of a fixed concentration of E₂ (10 pM) were additionally evaluated. As shown in figure 1B, exposure to AtRA significantly decreased both basal and E₂-induced proliferation in a concentration-dependent manner, the latter already in the nanomolar range. Thus, our proliferation assays show that an AtRA-mediated decrease in proliferation of MCF7/BUS occurs in both the absence and presence of E₂.

Figure 1. Effects of AtRA on the E₂-induced (A,B) and basal (B) cell proliferation of MCF7/BUS cells. Cell proliferation is expressed relative to the solvent control (0.2% DMSO) set at 100%. Results represent the average $\pm$ SEM of at least three independent experiments. For statistical analysis ANOVA with Bonferroni's (A) and Dunnett's (B) multiple comparison tests were performed. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared to the E₂-treated equivalent control. *** $p < 0.001$; ** $p < 0.01$ compared to the solvent control.

3.2 AtRA-mediated effects on ER α activity in the U2OS-ER α -Luc reporter gene cell line

In order to assess whether the AtRA-driven inhibition of MCF7/BUS proliferation could be (partially) attributed to the disruption of ER α -activity, a reporter gene study using the U2OS-ER α -Luc cell line was carried out (Figure 2). First, effects of E₂ (0.3-1000 pM) on ER α -mediated luciferase expression were assessed. As expected, ER α -driven gene expression increased in an E₂ concentration-dependent manner (Figure 2A) with an EC₅₀ of 16 pM. Upon co-incubation with 10 nM AtRA, the E₂-induced maximum response was remarkably reduced (Figure 2A). Effects of a higher concentration of AtRA (1000 nM) generated a decrease in the E₂-induced maximum response in the same order of magnitude as 10 nM AtRA (results not shown). Next, the effects on ER α -mediated luciferase expression of

different concentrations of AtRA alone and in the presence of a fixed concentration of E₂ (10 pM) were tested. As shown in Figure 2B, AtRA clearly decreased the E₂-induced ER α -mediated luciferase expression in a concentration-dependent manner. However, in the absence of E₂, the effects of AtRA differed when comparing low and high concentrations of the retinoid. In the low nanomolar range, AtRA inhibited ER α -mediated expression of luciferase. However, as the concentrations reached the low micromolar range (1-10 μ M), AtRA caused an increase in ER α -mediated luciferase expression. In order to reject the possibility of possible cytotoxic effects as underlying cause, viability of U2OS-ER α -Luc after 24h-exposure to the highest concentrations of AtRA both in absence/presence of E₂ was also tested showing no repercussions in terms of toxicity (results not shown). In summary, our results show that AtRA decreased E₂-driven ER α -mediated reporter gene expression at all concentrations tested while it exerted different concentration-related effects under E₂-free conditions. Compared to the solvent control, AtRA decreased basal ER α -mediated expression of luciferase at nanomolar concentrations while causing an increase at micromolar (10 μ M) concentrations. These results show that in the presence of E₂, AtRA counteracts the E₂-induced ER α -mediated gene expression, a mode of action that can underlie the AtRA-driven reduction of E₂-induced cell proliferation. In the absence of E₂, different modes of action influencing the ER α -mediated gene expression in opposite ways may be relevant. Whether these AtRA-induced effects are observed for other genes in more relevant cell models than the U2OS cells, and whether they are due to a direct binding to the ER α ligand binding domain or induced by more indirect modes of action was subsequently investigated.

Figure 2. Effects of retinoids on E₂-induced (A and B) and basal (B) ER α -mediated expression of the ERE-luciferase gene in U2OS-ER α -Luc cells. ER α activity after 24h-exposure is expressed relative to the solvent control (0.2% DMSO) set at 100%. Results represent the average $\pm$ SEM of at least three independent experiments. For statistical analysis ANOVA with Bonferroni's (A) and Dunnett's (B) multiple comparison tests were performed. "" p < 0.0001; "" p < 0.001 compared to the E₂-treated equivalent control. *** p < 0.001; ** p < 0.01; * p < 0.05 compared to the solvent control.

3.3 Assessment of the AtRA-driven modulation of the expression of ER α -related genes in MCF7/BUS cells.

In order to further investigate whether AtRA at micromolar concentrations can also affect expression of ER α -related genes in our biologically relevant cell model (MCF/BUS cells), RT-qPCR studies were carried out. Figure 3 firstly shows the effects of 10 μ M AtRA on the expression of two estrogen-responsive genes known to play important roles in cell proliferation, namely *TFF1* and *PKIB*, both in the presence and absence of E₂. As expected, exposure to E₂ potently induced the transcription of these genes in MCF7/BUS cells. In addition, co-exposure with the pan-RAR agonist AtRA led to a decrease

in the E₂-induced expression of both genes which was significant for *PKIB*. Interestingly, incubation with AtRA alone elicited an increase of the expression of *TFF1*. On the contrary, in the absence of E₂, the *PKIB* gene was slightly down-regulated upon exposure to AtRA. In addition, analysis of the expression levels of the *ERα* gene upon exposure to the respective test compounds was carried out in order to assess whether the AtRA-mediated effects on basal and E₂-induced ERα activity may be caused by mere up-/down-regulation of the receptor itself. However, our results showed that neither E₂ nor AtRA alone or in combination, exerted any remarkable effect on the expression of the *ERα* gene. In summary, our results clearly show that AtRA at micromolar concentrations is capable of modulating ERα-mediated transcription in a gene-specific manner, however these effects are probably not mediated via up-regulation or down-regulation of ERα expression levels in MCF7/BUS breast cancer cells.

Figure 3. Effects of AtRA (10 μ M) and E₂ (10 nM) on the transcription of TFF1, PKIB and ERα in the breast cancer cell line MCF7/BUS. Gene expression after 6h-exposure is expressed relative (fold-induction) to the solvent control (0.2% DMSO) set at 1. Results represent the average $\pm$ SEM (n=3) of three independent experiments. For statistical analysis Student's t-tests were carried out. * $p < 0.05$ compared to the solvent control. ' $p < 0.05$ compared to the E₂-treated sample.

3.4 Evaluation of the effects of AtRA on ERα coregulator binding profiles

An as yet highly unexplored mode of action for the observed AtRA-driven effects on ERα-mediated gene expression could be at the level of coregulator recruitment. In order to assess whether ERα-coregulator binding in MCF7/BUS cells may be affected by AtRA treatment, we performed the

MARCoNI assay using lysates from this cell line. To this end, lysates of MCF7/BUS cells were incubated on the arrays with E₂ (10⁻⁶ M) and/or AtRA (10⁻⁴ M) (post-lysis treatment). Figures 4A (binding signature) and 4B (heat map) display the effects of the tested compounds as modulation indexes (MI), i.e. the compound-induced log-fold change of ER α binding to each individual coregulator-derived peptide. Positive or negative MIs (log-scale) represent increase or decrease in coregulator motif binding in relation to the solvent control DMSO (MI = 0), respectively. As expected, E₂ mostly enhanced ER α -coactivator binding. Generally, co-incubation with the general RAR agonist AtRA did not markedly alter the E₂-induced response. Strikingly, upon incubation with AtRA alone, ER α -coregulator binding was significantly increased, similarly to E₂ although to a lower extent (Figure 4A-B).

In order to further test whether AtRA may elicit ER α -coregulator interactions by direct binding to this receptor, we performed the experiments using purified recombinant ER α -LBD (Figure 4C). To this end, the ER α -LBD was incubated with AtRA (10⁻⁴ M) and the respective coregulator binding profiles were compared to those generated by the solvent and positive control E₂ (10⁻⁶ M). As expected, incubation of the recombinant ER α -LBD with E₂ generated a clear increase in coactivator binding. However, the results upon exposure to AtRA showed no significant changes on the ER α coregulator binding signatures suggesting no direct (ligand-receptor) modulation.

In summary, the results using MCF7/BUS lysates generally showed that incubation with AtRA has no remarkable effect on the E₂-induced ER α binding to the coregulator peptides tested. In addition, basal ER α -coregulator binding was shown to be increased upon incubation of the lysate samples with AtRA. However, as shown by the experiments using the purified recombinant ER α -LBD, this modulation does not seem to be driven by direct binding (ligand-receptor) to the truncated ER α . Altogether, the results suggest the possibility of transcriptional complexes containing ER α which can be activated by AtRA via a mode of action different from ligand binding at the ER α -LBD.

3.5 Determination of cytoplasmic and nuclear ER α levels

The present study additionally evaluated possible rapid effects of AtRA on the subcellular localization of ER α , both in the absence or in the presence of E₂, as a possible mode of action underlying the effects of AtRA on ER α -related cellular events. Figure 5 depicts nuclear and cytoplasmic levels of ER α upon exposure for a 1-hour period to the test compounds. Incubation with 100 nM E₂ considerably decreased ER α levels in the cytoplasmic fraction while simultaneously increasing the ER α levels in the nuclear compartment. Co-treatment with 10 μ M AtRA did not exert any remarkable effect on the E₂-driven cytoplasmic decrease of ER α levels (8% and 7%, respectively). On the contrary, nuclear levels were slightly decreased when comparing incubation with E₂ alone (426%) to those with E₂ in combination with AtRA (356%) although not significantly. Interestingly, in the absence of any estrogenic stimulation, AtRA was also capable of decreasing cytoplasmic ER α levels while increasing their presence in the nucleus although to a lower extent than E₂. Thus, the results show that under basal conditions (E₂-free conditions), AtRA affects the subcellular localization of ER α .

Figure 5. Effects of AtRA (1-hour exposure) on the MCF7/BUS subcellular localization of ER α in the absence and presence of E₂. After fractionation, cytoplasmic and nuclear ER α was determined by western blot. Semi-quantitative determination of ER α levels was carried out correcting by the housekeeping proteins Cofilin (cytoplasmic fraction) and Histone H3 (nuclear fraction). Results are shown as percentage of the solvent control (Avg $\pm$ SEM) set at 100 and are representative of at least five independent experiments (A). In addition, the assessment of the purity of the respective fractions was carried out in singular by using Cofilin/GAPDH (Cytoplasmic) and Histone H3/Lamin A/C (nuclear) as fractionation controls (B). For statistical analysis Student's t-tests were carried out. *** $p < 0.001$ compared to the solvent control.

4 Discussion

ER α -mediated gene expression and the consequent proliferation of breast cancer cells has been reported to be affected upon exposure to RAR agonists [5,8,9,17]. However, the underlying molecular mechanisms of this interplay between ER and RAR signaling are not completely understood. The objective of the present study was to investigate the possible effects of the pan-agonist AtRA on ER α signaling by using two cell models that have not been used before for this purpose, being a highly E₂-responsive breast cancer MCF7 subline (MCF7/BUS) and the reporter gene cell line U2OS-ER α -Luc. To do so, a battery of assays testing several endpoints related to the ER α pathway such as cell proliferation, gene expression, coregulator binding and subcellular localization were carried out. Using these techniques, this study investigated possible modes of action of AtRA on the pro-proliferative ER α signaling, including a) action as a direct ligand, b) effects on gene expression, c) effects on coregulator interactions, and d) effects on ER α subcellular localization.

In accordance with previous studies using different ER⁺ breast cancer cell lines, our work showed that while E₂ remarkably increased cell proliferation, AtRA provoked a decrease of both E₂-induced and basal MCF7/BUS proliferation after long-term exposure [2,5,9]. These retinoid-mediated inhibitory effects were readily observed at therapeutic doses within the low micromolar range. In order to determine whether the retinoid-mediated effects observed in our proliferation assays may partially occur due to the modulation of ER α -mediated transcription, gene expression studies using both a reporter gene cell line (U2OS-ER α -Luc) and the breast cancer cell line model (MCF7/BUS) were performed. At the molecular level, inhibition of E₂-induced ER α activity by RAR agonists such as AtRA has been previously described for other reporter gene cell lines [8], and the effects found are in line with the results observed with U2OS-ER α -Luc cells. Interestingly, under basal conditions (E₂-free medium), our study revealed a bimodal effect depending on the concentration tested. While nanomolar concentrations of AtRA were capable of significantly decreasing ER α -mediated gene expression, concentrations in the low micromolar range were capable of stimulating ER α -mediated expression of

luciferase. Previous studies have shown that RARs can bind to estrogen responsive elements (EREs) in a way that depends on both the nature of the ERE and the concentrations of receptor and ligand [18]. Thus, the increased ER α -mediated luciferase expression mediated by high concentrations of AtRA might be explained by direct binding of liganded RARs to EREs. In order to acquire further insights in this topic, we performed gene expression studies on MCF7/BUS cells where we assessed the effects of E₂ and AtRA on two estrogen responsive genes namely *TFF1* and *PKIB*. These specific genes are known to promote cell proliferation and their expression has been described to be ER α -mediated [19,20]. In addition, previous studies evaluating ER α and RAR binding throughout the genome of MCF7 cells, reported that while *TFF1* can present both ER α and RARs in its promotor region, *PKIB* harbours only ER α . In the present study we found that AtRA can counteract E₂-induced expression of both genes, especially for *PKIB*. Interestingly, previous studies reported that E₂-induced expression of *PKIB* was not affected by treatment with AtRA [12]. This contradiction may be explained by differences in the concentrations tested (our work used therapeutic concentrations which are in the order of 100x higher) and/or the specific cell model/subline used. *PKIB* expression is modulated by ER α in cooperation with AP-1 [19]. Since retinoids have been shown to affect AP-1 signaling [21], possible effects on this specific pathway (ER α -AP-1 signaling) cannot be excluded. Next, our results showed that in the absence of E₂, AtRA can increase the expression of *TFF1*, whereas slight down-regulation of the basal *PKIB* expression was found. Previous studies have shown that retinoids are able to affect ER α -mediated expression of genes sharing binding regions with RARs in the genome [9]. In this study however, we show that these effects can also be seen on other types of ER α target genes that do not have a binding region for RARs (i.e. *PKIB*) leading to speculate that mechanisms other than the mere competition for binding to the promotor regions can be involved. One of the alternative modes of action by which AtRA may modulate ER α -related signaling may have been via up-/down-regulation of the receptor itself. However, the present study showed that exposure to both E₂ and AtRA has no appreciable effects on the expression levels of ER α . Considering other possibilities, AtRA-mediated

effects on ER α signaling may also be caused by other molecular events such as direct (ant)agonistic activity of AtRA on ER α , effects on ER α -coregulator binding, changes on ER α subcellular localization or processes indirectly related to ER α (e.g. modulation of AP-1 or kinase pathways).

In order to investigate some of these other possible mechanisms that might help to explain the retinoid-mediated effects on E₂-induced cell proliferation, the present study tested whether the retinoid-driven antagonism of E₂-induced ER α -mediated gene expression may be additionally explained by direct ER α (ant-)agonism and/or by a possible disruption of ER α coregulator binding driven by liganded RARs present in the ER α transcriptional complex. To this end, coregulator binding to ER α in either the presence or absence of E₂ was tested with a microarray method to assess a wide set of coregulator-nuclear receptor interactions, using lysates from the MCF7/BUS cell line or isolated recombinant ER α -LBDs. This microarray technology has previously shown to be able to characterize the binding of motifs belonging to more than 60 different coregulators to both ERs and RARs [16,22–24]. In a previous study, ChIP experiments showed that the E₂-induced recruitment of certain coregulators such as EP300 or MED1 in the promoter regions of several estrogen responsive genes was hampered by AtRA [9]. The results of the present study using the coregulator binding assay with MCF7/BUS cell lysates indicate however that in general terms, AtRA exerted no remarkable effects on the E₂-induced coregulator binding patterns of ER α . The present study did however clearly show that incubation with AtRA alone led to a significant increase on the binding of a subset of coregulator motifs that were also modulated by E₂, although the effect of E₂ was more pronounced. In a previous study it was shown that the apo-ER α -mediated gene expression and the consequent pro-proliferative activity could be enhanced by apo-RAR α activity [25], however similar effects upon AtRA incubation as seen in the present study, have not been described so far. To test whether the effects of retinoids may be due to mere activation of ER α by an agonist-like ligand-receptor binding, we also assessed ER α -coregulator binding of the purified recombinant ER α ligand binding domain (LBD) and we showed that AtRA is not able to activate ER α by direct interaction with the receptor ligand binding domain. Thus, one might

speculate that, in a cellular context (lysate), dimers/complexes between apo-ER α and retinoid-related proteins such as RARs or the Cellular Retinoic Acid-Binding Protein 2 (CRABP2) which also can bind other transcriptional cofactors in a ligand-dependent manner [26,27]) may contribute to the observed AtRA-induced increase in the coregulator binding of ER α . However, the relatively high resemblance (although at different intensities) between the coregulator binding signatures generated by E₂ and AtRA may also support a hypothetical E₂-independent transactivation of ER α and remains to be studied further in more detail.

Once we found that AtRA can affect ER α -related endpoints such as proliferation, gene expression and ER α coregulator binding profiles, we assessed whether these retinoids can also affect subcellular localization of ER α in the MCF7/BUS cell line. The results of our study showed no effects of AtRA on the E₂-induced decrease of cytoplasmic levels of ER α , and only a slight decrease of the E₂-induced nuclear ER α localization was observed. Interestingly, AtRA alone was also capable of decreasing ER α cytoplasmic levels while increasing its nuclear localization although these effects appeared at a lower extent than for E₂. Previous studies have shown that ER α shows a nucleocytoplasmic shuttle and that exposure to E₂ can rapidly decrease and increase cytoplasmic and nuclear ER α levels, respectively [28,29]. However, to the best of our knowledge, this phenomenon has not yet been described upon exposure to retinoids. Although a possible role of cytoplasmic ER α degradation cannot be ruled out, the observed dynamic change (decrease and increase) of ER α levels in cytoplasmic and nuclear compartments might point to induced translocation to or retention in the nucleus as likely underlying causes. Although RARs have been shown to mainly possess nuclear localization [4], cytoplasmic/membrane RAR pools have been described [30,31]. Thus, ER α -RAR interactions may potentially explain the migration to or retention in the nuclear compartment of ER α . Since AtRA is well-known to activate all RAR subtypes and other pathways in breast cancer cells [4], future studies using subtype-selective agonists for RAR α , RAR β or RAR γ may bring additional insights in determining the participation of the specific RARs in such effects. As potential non-RAR-mediated

events leading to the AtRA-induced nucleocytoplasmic shuttle of ER α , a possible transport to the nucleus mediated by the cytoplasmic CRABP II could be also considered. Finally, effects of retinoids on other elements or molecular pathways known to play a role in the subcellular localization of ER α may also explain the observed response. For example, possible retinoid-mediated effects on ER α nucleocytoplasmic transport proteins such as Crm1 [32] or kinase pathways leading to phosphorylation and the consequent nuclear accumulation of ER α [33,34], among others, should be further investigated.

The previously suggested genomic antagonism between ER and RARs [12] via competition for DNA binding regions supports our observed effects on the expression of ER α -related genes. Thus, the formation of a less efficient transcriptional complex on ERE regions would explain both the weak agonistic and strong antagonistic effects on estrogen responsive gene expression upon exposure to AtRA alone or in combination with E₂, respectively. The present study additionally showed that exposure to AtRA alone can affect ER α signaling by modulating different molecular events such as ER α -coregulator binding or ER α subcellular distributions. However, its relationship with the observed anti-proliferative effects still remains to be completely elucidated. On the one hand, the consistent depletion of cytoplasmic levels of ER α provoked by AtRA may partially hamper its non-genomic pro-proliferative activities. On the other hand, our results using coregulator binding microarrays point at the simultaneous presence of ER α in transcriptional complexes activated by AtRA. Thus, apart from a possible weak E₂-independent activation of ER α , our results lead to additionally hypothesize that a fraction of apo-ER α may be re-directed to specific RAREs leading to both up-regulation of the specific anti-proliferative retinoid responsive genes and the down-regulation of the pro-proliferative estrogen responsive genes. Since previous studies showed that in the presence of E₂, apo-RAR α is a crucial element (scaffold) within the ER α -ERE transcriptional complex [9], the opposed possibility of apo-ER α acting in a similar way on AtRA-activated RAR-RARE transcriptional complexes must be tested by future ChIP studies in the future.

In summary, the present work showed in the cell lines MCF7/BUS and U2OS-ER α -Luc, which have so far not been used to study RAR-ER α crosstalk, that parameters such as cell proliferation, ER α -mediated gene expression, ER α -coregulator binding and ER α subcellular localization can be in one way or another affected upon exposure to AtRA. However, the AtRA-driven antagonism of E₂-induced signaling observed in our proliferation and gene expression studies cannot be directly explained via competition (AtRA-E₂) for binding to ER α (direct antagonism), effects on E₂-mediated ER α -coregulator binding or changes in the subcellular localization of ER α . Strikingly, in the absence of E₂, the basal activity of ER α in terms of gene expression, coregulator binding and in relation with its subcellular distribution, was found to be affected when reaching micromolar (pharmacological) concentrations of AtRA. However, as previously mentioned, our results did not point at AtRA as a cognate ligand (agonist/antagonist) for ER α or as inducer/inhibitor of the expression of the *ER α* gene confirming that other mechanisms are involved in these processes. Future studies should acquire further insights in this indirect modulation of ER α activity which may play a main role in the interplay between estrogen and retinoid signaling and the final effectiveness of retinoids as anticancer agents.

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Disclosure of interest

The authors report no conflicts of interest.

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Chapter 5

General and subtype-selective Retinoic Acid Receptor agonists elicit differential effects on basal Estrogen Receptor signaling of the MCF7/BUS cell line

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Abstract

Retinoids selectively modulating the different Retinoic Acid Receptors (RAR) subtypes (α , β and γ) have shown to differentially affect Estrogen Receptor positive (ER+) breast cancer growth. Former research has reported extensive crosstalk between ER α and RARs where retinoids have been shown to affect the pro-proliferative E₂-ER α -driven signaling. In addition, we reported in a previous study that the general RAR agonist *all-trans* retinoic acid (AtRA) was also capable of affecting basal activity of ER α under estradiol-deprived conditions. Thus, we hypothesized that the different effects on breast cancer growth of subtype-selective retinoids could be explained in part by differential effects on ER α signaling. To test that, our objective was to evaluate the effects driven by the general agonist AtRA and the subtype-selective agonists Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ) on different ER α -related events. Thus, using the MCF7/BUS and the U2OS-ER α -Luc cell lines, we characterized the effects of these retinoids on basal ER α -related processes such as proliferation, gene expression, coregulator binding and subcellular receptor localization. The results showed that proliferation, ER α -mediated gene expression, ER α -coregulator binding and ER α subcellular localization can be differentially affected by the distinct chemicals. The present study showed that at micromolar concentrations all retinoids inhibited breast cancer growth after long-term exposure while some of them favored ER α -pro-proliferative events at the initial stage post-treatment. Among all compounds, the general RAR-agonist AtRA and the RAR β agonist CD2314 displayed the highest modulation of ER α activity. After AtRA treatment, ER α mobilization and coregulator binding were favored while upon exposure to CD2314, a significant up-regulation of estrogen-responsive genes was found. However, none of the chemicals showed to regulate ER α by direct binding suggesting other pathways as underlying reasons. Altogether, although general and subtype-selective retinoids differently affected several ER α -related molecular events, there was no evident correlation with their distinct effects on breast cancer cell proliferation. Future studies should unravel the underlying mechanisms leading to the variable modulations of ER α signaling exerted by the distinct retinoids in the present work.

1 Introduction

Breast cancer cell proliferation can be modulated by nuclear receptors (NRs) such as Retinoic Acid Receptors (RARs) and Estrogen Receptor alpha (ER α) which are transcription factors that modulate gene transcription upon binding to specific ligands. To do so, these NRs bind to their respective Hormone Responsive Elements (HRE) within the genome and to different coregulators (coactivators and corepressors) ultimately influencing gene expression [1]. *In vitro* studies have shown that while endogenous retinoids (RAR ligands) such as *all-trans* retinoic acid (AtRA) have been demonstrated to diminish proliferation rates, natural estrogens (ER α ligands) such as estradiol (E₂) have shown to favour growth of breast cancer cells [2].

There are three different RAR subtypes, namely RAR α , RAR β and RAR γ . RAR α and RAR γ expression levels are relatively high in breast cancer cell lines while RAR β expression is remarkably lower [3]. RAR subtypes have rather similar amino acid sequences [4] and have been shown to bind highly concordant DNA regions suggesting functional redundancy [5,6]. Despite this well-known structural and mechanistic resemblance, the different RAR subtypes and the treatment with their respective selective ligands have been shown to exert different responses on breast cancer cell growth depending on aspects such as concentration of ligand tested or the cellular context [2,3,7–10]. While RAR α agonists can decrease breast cancer growth, RAR β and RAR γ agonists have been shown to exert weak or no effects on the proliferative rates of breast cancer cells [10]. Apart from the classical induction of pro-apoptotic/anti-proliferative RAR-RARE-mediated genes [11,12], several studies have reported that molecular events not related to the RAR genomic pathway may play an important role in the final response to retinoids [13]. In fact, previous research has shown that retinoids can also affect many other pathways (RAR-mediated or not) which can lead to varied effects on proliferation [13]. For instance, retinoid-mediated effects on the functioning of other NRs that modulate cell proliferation such as ER α , has been proposed [5,14].

The effectiveness of RAR ligands in breast cancer treatment has been linked to the coexistence of ER α suggesting coupling between their respective signaling pathways [3]. In fact, previous studies have shown evidence of crosstalk between ER α and RARs. Thus, the inhibition of E₂-induced ER α activity by RAR agonists [15], the possible formation of ER α -RAR dimers [16], the existence of close/overlapping DNA binding regions for both receptors [5] or the simultaneous presence in the same transcriptional complex under estrogen conditions [14] are some of the findings that support this interrelationship. However, the underlying molecular mechanisms leading to the modulation of ER α activity by RAR-ligands are still not completely understood [17]. In a previous study we have showed that apart from the well-described antagonistic effect on E₂-induced ER α signaling, the general RAR agonist AtRA was capable of modulating basal activity of ER α (in the absence of any estrogenic stimulation) [18]. However, AtRA is a ligand that activates all RAR subtypes and can exert genomic and non-genomic effects via RAR- or non-RAR-mediated mechanisms [13]. Thus, it is yet not known whether RAR subtype-selective retinoids may affect basal ER α signaling and studies assessing their possible specific effects on hormone(E₂)-independent ER α signaling are required.

The aim of this study was to determine the effects driven by the general agonist AtRA and the subtype-selective agonists Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ) on ER α -related endpoints in order to test the hypothesis stating that the different effects on breast cancer cell proliferation observed upon exposure to distinct RAR subtype-selective ligands, may be partially due to a differential secondary modulation of the basal ER α pro-proliferative pathway. To this end, we used the highly E₂-responsive breast cancer MCF7/BUS cell subline and the U2OS-ER α -Luc reporter gene cell line to characterize the effects of the respective general and subtype specific RAR agonists on basal ER α -related events such as proliferation, gene expression, coregulator binding and subcellular receptor localization.

2 Materials and Methods

2.1 Chemicals

The general RAR-agonist *all-trans* Retinoic Acid (AtRA) and the ER α agonist 17 β -estradiol (E₂) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl] benzoic acid (Am80) was obtained from Abcam (Cambridge, UK). 5-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-3-thiophenecarboxylic acid (CD2314) and 3-fluoro-4-[[2-hydroxy-2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)acetyl]amino]-benzoic acid (BMS961) were purchased from Tocris Bioscience (Bristol, UK). Dimethylsulphoxide (DMSO, 99.9%) was obtained from Acros (Geel, Belgium).

2.2 Cell culture procedures

The present study made use of the human breast cancer MCF7/BUS subline provided by Rikilt Institute (Wageningen, The Netherlands) and the reporter (luciferase-based) cell line U2OS-ER α -Luc provided by BioDetection Systems BV (BDS) (Amsterdam, The Netherlands). Cells were grown in 150 cm² flasks using Dulbecco's Modified Eagle Medium DMEM/F12 (Gibco, Bleiswijk, The Netherlands) supplemented with 10% (v/v) FBS (PAA, Pasching, Austria) and were maintained in a humidified atmosphere at 37°C and 5% CO₂. Cells were passaged or used for the experiments when they reached 60-80% confluence. For U2OS-ER α -luc, Geneticine (G418) (Gibco, Bleiswijk, The Netherlands) was utilized for selection purposes. To strip the cells from any hormonal stimulation all assays were carried out using Dulbecco's Modified Eagle Medium (DMEM/F12) without phenol red (Gibco, Bleiswijk, The Netherlands) supplemented with 5% charcoal-dextran-treated serum foetal calf serum (Hyclone, Logan, USA). In all assays, medium containing retinoids (10⁻⁹-10⁻⁴ M), or E₂ (10⁻¹³-10⁻⁶ M) was freshly made with a maximum final concentration of solvent of 0.2% DMSO (unless otherwise stated).

2.3 MTT-Proliferation studies

MCF-7/BUS cells were seeded in flat-transparent 96-well plates (Corning, NY, USA) at a cell density per well of 1×10^4 (2-day exposure studies) and 2×10^3 cells (6-day exposure studies). 24h later, cells were exposed to the test compounds. After exposure during 2 or 6 days, the MTT proliferation assay was performed. To do so, cells were incubated for the last three hours of the exposure to the test compounds with tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, Chemie B.V., Zwijndrecht, the Netherlands). Next, after removal of the medium, 150 μ l of DMSO was added into each well. Absorbance of the dissolved formazan product was measured after 15 minutes incubation, at the wavelength of 562 nm with a Microplate Reader SpectraMax M2 (Molecular Devices, Sunnyvale CA, USA).

2.4 Reporter gene studies

U2OS-ER α -luc cells were seeded at a cell density of 1×10^4 cells/well in 96-well white opaque plates (PerkinElmer, Groningen, The Netherlands). After 24 hours, medium was renewed. On the second day, exposure was performed. After 24 h of exposure, cells were washed with half-diluted (water) Phosphate-Buffered Saline (PBS) (Gibco, Bleiswijk, The Netherlands). Next, cells were lysed with 30 μ l of hypotonic low-salt buffer (pH 7.8) containing 10 mM Tris (Sigma-Aldrich, St. Louis, MO, USA), 2 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM 1,2-diaminocyclohexanetetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA). After cooling down, the plates were stored at -80 °C for at least 6 hours. To assess luciferase expression, plates were thawed and luciferase activity (light) was measured by using a luminometer (GloMax, Promega Corporation, USA) after addition of 100 μ l of flashmix (pH 7.8) containing 20 mM tricine (Sigma-Aldrich, St. Louis, MO, USA), 1.07 mM (MgCO₃) 4Mg(OH)₂ 5H₂O (Aldrich, Milwaukee, WI, USA), 2.6 mM magnesium sulfate (MgSO₄, Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM ethylenediaminetetraacetic acid (EDTA 2H₂O, Merck, Darmstadt, Germany), 2 mM DTT, 0.47 mM

D-luciferin (Duchefa Biochemie, Haarlem, the Netherlands) and 5 mM adenosine triphosphate (ATP, Duchefa Biochemie, Haarlem, the Netherlands).

2.5 Gene expression (RT-qPCR) studies

For the RT-qPCR studies MCF-7/BUS cells were hormone-deprived in culture flasks during 3 days before exposure to the chemicals. Cells were seeded in 6-well plates (Corning, NY, USA) at a cell density of 6×10^5 cells per well. 24 hours later exposure was carried out by adding the test compounds. After 6h-exposure cells were lysed with RLT Lysis buffer (Qiagen, Venlo, The Netherlands). Next, isolation of RNA by using QIAshredder and RNeasy kits (Qiagen, Venlo, The Netherlands) was carried out according to the manufacturer's instructions. Quality of RNA was assessed spectrophotometrically by using a Nanodrop (ND-1000 Thermoscientific Wilmington, Delaware, USA). Total RNA was transformed in cDNA by using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). Expression of the genes *TFF1* (trefoil factor-1), *PKIB* (cAMP-dependent protein kinase inhibitor- β), *ER α* , and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) (housekeeping gene) was obtained by real-time PCR using Rotor-Gene SYBR[®] Green Kit (Qiagen, Venlo, The Netherlands) and the Rotor-Gene 6000 cycler (Qiagen, Venlo, The Netherlands) according to the manufacturer's guidelines. For this purpose, QuantiTect Primer Assay Hs_TFF1_1_SG, Hs_PKIB_1_SG, Hs_ESR1_1_SG and Hs_GAPDH_1_SG (Qiagen, Venlo, The Netherlands) were used.

2.6 Coregulator binding assays

To assess the potential effects of the respective chemicals on ER α coregulator binding patterns, the Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) was utilized. These experiments were carried out making use of partly purified recombinant His-tagged ER α -LBD (from *Escherichia coli*), or full length ER α present in MCF7/BUS lysates. For all assays, ligand concentrations with a maximum final concentrations of 2% DMSO (solvent) were used. Samples with

the same concentrations of DMSO without the respective test compounds, were utilized as solvent controls.

For the assays using recombinant proteins, samples were prepared in time-resolved fluorescence resonance energy transfer (TR-FRET) reaction buffer E (Life technologies, Ltd, UK, #PV4420) supplemented with 5 mM DTT (Sigma, Zwijndrecht, the Netherlands, #43819) and maintained on ice until the initiation of the assay. For these experiments using ER α -LBD partly purified from *E. coli*, mixtures containing 25 nM histidine (His)-tagged human ER α -LBD, 50 nM Alexa 488-conjugated anti-His (Qiagen, Venlo, The Netherlands) and the respective ligands were prepared. For the MCF7/BUS lysates assays, post-lysis treatment was carried out. In brief, cells were hormone-deprived in 150 cm² flasks for 72 hours. After trypsinization, assay medium was added to neutralize the remaining trypsin. Cells were spun down by centrifugation and medium (supernatant) was removed. Next, cells were washed with PBS and clean cell pellets were obtained by an additional centrifugation and removal of the remaining PBS (supernatant). Cell pellets were snap-frozen by direct immersion in liquid nitrogen and kept at -80°C. Next, lysis was carried out in TNRL01 lysis buffer using a dismembrator as previously reported [19] and the samples were maintained frozen at -80°C. For the coregulator binding assay, the lysate mixtures were made in 2xNR buffer containing 10 nM anti ER- α (D547) (Santa Cruz Biotechnology, CA, USA), 8 nM goat anti-Rat IgG2a (FITC) (Axxora, CA, USA), 2 μ M DTT (Sigma, Zwijndrecht, the Netherlands, #43819) and the respective ligands.

Coregulator binding profiles were determined by using PamChip[®] platforms (PamGene International B.V., 's-Hertogenbosch, the Netherlands) which contain 4 arrays each consisting of a three-dimensional porous metal oxide carrier containing 154 spots with different immobilized NR-binding motifs belonging to 65 coregulators. The automated platform PamStation[®] (PamGene International B.V., 's-Hertogenbosch, The Netherlands) and the EvolveHT software (PamGene International B.V., 's-Hertogenbosch, The Netherlands) were utilized applying 2 (up-down) pumping cycles per

minute at a temperature of 20°C. Before the initiation of the assay, incubation for 20 cycles with 25 µl blocking buffer composed of Tris buffered saline (Bio-Rad, Veenendaal, the Netherlands, #170-6435) supplemented with 1% BSA (Calbiochem, Merck, Darmstadt, Germany, #126609) and 0.01% Tween-20 (Bio-Rad, Veenendaal, the Netherlands, #170-6531) and 0.3% ELK (milk) blocking solution was performed to avoid unspecific binding. Next, samples were added to the Pamchip® arrays and incubated for 80 pumping cycles. For the MCF7/BUS cell lysate samples, an additional incubation for 40 cycles was performed. In the last step, unbound receptor was washed away with 25 µl Tris buffered saline and TIFF images were obtained by a charge coupled device camera (CCD) present in the PamStation®.

2.7 Determination of cytoplasmic and nuclear ERα levels

For the assessment of the cytoplasmic and nuclear ERα subcellular localization, MCF7/BUS cells were hormone-deprived in 150 cm² flasks (Corning, NY, USA) during 3 days prior to the exposure to the test compounds for 1 hour. After exposure, cells were washed with PBS and trypsinized. Next, neutralization of the trypsin was carried out by adding assay medium. After spinning down the cells at 2000 RCF for 10 minutes and discarding medium (supernatant), PBS was used following the same steps for washing purposes. The obtained cell pellet was processed in order to obtain nuclear and cytoplasmic fractions by using NE-PER Nuclear and cytoplasmic extraction reagents (ThermoFisher Scientific, Rockford Illinois, USA) according to the manufacturer's guidelines. Next, total protein was quantified by using the Pierce Bicinchoninic acid (BCA) protein assay (ThermoFisher Scientific, Rockford Illinois, USA) and samples were prepared in laemmli buffer (Bio-Rad, Veenendaal, The Netherlands) supplemented with dithiothreitol (DTT) (50 mM) (Sigma, Zwijndrecht, the Netherlands). After denaturation at 90°C for 5 minutes, 20 µl of the mixtures were loaded in 10% Mini-PROTEAN TGX™ Precast Protein Gels (Bio-Rad, Veenendaal, the Netherlands). Electrophoresis was run at 200 V for 40 minutes in Tris/Glycine/SDS Buffer (Bio-Rad, Veenendaal, the Netherlands). Proteins in the

gel were transferred to a nitrocellulose membrane (Bio-Rad, Veenendaal, the Netherlands) in Tris/Glycine Buffer containing 20% methanol (Biosolve, Valkenwaard, the Netherlands) at 70V for 75 minutes. Subsequently, a reversible Ponceau staining (Sigma, Zwijndrecht, the Netherlands) of the membrane was carried out to confirm proper blotting. Next, the membrane was exposed for 1 hour to Tris Buffered Saline (Bio-Rad, Veenendaal, the Netherlands) supplemented with Tween 20 (0.1%) (Bio-Rad, Veenendaal, the Netherlands) and Blotting Grade Blocker (50 g/l) (Bio-Rad, Veenendaal, the Netherlands) (blocking buffer) to avoid unspecific binding. Membranes were probed with the different antibodies which were diluted in blocking buffer according to the manufacturer's guidelines. First, membranes were incubated with the primary antibodies against ER α (sc-543), cofilin (sc-33779) and Lamin A/C (sc-20681) from Santa Cruz Biotechnologies (Santa Cruz Biotechnology, CA, USA); or against H3 Histone (ab176842) and GAPDH (ab128915) from Abcam (Abcam, Cambridge, UK). Membranes were washed with Tris Buffered Saline supplemented with Tween 20 (0.1%) and later incubated with the secondary goat-anti-rabbit IgG-HRP (sc-2004) (Santa Cruz Biotechnology, CA, USA) for 2 hours. After washing, Clarity Western ECL Substrate (Bio-Rad, Veenendaal, the Netherlands) was utilized for chemiluminescent detection. ER α subcellular levels were obtained by using Image Studio Lite (Li-COR) and ImageJ (National Institutes of Health; Bethesda, MD) softwares. Assessment of the purity of the respective pooled fractions (cytoplasmic or nuclear samples) was carried out making use of cytoplasmic (Cofilin and GAPDH) and nuclear (Histone H3 and Lamin A/C) specific markers.

3 Results.

3.1 Effects of retinoids on breast cancer cell proliferation.

The present study evaluated the concentration- (Figure 1A) and time- (Figure 1B) dependent effects of the general agonist AtRA and the subtype-selective agonists Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ) on MCF7/BUS cell proliferation. 10 nM Estradiol (E₂) was used as a positive control (known to induce proliferation in breast cancer cells). After a exposure of 6 days, all retinoids were able to diminish cell proliferation in a dose-dependent manner. When comparing retinoids, AtRA (general RAR agonist) and Am80 (RAR α -selective agonist) displayed the highest potency whilst the RAR γ -selective agonist BMS961 and especially the RAR β -selective agonist CD2314 showed lower EC₅₀ values (Figure 1A). Interestingly, when assessing these test compounds at a single concentrations of 10 uM and two different exposure periods, differential effects were observed (Figure 1B). During the first phase (after a 2-day exposure period), the number of viable MCF7/BUS cells was either not affected or slightly increased upon exposure to retinoids (especially for the case of CD2314 whose effects were similar to the ones generated by the pro-proliferative E₂). However, after a longer incubation period (6-day exposure studies), all RAR agonists showed a significant decrease in the MCF7/BUS cell proliferation and this effect was completely opposite when comparing to the remarkable proliferative response induced by the treatment with E₂. In summary, when comparing different exposure times at a single high concentration (10 uM) of retinoids, while no or slightly pro-proliferative effects were observed after a 2-day exposure period, anti-proliferative effects were generally seen in the 6-day proliferation studies. In addition, at this prolonged exposure, the concentration-response curves of the different test compounds pointed at the general RAR agonist ATRA and the RAR α -selective agonist Am80 as the most potent (lower EC₅₀) anti-proliferative agents in the MCF7/BUS breast cancer subline.

Figure 1. Effects of retinoids on the growth of MCF7/BUS cells at different concentrations (Figure 1A) and time points (Figure 1B). Cell proliferation is expressed relative to the solvent control (DMSO) set at 100%. Results represent the average $\pm$ SEM of at least three independent experiments. For statistical analysis, multiple paired t-tests between the respective treatments and the solvent control were carried out. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared to the solvent control.

3.2 Retinoid-driven effects on ER α activity in the U2OS-ER α -Luc reporter cell line

In order to test whether the retinoid-mediated effects shown in the proliferation studies could be partly attributed to the disruption or augmentation of ER α -activity, a reporter gene study using the U2OS-ER α -Luc cell line was performed (Figure 2). As a preliminary step, the cytotoxicity of the highest concentrations tested for all chemicals was assessed showing no remarkable effects on the viability of U2OS-ER α -Luc (results not shown). Figure 2 displays the effects of the retinoids on the ER α -mediated gene expression compared to E₂, which served as the positive control. The retinoids showed a biphasic effect when comparing low and high concentrations. In the low nanomolar range, all retinoids decreased ER α -driven expression of luciferase. However, as the dose reached the low micromolar range (1-10 μ M), AtRA, Am80 and more pronouncedly CD2314 significantly increased ER α -driven luciferase expression. In summary, our results showed that retinoids can modulate ER α activity exerting differential concentration-related effects under hormone (E₂)-free conditions. Thus, retinoids decreased ER α activity at the nanomolar range while increasing it at micromolar concentrations.

Figure 2. Effects of retinoids on basal ER α -mediated expression of the ERE-luciferase gene in U2OS-ER α -Luc cells. ER α -mediated luciferase expression after 24h-exposure is expressed relative to the solvent control (DMSO) set at 100%. Results represent the average $\pm$ SEM of at least three independent experiments. For statistical analysis, multiple paired t-tests between the respective treatments and the solvent control were carried out. ** $p < 0.01$; * $p < 0.05$ compared to the solvent control.

3.3 Assessment of the retinoid-driven modulation of the expression of ER α -related genes in the MCF7/BUS cell line.

Aiming at confirming whether retinoids at the micromolar range (10 μ M) can also favour expression of ER α mediated genes in our breast cancer cell model (MCF7/BUS), RT-qPCR studies were performed. Since nuclear receptor signaling is relatively rapid (few hours) and in order to avoid possible secondary (late) effects appearing due to long term exposure, a short treatment for 6 hours was carried out. Figure 3 depicts the effects of all retinoids on the expression of two estrogen-responsive genes known to be ER α -direct targets and play crucial roles in breast cancer cell proliferation (*TFF1* and *PKIB*) [5,14]. These genes were also chosen because previous research showed that their E₂-induced expression can be hampered by general retinoids such as AtRA [14,18]. Furthermore, the hypothesis of a potential up-/down-regulation of the *ER α* gene upon retinoid treatment was also tested (Figure 3). As foreseen, 10 nM E₂ clearly induced the transcription of the known estrogen-responsive *TFF1* and *PKIB* genes. Interestingly, incubation with all retinoids elicited up-regulation of the expression of *TFF1* especially for the RAR β -selective agonist CD2314. On the contrary, the *PKIB* gene was only up-regulated upon exposure to CD2314 while being not affected or slightly down-regulated upon exposure to the other retinoids. Thus, RAR-ligands at high concentrations showed to be capable of differentially modulating ER α -mediated transcription in a gene-specific manner. Finally, the results showed no retinoid-mediated effects on ER α expression discarding a possible increase/decrease on ER α levels as underlying mechanism of action involved in the interplay between estrogen and retinoid signaling.

Figure 3. Effects of retinoids (10 μ M) and E₂ (10 nM) on ER α -mediated transcription of *TFF1* and *PKIB* in the breast cancer cell line MCF7/BUS. In addition, effects of the retinoids (10 μ M) on the expression of the *ERα* gene itself were also tested. Expression of the genes of interest was assessed after 6h-exposure. The respective gene expression modulation is expressed relative (fold-induction) to the solvent control (DMSO) set at 1. Results represent the average $\pm$ SEM of at least three independent experiments. For statistical analysis, multiple paired t-tests between the treatments and the solvent control were carried out. * $p < 0.05$ compared to the solvent control.

3.4 Evaluation of the effect of retinoids on ER α coregulator binding profiles in the MCF7/BUS cell lysates

In order to assess whether the retinoid mediated effects on ER α activity may be due to direct ligand-receptor binding of the RAR agonists to the ER α , a coregulator binding assay was performed (Figure 4) using full length ER α obtained from MCF7/BUS lysates (Figure 4A) and partially purified recombinant ER α -LBD (Figure 4B). To this end, coregulator binding profiles generated with both forms of ER α upon exposure to E₂ (positive control) and the respective general and subtype-selective retinoids were analyzed and compared. Coregulator binding patterns generated by the test compounds are represented as modulation indexes (MI). Positive or negative MIs represent increase or decrease in coregulator motif binding in relation to the solvent control (MI = 0), respectively.

As one could foresee, estradiol increased coactivator binding to ER α when using both lysate samples and the ER α -LBD. When assessing the effects upon incubation with the RAR-subtype selective ligands, no clear modulation of ER α -coregulator binding was observed in any of the samples (lysates or isolated ER α -LBD). Instead of this, coregulator binding to ER α was consistently increased upon exposure of the lysate samples to the general RAR agonist AtRA, although to a lower extent than with E₂. However, the results showed no modulation by AtRA of the isolated ER α -LBD coregulator binding profiles suggesting no direct (ligand-receptor) regulation. In summary, the results principally indicate that retinoids cannot directly bind and activate the ER α -LBD since no appreciable modulation on coregulator binding signatures was observed when testing the RAR ligands with the isolated ER α -LBD.

3.5 Determination of cytoplasmic and nuclear ER α levels

The present work further tested potential effects of the different retinoids on the subcellular localization of ER α which may provide additional understanding on the previously observed effects of retinoids on ER α signaling. Figure 5 depicts nuclear and cytoplasmic levels of ER α upon exposure of 1 hour to 10 μ M of the respective general and subtype-selective retinoids. As expected, E₂ was capable of rapidly decreasing cytoplasmic ER α levels while increasing their presence in the nucleus. Compared to the solvent control, the general RAR agonist AtRA elicited a significant decrease in cytoplasmic ER α levels while increasing their presence in the nuclear compartment in a similar way although at a lower intensity than E₂. On the contrary, when assessing the results obtained upon exposure to all RAR subtype-selective retinoids, only variable (BMS961) or weak (Am80 and CD2314) effects on ER α subcellular localization were generally observed without showing any clear pattern of ER α translocation to or retention in the nuclear compartment.

Figure 5. Effects of retinoids on the subcellular localization of ER α upon exposure of 1 hour to 10 μ M of both general and subtype-selective retinoids. After fractionation, cytoplasmic and nuclear levels of ER α were assessed by western blot. Semi-quantitative determination of ER α levels was performed correcting by the housekeeping proteins Cofilin (cytoplasmic fraction) and Histone H3 (nuclear fraction). Results are shown as percentage of the solvent control (Avg $\pm$ SEM) set at 100. Blots are representative of at least four independent experiments (A). Purity of the respective samples was confirmed by using Cofilin/GAPDH (Cytoplasmic) and Histone H3/Lamin A/C (nuclear) as fractionation controls (B). For statistical analysis multiple paired t-tests between the treatments and the solvent control were carried out. *** $p < 0.001$ compared to the solvent control.

4 Discussion

Basal proliferation of breast cancer cells has been reported to be differentially modulated upon exposure to different RAR subtype-selective agonists [3,15,20]. However, the underlying molecular mechanisms by which these specific retinoids exert their distinct actions are not completely known. The present study tested the hypothesis stating that the differential effects observed upon exposure to different RAR-subtype selective ligands may be partially due to a secondary modulation of the ER α pro-proliferative pathway. Thus, the aim of the present work was to investigate possible effects of general and subtype-selective retinoids on estrogen-independent ER α activity in order to acquire further understanding on the crosstalk between estrogen and retinoid signaling. To do so, a battery of

different assays testing several endpoints related to ER α signaling such as cell proliferation, ER α -mediated gene expression, ER α coregulator binding and ER α subcellular localization were carried out.

In the present study we first evaluated the effects of retinoids on the basal proliferation of the breast cancer cell line MCF7/BUS. After a 6-day exposure, the RAR α -selective agonist Am80 showed to be the most potent retinoid (lowest EC₅₀) showing anti-proliferative effects at concentrations in the nanomolar range. On the contrary, the RAR β - and RAR γ -selective retinoids CD2314 and BMS961 showed mild anti-proliferative effects and were effective only at the highest concentrations tested (1-10 μ M). These results are in agreement with previous research showing that RAR α -selective ligands are the most effective anti-proliferative retinoids while RAR β and RAR γ agonists are weaker anticancer agents [10]. The effects on proliferation observed for retinoids such as CD2314 may be partly explained by the lower expression levels of its target receptor (RAR β) [3]. However, other molecular mechanisms (e.g. effects on the pro-proliferative ER α signaling) may additionally drive such response. Interestingly, when comparing effects of all retinoids in both the short 2-day and the prolonged 6-day exposure studies a different trend was observed. Thus, while all ligands tested at concentrations of 10 μ M could significantly inhibit breast cancer cell proliferation after 6-day exposure, these compounds elicited no effect or even slightly pro-proliferative behaviour (although not significant) in the early stage after treatment (2-day exposure). In accordance with these results, previous studies have found that the inhibitory effects of retinoids in breast cancer growth can normally be observed after prolonged exposure (from three days onwards) [21].

As a first step to test our hypothesis stating that the differential effects on the basal breast cancer proliferation provoked by the distinct subtype-selective retinoids may partially occur due to a possible differential modulation of ER α -signaling, we carried out studies focusing on ER α -mediated gene expression. For this purpose, both a reporter gene cell line (U2OS-ER α -Luc) and our breast cancer cell model (MCF7/BUS) were utilized. Using other reporter cell systems, inhibition of estradiol-induced ER α activity by general RAR agonists has been described [15]. Under estradiol-free conditions, our

study clearly showed differential effects of the general and subtype-selective retinoids on ER α -mediated luciferase expression which depended on the concentration tested. While low concentrations of all chemicals (nanomolar range) were capable of decreasing ER α -activity, AtRA, Am80 and especially CD2314 were able to significantly favour the ER α -mediated expression of luciferase when used at micromolar concentrations. Previous research showed that RARs can bind to estrogen responsive elements (EREs) what will depend on both the levels of receptor and ligand and the specific nature of the ERE [22]. Thus, the retinoid-induced luciferase expression in the U2OS-ER α -Luc reporter cell line could be explained by direct binding of liganded RARs to EREs. In this context, it has to be noted that both the unique nature of the reporter ERE-Luc sequence and the potential different levels of the respective RAR variants in U2OS made necessary the confirmation of relevant gene expression in the breast cancer MCF7/BUS cell model. Therefore in a follow-up study, we assessed the expression of the ER α -mediated *TFF1* and *PKIB* genes which has been associated with regulation of proliferation in previous research [23,24]. The election of these specific genes was mainly based on previous studies which reported that ER α and RARs can be present in the promotor region of *TFF1* while *PKIB* is a direct binding target for only ER α . Firstly, our results showed that in the absence of E₂, all retinoids increased the expression of *TFF1*, with CD2314 being the highest inducer. CD2314 was also capable of significantly up-regulating the basal *PKIB* expression. Previous studies have shown the capacity of general RAR agonists such as AtRA to affect ER α -mediated expression of genes sharing binding regions with RARs in the genome [5,14,18]. However, to the best of our knowledge, effects on the basal ER α -driven gene expression elicited by retinoids such as CD2314 in both, genes known to harbor or lack RARs in their promoter regions, have not been described before.

One of the modes of action possibly explaining the retinoid-mediated effects on the basal ER α -mediated proliferation or transcription could be the regulation of the *ER α* gene itself. Previous studies have shown that basal proliferation of breast cancer cells is also supported by apo-ER α activity [25] so that chemicals favouring or hampering the expression of this nuclear receptor may modulate the

eventual biological outcome. The present work clearly showed that neither the general agonist AtRA nor the respective subtype-selective retinoids Am80 (RAR α agonist), CD2314 (RAR β agonist) and BMS961 (RAR γ agonist) can affect the expression of ER α . In consequence, the possibility of up- or down-regulation of ER α as underlying molecular mechanism leading to the observed effects on the expression of ER α -driven genes and the consequent proliferation of breast cancer cells seems unlikely.

In order to test whether the observed retinoid-mediated effects on ER α -mediated gene transcription at micromolar concentrations may be due to regulation of ER α activity by direct ligand-receptor binding, we performed ER α coregulator binding assays with both MCF7/BUS lysates and the partly purified recombinant ER α -LBD. Our results with the isolated protein suggested that none of the retinoids are able to modulate ER α by direct binding to the receptor. In accordance to our previous research [18], the experiments performed with lysates containing the full length ER α showed that incubation with AtRA consistently elevated coactivator binding. The effect, although less potent, was similar to the response generated by E₂. Since this specific effect was not seen upon exposure to the respective subtype-selective ligands, it is suggested that processes which are not RAR-mediated may be occurring. We can speculate that in a lysate sample where many other cellular components could drive the observed response, dimers/complexes between ER α and other (no RARs) proteins to which AtRA can bind with high affinity (e.g. CRABP II) may explain the up-regulation of the coregulator binding signature of ER α . In fact, apart from its well-known function as an AtRA transporter, it has been shown that CRABP II can act as a ligand-dependent protein capable of binding other transcriptional cofactors [26]. The possible implication of AtRA-related proteins such as CRABP II in the formation of active ER α transcriptional complexes remains to be elucidated.

Once we showed that retinoids can differentially affect ER α -related endpoints such as cell proliferation, gene expression or its coregulator binding profiles (e.g. AtRA), we also assessed whether retinoids can affect the subcellular localization of ER α . Among all retinoids, AtRA was the only chemical that although at a lower extent than E₂, was capable of significantly decreasing cytoplasmic

levels of ER α while increasing its nuclear localization. It is known that ER α experiences a continuous nucleocytoplasmic shuttle and exposure to E₂ can cause changes on the usual shuttle of ER α such as translocation to or retention in the nucleus [27,28]. The results of the present work point at AtRA as the only retinoid provoking these changes on ER α subcellular distribution showing variable or weak effects upon incubation with the respective RAR subtype-selective agonists. Consequently, we anticipate that the AtRA-driven observed effects on this process may be guided by non-RAR-mediated events. Firstly, effects of AtRA on other molecular pathways or elements known to play a role in the eventual subcellular localization of ER α such as kinase pathways [29,30] or ER α Nucleocytoplasmic transport proteins [31] should be assessed in follow-up studies. Secondly, possible AtRA-driven molecular events leading to a final degradation of cytoplasmic ER α or accumulation in other localizations different to the nuclear and cytoplasmic compartments should be also considered. Furthermore, as mentioned earlier other proteins which are known to experience an AtRA-driven migration from the cytoplasm to the nucleus (e.g. CRABP II) may form complexes with ER α and subsequently serve as transporters of ER α to the nucleus.

In summary, the results of this study showed that parameters such as proliferation, ER α -mediated gene expression, ER α -coregulator binding and ER α subcellular localization can be differentially affected by RAR agonists. The general RAR-agonist AtRA and the RAR β agonist CD2314 showed to exert the most pronounced effects. After AtRA treatment, ER α mobilization and coregulator binding were favoured in contrast to the lower or no effects observed upon exposure to the subtype-selective retinoids. Thus, we speculate that the increase on ER α -mediated gene expression observed after exposure with AtRA may be partially related to a possible association (complexes) of ER α with proteins known to specifically respond to this retinoid in terms of nuclear translocation or coregulator binding (e.g. CRABP II). Concerning CD2314, while it was found a significant up-regulation of ER-target genes known to harbor (*TFF1*) or lack (*PKIB*) RARs in their promotor regions, only weak nuclear translocation and no increase on coregulator binding of ER α were observed. Thus, effects on

other hormone-independent pathways able to activate ER α (e.g. kinase pathways) or ER α -related transcription factors (e.g. AP-1) should be investigated in future research. Altogether, the present study showed highly variable retinoid-mediated effects on ER α signaling which depended on the compound or the endpoint tested. However, no evident connection between the effects of general and subtype-selective retinoids on ER α signaling and proliferation was observed. Future research should further elucidate the underlying mechanisms driving the different modulations of ER α activity shown by the distinct retinoids in this study in order to understand and consequently develop better retinoid-based anticancer therapies.

Conflicts of Interest

The authors declare no conflicts of interest.

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Chapter 6

**Insights in the interference exerted by retinoic acid signaling on the
estradiol-induced proliferation and transcriptomic signatures of the T47D
breast cancer cell line**

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In preparation

Abstract

Previous studies have reported interplay between estrogen and retinoid signaling in the context of breast cancer growth. However, the molecular events involved in this crosstalk are not completely characterized. Additionally, most of these studies were performed using the ER⁺ MCF7 breast cancer cell line while information on the retinoid-driven interference on estrogen signaling in the other widely used ER⁺ breast cancer cell line T47D is lacking. The aim of this study was to evaluate the effects of the general retinoic acid receptor (RAR) agonist *all-trans* retinoic acid (AtRA) and the estrogen receptor (ER) agonist estradiol (E₂), alone or in combination, on the proliferation and transcriptomic profiles of T47D cells. To this end, the effects on cell proliferation and gene expression were characterized. The results show that incubation with AtRA reduced the basal and E₂-induced proliferation in T47D cells. Next, gene expression studies were performed to assess the possible molecular interference between retinoid and estrogen signaling. The results using reporter gene cell lines revealed that AtRA decreased E₂-induced ER α - and ER β - mediated transcription of luciferase while exposure to AtRA alone increased the gene expression of both ERs. In line with it, the RNA-seq study confirmed the induction of many estrogen-responsive genes by the single administration of AtRA. Further analysis showed that the modulation of the E₂-induced transcriptome upon co-treatment with AtRA appeared in both directions (inhibition/enhancement). However, inhibiting/counteracting events were more frequent in genes involved in breast cancer growth. Thus, this study showed that the differential effects of AtRA on E₂ signaling are highly gene-dependent. However, the higher prevalence of inhibiting/counteracting patterns mediated by AtRA on estrogen-responsive genes related to proliferation, differentiation and apoptosis may explain its hampering effects on E₂-induced breast cancer growth.

1 Introduction

Estrogen Receptors (ERs), as all nuclear receptors (NRs), are ligand-induced transcription factors binding to specific sequences in the DNA called Estrogen Responsive Elements (EREs) while attracting coregulators and other transcription enzymes necessary for modulating gene expression. In relation to breast cancer growth and progression, ERs and their natural cognate ligands such as 17 β -estradiol (E₂) have been reported to play a crucial role. There are two types of ERs, namely ER α and ER β which have been shown to have pro-proliferative and anti-proliferative effects on breast cancer cells, respectively [1]. Breast cancer cell proliferation induced by estrogens can be counteracted by the use of ER antagonists. However, many other signaling pathways have been shown to also counteract the pro-proliferative estrogen signaling. For instance, treatment with retinoids such as *all-trans* retinoic acid (AtRA) has been shown to decrease the E₂-induced breast cancer proliferative rates [2,3]. AtRA mainly exerts its actions via binding to the Retinoic Acid Receptor (RAR) alpha, beta or gamma (α , β and γ) although plenty of non-RAR-mediated effects have also been described [4]. Interplay between estrogen and retinoid signaling was initially suggested when several studies found that the effects of retinoids can greatly differ depending on the presence of ERs in breast cancer cells [5]. Thus, retinoids were demonstrated to decrease proliferation of ER⁺ breast cancer cells whereas no effect was generally detected for ER⁻ breast cancer cells [5–7]. In addition, evidence of crosstalk between estradiol (ER) and retinoid (RAR) signaling has been reported at different levels such as protein-protein interactions between ERs and RARs, competition between these receptors for overlapping DNA binding regions or as showed by the effects of retinoids on different ER-related processes like ER α coregulator binding or subcellular mobilization [2,8,9,3]. However, the molecular events underlying such interplay are not fully understood yet.

When focussing on the specific repercussions of retinoid signaling on the E₂-induced (activated) ER pathway, retinoid-driven inhibition of estradiol-induced ER α -mediated transcription and proliferation in breast cancer cells has been reported [2,10]. Interestingly, Ross Innes et al., 2010 discovered a dual

role of RARs being both promoter and repressor of ER α -mediated transcription and proliferation depending on the ligand conditions [2]. Thus, while apo-RAR α proved to be necessary for ER α -driven gene expression (acting as scaffold protein within the ER transcriptional complex), AtRA-bound RAR α remarkably decreased such transcription [2]. Up till now, most of these mechanistic studies were carried out using the MCF7 cell line as an ER $^{+}$ breast cancer model [2,9]. However, recent studies showed significantly different proteomic profiles in this cell line when compared to other widely used ER $^{+}$ cell lines such as the T47D cell line, especially in proteins which affect biological processes related to breast cancer growth [11]. In addition, transcriptomic studies characterizing in detail the influence of retinoids on estrogen-induced signaling and its relationship with the potential retinoid-driven effects on the E $_2$ -induced proliferation in the T47D breast cancer cell line are lacking.

The main objective of the present study was to investigate the effects of the general RAR agonist AtRA on the proliferative and transcriptomic patterns induced upon treatment with E $_2$ and AtRA alone and in combination in the ER $^{+}$ T47D breast cancer cell line. To do so, methods evaluating the effects on cell proliferation such as the MTT and BrdU assays and techniques assessing E $_2$ -driven gene expression in the T47D cell line such as RNA-seq and RT-qPCR were utilized. In addition, reporter gene studies using the T47D-ER α -Luc and U2OS-ER β -Luc which allow to determine the specific effects on each of the two types of ER (ER α and ER β) were carried out.

2 Materials and Methods

2.1 Chemicals

The general ER agonist 17- β -estradiol (E $_2$) and the general RAR agonist *all-trans* retinoic acid (AtRA) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Dimethylsulphoxide (DMSO, 99.9%) was obtained from Acros (Geel, Belgium).

2.2 Cell lines and cell culture conditions

The human breast cancer cell line T47D (ATCC, Manassas, VA) and the reporter cell lines T47D-ER α -Luc [12] and U2OS-ER β -Luc (provided by BDS, Amsterdam, The Netherlands) were used for the experiments. Cells were maintained in 150 cm² flasks (Corning, NY, USA) in Dulbecco's Modified Eagle Medium DMEM/F12 (Gibco, Bleiswijk, The Netherlands) supplemented with 10% (v/v) foetal bovine serum provided by Sigma-Aldrich (St Louis, MO, USA). Cells were kept in a humidified atmosphere at 37°C and 5% CO₂ and subcultured when they reached 60-80% confluence. For the U2OS reporter cells, 0.2 mg/ml geneticine (G418) (Gibco, Bleiswijk, The Netherlands) was utilized as selection agent. In order to avoid estrogenicity from the assay medium, all experiments were performed using Dulbecco's Modified Eagle Medium (DMEM/F12) without phenol red (Gibco, Bleiswijk, The Netherlands) supplemented with 5% charcoal-dextran-treated foetal calf serum provided by Gibco (Bleiswijk, The Netherlands). For all assays, exposure medium containing E₂ (0.1-10 nM) and AtRA (1 μ M), alone or in combination, was freshly prepared with a maximum final concentration of 0.2% DMSO. The same concentration of DMSO (0.2%) was used for comparison as a solvent control.

2.3 MTT and BrdU proliferation studies

T47D cells were seeded in flat-transparent 96-well plates (Corning, NY, USA) with assay medium at a cell density of 5 x 10³ cells/well (72-hour BrdU proliferation study) or 3 x 10³ cells/well (6-day MTT proliferation study). Cells were allowed to attach and 24 hours later exposed to the test compounds. Exposure medium containing the test compounds was replenished after 72h for the 6-day exposure study. For the MTT assay, the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) was diluted in exposure medium (10% v/v) and added to the wells for the last three hours of exposure. After removal of the medium containing the MTT dye, 150 μ l of DMSO was added to the wells for dissolving the formazan crystals. Absorbance was determined after 15 minutes incubation, at a

wavelength of 562 nm with a Microplate Reader SpectraMax M2 (Molecular Devices, Sunnyvale CA, USA). For the BrdU assay (Roche, Mannheim, Germany), BrdU labelling solution diluted in exposure medium (10% v/v) was added to the cells during the last 4 hours of exposure. Next, BrdU incorporation was determined by fixation-denaturation of the cells followed by incubation with BrdU detection antibodies and the corresponding substrate following manufacturer's instructions (Roche, Mannheim, Germany). Colorimetric measurements were carried out at the wavelength of 370 nm with a Microplate Reader SpectraMax M2 (Molecular Devices, Sunnyvale CA, USA).

2.4 ER reporter gene studies

Cells were seeded in 96-well white opaque plates (PerkinElmer, Groningen, The Netherlands) at a cell density of 1×10^4 cells/well (T47D-ER α -Luc) or 7.5×10^3 cells/well (U2OS-ER β -Luc). On the next day, assay medium was renewed. After 48h post-seeding, exposure was carried out by adding the respective chemicals. After 24 h of exposure, cells were washed with half-diluted (H₂O) Phosphate-Buffered Saline (PBS) (Gibco, Bleiswijk, The Netherlands) and lysed with 30 μ l of hypotonic low-salt buffer (pH 7.8) containing 10 mM Tris (Sigma-Aldrich, Zwijndrecht, The Netherlands), 2 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM 1,2-diaminocyclohexanetetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA). Plates were cooled down and kept at -80 °C until analysis. To measure luciferase activity, plates were thawed and the light emission generated due to luciferase activity was measured by using a luminometer (GloMax, Promega Corporation, USA) after addition of 100 μ l of flashmix (pH 7.8) composed of 20 mM tricine (Sigma-Aldrich, St. Louis, MO, USA), 1.07 mM (MgCO₃) 4Mg(OH)₂·5H₂O (Aldrich, Milwaukee, WI, USA), 2.6 mM magnesium sulfate (MgSO₄, Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM ethylenediaminetetraacetic acid (EDTA·2H₂O, Merck, Darmstadt, Germany), 2 mM dithiothreitol (DTT) (Sigma, Zwijndrecht, The Netherlands, #43819), 0.47 mM D-luciferin (Duchefa Biochemie, Haarlem, The Netherlands) and 5 mM adenosine triphosphate (ATP, Duchefa Biochemie, Haarlem, The Netherlands).

2.5 Next-generation sequencing (RNA-seq) and transcriptome analysis.

T47D cells were maintained in 25 cm² flasks (Corning, NY, USA) and when they reached 50-60% confluence, growth medium was replaced by hormone-free medium. After 24 hours of hormone deprivation, treatment with the respective chemicals for a period of 6 hours was carried out. Next, cell lysis and extraction of total RNA was performed using the Quick RNA Miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. To ensure quality of the samples, spectrophotometric analysis using a Nanodrop (ND-1000 Thermoscientific Wilmington, Delaware, USA) and determination of the RNA Integrity number (RIN) using the 2100 Bioanalyzer (Agilent Technologies California, EE. UU) were carried out. Only samples with RIN values higher than 8 were accepted for the study.

RNA-seq library preparation and sequencing was performed by BaseClear BV (Leiden, the Netherlands). The mRNA was purified by polyA capture, fragmented and converted to cDNA which was amplified by PCR. Illumina libraries were created, clustered and sequenced on the Illumina HiSeq2500 with a single-end sequencing protocol (10M Reads). Colorimetric sequencing signals were translated into base calls by means of an internal Illumina software (CASAVA). Next, making use of the tool bcl2fastq2 (version 2.18), the per-cycle basecall (BCL) files were demultiplexed and transformed into per-read FASTQ sequence files for downstream analysis. Subsequently, reads containing PhiX control signal were removed by BaseClear BV using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to minimum read length of 50bp). Finally, the quality of the FASTQ sequences was evaluated by the tool FastQC [13] (version 0.11.5), and enhanced by trimming off low-quality bases by setting the cut-off of the error probability limit of the modified-Mott algorithm [14] to 0.02. The RNA-seq reads were later used to quantify transcript abundances. The tool Salmon [15] (version 0.8.2) was used to map the reads to the GRCh38.p10 genome assembly-based transcriptome sequences as annotated by the Ensembl genome database project [16] (Ensembl release v90). The obtained transcript abundance estimates and lengths were then

imported in R by the package tximport [17] (version 1.6.0), and summarized on the gene-level. Differential gene expression was obtained using the package edgeR [18] (version 3.20.5) using the estimated gene-level counts and offsets based on the transcript-level abundance estimates. The latter corrects for changes to the average transcript length across samples and incorporation of such offsets was reported to improve the accuracy of differential gene expression analysis [18].

The complete initial RNA-seq dataset consisted of 16 samples (8 treatments x 2 replicates). Despite not all treatments are addressed in this paper, all samples were included in the statistical analyses to improve the gene-wise dispersion (variability) estimates, that is advantageous when only having a duplicate per treatment. Before statistics, non-specific filtering of the count table was carried out to enhance detection power [19], based on the requirement that a gene should have an expression level greater than 10 counts, i.e. 0.65 count per million reads (cpm) mapped, for at least 2 libraries across all 16 samples. Differences in library size were adjusted by the trimmed mean of M-values normalization method [20]. Differentially expressed genes (DEGs) were identified by using generalized linear models that incorporate empirical Bayes methods that allow the estimation of gene-specific biological variation, thereby improving testing power [21–23]. For the RNA-seq study, thresholded hypotheses testing using a fold-change of 1.5 was performed to identify robustly regulated genes, i.e. genes modulated by a fold-change below this threshold were considered not to be biologically meaningful [24]. For all cases, genes that satisfied the criterion of a p-value lower than 0.05 were considered significantly regulated.

To acquire insights on the transcriptomic signature of the differentially expressed genes (DEGs), gene ontology (GO) and pathway analysis were performed using the Consensus Path Database (cpdb.molgen.mpg.de) tool. To do so, only GO terms and pathways with p-values lower than 0.005 were considered for analysis. In addition, other web tools such as Heatmapper [25] and Interactivenn [26] were utilized to create the heatmap and Venn diagrams presented in this work.

2.6 Gene expression (RT-qPCR) studies

In the RT-qPCR studies (performed for RNA-seq confirmation purposes), T47D cells were seeded in 25 cm² flasks (Corning, NY, USA) in growth medium and when reaching 50-60% confluence, medium was replaced by assay medium (hormone-free conditions). After 24 hours, treatment with 10 nM E₂ and 1 μ M AtRA, alone and in combination, was carried out for 6 hours. Next, cell lysis was performed by using RLT Lysis buffer (Qiagen, Venlo, The Netherlands). Total RNA was extracted and purified by using QIAshredder and RNeasy kits (Qiagen, Venlo, The Netherlands) following manufacturer's guidelines. To guarantee the quality of the RNA samples, spectrophotometric analysis was performed by using a Nanodrop (ND-1000 Thermoscientific Wilmington, Delaware, USA) with an A260/280 ratio limit of 1.8–2.1. Next, RNA was transformed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). Expression of the specific genes *ADORA1* (adenosine A1 receptor), *CXCL12* (chemokine CXCL12), *TFF1* (trefoil factor-1), *BTG2* (p53-controlled antiproliferative gene B-cell translocation gene, member 2), and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) (housekeeping gene) was determined by real-time PCR using Rotor-Gene SYBR® Green Kit (Qiagen, Venlo, The Netherlands) and the Rotor-Gene 6000 cyclor (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. For this purpose, the QuantiTect Primer Assay Hs_ADORA1_2_SG, Hs_CXCL12_1_SG, Hs_TFF1_1_SG, Hs_BTG2_1_SG, and Hs_GAPDH_1_SG (Qiagen, Venlo, The Netherlands) were used.

2.7 Further data representation, analysis and statistics

Apart from the type of analysis and tools described in previous sections for the RNA-seq data, all the other graphs, analysis and statistics were elaborated by using the software GraphPad Prism 5 (San Diego, Ca, USA). For all the proliferation, reporter gene and RT-qPCR studies a minimum of three independent experiments were carried out. For comparison in these studies, statistics between the different treatment conditions were evaluated by multiple paired t-tests where a criterion of a p-value

lower than 0.05 was considered to be significant. All along this work, data are expressed as percentage/fold induction of the solvent control DMSO (Avg $\pm$ SEM) or as log2 Fold Changes (FC) in comparison with the solvent control DMSO.

3 Results

3.1 Proliferation studies

In this work we initially evaluated the effects of the tested compounds, alone or in combination, on the proliferation of T47D cells (Figure 1). To do so, 3-day BrdU and 6-day MTT proliferation assays were carried out. Results showed an increase in proliferation of T47D cells after exposure to 10 nM E₂ confirming the hormone-responsiveness of the cells. Upon co-exposure with AtRA, the E₂-induced proliferation was counteracted showing a higher reduction of proliferation in the 6-day MTT study than in the 3-day BrdU assay. When analyzing the results obtained upon incubation with AtRA, it was shown that this compound by itself remarkably hampered cell proliferation in T47D cells. In summary, the results of the proliferation studies in the T47D breast cancer cell line pointed at AtRA as a repressor of the E₂-induced but also of the basal breast cancer cell proliferation, hampering a clear conclusion on the occurrence of interplay between estrogen and retinoid signaling. Thus, in the next steps we studied this possible crosstalk at the gene expression level.

Figure 1. Effects on the proliferation of T47D cells induced by 10 nM E₂ and 1 μ M AtRA, alone or in combination, after a 3-day (BrdU assay) or a 6-day (MTT assay) exposure period. All results are expressed as percentage in relation to the solvent control. Bars represent the average $\pm$ SEM of at least three independent experiments. For statistical analysis, multiple paired t-tests between the different condition were performed. ** $p < 0.01$; * $p < 0.05$ compared to the solvent control; † $p < 0.05$ compared to the E₂ treatment.

3.2 Reporter gene studies

In order to evaluate whether AtRA can interfere with the functioning of ER α in T47D cells, we performed reporter gene studies (Figure 2) using the T47D-ER α -Luc reporter gene cell line which allows determining ER α -mediated transcription in the cell line of interest. In addition, the U2OS-ER β -Luc reporter gene cell line was used to acquire further insights in the possible crosstalk between retinoids and ER β genomic signaling. As expected, the results show that E₂ (positive control) was capable of increasing ER-mediated luciferase expression in both cell models. Next, co-incubation with AtRA reduced the E₂-induced ER α - and ER β -mediated transcription although to a different extent. Strikingly, exposure to AtRA alone enhanced basal ER-mediated transcription in both types of ERs, a phenomenon that was more pronounced for ER β . Altogether, the results using reporter gene cell lines showed that treatment with AtRA can lead to both the decrease of the E₂-induced ER-mediated expression and the increase of basal ER-driven transcription at concentrations where clear inhibitory effects on cell proliferation, both in the presence and absence of E₂, were observed

Figure 2. Effects of E₂ and AtRA, alone or in combination, on ER α - and ER β -mediated luciferase expression. For these studies, the cell lines T47D-ER α -Luc and U2OS-ER β -Luc were used. For the assays with T47D-ER α -Luc cells, concentrations of 10 nM E₂ and 1 μ M AtRA were utilized. For the experiments with U2OS-ER β -Luc, concentrations of 100 pM E₂ and 1 μ M AtRA were used. All results are expressed as percentage in relation to the solvent control. Bars represent the average $\pm$ SEM of at least three independent experiments. For statistic analysis, multiple paired t-tests between the different conditions were performed. *** $p < 0.001$ ** $p < 0.01$; * $p < 0.05$ compared to the solvent control. ' ' ' $p < 0.001$ compared to the E₂ treatment.

3.3 Evaluation of the transcriptome generated by E₂ and AtRA alone

To acquire further insights on the gene expression induced by E₂ and AtRA in the cell line of interest (T47D), we performed a genome-wide study by means of RNA-seq. Figure 3 presents an overview of the effects of the respective test compounds. As shown by the distance between the treatments in Principal Component Analysis (PCA), the gene expression profiles generated after 6-hour exposure to E₂ or AtRA greatly differ (Figure 3A). In fact, analysis of the number of Differentially Expressed Genes (DEGs), reveals that E₂ triggered changes in gene expression of 635 genes, while AtRA was capable of modulating the expression of a much higher number of transcripts, being 1623 (Figure 3B). When focusing on the overlap between both compounds though, multiple estrogen-responsive genes were also modulated by AtRA (Figure 3C). In fact, when analyzing all DEGs, up to one third of the genes that were regulated by E₂, were also modulated upon exposure to AtRA. Next, Gene Ontology (GO) analysis was carried out to identify the biological processes (BPs) that were affected upon exposure to E₂ and AtRA, respectively. Lists with all GO (BP) terms over-represented by up-regulated, down-regulated and all DEGs are included in Supplementary Table A. Multiple genes involved in biological processes influencing breast cancer growth such as “regulation of epithelial cell proliferation”, “epithelial cell differentiation” or “apoptotic process” were modulated by both test compounds (Figure 3D and Suppl. Table A). Finally, the effects of E₂ and AtRA on different signaling processes were determined by pathway analysis. All pathways over-represented upon assessment of up-regulated, down-regulated and all DEGs are included in Supplementary Table B. Interestingly, pathway analysis showed that treatment with E₂ but also with AtRA greatly affected the “validated nuclear estrogen receptor alpha network” when analyzing both up-regulated and all DEGs (Figure 3E). When assessing down-regulated DEGs though, this pathway did not appear in the analysis for any of the chemicals. In summary, the gene expression data showed that despite the striking differences in their general transcriptomic signatures, AtRA was capable of modulating a high proportion of genes, biological processes and molecular pathways that were significantly affected upon treatment with E₂.

Figure 3. RNA-seq analysis of the transcriptome generated by E₂ and AtRA. Genes whose expression was significantly different (ANOVA) to the solvent control in at least one treatment were included in the PCA plot (A). An overview of the number (B) and overlap (C) of DEGs (up-regulated, down-regulated and all DEGs) upon E₂ and AtRA treatment is presented by bar and Venn diagrams, respectively. An illustration showing biological process (GO terms) related to breast cancer growth affected by both compounds is also included (D). A bar graph showing the significance (p-value) of the effects of E₂ and AtRA in the pathway entitled “validated nuclear estrogen receptor alpha network” is presented (E).

3.4 Effects of co-exposure with AtRA on the E₂-induced transcriptome

Once we evaluated the effects of E₂ and AtRA separately, the effects generated by co-incubation with AtRA on the E₂-induced gene expression were assessed. To do so, we analyzed all the E₂-responsive genes that were significantly different upon co-exposure with AtRA (Figure 4). As shown in Figure 4A, a relatively high number of the E₂-regulated genes (26-36%) were affected when co-exposing with AtRA. However, this modulation was not always unidirectional since both reduction and enhancement of the E₂-induced responses were similarly seen in the heatmap plot (Figure 4B). In fact, the E₂-responsive genes that were affected upon co-exposure with AtRA showed 57% inhibiting/counteracting and 43 % enhancing effects of AtRA on the E₂-induced response, respectively (results not shown). However, upon GO analysis of both categories of genes (Supplementary table C), biological processes related to breast cancer growth such as proliferation, differentiation and apoptosis were more affected for the case of estrogen-responsive genes whose E₂-induced expression responses were reduced/counteracted upon co-exposure with AtRA. Thus, as represented by the diagram and the illustrative E₂-responsive genes depicted in Figure 4C, AtRA counteracted or enhanced the initial effects of E₂ on the expression of different genes. However, genes where co-exposure with AtRA counteracted the initial response to E₂ showed to be more related to breast cancer growth regulation processes. It has to be noted though, that in many of the cases, AtRA by itself could highly modulate the expression of those genes thereby playing a dominant role in the final response upon co-exposure with E₂ and AtRA. Altogether, the results showed that AtRA can exert both enhancement and reduction/counteraction of the E₂-induced gene expression in a gene-dependent manner where the later seems to be more prevalent in those genes related to cell proliferation, differentiation and apoptosis.

Figure 4. Determination of the effects of co-incubation with AtRA on the transcriptomic profiles generated by E₂. In Figure A, E₂-induced genes whose expression was significantly different upon co-exposure with AtRA are quantified (%) for up-regulated, down-regulated and all DEGs. Figure B shows by a heatmap all DEGs modulated by E₂ which presented significant changes upon co-exposure with AtRA. Figure C presents that estrogen-responsive genes whose expression was reduced/counteracted by co-exposure with AtRA were more related to biological processes regulating breast cancer growth such as proliferation, differentiation and apoptosis. In addition, Figure C provides some examples of E₂-induced genes whose expression was enhanced or reduced/counteracted upon co-incubation with AtRA. In both, heatmap and bar graphs, results are shown as log₂ fold changes compared to the solvent control.

3.5 Evaluation of the effects of AtRA on ER-target genes known to affect breast cancer cell proliferation

After showing that a high proportion of E₂-responsive genes were differentially affected when combining E₂ and AtRA treatment, and that this modulation was more related to BPs regulating breast cancer growth for those genes whose response to E₂ was reduced or counteracted by co-exposure with

AtRA, we evaluated whether some of the ER-target genes known to influence breast cancer cell proliferation would show a similar trend (Figure 5). Some of these known ER-mediated genes representing examples of pro-proliferative (TFF1, CXCL12, ADORA1) [2,27–30] and anti-proliferative (BTG2) [9,31] effectors were further analyzed by RT-qPCR studies (for confirmation purposes). As displayed in figure 5, different effects of co-incubation with AtRA on the E₂-induced expression of these genes can be observed. While the effects of the different test compounds on the expression of the ER-target PKIB or ER α itself were very slight, the effects on most of the ER target genes tested were pronounced. Firstly, the estrogen-induced expression of pro-proliferative genes like CXCL12 [27,28], KCNK5 [32], GREB1 [2,33] and TFF1 [2,29] was counteracted by co-exposure with AtRA. Secondly, for some other pro-proliferative ER-target genes such as MYC [2,34] and CCND1 [2,35], there was no effect on the E₂-driven response when co-exposing with AtRA. Oppositely, the E₂-induced transcription of other ER-mediated genes involved in breast cancer growth such as the pro-proliferative ADORA1 [30] or the anti-proliferative STC2 [28,36], was increased upon co-treatment with AtRA. Finally, the present study tested the effects of E₂ and AtRA, alone or in combination, on the anti-proliferative ER- and RAR-target gene BTG2 [9,31]. As shown in figure 5, the preponderant effects of AtRA on the BTG2 expression, were also affected upon co-incubation with E₂ although, not significantly. In summary, the results of this analysis suggested that AtRA can differentially modulate the E₂-driven effects on the expression of ER-target genes previously described to influence breast cancer growth and progression. However, in line with the previously shown results, co-exposure with AtRA reduced/counteracted the expression of pro-proliferative E₂-induced genes more often whereas enhancing effects were less frequently observed.

Figure 5. Analysis (RNA-seq) of the effects of co-exposure with AtRA on the expression of E₂-modulated ER-target genes influencing breast cancer growth. The ER-target genes CXCL12, TFF1, ADORA1 and BTG2 were evaluated also by RT-qPCR. All results are shown as log₂ fold changes compared to the solvent control. Statistical analysis for the RT-qPCR results was performed by multiple paired t-tests between E₂ and E₂ + AtRA treatments. Differences were considered significant if p-value < 0.05. ** = p-value < 0.01; ' = p-value < 0.05 compared to the E₂ treatment.

4. Discussion

While several studies have reported crosstalk between estrogen (ER) and retinoid (RAR) signaling [2,9], the molecular mechanisms involved in this interplay are still not completely understood. Thus, the principal objective of the present work was to study the effects of the general RAR agonist AtRA on the proliferative and transcriptomic profiles triggered after treatment with E₂ in the ER⁺ T47D breast cancer cell line. For this purpose, methods evaluating the effects on cell proliferation and on gene expression in the presence of E₂ and AtRA, alone or in combination, were carried out.

First, the effects of the different treatments on the proliferation of T47D cells after exposure for 3 days (BrdU assay) and 6 days (MTT assay) were characterized. These different exposure periods were chosen since effects on DNA synthesis and replication (measured with the BrdU assay) can be detected prior to the changes in cell number (deducted from the MTT assay) [37]. In addition, significant retinoid-driven effects on cell proliferation are mainly observed after exposure times of three days onwards [38]. The results of these studies clearly showed that E₂ promotes cell proliferation in both assays which is in accordance with previous research [39]. Next, co-incubation with AtRA was shown

to reduce the E₂-induced effects as described for other breast cancer cell lines [2]. However, the results observed with AtRA showed that this compound can also, by itself, highly decrease the proliferation of T47D cells, which is in line with our earlier studies using the ER⁺ MCF7/BUS breast cancer cell line [3]. To determine a possible interference of AtRA on E₂ signaling (ER-mediated or not), a deep gene expression study using reporter gene, RNA-seq and RT-qPCR assays was carried out.

In order to assess the possible effects of AtRA treatment on the basal and E₂-induced gene expression mediated by ER α and ER β , the T47D-ER α -Luc and the U2OS-ER β -Luc reporter gene cell lines were utilized. The results of these studies displayed that E₂ (positive control) increased the ER α - and ER β -mediated expression of luciferase in both cell models which is in line with literature data [12,40]. Similar to the outcomes seen in the proliferation studies, co-treatment with AtRA hampered the E₂-induced transcription driven by ER α and ER β . Strikingly, treatment with AtRA alone enhanced the basal mediated expression of luciferase by both ER α and ER β . While previous studies have shown that retinoids can decrease E₂-induced ER α -mediated transcription [2,10,41], this study displayed a similar trend in the ER β subtype, and interestingly, an increase in the basal activity of both ERs, that was more pronounced for the case of ER β . Although the ratio of ER α /ER β is 9:1 in T47D cells [42], given the anti-proliferative effects exerted by ER β [42], it may be possible that the AtRA-mediated induction of ER β -mediated gene expression in the absence of E₂ could provide an additional mode of action possibly explaining its reduction of breast cancer growth. Nevertheless, it has to be noted, that the observed changes in the basal and E₂-induced expression of luciferase may be influenced by cell- and promoter-dependent factors. Thus, differences in the cellular levels of receptors or other proteins of the transcription machinery (e.g. coregulators) in a reporter gene cell such as the U2OS (osteosarcoma cells), could influence the final results. In addition, reporter gene cell lines make use of single sequences on their promoter regions while ERs can bind to different hormone responsive elements (EREs) in a physiological scenario. Altogether we showed that AtRA can affect both basal and E₂-induced ER α - and ER β -mediated transcription in the reporter gene cell lines T47D-ER α -Luc and

U2OS-ER β -Luc. However, further studies of the effects of AtRA on the estrogen-induced gene expression in our physiologically relevant breast cancer model (wild type T47D) were required.

In order to acquire more insights in the effects on the transcriptomic profiles of T47D cells upon treatment with AtRA and E₂, alone and in combination, an RNA-seq study after a 6-hour exposure was carried out. The election of this short period was based on previous studies evaluating the effects on gene expression of estrogens and retinoids which showed that 4-8 hours are enough to detect direct genomic events while avoiding as much as possible secondary effects appearing after longer exposure periods [43,44]. The analysis of the transcriptomes generated by E₂ and AtRA were found to be different as represented by the PCA and the number of differentially expressed genes (DEGs). However, when focusing on the estrogen-responsive genes a high amount of DEGs generated upon exposure to E₂, were also affected by AtRA treatment. Thus, a remarkable overlap of DEGs was observed when comparing E₂- and AtRA-treated conditions. In addition, when performing pathway analysis, the “validated nuclear estrogen receptor alpha network” was highly modulated upon exposure to AtRA although to a lower extent than for E₂. Previous studies have shown genomic antagonism between ERs and RARs where both receptors showed to bind and compete for close or overlapping regions within the genome [9]. However, the results of the present study show that AtRA was also capable of modulating (up-/down-regulating) ER genomic signaling. Finally, analysis of the transcriptomic signatures induced by both compounds showed, as expected, multiple effects on genes related to biological processes influencing breast cancer growth such as epithelial proliferation, differentiation and apoptosis. In summary, the effects of the test compounds (E₂ and AtRA), when administered individually, highlighted that despite generating a rather different transcriptomic profile in global terms, a high proportion of E₂-responsive genes were also affected by AtRA.

Once we showed that the single treatment with AtRA could affect the expression of many of the genes that were differentially expressed upon E₂ treatment, we analysed the effects of a co-exposure with AtRA on the estrogen-responsive genes. Thus, the results obtained showed that a significant portion

reaching up to one third of all the E₂-modulated genes was significantly affected by co-treatment with AtRA. Next, we analyzed this sub-group within the differentially E₂-driven genes to characterize what kind of effect was mostly observed. Interestingly, the E₂-induced gene expression was either enhanced or counteracted by co-treatment with AtRA in similar proportions. Interestingly, GO analysis showed that E₂-induced genes related to biological processes influencing breast cancer growth appeared more frequently among the list of genes where AtRA exerted reducing/counteracting effects. In accordance with our data, previous research in different breast cancer cell lines (e.g. MCF7) also showed that the response to E₂ of different estrogen-responsive genes can be differently modulated upon co-exposure with AtRA [2,9]. It must be noted though, that exposure to AtRA alone highly affected many of the E₂-responsive genes influencing thereby the responses upon co-exposure with E₂.

The AtRA-driven modulation of estrogen-responsive genes does not directly imply effects on the classical ER-ERE-mediated pathway since E₂ can act via both genomic and non-genomic signaling [45]. To determine how co-treatment with AtRA affects the pro-proliferative ER-mediated transcription, we assessed the effects of this compound on previously described ER target genes playing vital roles in breast cancer cell proliferation such as CXCL12 [27,28], KCNK5 [32], GREB1 [2,33], TFF1 [2,29], MYC [2,34], CCND1 [2], ADORA1 [30], STC2 [28,36] and BTG2 [9,31]. As quality control measure for the RNA-seq data, some illustrative examples of pro-proliferative (TFF1, CXCL12, ADORA1) [2,27–30] and anti-proliferative (BTG2) [9,31] ER target genes were confirmed by additional RT-qPCR studies. The results showed, in line with the previous section, that AtRA can also affect in different directions ER-target genes known to affect proliferation although inhibiting/counteracting effects were again more frequent. Thus, co-exposure with AtRA hampered the E₂-induced expression of some genes increasing proliferation like CXCL12 [27] KCNK5 [32] GREB1 [2,33] or TFF1 [2,29] while also enhancing the E₂-driven expression of other ER pro-proliferative genes such as ADORA1. In relation to this phenomenon, a previous study demonstrated that AtRA cannot act as cognate ligand (neither agonist nor antagonist) of ER [3] so that other kind of

molecular mechanisms at the specific promoter regions are likely to lead to these divergent gene expression patterns. Finally, when assessing a retinoid-responsive gene known to be a direct target for both ER and RAR like BTG2 [9,31] we also saw that the preponderant up-regulation triggered by AtRA can be hampered by co-exposure with E₂ suggesting that the crosstalk between estrogen and retinoid signaling can function in both ways. Altogether, multiple E₂-induced ER target genes showed significant changes in distinct directions in their expression levels after co-treatment with AtRA. Nevertheless, the more prevalent inhibiting/counteracting patterns occurring on ER target genes linked to breast cancer cell proliferation may partially explain the pharmacological inhibitory effects seen on breast cancer growth.

In summary this work showed that AtRA decreases both basal and E₂-induced proliferation of T47D cells. Although the RNA-seq study displayed distinct global transcriptomic signatures, crosstalk between AtRA and E₂ signaling was reflected by the high overlap between the genes modulated by these compounds and by the high proportion of E₂-responsive genes affected upon co-exposure with AtRA. The results also showed that the effects of AtRA on the E₂-induced gene expression levels (ER-mediated or not) appeared in both directions (counteracting and enhancing effects). However, events where co-exposure with AtRA generated counteracting effects or reduced the magnitude of response induced by E₂, were more often found in genes related to breast cancer growth regulation. This supports in our view that AtRA can hamper E₂-induced proliferation of breast cancer cells by predominantly counteracting the E₂-driven expression of pro-proliferative genes.

Disclosure of Interest

The authors declare no conflict of interest.

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Appendix. Supplementary Material

Supplementary material can be found in the end of this chapter.

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Appendix. Supplementary Table A. List containing the biological processes (p-value < 0.005) over-represented upon GO analysis of all DEGs, up-regulated DEGs, and down-regulated DEGs for the treatments with AtRA or E₂

GO analysis (Biological Processes) AtRA (all DEGs)			
term_goid	p-value	term_name	size
GO:0022008	5.22E-13	neurogenesis	1433
GO:0030182	2.12E-12	neuron differentiation	1231
GO:0051960	9.00E-11	regulation of nervous system development	767
GO:0045597	1.16E-10	positive regulation of cell differentiation	830
GO:0031344	3.00E-10	regulation of cell projection organization	575
GO:0060284	1.22E-09	regulation of cell development	782
GO:0043067	8.01E-09	regulation of programmed cell death	1409
GO:0006915	1.98E-08	apoptotic process	1817
GO:0000904	9.44E-08	cell morphogenesis involved in differentiation	638
GO:0010942	5.30E-07	positive regulation of cell death	612
GO:0051046	1.25E-06	regulation of secretion	691
GO:0015711	1.90E-06	organic anion transport	425
GO:0051130	2.15E-06	positive regulation of cellular component organization	1142
GO:0051272	4.04E-06	positive regulation of cellular component movement	416
GO:0051962	4.23E-06	positive regulation of nervous system development	447
GO:0051174	4.86E-06	regulation of phosphorus metabolic process	1632
GO:0045834	5.46E-06	positive regulation of lipid metabolic process	130
GO:1902531	5.66E-06	regulation of intracellular signal transduction	1683
GO:0070371	7.05E-06	ERK1 and ERK2 cascade	258
GO:0042886	7.06E-06	amide transport	295
GO:0010647	7.67E-06	positive regulation of cell communication	1525
GO:0033500	8.67E-06	carbohydrate homeostasis	221
GO:0010631	8.67E-06	epithelial cell migration	222
GO:0006721	8.78E-06	terpenoid metabolic process	110
GO:0019216	8.87E-06	regulation of lipid metabolic process	288
GO:0061564	1.11E-05	axon development	460
GO:0022604	1.23E-05	regulation of cell morphogenesis	443
GO:0071300	1.62E-05	cellular response to retinoic acid	67
GO:0010648	1.81E-05	negative regulation of cell communication	1207
GO:0006939	1.87E-05	smooth muscle contraction	98
GO:0015833	2.27E-05	peptide transport	270
GO:0014706	2.68E-05	striated muscle tissue development	341
GO:0006820	3.02E-05	anion transport	561
GO:0050801	3.36E-05	ion homeostasis	683
GO:0045596	4.11E-05	negative regulation of cell differentiation	624
GO:0072073	4.20E-05	kidney epithelium development	137
GO:0009967	4.33E-05	positive regulation of signal transduction	1396
GO:0046879	4.55E-05	hormone secretion	308
GO:0008285	4.57E-05	negative regulation of cell proliferation	653
GO:0051271	4.90E-05	negative regulation of cellular component movement	249
GO:0034110	5.18E-05	regulation of homotypic cell-cell adhesion	26
GO:0071396	5.32E-05	cellular response to lipid	528
GO:0001657	5.44E-05	ureteric bud development	97
GO:0043010	5.68E-05	camera-type eye development	290
GO:1902284	5.76E-05	neuron projection extension involved in neuron projection guidance	45
GO:0006023	5.84E-05	aminoglycan biosynthetic process	114
GO:0090087	5.99E-05	regulation of peptide transport	213
GO:0016310	6.35E-05	phosphorylation	2230
GO:0002790	6.87E-05	peptide secretion	254
GO:0090257	7.10E-05	regulation of muscle system process	196
GO:0050678	7.16E-05	regulation of epithelial cell proliferation	293
GO:0034284	7.81E-05	response to monosaccharide	197
GO:0071345	8.51E-05	cellular response to cytokine stimulus	692
GO:0002521	0.0001002	leukocyte differentiation	466
GO:0007169	0.00010058	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0009968	0.00011135	negative regulation of signal transduction	1102
GO:0071322	0.00012986	cellular response to carbohydrate stimulus	129
GO:0030855	0.00013343	epithelial cell differentiation	567
GO:0007411	0.00014486	axon guidance	223
GO:0042692	0.00014518	muscle cell differentiation	355
GO:0032570	0.00017389	response to progesterone	50
GO:0001894	0.00017694	tissue homeostasis	206
GO:0045995	0.00019672	regulation of embryonic development	116
GO:0034762	0.00019797	regulation of transmembrane transport	402
GO:0031032	0.00020237	actomyosin structure organization	151
GO:0022408	0.00020483	negative regulation of cell-cell adhesion	133
GO:0051961	0.00022224	negative regulation of nervous system development	268
GO:1901342	0.00023962	regulation of vasculature development	239
GO:0030038	0.00025016	contractile actin filament bundle assembly	83
GO:0072088	0.00025402	nephron epithelium morphogenesis	75
GO:0061572	0.00025419	actin filament bundle organization	127

GO:0071634	0.00025421	regulation of transforming growth factor beta production	25
GO:0071407	0.00025866	cellular response to organic cyclic compound	527
GO:0050807	0.00030793	regulation of synapse organization	111
GO:0051592	0.00031509	response to calcium ion	120
GO:0060600	0.00035786	dichotomous subdivision of an epithelial terminal unit	10
GO:0043587	0.00035786	tongue morphogenesis	10
GO:0045444	0.0003701	fat cell differentiation	205
GO:0045778	0.00038128	positive regulation of ossification	86
GO:0032268	0.00038315	regulation of cellular protein metabolic process	2327
GO:0071248	0.00038855	cellular response to metal ion	140
GO:1902903	0.0004223	regulation of supramolecular fiber organization	278
GO:0048048	0.00051493	embryonic eye morphogenesis	34
GO:0030203	0.00064165	glycosaminoglycan metabolic process	163
GO:1901862	0.00064932	negative regulation of muscle tissue development	35
GO:0048635	0.00064932	negative regulation of muscle organ development	35
GO:0051246	0.00083067	regulation of protein metabolic process	2476
GO:0010811	0.00084751	positive regulation of cell-substrate adhesion	101
GO:0034220	0.00085947	ion transmembrane transport	1007
GO:0043269	0.00087349	regulation of ion transport	599
GO:0045765	0.00094269	regulation of angiogenesis	217
GO:0051345	0.00099156	positive regulation of hydrolase activity	916
GO:0007351	0.00099537	tripartite regional subdivision	12
GO:0001678	0.00100896	cellular glucose homeostasis	130
GO:0007044	0.00101765	cell-substrate junction assembly	85
GO:0001649	0.00110048	osteoblast differentiation	209
GO:1903035	0.00115794	negative regulation of response to wounding	69
GO:0030279	0.00115794	negative regulation of ossification	70
GO:0032956	0.00115923	regulation of actin cytoskeleton organization	282
GO:0051965	0.00119606	positive regulation of synapse assembly	62
GO:0034114	0.0012091	regulation of heterotypic cell-cell adhesion	18
GO:0006941	0.00121675	striated muscle contraction	151
GO:0032102	0.00123242	negative regulation of response to external stimulus	262
GO:2000736	0.00125806	regulation of stem cell differentiation	31
GO:0035282	0.00132447	segmentation	96
GO:0031325	0.00134884	positive regulation of cellular metabolic process	2789
GO:0050880	0.00140411	regulation of blood vessel size	143
GO:0010594	0.00144333	regulation of endothelial cell migration	115
GO:0040036	0.00150695	regulation of fibroblast growth factor receptor signaling pathway	25
GO:1902285	0.00152191	semaphorin-plexin signaling pathway involved in neuron projection guidance	13
GO:0060548	0.00156757	negative regulation of cell death	895
GO:0016311	0.00158784	dephosphorylation	440
GO:0035296	0.00161811	regulation of tube diameter	135
GO:0007265	0.00163437	Ras protein signal transduction	309
GO:0006687	0.00173787	glycosphingolipid metabolic process	72
GO:0031324	0.00193	negative regulation of cellular metabolic process	2323
GO:1903053	0.00193535	regulation of extracellular matrix organization	26
GO:1903729	0.00197768	regulation of plasma membrane organization	73
GO:0090184	0.00217943	positive regulation of kidney development	41
GO:0002718	0.00222092	regulation of cytokine production involved in immune response	57
GO:0034308	0.0022542	primary alcohol metabolic process	49
GO:0032233	0.0022542	positive regulation of actin filament bundle assembly	49
GO:0070555	0.00234955	response to interleukin-1	121
GO:0007435	0.00238729	salivary gland morphogenesis	34
GO:0006464	0.00238768	cellular protein modification process	3772
GO:1901203	0.00252184	positive regulation of extracellular matrix assembly	9
GO:0043087	0.00253733	regulation of GTPase activity	683
GO:0046890	0.00267339	regulation of lipid biosynthetic process	132
GO:0016202	0.00279669	regulation of striated muscle tissue development	103
GO:0099623	0.00293194	regulation of cardiac muscle cell membrane repolarization	21
GO:0045667	0.00296738	regulation of osteoblast differentiation	113
GO:1900024	0.00308105	regulation of substrate adhesion-dependent cell spreading	43
GO:0010634	0.00308933	positive regulation of epithelial cell migration	104
GO:1903522	0.0031288	regulation of blood circulation	299
GO:0099537	0.0031612	trans-synaptic signaling	607
GO:0060538	0.00318344	skeletal muscle organ development	183
GO:0010605	0.00324931	negative regulation of macromolecule metabolic process	2297
GO:0043435	0.00334446	response to corticotropin-releasing hormone	6
GO:0008582	0.00334446	regulation of synaptic growth at neuromuscular junction	5
GO:0051402	0.0034009	neuron apoptotic process	216
GO:0048634	0.0034068	regulation of muscle organ development	105
GO:0048592	0.00350424	eye morphogenesis	144
GO:0009792	0.00353718	embryo development ending in birth or egg hatching	574
GO:0051492	0.00355667	regulation of stress fiber assembly	69
GO:0050994	0.00356594	regulation of lipid catabolic process	52
GO:0000271	0.0036207	polysaccharide biosynthetic process	78
GO:0060445	0.00378876	branching involved in salivary gland morphogenesis	22
GO:0021561	0.0039645	facial nerve development	10

GO:0048640	0.0040075	negative regulation of developmental growth	88
GO:0043255	0.0040075	regulation of carbohydrate biosynthetic process	89
GO:0008284	0.00406703	positive regulation of cell proliferation	839
GO:1903034	0.00410939	regulation of response to wounding	146
GO:0060443	0.00425339	mammary gland morphogenesis	45
GO:1900115	0.00430601	extracellular regulation of signal transduction	16
GO:0030148	0.00492666	sphingolipid biosynthetic process	90
GO analysis (Biological Processes) AtRA (Up-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0045597	5.43E-08	positive regulation of cell differentiation	830
GO:0006915	2.70E-07	apoptotic process	1817
GO:0051046	3.31E-07	regulation of secretion	691
GO:0043067	5.58E-07	regulation of programmed cell death	1409
GO:0022008	1.21E-06	neurogenesis	1433
GO:0031344	1.42E-06	regulation of cell projection organization	575
GO:0072073	2.31E-06	kidney epithelium development	137
GO:0060284	2.31E-06	regulation of cell development	782
GO:0045834	4.04E-06	positive regulation of lipid metabolic process	130
GO:0001657	4.72E-06	ureteric bud development	97
GO:0071300	5.83E-06	cellular response to retinoic acid	67
GO:0008285	9.20E-06	negative regulation of cell proliferation	653
GO:0030855	1.12E-05	epithelial cell differentiation	567
GO:0001894	1.33E-05	tissue homeostasis	206
GO:0033500	1.48E-05	carbohydrate homeostasis	221
GO:0050678	1.58E-05	regulation of epithelial cell proliferation	293
GO:0051960	1.95E-05	regulation of nervous system development	767
GO:0019216	2.78E-05	regulation of lipid metabolic process	288
GO:0071345	3.08E-05	cellular response to cytokine stimulus	692
GO:0030182	3.32E-05	neuron differentiation	1231
GO:0043587	3.39E-05	tongue morphogenesis	10
GO:1901342	5.55E-05	regulation of vasculature development	239
GO:0090184	5.77E-05	positive regulation of kidney development	41
GO:0006687	6.48E-05	glycosphingolipid metabolic process	72
GO:0051130	6.56E-05	positive regulation of cellular component organization	1142
GO:0022408	7.45E-05	negative regulation of cell-cell adhesion	133
GO:0010942	7.45E-05	positive regulation of cell death	612
GO:0045765	8.88E-05	regulation of angiogenesis	217
GO:0034110	9.75E-05	regulation of homotypic cell-cell adhesion	26
GO:0072088	9.77E-05	nephron epithelium morphogenesis	75
GO:0042886	0.00010733	amide transport	295
GO:0090183	0.0001233	regulation of kidney development	55
GO:0060443	0.00012468	mammary gland morphogenesis	45
GO:0051962	0.00016828	positive regulation of nervous system development	447
GO:0002521	0.00017945	leukocyte differentiation	466
GO:1903035	0.00019553	negative regulation of response to wounding	69
GO:0010648	0.0002205	negative regulation of cell communication	1207
GO:0014706	0.00024012	striated muscle tissue development	341
GO:0007169	0.00025276	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0006721	0.00029816	terpenoid metabolic process	110
GO:0010631	0.00030783	epithelial cell migration	222
GO:0071396	0.00031182	cellular response to lipid	528
GO:2000736	0.00032161	regulation of stem cell differentiation	31
GO:0019752	0.00034903	carboxylic acid metabolic process	961
GO:0015833	0.00035715	peptide transport	270
GO:0051272	0.000383	positive regulation of cellular component movement	416
GO:0070371	0.00040934	ERK1 and ERK2 cascade	258
GO:0010647	0.00045758	positive regulation of cell communication	1525
GO:0045995	0.00051947	regulation of embryonic development	116
GO:0051174	0.00054632	regulation of phosphorus metabolic process	1632
GO:0040036	0.00061079	regulation of fibroblast growth factor receptor signaling pathway	25
GO:0071634	0.00061079	regulation of transforming growth factor beta production	25
GO:0051271	0.00065788	negative regulation of cellular component movement	249
GO:0060548	0.00068959	negative regulation of cell death	895
GO:0097300	0.00070579	programmed necrotic cell death	35
GO:1902531	0.00070721	regulation of intracellular signal transduction	1683
GO:1902105	0.00075542	regulation of leukocyte differentiation	238
GO:0002790	0.00081874	peptide secretion	254
GO:0034114	0.00085981	regulation of heterotypic cell-cell adhesion	18
GO:0046394	0.00087514	carboxylic acid biosynthetic process	302
GO:0009108	0.0008805	coenzyme biosynthetic process	136
GO:0050801	0.00093834	ion homeostasis	683
GO:0031643	0.00095361	positive regulation of myelination	11
GO:0032268	0.00098157	regulation of cellular protein metabolic process	2327
GO:0051246	0.00108382	regulation of protein metabolic process	2476
GO:0046879	0.00111112	hormone secretion	308
GO:0097421	0.001125	liver regeneration	19
GO:0072074	0.001125	kidney mesenchyme development	19

GO:0034284	0.00112747	response to monosaccharide	197
GO:0009968	0.00114761	negative regulation of signal transduction	1102
GO:0090087	0.00117782	regulation of peptide transport	213
GO:0034308	0.00122398	primary alcohol metabolic process	49
GO:1903729	0.00128621	regulation of plasma membrane organization	73
GO:0001973	0.00138089	adenosine receptor signaling pathway	12
GO:0033003	0.001386	regulation of mast cell activation	39
GO:0030195	0.00140195	negative regulation of blood coagulation	50
GO:0060602	0.00144622	branch elongation of an epithelium	20
GO:0016310	0.00149097	phosphorylation	2230
GO:0002699	0.00152377	positive regulation of immune effector process	157
GO:0061550	0.00153062	cranial ganglion development	6
GO:0009792	0.00154527	embryo development ending in birth or egg hatching	574
GO:0050880	0.00158378	regulation of blood vessel size	143
GO:0010594	0.00162077	regulation of endothelial cell migration	115
GO:0006664	0.00175372	glycolipid metabolic process	116
GO:0045444	0.00179617	fat cell differentiation	205
GO:0050994	0.00181925	regulation of lipid catabolic process	52
GO:0009310	0.00183019	amine catabolic process	21
GO:009623	0.00183019	regulation of cardiac muscle cell membrane repolarization	21
GO:0009967	0.00184445	positive regulation of signal transduction	1396
GO:0002221	0.00185502	pattern recognition receptor signaling pathway	160
GO:0010813	0.00192902	neuropeptide catabolic process	2
GO:0002386	0.00192902	immune response in mucosal-associated lymphoid tissue	2
GO:0031349	0.00194286	positive regulation of defense response	405
GO:0008284	0.00196539	positive regulation of cell proliferation	839
GO:0050819	0.0020617	negative regulation of coagulation	53
GO:0051092	0.00216349	positive regulation of NF-kappaB transcription factor activity	133
GO:0031347	0.00222437	regulation of defense response	769
GO:0043010	0.00229688	camera-type eye development	290
GO:0070555	0.0023789	response to interleukin-1	121
GO:0035296	0.00248746	regulation of tube diameter	135
GO:0070587	0.00259119	regulation of cell-cell adhesion involved in gastrulation	7
GO:0019441	0.00259119	tryptophan catabolic process to kynurenine	7
GO:2000027	0.00277157	regulation of organ morphogenesis	246
GO:0032102	0.00286912	negative regulation of response to external stimulus	262
GO:0030279	0.00312037	negative regulation of ossification	70
GO:1902284	0.0032559	neuron projection extension involved in neuron projection guidance	45
GO:0002718	0.00329566	regulation of cytokine production involved in immune response	57
GO:0033209	0.00347565	tumor necrosis factor-mediated signaling pathway	155
GO:0001819	0.00348446	positive regulation of cytokine production	386
GO:0034612	0.00377048	response to tumor necrosis factor	269
GO:0006939	0.00380307	smooth muscle contraction	98
GO:0002688	0.00380307	regulation of leukocyte chemotaxis	99
GO:0022604	0.00392244	regulation of cell morphogenesis	443
GO:0042692	0.00398891	muscle cell differentiation	355
GO:0008300	0.00401096	isoprenoid catabolic process	8
GO:0015711	0.00415538	organic anion transport	425
GO:0071322	0.00446537	cellular response to carbohydrate stimulus	129
GO:0002448	0.00471576	mast cell mediated immunity	48
GO:0001678	0.0047678	cellular glucose homeostasis	130
GO:0010811	0.00479352	positive regulation of cell-substrate adhesion	101
GO analysis (Biological Processes) AtRA (Down-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0030182	5.16E-09	neuron differentiation	1231
GO:0022008	1.08E-07	neurogenesis	1433
GO:0000904	3.02E-07	cell morphogenesis involved in differentiation	638
GO:0051960	1.10E-06	regulation of nervous system development	767
GO:0061564	4.81E-05	axon development	460
GO:0051965	4.85E-05	positive regulation of synapse assembly	62
GO:0050807	6.23E-05	regulation of synapse organization	111
GO:0015711	7.66E-05	organic anion transport	425
GO:0031344	8.66E-05	regulation of cell projection organization	575
GO:0006820	0.00013181	anion transport	561
GO:0045778	0.00014823	positive regulation of ossification	86
GO:0060284	0.00019511	regulation of cell development	782
GO:0045667	0.00034359	regulation of osteoblast differentiation	113
GO:0006023	0.00037085	aminoglycan biosynthetic process	114
GO:0034762	0.00045334	regulation of transmembrane transport	402
GO:0051592	0.00053591	response to calcium ion	120
GO:0071248	0.00056652	cellular response to metal ion	140
GO:0051961	0.00061412	negative regulation of nervous system development	268
GO:0045597	0.00065744	positive regulation of cell differentiation	830
GO:0022604	0.00076564	regulation of cell morphogenesis	443
GO:0045596	0.00077535	negative regulation of cell differentiation	624
GO:1902359	0.00078959	Notch signaling pathway involved in somitogenesis	2
GO:0071608	0.00078959	macrophage inflammatory protein-1 alpha production	2

GO:0003365	0.00078959	establishment of cell polarity involved in ameboidal cell migration	2
GO:0090257	0.0013448	regulation of muscle system process	196
GO:0007411	0.00167467	axon guidance	223
GO:0006939	0.00176219	smooth muscle contraction	98
GO:0009890	0.00193948	negative regulation of biosynthetic process	1492
GO:0042475	0.00197557	odontogenesis of dentin-containing tooth	81
GO:1902531	0.00222765	regulation of intracellular signal transduction	1683
GO:0030203	0.00225381	glycosaminoglycan metabolic process	163
GO:0010942	0.00233723	positive regulation of cell death	612
GO:0034220	0.00247145	ion transmembrane transport	1007
GO:0051174	0.00252533	regulation of phosphorus metabolic process	1632
GO:0071398	0.00327143	cellular response to fatty acid	52
GO:0043255	0.00333001	regulation of carbohydrate biosynthetic process	89
GO:0043067	0.00336247	regulation of programmed cell death	1409
GO:0051272	0.00352464	positive regulation of cellular component movement	416
GO:0007156	0.00468931	homophilic cell adhesion via plasma membrane adhesion molecules	156
GO:0010647	0.00485684	positive regulation of cell communication	1525
GO analysis (Biological Processes) E ₂ (all DEGs)			
term_goid	p-value	term_name	size
GO:0001649	1.35E-06	osteoblast differentiation	209
GO:0022008	2.96E-06	neurogenesis	1433
GO:0003197	3.72E-06	endocardial cushion development	35
GO:0051174	5.64E-06	regulation of phosphorus metabolic process	1632
GO:0061156	8.04E-06	pulmonary artery morphogenesis	6
GO:0090257	8.07E-06	regulation of muscle system process	196
GO:0043269	8.83E-06	regulation of ion transport	599
GO:0009967	9.05E-06	positive regulation of signal transduction	1396
GO:0030182	9.61E-06	neuron differentiation	1231
GO:0060602	1.16E-05	branch elongation of an epithelium	20
GO:0010647	1.28E-05	positive regulation of cell communication	1525
GO:0010604	1.28E-05	positive regulation of macromolecule metabolic process	2795
GO:0045667	1.35E-05	regulation of osteoblast differentiation	113
GO:2000027	1.50E-05	regulation of organ morphogenesis	246
GO:0007169	1.52E-05	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:1902531	1.69E-05	regulation of intracellular signal transduction	1683
GO:0045597	2.33E-05	positive regulation of cell differentiation	830
GO:0071407	2.34E-05	cellular response to organic cyclic compound	527
GO:0071363	3.22E-05	cellular response to growth factor stimulus	609
GO:0010648	3.99E-05	negative regulation of cell communication	1207
GO:0030855	4.37E-05	epithelial cell differentiation	567
GO:0048762	5.31E-05	mesenchymal cell differentiation	185
GO:0019901	5.35E-05	protein kinase binding	549
GO:0035051	5.37E-05	cardiocyte differentiation	112
GO:0007188	5.43E-05	adenylate cyclase-modulating G-protein coupled receptor signaling pathway	148
GO:0031325	7.91E-05	positive regulation of cellular metabolic process	2789
GO:0016310	9.10E-05	phosphorylation	2230
GO:1905207	9.33E-05	regulation of cardiocyte differentiation	28
GO:0051173	9.78E-05	positive regulation of nitrogen compound metabolic process	1721
GO:0032640	0.00010204	tumor necrosis factor production	101
GO:0072073	0.00010338	kidney epithelium development	137
GO:0008285	0.00010511	negative regulation of cell proliferation	653
GO:0030279	0.00010769	negative regulation of ossification	70
GO:1903555	0.00012191	regulation of tumor necrosis factor superfamily cytokine production	103
GO:0071396	0.00014783	cellular response to lipid	528
GO:0051960	0.0001588	regulation of nervous system development	767
GO:0007178	0.00018397	transmembrane receptor protein serine/threonine kinase signaling pathway	316
GO:0070167	0.00020641	regulation of biomineral tissue development	76
GO:0060047	0.00022896	heart contraction	253
GO:1903522	0.00023883	regulation of blood circulation	299
GO:0045596	0.00024172	negative regulation of cell differentiation	624
GO:0043067	0.00024414	regulation of programmed cell death	1409
GO:0070252	0.00025414	actin-mediated cell contraction	94
GO:0060350	0.00027274	endochondral bone morphogenesis	47
GO:0061035	0.00027951	regulation of cartilage development	63
GO:0006915	0.00028555	apoptotic process	1817
GO:0009968	0.00031845	negative regulation of signal transduction	1102
GO:0002062	0.00032861	chondrocyte differentiation	98
GO:0001657	0.00032861	ureteric bud development	97
GO:1901862	0.00034149	negative regulation of muscle tissue development	35
GO:0048635	0.00034149	negative regulation of muscle organ development	35
GO:0051046	0.00035688	regulation of secretion	691
GO:0034284	0.00036561	response to monosaccharide	197
GO:0000904	0.00038062	cell morphogenesis involved in differentiation	638
GO:0071383	0.00039253	cellular response to steroid hormone stimulus	242
GO:0030282	0.00042046	bone mineralization	101
GO:0001819	0.00043055	positive regulation of cytokine production	386
GO:0043010	0.00043862	camera-type eye development	290

GO:0050678	0.00049773	regulation of epithelial cell proliferation	293
GO:0031960	0.00051306	response to corticosteroid	182
GO:0040036	0.00051374	regulation of fibroblast growth factor receptor signaling pathway	25
GO:0051272	0.0005188	positive regulation of cellular component movement	416
GO:0030203	0.00057239	glycosaminoglycan metabolic process	163
GO:0045778	0.00057948	positive regulation of ossification	86
GO:0030500	0.00058478	regulation of bone mineralization	70
GO:1903708	0.0006065	positive regulation of hemopoiesis	164
GO:0034762	0.00075322	regulation of transmembrane transport	402
GO:1902105	0.00084469	regulation of leukocyte differentiation	238
GO:0030515	0.00088805	snoRNA binding	29
GO:0001678	0.00091685	cellular glucose homeostasis	130
GO:0006941	0.00095072	striated muscle contraction	151
GO:1901342	0.0009658	regulation of vasculature development	239
GO:0061564	0.00097781	axon development	460
GO:0061036	0.00104913	positive regulation of cartilage development	29
GO:0022408	0.00110627	negative regulation of cell-cell adhesion	133
GO:0051246	0.00124341	regulation of protein metabolic process	2476
GO:0009891	0.00130179	positive regulation of biosynthetic process	1726
GO:1904018	0.00132683	positive regulation of vasculature development	136
GO:0032330	0.00135973	regulation of chondrocyte differentiation	46
GO:0032268	0.0014243	regulation of cellular protein metabolic process	2327
GO:2000736	0.00143492	regulation of stem cell differentiation	31
GO:0061314	0.00152391	Notch signaling involved in heart development	9
GO:0060290	0.00152391	transdifferentiation	9
GO:0048541	0.00152391	Peyer,s patch development	9
GO:1901203	0.00152391	positive regulation of extracellular matrix assembly	9
GO:0043392	0.00152804	negative regulation of DNA binding	46
GO:0072077	0.00156317	renal vesicle morphogenesis	19
GO:0031690	0.00156317	adrenergic receptor binding	19
GO:0060548	0.00161888	negative regulation of cell death	895
GO:1903115	0.00166279	regulation of actin filament-based movement	32
GO:0006464	0.00202851	cellular protein modification process	3772
GO:0072190	0.00221239	ureter urothelium development	3
GO:0060584	0.00221239	regulation of prostaglandin-endoperoxide synthase activity	3
GO:0010621	0.00221239	negative regulation of transcription by transcription factor localization	3
GO:0071880	0.00231119	adenylate cyclase-activating adrenergic receptor signaling pathway	21
GO:0010717	0.00235651	regulation of epithelial to mesenchymal transition	67
GO:0032890	0.00236417	regulation of organic acid transport	50
GO:0050707	0.00246524	regulation of cytokine secretion	147
GO:0061180	0.00256617	mammary gland epithelium development	68
GO:0006026	0.00256617	aminoglycan catabolic process	68
GO:0005160	0.00261848	transforming growth factor beta receptor binding	51
GO:0046717	0.00272801	acid secretion	107
GO:0051223	0.0027497	regulation of protein transport	746
GO:0003094	0.00276422	glomerular filtration	22
GO:1903035	0.00278988	negative regulation of response to wounding	69
GO:0010039	0.00284532	response to iron ion	36
GO:0071322	0.00295806	cellular response to carbohydrate stimulus	129
GO:0014032	0.00302824	neural crest cell development	70
GO:0008284	0.00310219	positive regulation of cell proliferation	839
GO:0051961	0.00310412	negative regulation of nervous system development	268
GO:0007411	0.00352259	axon guidance	223
GO:0098801	0.00362226	regulation of renal system process	38
GO:0003214	0.00375346	cardiac left ventricle morphogenesis	12
GO:0090183	0.00384504	regulation of kidney development	55
GO:1903846	0.00384531	positive regulation of cellular response to transforming growth factor beta stimulus	24
GO:0003203	0.00384531	endocardial cushion morphogenesis	24
GO:0051147	0.00389592	regulation of muscle cell differentiation	157
GO:0070201	0.00392004	regulation of establishment of protein localization	821
GO:0070169	0.00406294	positive regulation of biomineral tissue development	39
GO:0042551	0.00406294	neuron maturation	39
GO:0010001	0.00414365	glial cell differentiation	180
GO:0003099	0.00434435	positive regulation of the force of heart contraction by chemical signal	4
GO:0001996	0.00434435	positive regulation of heart rate by epinephrine-norepinephrine	4
GO:0050801	0.0044112	ion homeostasis	683
GO:0001077	0.00445594	transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	229
GO:0061037	0.0044799	negative regulation of cartilage development	26
GO:0002521	0.00482154	leukocyte differentiation	466
GO analysis (Biological Processes) E ₂ (Up-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0001819	2.82E-06	positive regulation of cytokine production	386
GO:0043269	1.11E-05	regulation of ion transport	599
GO:1903708	2.39E-05	positive regulation of hemopoiesis	164
GO:0007188	4.24E-05	adenylate cyclase-modulating G-protein coupled receptor signaling pathway	148

GO:0051046	4.80E-05	regulation of secretion	691
GO:0051174	0.00010978	regulation of phosphorus metabolic process	1632
GO:0090257	0.0001344	regulation of muscle system process	196
GO:0022008	0.00016999	neurogenesis	1433
GO:0045597	0.00018818	positive regulation of cell differentiation	830
GO:0032890	0.00019124	regulation of organic acid transport	50
GO:0032640	0.00032032	tumor necrosis factor production	101
GO:0010648	0.00033066	negative regulation of cell communication	1207
GO:0010604	0.00035147	positive regulation of macromolecule metabolic process	2795
GO:0006464	0.00036358	cellular protein modification process	3772
GO:1903555	0.00036603	regulation of tumor necrosis factor superfamily cytokine production	103
GO:0061314	0.00037496	Notch signaling involved in heart development	9
GO:0060290	0.00037496	transdifferentiation	9
GO:0071880	0.00038503	adenylate cyclase-activating adrenergic receptor signaling pathway	21
GO:0003094	0.00046435	glomerular filtration	22
GO:0046717	0.0004734	acid secretion	107
GO:0030182	0.0004807	neuron differentiation	1231
GO:0072073	0.0005518	kidney epithelium development	137
GO:0034660	0.00055284	ncRNA metabolic process	559
GO:0034284	0.00055621	response to monosaccharide	197
GO:1903522	0.00061361	regulation of blood circulation	299
GO:0010647	0.00063347	positive regulation of cell communication	1525
GO:0030855	0.00071071	epithelial cell differentiation	567
GO:0009967	0.00072649	positive regulation of signal transduction	1396
GO:1902105	0.00072768	regulation of leukocyte differentiation	238
GO:0016310	0.00088982	phosphorylation	2230
GO:0034110	0.00089974	regulation of homotypic cell-cell adhesion	26
GO:0001649	0.00090666	osteoblast differentiation	209
GO:0050707	0.00091524	regulation of cytokine secretion	147
GO:0009968	0.00105324	negative regulation of signal transduction	1102
GO:0048762	0.00125694	mesenchymal cell differentiation	185
GO:0001657	0.00130912	ureteric bud development	97
GO:0060047	0.00132637	heart contraction	253
GO:0051952	0.00137782	regulation of amine transport	72
GO:0051246	0.00143769	regulation of protein metabolic process	2476
GO:0032268	0.0014578	regulation of cellular protein metabolic process	2327
GO:0050678	0.00154674	regulation of epithelial cell proliferation	293
GO:0071407	0.00164542	cellular response to organic cyclic compound	527
GO:0001996	0.00167802	positive regulation of heart rate by epinephrine-norepinephrine	4
GO:0001678	0.00168925	cellular glucose homeostasis	130
GO:0071396	0.00175521	cellular response to lipid	528
GO:0010574	0.00177185	regulation of vascular endothelial growth factor production	31
GO:0002070	0.00188339	epithelial cell maturation	15
GO:0022408	0.00195114	negative regulation of cell-cell adhesion	133
GO:1903115	0.00199827	regulation of actin filament-based movement	32
GO:0015837	0.0022214	amine transport	79
GO:0003215	0.00228913	cardiac right ventricle morphogenesis	16
GO:0031325	0.0023299	positive regulation of cellular metabolic process	2789
GO:1902531	0.00245431	regulation of intracellular signal transduction	1683
GO:0060548	0.00264628	negative regulation of cell death	895
GO:0001977	0.00274504	renal system process involved in regulation of blood volume	17
GO:0042695	0.0027654	thelarche	5
GO:0046813	0.0027654	receptor-mediated virion attachment to host cell	5
GO:0010912	0.0027654	positive regulation of isomerase activity	5
GO:0003197	0.00279613	endocardial cushion development	35
GO:0071383	0.00289337	cellular response to steroid hormone stimulus	242
GO:0050867	0.00306951	positive regulation of cell activation	318
GO:0010039	0.00310427	response to iron ion	36
GO:2000027	0.00317859	regulation of organ morphogenesis	246
GO:0002526	0.00333379	acute inflammatory response	145
GO:0045778	0.00340578	positive regulation of ossification	86
GO:0051173	0.00348558	positive regulation of nitrogen compound metabolic process	1721
GO:0098801	0.00378842	regulation of renal system process	38
GO:0032674	0.003815	regulation of interleukin-5 production	19
GO:0006260	0.00385349	DNA replication	288
GO:0031960	0.00393918	response to corticosteroid	182
GO:1904029	0.00403625	regulation of cyclin-dependent protein kinase activity	89
GO:0061156	0.00410174	pulmonary artery morphogenesis	6
GO:0006941	0.00426254	striated muscle contraction	151
GO:0051223	0.0043219	regulation of protein transport	746
GO:0060602	0.00443249	branch elongation of an epithelium	20
GO:0003416	0.00443249	endochondral bone growth	20
GO:0007169	0.0044931	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0008284	0.00453465	positive regulation of cell proliferation	839
GO:0010605	0.0047618	negative regulation of macromolecule metabolic process	2297
GO:2000377	0.00479584	regulation of reactive oxygen species metabolic process	154
GO:0051272	0.0049856	positive regulation of cellular component movement	416

GO analysis (Biological Processes) E ₂ (Down-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0071363	6.62E-06	cellular response to growth factor stimulus	609
GO:0045667	0.00019037	regulation of osteoblast differentiation	113
GO:0072190	0.00032642	ureter urothelium development	3
GO:0001649	0.00037313	osteoblast differentiation	209
GO:1901862	0.00047738	negative regulation of muscle tissue development	35
GO:0048635	0.00047738	negative regulation of muscle organ development	35
GO:0003197	0.00047738	endocardial cushion development	35
GO:0007178	0.00052779	transmembrane receptor protein serine/threonine kinase signaling pathway	316
GO:0007169	0.00062263	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0030500	0.00078886	regulation of bone mineralization	70
GO:0030279	0.00078886	negative regulation of ossification	70
GO:0035051	0.00112473	cardiocyte differentiation	112
GO:0060602	0.00113736	branch elongation of an epithelium	20
GO:0000904	0.00115088	cell morphogenesis involved in differentiation	638
GO:0070167	0.00115167	regulation of biomineral tissue development	76
GO:2000027	0.00116036	regulation of organ morphogenesis	246
GO:0051960	0.00119862	regulation of nervous system development	767
GO:0032330	0.00125189	regulation of chondrocyte differentiation	46
GO:0051147	0.00131562	regulation of muscle cell differentiation	157
GO:1902531	0.00140327	regulation of intracellular signal transduction	1683
GO:0061156	0.0015985	pulmonary artery morphogenesis	6
GO:0005160	0.00199956	transforming growth factor beta receptor binding	51
GO:0051961	0.0021472	negative regulation of nervous system development	268
GO:0061037	0.00220791	negative regulation of cartilage development	26
GO:0045596	0.00238351	negative regulation of cell differentiation	624
GO:2000020	0.00294283	positive regulation of male gonad development	8
GO:0010001	0.00295911	glial cell differentiation	180
GO:0009967	0.00299421	positive regulation of signal transduction	1396
GO:1905207	0.00307307	regulation of cardiocyte differentiation	28
GO:0061036	0.00340141	positive regulation of cartilage development	29
GO:0008285	0.00340379	negative regulation of cell proliferation	653
GO:0002695	0.00350628	negative regulation of leukocyte activation	139
GO:0043010	0.00362883	camera-type eye development	290
GO:0071407	0.00372976	cellular response to organic cyclic compound	527
GO:0010092	0.00375042	specification of animal organ identity	30
GO:1901203	0.00375756	positive regulation of extracellular matrix assembly	9
GO:0030282	0.00407151	bone mineralization	101
GO:0061035	0.00408204	regulation of cartilage development	63
GO:0071868	0.0041205	cellular response to monoamine stimulus	31
GO:0022008	0.00423929	neurogenesis	1433
GO:0010647	0.00496162	positive regulation of cell communication	1525

Appendix. Supplementary Table B. List containing the signaling pathways (p-value < 0.005) over-represented upon analysis of all DEGs, up-regulated DEGs, and down-regulated DEGs for the treatments with AtRA or E₂

Pathway Analysis AtRA (All DEGs)			
pathway	p-value	source	Size
Vitamin D Receptor Pathway	3.66E-07	Wikipathways	184
Axon guidance	2.93E-05	Reactome	487
Non-integrin membrane-ECM interactions	3.09E-05	Reactome	46
Vitamin A and Carotenoid Metabolism	4.00E-05	Wikipathways	43
PPAR signaling pathway - Homo sapiens (human)	4.93E-05	KEGG	72
Extracellular matrix organization	6.73E-05	Reactome	295
Adipogenesis	7.01E-05	Wikipathways	131
Axon guidance - Homo sapiens (human)	7.53E-05	KEGG	177
ECM proteoglycans	0.000127262	Reactome	57
Developmental Biology	0.000134216	Reactome	748
Metabolism of lipids and lipoproteins	0.000143609	Reactome	728
Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)	0.00015698	Wikipathways	42
Pathways in cancer - Homo sapiens (human)	0.000201855	KEGG	397
Metabolism	0.000208405	Reactome	2035
Signaling by Retinoic Acid	0.000245755	Reactome	44
PPAR signaling pathway	0.000337252	Wikipathways	68
Rap1 signaling pathway - Homo sapiens (human)	0.000360863	KEGG	212
Phase 1 - Functionalization of compounds	0.000631307	Reactome	110
Nucleotide GPCRs	0.000658554	Wikipathways	11
NHR	0.000666814	Signalink	49
Beta1 integrin cell surface interactions	0.000983142	PID	70
Integrin cell surface interactions	0.000983142	Reactome	68
Pre-NOTCH Processing in Golgi	0.00106125	Reactome	12
Import of palmitoyl-CoA into the mitochondrial matrix	0.00106125	Reactome	12
Pathway_PA165986194 - need delete	0.001254317	PharmGKB	24
Acetaminophen Pathway, Pharmacokinetics	0.001254317	PharmGKB	24
Adenosine P1 receptors	0.001474588	Reactome	4
Fosphenytoin (Antiarrhythmic) Metabolism Pathway	0.001474588	SMPDB	4
Neurophilin interactions with VEGF and VEGFR	0.001474588	Reactome	4
Spinal Cord Injury	0.002034495	Wikipathways	117
VEGFA-VEGFR2 Signaling Pathway	0.002096082	Wikipathways	236
Vitamin A (retinol) metabolism	0.002096177	EHMN	26
RXR and RAR heterodimerization with other nuclear receptor	0.002096177	PID	26
A tetrasaccharide linker sequence is required for GAG synthesis	0.002096177	Reactome	26
Alpha 6 Beta 4 signaling pathway	0.002124419	Wikipathways	33
Sialic acid metabolism	0.002124419	Reactome	33
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0.002131459	KEGG	56
ECM-receptor interaction - Homo sapiens (human)	0.002317444	KEGG	82
Validated nuclear estrogen receptor alpha network	0.002371623	PID	65
Syndecan interactions	0.00239323	Reactome	21
Visual phototransduction	0.002603667	Reactome	102
Nuclear Receptors Meta-Pathway	0.002810991	Wikipathways	316
Transmembrane transport of small molecules	0.003112599	Reactome	628
Synthesis of IP3 and IP4 in the cytosol	0.003318808	Reactome	28
Fatty Acid Omega Oxidation	0.003345048	Wikipathways	15
Negative regulation of TCF-dependent signaling by WNT ligand antagonists	0.003345048	Reactome	15
Aromatase Inhibitor Pathway (Multiple Tissues), Pharmacodynamics	0.003485645	PharmGKB	5
Hippo signaling pathway - Homo sapiens (human)	0.003783138	KEGG	154
Integrin	0.003883399	INOH	124
EPH-Ephrin signaling	0.004060299	Reactome	78
Transcription factor regulation in adipogenesis	0.004062006	Wikipathways	22
The canonical retinoid cycle in rods (twilight vision)	0.004062006	Reactome	22
EV release from cardiac cells and their functional effects	0.004174833	Wikipathways	10
Bicarbonate transporters	0.004174833	Reactome	10
Leukocyte transendothelial migration - Homo sapiens (human)	0.004470534	KEGG	116
Nucleotide-like (purinergic) receptors	0.004576067	Reactome	16
Pathway Analysis AtRA (Up-regulated DEGs)			
pathway	p-value	source	size
Vitamin A and Carotenoid Metabolism	2.47E-05	Wikipathways	43
Adipogenesis	4.51E-05	Wikipathways	131
Vitamin D Receptor Pathway	5.51E-05	Wikipathways	184
Metabolism	5.63E-05	Reactome	2035
Metabolism of lipids and lipoproteins	8.28E-05	Reactome	728
Adenosine P1 receptors	0.000409583	Reactome	4
Neurophilin interactions with VEGF and VEGFR	0.000409583	Reactome	4
The canonical retinoid cycle in rods (twilight vision)	0.000431205	Reactome	22
Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)	0.000689127	Wikipathways	42
Sialic acid metabolism	0.00075661	Reactome	33
Validated nuclear estrogen receptor alpha network	0.000903314	PID	65

Signaling by Retinoic Acid	0.000951999	Reactome	44
Aromatase Inhibitor Pathway (Multiple Tissues), Pharmacodynamics	0.000987798	PharmGKB	5
Nucleotide GPCRs	0.001265066	WikiPathways	11
Phase 1 - Functionalization of compounds	0.001485639	Reactome	110
Type II interferon signaling (IFNG)	0.001547016	WikiPathways	37
Caspase activation via extrinsic apoptotic signalling pathway	0.001708416	Reactome	28
antigen processing and presentation	0.001826872	BioCarta	12
Vitamins	0.001906	Reactome	6
PPAR signaling pathway - Homo sapiens (human)	0.002011123	KEGG	72
Nuclear Receptors Meta-Pathway	0.002158666	WikiPathways	316
Axon guidance	0.002540122	Reactome	487
Tryptophan catabolism	0.002540626	Reactome	13
Type II diabetes mellitus	0.00317011	WikiPathways	22
tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde	0.003218282	HumanCyc	7
Non-integrin membrane-ECM interactions	0.003303352	Reactome	46
Activation of IRF3/IRF7 mediated by TBK1/IKK epsilon	0.003424753	Reactome	14
Spinal Cord Injury	0.003599698	WikiPathways	117
Folate Metabolism	0.003771861	WikiPathways	66
RA biosynthesis pathway	0.003895077	Reactome	23
Copper homeostasis	0.004142672	WikiPathways	55
Beta1 integrin cell surface interactions	0.004178768	PID	70
Integrin cell surface interactions	0.004178768	Reactome	68
VEGFA-VEGFR2 Signaling Pathway	0.004183357	WikiPathways	236
Thiamine metabolism - Homo sapiens (human)	0.00449694	KEGG	16
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0.004637407	KEGG	56
Signaling by VEGF	0.00484328	Reactome	290
Nifedipine Activity	0.004968739	WikiPathways	8
Axonal growth inhibition (RHOA activation)	0.004968739	Reactome	10
Glial Cell Differentiation	0.004968739	WikiPathways	8
NAD Biosynthesis II (from tryptophan)	0.004968739	WikiPathways	8
Pathway Analysis AtRA (Down-regulated DEGs)			
pathway	p-value	source	size
Hippo signaling pathway - Homo sapiens (human)	3.98E-05	KEGG	154
Ectoderm Differentiation	7.45E-05	WikiPathways	142
Pathways in cancer - Homo sapiens (human)	9.33E-05	KEGG	397
Ca2+ pathway	0.000489106	Reactome	55
Synthesis of IP3 and IP4 in the cytosol	0.000657632	Reactome	28
Thyroid hormone signaling pathway - Homo sapiens (human)	0.000918703	KEGG	118
Phosphatidylinositol signaling system - Homo sapiens (human)	0.000997774	KEGG	99
Inflammatory mediator regulation of TRP channels - Homo sapiens (human)	0.000997774	KEGG	99
Signaling pathways regulating pluripotency of stem cells - Homo sapiens (human)	0.001092023	KEGG	142
Rap1 signaling pathway - Homo sapiens (human)	0.001173489	KEGG	212
Prostanoid ligand receptors	0.001260231	Reactome	9
Elastic fibre formation	0.001875317	Reactome	36
G alpha (12/13) signalling events	0.001887001	Reactome	90
Axon guidance - Homo sapiens (human)	0.001983494	KEGG	177
Inositol phosphate metabolism - Homo sapiens (human)	0.002259666	KEGG	71
Phosphatidylinositol phosphate metabolism	0.002335199	EHMN	91
cxcr4 signaling pathway	0.002382519	BioCarta	11
Adrenaline,noradrenaline inhibits insulin secretion	0.002579116	Reactome	23
Vitamin D Receptor Pathway	0.002742832	WikiPathways	184
ECM proteoglycans	0.00276828	Reactome	57
Pathway_PA165986194 -need delete	0.003033055	PharmGKB	24
Acetaminophen Pathway, Pharmacokinetics	0.003033055	PharmGKB	24
phospholipases	0.003058975	HumanCyc	39
Pre-NOTCH Processing in Golgi	0.003116606	Reactome	12
Axon guidance	0.003192113	Reactome	487
Extracellular matrix organization	0.003321536	Reactome	295
Wnt Canonical	0.003374472	INOH	120
Wnt Mammals	0.003374472	INOH	120
Glycosaminoglycan metabolism	0.003775006	Reactome	122
HCN channels	0.003820173	Reactome	4
Imipramine/Desipramine Pathway, Pharmacokinetics	0.003820173	PharmGKB	4
Clomipramine Pathway, Pharmacokinetics	0.003820173	PharmGKB	4
Clomipramine Metabolism Pathway	0.003820173	SMPDB	4
D-Glutamine and D-glutamate metabolism - Homo sapiens (human)	0.003820173	KEGG	4
Serotonin and anxiety-related events	0.003975054	WikiPathways	13
ID	0.004098563	NetPath	26
A tetrasaccharide linker sequence is required for GAG synthesis	0.004098563	Reactome	26
Non-integrin membrane-ECM interactions	0.00424522	Reactome	46
NRAGE signals death through JNK	0.004296209	Reactome	62
Developmental Biology	0.004913148	Reactome	748
Pathway Analysis E2 (all DEGs)			
pathway	p-value	source	size
HIF-1-alpha transcription factor network	2.58E-07	PID	67
Adipogenesis	6.54E-07	WikiPathways	131
Notch-mediated HES/HEY network	6.69E-06	PID	51

TGF-beta signaling pathway - Homo sapiens (human)	1.92E-05	KEGG	84
RAF-independent MAPK1/3 activation	2.93E-05	Reactome	23
Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors	0.000117706	Reactome	42
Interleukin-6 signaling	0.00016376	Reactome	11
Notch Signaling Pathway	0.000261871	WikiPathways	61
rRNA processing	0.000366253	Reactome	67
Hepatitis C and Hepatocellular Carcinoma	0.000371965	WikiPathways	50
IL-6 signaling	0.000396221	INOH	6
Validated nuclear estrogen receptor alpha network	0.000407758	PID	65
tumor suppressor arf inhibits ribosomal biogenesis	0.000436541	BioCarta	24
G1 to S cell cycle control	0.000555734	WikiPathways	68
Ectoderm Differentiation	0.000582945	WikiPathways	142
ID	0.000645559	NetPath	26
ID signaling pathway	0.000809165	WikiPathways	16
Validated targets of C-MYC transcriptional repression	0.000981503	PID	75
rRNA modification in the nucleus and cytosol	0.001052336	Reactome	61
rRNA processing in the nucleus and cytosol	0.001052336	Reactome	61
Interleukin-11 Signaling Pathway	0.001256524	WikiPathways	44
IL17 signaling pathway	0.001488678	WikiPathways	31
Spinal Cord Injury	0.001510861	WikiPathways	117
MAPK1 (ERK2) activation	0.001563904	Reactome	9
IL6-mediated signaling events	0.001782908	PID	48
MAPK3 (ERK1) activation	0.002188492	Reactome	10
LIF signaling	0.002188492	INOH	10
Mammary gland development pathway - Involution (Stage 4 of 4)	0.002188492	WikiPathways	10
Organic cation transport	0.002188492	Reactome	11
Transcription factor regulation in adipogenesis	0.00285234	WikiPathways	22
Glucocorticoid Receptor Pathway	0.003162903	WikiPathways	71
mechanism of gene regulation by peroxisome proliferators via ppara	0.003318134	BioCarta	53
Type II interferon signaling (IFNG)	0.003334849	WikiPathways	37
Sympathetic Nerve Pathway (Neuroeffector Junction)	0.003378401	PharmGKB	23
Endoderm Differentiation	0.003427426	WikiPathways	71
melatonin degradation I	0.003850258	HumanCyc	13
Mitotic G1-G1/S phases	0.003947158	Reactome	92
TGF-beta Receptor Signaling	0.004001049	WikiPathways	55
Oncostatin_M	0.004210748	NetPath	39
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0.004379061	KEGG	56
BMP2 signaling TGF-beta MV	0.004379061	INOH	56
TFAP2 (AP-2) family regulates transcription of other transcription factors	0.00442541	Reactome	4
Estrogen Receptor Pathway	0.004903476	WikiPathways	13
Activation of the TFAP2 (AP-2) family of transcription factors	0.004903476	Reactome	13
superpathway of melatonin degradation	0.004903476	HumanCyc	14
Pathway Analysis E2 (Up-regulated DEGs)			
pathway	p-value	source	size
RAF-independent MAPK1/3 activation	2.3336E-06	Reactome	23
rRNA processing	1.672E-05	Reactome	67
Validated nuclear estrogen receptor alpha network	1.8778E-05	PID	65
Interleukin-6 signaling	2.9263E-05	Reactome	11
rRNA modification in the nucleus and cytosol	7.175E-05	Reactome	61
rRNA processing in the nucleus and cytosol	7.175E-05	Reactome	61
Notch Signaling Pathway	9.9508E-05	WikiPathways	61
IL-6 signaling	0.00010705	INOH	6
Adipogenesis	0.00011537	WikiPathways	131
Interleukin-11 Signaling Pathway	0.00011943	WikiPathways	44
G1 to S cell cycle control	0.00019902	WikiPathways	68
Notch-mediated HES/HEY network	0.00021971	PID	51
Hepatitis C and Hepatocellular Carcinoma	0.00021971	WikiPathways	50
Mitotic G1-G1/S phases	0.00022953	Reactome	92
Cell cycle - Homo sapiens (human)	0.00036895	KEGG	124
MAPK1 (ERK2) activation	0.00043212	Reactome	9
Type II interferon signaling (IFNG)	0.00046859	WikiPathways	37
TGF-beta Signaling Pathway	0.0005837	WikiPathways	132
MAPK3 (ERK1) activation	0.00060923	Reactome	10
LIF signaling	0.00060923	INOH	10
Mammary gland development pathway - Involution (Stage 4 of 4)	0.00060923	WikiPathways	10
Sympathetic Nerve Pathway (Neuroeffector Junction)	0.00066382	PharmGKB	23
Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors	0.00076041	Reactome	42
tumor suppressor arf inhibits ribosomal biogenesis	0.00078555	BioCarta	24
Ectoderm Differentiation	0.00098621	WikiPathways	142
IL27-mediated signaling events	0.00107484	PID	26
HIF-1-alpha transcription factor network	0.00120214	PID	67
Interleukin-6 family signaling	0.00124432	Reactome	27
Proton Pump Inhibitor Pathway, Pharmacodynamics	0.00129425	PharmGKB	46
IL6-mediated signaling events	0.00142764	PID	48
G1/S Transition	0.00151124	Reactome	70
Cyclin E associated events during G1/S transition	0.00163778	Reactome	29
Regulation of IFNG signaling	0.00175316	Reactome	14

IL17 signaling pathway	0.00211026	Wikipathways	31
E2F transcription factor network	0.00230722	PID	79
Cell Cycle	0.00240863	Wikipathways	103
Th17 cell differentiation - Homo sapiens (human)	0.00298655	KEGG	107
ifn gamma signaling pathway	0.00304607	BioCarta	6
TFAP2 (AP-2) family regulates transcription of cell cycle factors	0.00304607	Reactome	5
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0.00312421	KEGG	56
TGF_beta_Receptor	0.0039603	NetPath	176
IL2 signaling events mediated by PI3K	0.00407599	PID	37
Small cell lung cancer - Homo sapiens (human)	0.00428564	KEGG	86
Interferon gamma signaling	0.00437078	Reactome	19
IFN gamma signaling	0.00451558	INOH	6
deregulation of cdk5 in alzheimers disease	0.00451558	BioCarta	6
Signaling by NOTCH1	0.00451558	Wikipathways	6
Coregulation of Androgen receptor activity	0.00452975	PID	61
Leptin	0.00485717	NetPath	62
Spinal Cord Injury	0.00489533	Wikipathways	117
Oncostatin_M	0.0049377	NetPath	39
Pathway Analysis E ₂ (Down-regulated DEGs)			
pathway	p-value	source	size
HIF-1-alpha transcription factor network	5.081E-05	PID	67
TGF-beta signaling pathway - Homo sapiens (human)	0.00018049	KEGG	84
Monoamine Transport	0.00026554	Wikipathways	32
ID signaling pathway	0.00048271	Wikipathways	16
Glucocorticoid Receptor Pathway	0.00063495	Wikipathways	71
Validated targets of C-MYC transcriptional repression	0.00081895	PID	75
GRB7 events in ERBB2 signaling	0.00095351	Reactome	5
miR-517 relationship with ARCN1 and USP1	0.00095351	Wikipathways	5
Transcription factor regulation in adipogenesis	0.00127113	Wikipathways	22
Imipramine Metabolism Pathway	0.00142099	SMPDB	6
Adipogenesis	0.00190389	Wikipathways	131
ID	0.00208507	NetPath	26
BMP2 signaling TGF-beta MV	0.00225916	INOH	56
Direct p53 effectors	0.00295609	PID	147
Nuclear Receptors Meta-Pathway	0.00406333	Wikipathways	316
BMP signaling Dro	0.00453064	INOH	34
DNA Damage Response (only ATM dependent)	0.00469413	Wikipathways	110

Appendix. Supplementary Table C. List containing the biological processes (p-value < 0.005) over-represented upon GO analysis of all E₂ DEGs whose expression pattern was significantly reduced/counteracted or enhanced upon co-exposure with AtRA.

GO analysis (AtRA-mediated reducing/counteracting effects on E ₂ DEGs)			
term_goid	p-value	term_name	size
GO:0060584	5,64E-05	regulation of prostaglandin-endoperoxide synthase activity	3
GO:0006915	7,35E-05	apoptotic process	1817
GO:0032640	8,02E-05	tumor necrosis factor production	101
GO:1903555	8,80E-05	regulation of tumor necrosis factor superfamily cytokine production	103
GO:1902531	8,93E-05	regulation of intracellular signal transduction	1683
GO:0043067	0,00010108	regulation of programmed cell death	1409
GO:0032103	0,000151911	positive regulation of response to external stimulus	264
GO:0009967	0,00030057	positive regulation of signal transduction	1396
GO:0051272	0,000455662	positive regulation of cellular component movement	416
GO:0060548	0,000474489	negative regulation of cell death	895
GO:0010647	0,00079651	positive regulation of cell communication	1525
GO:0008631	0,000998557	intrinsic apoptotic signaling pathway in response to oxidative stress	45
GO:0008630	0,001116046	intrinsic apoptotic signaling pathway in response to DNA damage	104
GO:0001819	0,001467567	positive regulation of cytokine production	386
GO:0010648	0,002069169	negative regulation of cell communication	1207
GO:0032652	0,002084629	regulation of interleukin-1 production	58
GO:0051046	0,003053213	regulation of secretion	691
GO:0070168	0,003071124	negative regulation of biomineral tissue development	20
GO:0045597	0,003219535	positive regulation of cell differentiation	830
GO:0051767	0,003402717	nitric-oxide synthase biosynthetic process	20
GO:0060602	0,003402717	branch elongation of an epithelium	20
GO:0008284	0,003476704	positive regulation of cell proliferation	839
GO:0051952	0,003856648	regulation of amine transport	72
GO:0050707	0,003934358	regulation of cytokine secretion	147
GO:0048011	0,00411365	neurotrophin TRK receptor signaling pathway	22
GO:0032092	0,004165772	positive regulation of protein binding	74
GO:0051174	0,004274859	regulation of phosphorus metabolic process	1632
GO:0070167	0,004325819	regulation of biomineral tissue development	76
GO analysis (AtRA-mediated additive/enhancing effects on E ₂ DEGs)			
term_goid	p-value	term_name	size
GO:0008300	0.00041133	isoprenoid catabolic process	8
GO:0090257	0.00099642	regulation of muscle system process	196
GO:0016311	0.00159364	dephosphorylation	440
GO:0006941	0.00284731	striated muscle contraction	151

Chapter 7

Relating transcriptomic profiles induced by general and subtype-selective retinoic acid receptor agonists to their divergent proliferative effects in the T47D cell line

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Abstract

Retinoic Acid Receptors (RARs) are retinoid-modulated transcription factors regulating breast cancer cell proliferation via the classical genomic signaling or via crosstalk with other molecular pathways. There are three RAR subtypes (RAR α , RAR β and RAR γ) that have proven to elicit differential effects on breast cancer growth. However, the molecular mechanisms underlying these distinct roles are still not completely understood. The objective of this study was to characterize and compare the effects of general and subtype-selective agonists on the proliferation and the transcriptomic profiles of the T47D breast cancer cell line. Thus, potential effects on RAR or other NR pathways of importance in breast cancer growth were analysed. For this purpose, methods assessing proliferation (BrdU and MTT assays) and gene expression (reporter gene, RNA-seq and RT-qPCR assays) were performed. The results show that the general RAR agonist AtRA and the RAR α selective Am80 highly inhibited breast cancer cell proliferation while the RAR β agonist CD2314 and the RAR γ agonist BMS961 were ineffective showing even weakly increased proliferation. When analyzing global gene expression profiles, effective (anti-proliferative) and ineffective retinoids were distinguished upon hierarchical clustering. In addition, the larger effects on gene expression elicited by AtRA and Am80 pointed at RAR α as the main RAR subtype involved in the effects of retinoids on gene expression. Differences in the expression patterns between effective and ineffective retinoids were additionally observed when assessing specific genes involved in relevant processes such as proliferation, differentiation and apoptosis. Strikingly, pathways of multiple NRs which can influence breast cancer growth such as RAR, ER, VDR and GR were highly affected showing differences in the magnitude of response elicited by the effective and the ineffective retinoid treatments. In summary, the results of this study indicated that various small differences on many pathways regulating breast cancer growth may sum up leading to the differential responses elicited by the different retinoids in breast cancer cells.

1 Introduction

Nuclear Receptors (NRs) such as Retinoic Acid Receptors (RARs) are ligand-driven transcription factors regulating gene expression that play a crucial role in a wide variety of physiological and pathological processes. For instance, RARs and their ligands (retinoids) have been related to breast cancer growth modulation [1–3]. By the classical pathway, RARs bind to sequences called retinoic acid responsive elements (RARE) located in promoter regions of genes which expression leads to differentiation/apoptosis [4,5]. However, retinoids, like *all-trans* retinoic acid (AtRA), play roles in many other pathways (RAR-mediated or not) modulating gene expression [6,7]. Thus, crosstalk between retinoids and other NR pathways affecting proliferation (e.g. the Estrogen Receptor (ER) pathway) has been reported [8,9].

Three different RAR subtypes (RAR α , RAR β and RAR γ) have been described and their expression is variable in breast cancer cells [2]. While RAR α and RAR γ are often highly expressed in breast cancer cells, RAR β expression levels are considerably lower [2]. Both amino acid sequences [7] and DNA binding regions of the RAR subtypes have been proven to be highly similar leading to an expected functional redundancy [8,10]. However, the different RAR subtypes can exert different and even opposite effects on breast cancer cell proliferation [11,12]. In fact, activation of the different RARs by subtype-selective agonists has been shown to elicit different effects on breast cancer cell proliferation [13]. While RAR α -selective agonists have been shown to hamper proliferation, both RAR β - and RAR γ -selective agonists have shown to exert weak or no remarkable effects on breast cancer growth [13]. Up to now, the molecular mechanisms underlying these differential effects observed upon retinoid treatment of breast cancer cells are still not completely understood. In consequence, studies evaluating broad gene expression signatures elicited upon exposure to the different RAR subtype-selective ligands may be of use to obtain further insights.

Previous research comparing the widely used ER+ breast cancer cell lines, MCF7 and T47D, showed significant differences in the basal expression of proteins related to biological processes such as proliferation, differentiation or apoptosis [14]. Despite this dissimilarity, most studies assessing retinoids for breast cancer treatment have been performed using only MCF7 as ER+ breast cancer cell model. Thus, while previous research has characterized the whole retinoid-driven transcriptome in MCF7 cells [4,5,8,9,15], only a low number of studies have assessed a few retinoid-responsive genes in the ER+ breast cancer cell line T47D [16–19]. Consequently, studies on the transcriptome triggered by exposure to general and subtype-selective retinoids in the T47D breast cancer cell line may bring new knowledge in the molecular mechanisms leading to the retinoid specific modulation of breast cancer growth.

The aim of this study was to characterize and compare the effects of the general agonist AtRA and the RAR α -, RAR β - and RAR γ -selective agonists Am80, CD2314 and BMS961, on the proliferation and the whole transcriptome of T47D cells. Apart from the effects on RAR signaling, possible crosstalk with other NR pathways playing important roles in proliferation, differentiation and apoptosis was analysed in detail.

2 Materials and Methods

2.1 Chemicals

17 β -estradiol (E2) and *all-trans* retinoic acid (AtRA) were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid (Am80) was acquired from Abcam (Cambridge, UK). 5-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-3-thiophenecarboxylic-acid (CD2314) and 3-fluoro-4-[[2-hydroxy-2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl) acetyl]amino]-benzoic acid (BMS961) were obtained from Tocris Bioscience (Bristol, UK). Dimethylsulphoxide (DMSO) was obtained from Acros (Geel, Belgium).

2.2 Cell culture procedures

In the present study, the human T47D breast cancer cell line (ATCC, Manassas, VA) and the reporter (luciferase-based) cell lines T47D-ER α -Luc [20] and U2OS-ER β -Luc (BDS, Amsterdam, The Netherlands) were utilized. Cell culture maintenance was carried out in 150 cm² flasks (Corning, NY, USA) using Dulbecco's Modified Eagle Medium DMEM/F12 (Gibco, Bleiswijk, The Netherlands) supplemented with 10% (v/v) FBS (Sigma-Aldrich, St Louis, MO, USA) (growth medium) and cells were kept in a humidified atmosphere at 37°C and 5% CO₂. Cells were passaged or used for the different assays when they reached 60-80% confluence. For the U2OS reporter gene cell line, Geneticine (G418) (Gibco, Bleiswijk, The Netherlands) was used for selection pressure. To ensure that the cells were not affected by any potential hormonal stimulation originating from the medium itself, all assays were performed using Dulbecco's Modified Eagle Medium (DMEM/F12) without phenol red (Gibco, Bleiswijk, The Netherlands) supplemented with 5% charcoal-dextran-treated serum foetal calf serum (Gibco, Bleiswijk, The Netherlands). For all the assays, exposure medium containing the test compounds was freshly prepared and contained a final concentration of 0.2% DMSO (solvent). A solvent control also containing 0.2% DMSO was utilized.

2.3 MTT and BrdU proliferation studies

T47D cells were seeded with hormone-deprived medium in flat-transparent 96-well plates (Corning, NY, USA) at a cell density per well of 1×10^4 (24- and 48-hour exposure studies), 5×10^3 (72-hour exposure studies) and 3×10^3 (6-day exposure studies) cells. Cells were allowed to attach and 24h later exposed to the test compounds. Exposure medium containing retinoids was replenished after 72h for the 6-day exposure study. After exposure for 24h, 48h, 72h and 6 days, the MTT assay was carried out. For the BrdU experiments a single exposure period of 72 h was applied.

For the MTT proliferation assays, cells were incubated for the last three hours of exposure with the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich

Chemie B.V., Zwijndrecht, the Netherlands) diluted in exposure medium (10% v/v). In order to dissolve the formazan crystals, medium was removed and 150 μ l of DMSO was added into each well. Absorbance was measured after 15 minutes incubation, at a wavelength of 562 nm with a Microplate Reader SpectraMax M2 (Molecular Devices, Sunnyvale CA, USA).

For the BrdU proliferation assay (Roche, Mannheim, Germany), BrdU labelling solution diluted in exposure medium (10% v/v) was added to the cells during the last 4 hours of exposure. Next, BrdU incorporation was measured by fixation-denaturation of the cells followed by incubation with BrdU detection antibodies and the corresponding substrate according to the manufacturer's guidelines (Roche, Mannheim, Germany). Colorimetric measurements were carried out at a wavelength of 370 nm with a Microplate Reader SpectraMax M2 (Molecular Devices, Sunnyvale CA, USA).

2.4 Next-generation sequencing (RNA-seq) and transcriptome analysis.

T47D cells were grown in 25 cm² flasks (Corning, NY, USA) and when they reached 50-60% confluence, growth medium was replaced by hormone-deprived medium. After 24 hours, cells were exposed to the compounds for a period of 6 hours. This period was chosen based on previous literature [15,21] and on the fact that at this time period no effects on cell viability were observed (results not shown). Next, cells were lysed and total RNA was extracted and purified with the Quick RNA Miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's guidelines. For quality control, spectrophotometric analysis using a Nanodrop (ND-1000 Thermoscientific Wilmington, Delaware, USA) and RIN analysis 2100 Bioanalyzer (Agilent Technologies California, EE. UU) were utilized. Only samples with RNA integrity number (RIN) values higher than 8 were accepted for analysis.

RNA-seq library preparation and sequencing was commissioned to BaseClear BV (Leiden, the Netherlands). The mRNA was purified by polyA capture, fragmented and converted to cDNA which was amplified by PCR. Illumina libraries were generated, clustered and sequenced on the Illumina

HiSeq2500 with a single-end sequencing protocol (10M Reads). Colorimetric sequencing signals were translated into base calls by an internal Illumina software (CASAVA). Subsequently, using the tool bcl2fastq2 (version 2.18), the per-cycle basecall (BCL) files were demultiplexed and converted into per-read FASTQ sequence files for downstream analysis. Next, reads containing PhiX control signals were removed by BaseClear BV using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to minimum read length of 50bp). Finally, the quality of the FASTQ sequences was assessed by the tool FastQC [22] (version 0.11.5), and enhanced by trimming off low-quality bases by setting the cut-off of the error probability limit of the modified-Mott algorithm [23] to 0.02. The RNA-seq reads were then used to quantify transcript abundances. To this end the tool Salmon [24] (version 0.8.2) was used to map the reads to the GRCh38.p10 genome assembly-based transcriptome sequences as annotated by the Ensembl genome database project [25] (Ensembl release v90). The obtained transcript abundance estimates and lengths were then imported in R using the package tximport [26] (version 1.6.0), and summarized on the gene-level. Differential gene expression was determined using the package edgeR [27] (version 3.20.5) utilizing the obtained estimated gene-level counts and offsets based on the transcript-level abundance estimates. The latter corrects for changes to the average transcript length across samples, and incorporation of such offsets was reported to improve the accuracy of differential gene expression analysis [27].

The complete RNA-seq dataset that was generated consisted of 16 samples (8 treatments x 2 replicates). Although not all treatments are of relevance to address the research question posed in this manuscript, all samples were included in the statistical analyses. This was done because this improves the gene-wise dispersion (variability) estimates, as detailed below, which is advantageous when having only two replicates per group. Before statistical analyses, non-specific filtering of the count table was performed to increase detection power [28], based on the requirement that a gene should have an expression level greater than 10 counts, i.e. 0.65 count per million reads (cpm) mapped, for at least 2 libraries across all 16 samples. Differences in library size were adjusted by the trimmed mean of M-

values normalization method [29]. Differentially expressed genes (DEGs) were identified using generalized linear models that incorporate empirical Bayes methods that permit the estimation of gene-specific biological variation, thereby improving testing power [30–32]. For the RNA-seq study, thresholded hypotheses testing using a fold-change of 1.5 was performed to identify robustly regulated genes, i.e. genes regulated by a fold-change below this threshold were considered not to be biologically meaningful [33]. In all cases, genes that satisfied the criterion of a p-value lower than 0.05 were deemed to be significantly regulated.

To acquire an overview on the transcriptomic signature of DEGs gene ontology (GO) and pathway analysis were carried out using the Consensus Path Database tool [34]. In all cases, only GO terms and pathways with p-values lower than 0.005 were included for analysis. For NR pathway analysis, lists containing all cpdb and the top 100 genes of the NURSA data base Transcriptomine (Consensome) [35] were also used. In addition, all along this work, other web tools such as Heatmapper [36], Interactivenn [37] and Revigo [38] were utilized to create the heatmaps, the Venn diagrams and the plots clustering gene ontology terms, respectively.

2.5 Reporter gene studies

T47D-ER α -luc and U2OS-ER β -luc cells were seeded in 96-well white opaque plates (PerkinElmer, Groningen, The Netherlands) at cell densities of 1×10^4 and 7.5×10^3 cells/well, respectively. The next day the hormone-deprived medium was renewed. On the second day, cells were exposed. After 24h exposure, washing of cells with half-diluted (H₂O) phosphate-buffered saline (PBS) (Gibco, Bleiswijk, The Netherlands) was carried out. Next, cell lysis was performed with 30 μ l of hypotonic low-salt buffer (pH 7.8) containing 10 mM Tris (Sigma-Aldrich, St. Louis, MO, USA), 2 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM 1,2-diaminocyclohexanetetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA). Plates were cooled down and stored at -80 °C. Next, plates were thawed and luciferase activity (light) was measured with a luminometer (GloMax, Promega

Corporation, USA) upon adding 100 μ l of flashmix (pH 7.8) containing 20 mM tricine (Sigma–Aldrich, St. Louis, MO, USA,), 1.07 mM (MgCO_3) $4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ (Aldrich, Milwaukee, WI, USA), 2.6 mM magnesium sulfate (MgSO_4 , Sigma–Aldrich, St. Louis, MO, USA), 0.1 mM ethylenediaminetetraacetic acid ($\text{EDTA} \cdot 2\text{H}_2\text{O}$, Merck, Darmstadt, Germany), 2 mM DTT, 0.47 mM D-luciferin (Duchefa Biochemie, Haarlem, the Netherlands) and 5 mM adenosine triphosphate (ATP, Duchefa Biochemie, Haarlem, the Netherlands).

2.6 Gene expression (RT-qPCR) studies

For the gene expression (RT-qPCR) studies T47D cells were seeded in 25 cm^2 flasks (Corning, NY, USA) using growth medium and when they reached 50-60% confluence, medium was replaced by hormone-deprived medium. 24h later, cells were exposed by adding the different chemicals. After 6h exposure, cell lysis was carried out using RLT Lysis buffer (Qiagen, Venlo, The Netherlands). Total RNA was extracted using QIAshredder and RNeasy kits (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. To ensure the quality of the RNA, spectrophotometric analysis was performed using a Nanodrop (ND-1000 Thermoscientific Wilmington, Delaware, USA). Next, RNA was converted into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). Expression of *SOX9*, *SMAD3*, *SULF1*, *BTG2*, *CXCL12*, *SERPINB1*, *PLK2* and *GAPDH* (housekeeping gene) was quantified by real-time PCR using Rotor-Gene SYBR® Green Kit (Qiagen, Venlo, The Netherlands) and the Rotor-Gene 6000 cycler (Qiagen, Venlo, The Netherlands) following the manufacturer's protocol. To do so, this study made use of the QuantiTect Primer Assays Hs_SOX9_1_SG, Hs_SMAD3_1_SG, Hs_SULF1_1_SG, Hs_BTG2_1_SG, Hs_CXCL12_1_SG, Hs_SERPINB1_1_SG, Hs_PLK2_1_SG and Hs_GAPDH_1_SG (Qiagen, Venlo, The Netherlands).

2.7 Further data representation, analysis and statistics

Apart from the type of analysis and tools described in previous sections for the RNA-seq data, all the other graphs, analysis and statistics were elaborated by using the software GraphPad Prism 5 (San

Diego, Ca, USA). For all the proliferation, reporter gene and RT-qPCR studies a minimum of three independent experiments were carried out. For comparison in these studies, statistics between the different treatment conditions were evaluated by multiple paired t-tests where a criterion of a p-value lower than 0.05 was considered to be significant. All along this work, data are expressed as percentage/fold induction of the solvent control DMSO ($\text{Avg} \pm \text{SEM}$) or as log₂ Fold Changes (FC) in comparison with the solvent control DMSO.

3 Results

3.1 Cell proliferation studies

As a first step, the present work evaluated the effects of the different general and subtype-selective RAR agonists on T47D cell proliferation. The pro-proliferative effects of E₂ were also tested for comparison and as a positive control. Figure 1A shows the effects of the retinoids on DNA synthesis as detected in the BrdU assay. As clearly shown, upon exposure during 72 hours to 1 μM of the respective retinoids, differential effects on the incorporation of BrdU were found indicating distinct compound-specific effects on the proliferative rates of the T47D cells. While exposure to AtRA and Am80 significantly decreased cell proliferation, exposure to CD2314 and BMS961 was ineffective and resulted in a slight increase in the proliferative rates. When assessing different time points in the MTT assay (Figure 1B), similar effects were observed. All test compounds showed to exert their maximal effects after a long-term exposure of 6 days. Thus, in accordance with the results obtained in the BrdU assays, the number of viable T47D cells was either decreased upon exposure to AtRA and Am80 or weakly increased after incubation with CD2314 and BMS961. Altogether, the results showed that distinct general and selective retinoids can elicit different effects on the T47D cell proliferation.

A

B

Figure 1. Effects of retinoids on the proliferation of T47D cells measured by the BrdU proliferation assay (A) and the MTT proliferation assay (B). E₂-driven proliferative effects were also included for comparison. Figure A shows the different effects of 1 μ M retinoids on the T47D cell proliferation after exposing cells for a period of 72 hours. Figure B additionally shows the effects of the same compounds at different time points (Day 1, 2, 3 and 6). All results are expressed as percentage in relation to the solvent control (Average $\pm$ SEM of at least three independent experiments). For statistical analysis, multiple paired t-tests between the treatments and the solvent control were performed. ** $p < 0.01$; * $p < 0.05$ compared to the solvent control. Ret = Retinoid

3.2 Overview of the transcriptome generated by the general and selective retinoids

Once it was shown that the distinct general and subtype-selective retinoids can elicit differential effects on the proliferation of T47D cells, we performed an RNA-seq study to characterize and compare the transcriptomic profiles generated by these compounds (Figure 2). To do so, we first acquired a general

overview of the gene expression changes elicited by the respective retinoids by comparing gene expression profiles (Figure 2A and 2B). As clearly shown, both the heatmap (Figure 2A) and principal component analysis (PCA) (Figure 2B) indicate differential modulation of transcription driven by the effective (anti-proliferative) retinoids (AtRA and Am80) in comparison with the ineffective retinoids (CD2314 and BMS961).

After comparing the general expression profiles, we next characterized and compared the respective DEGs. The present work considered as DEGs those genes presenting a minimum fold change of 1.5 compared to the solvent control and a p-value lower than 0.05. Figure 2C shows a bar plot presenting the total number and both up- and down-regulated genes upon exposure to the different retinoids. When analyzing DEGs per retinoid, AtRA and Am80 showed to remarkably affect the transcriptomic profiles of T47D cells with more genes being modulated compared to CD2314 and BMS961. When evaluating the similarity in responses in the Venn diagrams (Figure 2D), several interesting patterns were observed. High overlap between DEGs for AtRA and Am80 was observed which was not that evident for CD2314 and BMS961. Thus, treatment with AtRA and Am80 generated expression changes in a high number of coincident genes whereas CD2314 and BMS961 affected a relatively lower number of genes.

Figure 2. RNA-seq assessment of the general transcriptomic profiles elicited by the retinoids. Genes whose expression was significantly different (ANOVA) to the solvent control in any treatment are represented by a heatmap (expression values scaled per gene) (A) and a PCA plot (B). An overview of the number of DEGs (all DEGs, up-regulated and down-regulated) and the overlap upon retinoid-treatment is additionally presented by bar and Venn diagrams (C and D).

After getting a global insight on the general transcriptomic profile by characterizing and comparing gene expression patterns and DEGs generated upon exposure to the different test compounds, Gene Ontology (Biological Processes, BPs) and Pathway analysis were carried out. For this purpose, the DEGs lists appearing upon each retinoid treatment were analyzed using the Consensus Path Database tool. To do so, only gene ontology (GO) terms and pathways with p-values lower than 0.005 were deemed as relevant and included in this work.

The totality of the GO terms (BPs) for the lists of DEGs (all DEGs, up- and down-regulated) generated upon exposure to the retinoids is presented in the Suppl. Table A. Suppl. Figure A displays the distribution of GO (BP) terms over-represented upon analysis of all DEGs per each retinoid (clustered by semantic similarity) where it can be observed that more GO (BP) terms were enriched for AtRA and Am80 when compared to CD2314 and BMS961. In addition, Suppl. Figure A presents an analysis of important BPs playing a role in breast cancer growth, namely, “regulation of epithelial cell proliferation”, “epithelial cell differentiation” and “apoptotic process”. All retinoids elicited changes in the expression of genes related to these GO terms. However, both AtRA and Am80 presented generally lower p-values for these specific biological processes and displayed a higher number of relevant BP terms semantically similar to them (Suppl. Figure A).

To determine possible modes of action, pathways over-represented in the DEG lists of each retinoid were further analyzed. All significantly over-represented pathways (p-value < 0.005) for the lists of DEGs (all, up- and down-regulated) generated upon treatment with the different retinoids are presented in the Suppl. Table B. In line with the differential number of retinoid-driven DEGs and biological processes, multiple pathways were modulated for the cases of AtRA and Am80 while a lower number was found for CD2314 and especially BMS961. Despite multiple molecular pathways were affected, this work showed potential effects on nuclear receptor signaling as possible mechanisms of action partially underlying the distinct effects in breast cancer cell proliferation observed upon exposure to retinoids. In fact, apart from the expected modulation of Retinoic acid, Vitamin A and RAR-RXR

related processes, effects on other NR pathways regulating breast cancer growth was observed. Thus, retinoid-mediated interference on the pro-proliferative ER, the anti-proliferative VDR and the dual (pro-/anti-proliferative) GR pathways was found.

In summary, hierarchical clustering and PCA differentiated gene expression patterns between the effective anticancer agents AtRA and Am80 and the ineffective CD2314 and BMS961. This differential transcriptomic signature was also evident after comparing DEGs (including biological processes and pathways affected). After screening the biological processes and NR pathways that can play important roles in the retinoid-mediated modulation of T47D breast cancer growth, we will characterize them in detail in subsequent sections by comparing gene expression resulting from the treatment with the effective (AtRA and Am80) and the ineffective (CD2314 and BMS961) retinoids.

3.3 Characterization of the retinoid-mediated effects on gene expression related to the relevant GO biological processes proliferation, differentiation and apoptosis.

Since all retinoids, to a higher or lower extent, were able to modulate GO biological processes playing important roles in breast cancer growth, the specific genes regulating epithelial proliferation, differentiation and apoptosis were analyzed in more detail in the next step of the data analysis (Figure 3). First, an overview of the genes related to these events that were significantly changed in at least one of the treatments is presented by a heatmap (Figure 3A). Despite the relatively high similarity observed, hierarchical clustering was able to separate the anti-proliferative AtRA and Am80 from the ineffective CD2314 and BMS961. The main difference observed appeared in the magnitude of response, being generally higher for the anti-proliferative retinoids AtRA and Am80. Next, a selection of genes known to specifically affect breast cancer growth and progression were further analyzed and confirmed by RT-qPCR (Figure 3B, 3C and 3D). In terms of proliferation (Figure 3B), the expression of pro-proliferative genes such as the gene SCN5A was decreased upon incubation with AtRA and Am80 while increased after exposure to CD2314 and BMS961. However, for most of the cases such

as the pro-proliferative PRKCA and the anti-proliferative CEACAM1 and SOX9, the difference among the effective and ineffective retinoids was mainly based on the level of response. Concerning differentiation (Figure 3C), a similar trend was observed since plenty of genes promoting differentiation such as DMBT1, DAB2, GJA1 and SMAD3 presented higher FC values for AtRA and Am80 when comparing to CD2314 and BMS961. Interestingly when assessing the modulation of apoptotic genes (Figure 3D), we observed the biggest difference between effective and ineffective retinoid treatments. Thus, genes involved in apoptosis like TLR3, TRAIL, BIN1, CASP4 and SULF1 showed a higher expression upon treatment with AtRA and Am80 when compared to the expression levels seen with CD2314 and BMS961. In summary, analysis of genes modulating proliferation, differentiation and apoptosis pointed at several marker genes showing to be differentially modulated by the different effective (anti-proliferative) and ineffective retinoid treatments.

Figure 3. RNA-seq characterization of the modulation by the respective retinoids of the BPs “regulation of epithelial cell proliferation”, “epithelial cell differentiation” and “apoptotic process”. Genes included in these GO terms presenting significant changes in at least one of the conditions are displayed in the heat map (A). In addition, genes known to be determinant on proliferation (B), differentiation (C) and apoptosis (D) of breast cancer cells are further characterized and, in some cases, confirmed by RT-qPCR. All results are expressed as log2 Fold Changes (FC) in comparison with the solvent control. For RNA-seq data (edgeR analysis), differences between retinoid treatments were deemed significant if $FC > 1.5$ and $p\text{-value} < 0.05$. For RT-qPCR results, multiple paired t-tests between treatments were performed and differences between retinoids were considered significant if $p\text{-value} < 0.05$. ^A = Significantly different to AtRA; ^B = Significantly different to Am80; ^C = Significantly different to CD2314

3.4 Characterization of the differential retinoid-mediated effects on the RAR signaling.

After assessing retinoid-driven BPs regulating cancer growth, analysis of their effects on the RAR pathway was carried out (Figure 4). Evaluation of the retinoid-driven effects on RAR signaling is shown in Figure 4A where RAR-related genes presenting significant changes in at least one condition

are included. As shown in the heatmap, except for a few genes, differences among treatments were again mainly observed in terms of magnitude of effects. One exception was seen for the proliferative *CDK1* gene where CD2314 and BMS961 increased its transcription while Am80 and AtRA decreased it or had no effect, respectively (Figure 4B). However, for most of the anti-proliferative genes reported in the context of breast cancer, which are displayed in Figure 4B (*RARβ*, *TINAGL1*, *IGFBP6*, *IRF1* and *BTG2*), AtRA and Am80 showed to induce higher expression rates than CD2314 and BMS961. Altogether, the inhibitors of breast cancer growth AtRA and Am80 presented larger effects on the RAR pathway.

Figure 4. RNA-seq analysis of the effects of retinoids on RAR-related gene expression (genes from cpdb “RAR-mediated signaling” + top 100 RAR NURSA). Figure A shows by a heatmap all genes linked to the RAR pathway presenting significant changes in at least one of the treatments tested. Figure B zooms in some examples of genes reported to affect breast cancer growth. For the case of the gene *BTG2*, RNA-seq results were further supported by RT-qPCR. All results are shown as log2 fold changes compared to the solvent control. For RNA-seq data (edgeR analysis), differences between retinoid treatments were deemed significant if FC>1.5 and p-value < 0.05. For RT-qPCR results, multiple paired t-tests between treatments were performed and differences between retinoids were considered significant if p-value < 0.05. ^A = Significantly different to AtRA; ^B = Significantly different to Am80; ^C = Significantly different to CD2314

3.5 Assessment of the distinct retinoid-driven effects on the ER signaling pathway.

Once the changes in gene expression linked to the RAR pathway were characterized, the differential effects of retinoids on genes related to ER signaling were evaluated (Figure 5). Figure 5A displays in a heatmap the fold changes elicited by the respective retinoids on the transcription of genes known to play important roles in the ER pathway and compared them to the expression profile generated by E₂ (positive control). In addition, Figure 5B focuses on ER-related genes previously reported to affect proliferative rates of breast cancer cells. As shown in the heatmap and the bar graphs, many E₂-induced genes such as GREB1, STC or MYC were also up-regulated upon treatment with all retinoids at different intensities. However, the E₂-responsive gene IL17RB was only up-regulated by CD2314 and BMS961. To get further insights on this matter, the present study assessed the effects of the retinoids on the specific expression driven by the two subtypes of ER (ER α and ER β) by using the T47D-ER α -Luc and U2OS-ER β -Luc cell lines (Figures 5C and 5D). Interestingly, all retinoids were able to increase ER α -driven luciferase expression at different levels in the T47D reporter cell line. However, only AtRA was able of up-regulating ER β -mediated luciferase expression in the U2OS-ER β -Luc cells. Contrary to the above mentioned, some ER-target genes involved in breast cancer growth such as CXCL12 were down-regulated after exposure to all the different RAR ligands, response that was more pronounced for the anti-proliferative AtRA and Am80 (Figure 5B). In general terms, the results of the present study indicated that the general RAR agonist AtRA and the RAR-subtype selective retinoids Am80, CD2314 and BMS961 can exert differential effects on both ER α and ER β pathways in a gene-dependent manner which may influence the final behaviour of breast cancer cells regarding growth progression or regression.

Figure 5. RNA-seq characterization of the effects of retinoids on gene expression associated to the ER pathway (genes from cpdb “Validated nuclear ER α network” + top 100 ER NURSA). Figure A displays via heatmap all genes related to ER signaling presenting significant changes in at least one of the retinoid treatments. Figure B focuses on some representative genes described to modulate breast cancer cell proliferative rates. In addition, the results of reporter gene studies assessing the specific effects of retinoids on ER α and ER β activity are displayed in Figures C and D. For this purpose, the cell lines T47D-ER α -Luc and U2OS-ER β -Luc were utilized. All results are expressed as log2 fold changes (RNA-seq) or percentage (reporter gene studies) in relation to the solvent control. For RNA-seq data (edgeR analysis), differences between retinoid treatments were deemed significant if FC>1.5 and p-value <0.05. For RT-qPCR results, bars represent Average $\pm$ SEM from at least three independent experiments. For statistical analysis of the RT-qPCR data multiple paired t-tests between retinoid treatments were performed and differences were considered significant if p-value <0.05. ^A = Significantly different to AtRA; ^B = Significantly different to Am80; ^C = Significantly different to CD2314. For reporter gene studies: ** p < 0.01; * p < 0.05 compared to the solvent control. Ret = Retinoid

3.6 Characterization of the potential crosstalk of retinoid signaling with other NR (VDR and GR) pathways regulating breast cancer growth.

After showing the retinoid-driven interference on ER signaling, this work also evaluated two other NR pathways which were affected by the different retinoids (see Supplementary Table B), namely the

VDR pathway and the GR pathway, which have been linked to modulation of proliferative rates in breast cancer cells. As shown in Supplementary Table B, upon treatment with the retinoids AtRA and Am80, the VDR pathway was highlighted appearing as one of the top hits of the analysis. In consequence, characterization of the specific VDR-related genes significantly modulated by at least one of the retinoids was carried out (Figure 6A and 6B). Both heatmap and bar plots showed multiple genes responding differently to the anti-proliferative AtRA and Am80 when compared to the ineffective CD2314 and BMS961. Thus, VDR itself and VDR-related genes previously described to inhibit breast cancer growth (e.g. CLMN, STS and SERPINB1), showed to be clearly up-regulated upon exposure with AtRA and Am80 whereas treatment with CD2314 and BMS961 presented much weaker effects.

Concerning effects on other nuclear receptor pathways, retinoids showed to affect genes linked to the GR pathway (Figure 6C and 6D). Again, hierarchical clustering was capable of discriminating the effective (AtRA and Am80) from the ineffective (CD2314 and BMS961) treatments. A closer analysis of several genes related to GR signaling and known to be involved in breast cancer cell proliferation, differentiation and/or apoptosis was performed. On one hand, genes promoting breast cancer progression such as EDN2 and TNS4 were up-regulated by all test compounds without a clear trend discerning between the effective and ineffective retinoid treatments. On the other hand, GR-related genes reported to inhibit breast cancer growth, such as FOXO3 or PLK2 showed to be more up-regulated upon treatment with AtRA and Am80 in comparison with CD2314 and BMS961.

Overall, interference of retinoids with other NRs pathways such as the VDR and the GR pathways was seen after analyzing the transcriptomic profiles generated by the distinct retinoids. Thus, multiple genes related to the signalling of these receptors and also known to play important roles in breast cancer growth were found to be differentially affected by the anti-proliferative and the ineffective retinoid treatments.

Figure 6. RNA-seq analysis of the effects of retinoids on gene expression linked to the VDR and GR pathways (genes from cpdb “VDR Pathway” and “GR Pathway” + top 100 VDR and GR NURSA). All genes associated to VDR (A) and GR (C) signaling presenting significant modulation in at least one of the retinoid treatments are included in the respective heatmaps. A selection of genes previously reported to affect breast cancer growth linked to VDR (B) and GR (D) are also presented. For the case of the VDR-related gene SerpinB1 and the GR-related gene PLK2, RNA-seq results were additionally confirmed by RT-qPCR. All results are expressed as log2 fold changes compared to the solvent control. For RNA-seq data (edgeR analysis), differences between retinoid treatments were deemed significant if $FC > 1.5$ and $p\text{-value} < 0.05$. For RT-qPCR, multiple paired t-tests between treatments were performed and differences between retinoids were considered significant if $p\text{-value} < 0.05$. ^A = Significantly different to AtRA; ^B = Significantly different to Am80; ^C = Significantly different to CD2314.

4 Discussion

Several decades ago, retinoids such as AtRA were found to inhibit breast cancer cell proliferation *in vitro* [2,39] and this elicited new research on the protective role of retinoids against breast cancer. However, clinical trials testing the effectiveness of retinoid-based breast cancer therapies have not been successful yet [6,40]. Little is known about the molecular mechanisms underlying the observed differences in sensitivity/resistance upon treatment with retinoids and about the specific role played

by their cognate receptor subtypes RAR α , RAR β and RAR γ . In our view, this merited new studies characterizing and comparing the differential effects of general and subtype-selective retinoids on breast cancer cell proliferation and their concomitant modulation of the transcriptomic profiles. Thus, the aim of this work was to assess and compare the effects on the proliferation and gene expression of the T47D breast cancer cell line elicited by treatments with the general agonist AtRA, the RAR α agonist Am80, the RAR β agonist CD2314 and the RAR γ agonist BMS961.

Retinoid-mediated effects on the proliferation of breast cancer cells have been reported to vary depending on the compound tested [41]. Thus, while previous research has proven the potential of general and RAR α -selective agonists to hamper breast cancer cell proliferation, RAR β and RAR γ have shown weaker or no effects [41]. In accordance with this, our study showed that the general agonist AtRA and the RAR α -selective ligand Am80 were capable of significantly decreasing proliferation of T47D cells. In addition, the RAR β -selective CD2314 and the RAR γ -selective BMS961 were not effective as anti-proliferative agents since these compounds even slightly induced proliferation on T47D cells. The role of RAR γ as a pro-proliferative effector is more and more recognized [11,12], however, RAR β is widely considered a tumour suppressor protein [6]. To acquire insights in the underlying molecular events leading to the distinct effects of retinoids, a complete gene expression analysis using different methodologies was carried out.

To evaluate the effects on the transcriptomic profiles of T47D cells upon treatment with the different retinoids, an RNA-seq study after a 6-hour exposure period was performed. This short exposure period was based on previous studies evaluating the effects on gene expression of this type of chemicals (NR ligands) which suggested that 4-8 hours are sufficient to detect direct genomic events while reducing possible secondary effects occurring after longer exposure [15,21]. In addition, effects on viability/proliferation were only seen after longer periods (results not shown) further supporting the suitability of this exposure time. An initial view of the global transcriptomes generated by the test compounds showed that anti-proliferative and ineffective retinoids were clearly grouped apart by

hierarchical clustering (heatmap) or by PCA. Moreover, the anti-proliferative AtRA and Am80 presented considerably similar and much higher number of DEGs. Previous research reported AtRA as a chemical able of eliciting multiple RAR-mediated and non-RAR-mediated molecular events [6]. However, we showed that not only AtRA but also the RAR α -selective Am80 modulated a remarkably high number of BPs and pathways when compared with the RAR β -selective (CD2314) and the RAR γ -selective (BMS961) agonists. It has to be noted that the low expression levels of RAR β in relation to the highly expressed RAR α or RAR γ [2] could explain the results for CD2314 (RAR β -ligand) but not for BMS961 (RAR γ -ligand). Thus, the more abundant and similar DEGs, BPs and pathways affected upon treatment with AtRA and Am80 point at RAR α as the principal effector involved in the genomic effects of retinoids in breast cancer cells.

In the context of breast cancer, BPs such as proliferation, differentiation and programmed cell death play crucial roles. In line with the proliferation results, the present study showed a generally higher number and statistical significance of GO terms closely related to these BPs upon treatment with the anti-proliferative AtRA and Am80 when compared to the ineffective CD2314 and BMS961. However, assessment of the genes involved in “Regulation of epithelial cell proliferation”, “epithelial cell differentiation” and “Apoptotic process” indicated similar gene expression modulation where most of the discrepancies were mainly based on magnitudes of response. Thus, despite few exceptions like the pro-proliferative SCN5A [42], where opposite effects were seen upon treatment with the anti-proliferative and the ineffective retinoids, most of the differences were based on a similar but lower modulation of gene expression. Thus, down-regulation of pro-proliferative genes such as PRKCA [43] as well as up-regulation of the anti-proliferative CEACAM1 [44] and SOX9 [17] was found systematically weaker upon exposure to CD2314 and BMS961 in comparison with the treatments with AtRA and Am80. In concordance with the genes known to regulate cell proliferation, the other GO terms related to differentiation and apoptosis presented similar trends. Thus, genes supporting differentiation like DMBT1 [45], DAB2 [46], GJA1 [47] and SMAD3 [48] or leading to programmed

cell death like TLR3 [49], TRAIL [50], BIN1 [51], CASP4 [52] and SULF1 [53,54], were found to be highly up-regulated by AtRA and Am80 while more weakly affected upon treatment with CD2314 and BMS961.

Once having characterized the main differences between the BPs promoted after treatment with the different retinoids, this study analyzed the molecular pathways affected to better understand the mechanisms of action that may be involved in their distinct effects on breast cancer cell proliferation. Interestingly, apart from the expected modulation of RAR-related genes, pathway analysis highlighted potential crosstalk between retinoid signaling and other NR pathways such as ER, VDR or GR which are widely reported to be relevant in the context of breast cancer growth [55]. Previous research reported that retinoid efficacy often depends on the simultaneous presence and actions of other NRs (e.g. ER) [2,9]. Consequently, the present work focused on retinoid effects on the RAR and these other NR pathways.

Previous studies have shown that RARs bind to highly coincident regions in the genome suggesting functional redundancy. However, plenty of research has shown that specific activation or changes in the expression of the different RAR subtypes can lead to different and even opposite biological outcomes [11,41]. The present study compared gene expression related to the RAR pathway upon exposure to general and subtype-selective retinoids. All retinoids up-regulated multiple RAR-related genes reported to decrease cancer growth. However, as shown for the retinoid-responsive genes TINAGL1 [56], IGFBP6 [57] and IRF1 [58] or the direct RAR-target genes BTG2 [5] and RARB [2], AtRA and Am80 highly increased their transcription while CD2314 and BMS961 usually presented lower or in certain cases opposite expression patterns. Since selectivity is mainly based on ligand affinity for a specific RAR subtype, residual activation of the non-targeted RAR subtypes upon exposure to high concentrations of a selective retinoid cannot be excluded. In fact, in a previous biochemical study using the isolated recombinant RAR subtypes we showed that this selective compounds could also, although weakly, activate the non-targeted RAR subtypes [59]. Accepting this

limitation, the trends of the results clearly support a principal role of RAR α on the RAR-related gene expression promoted by retinoids. One of the few pro-proliferative genes linked to the RAR pathway, namely CDK1 [60], was up-regulated upon exposure to CD2314 and BMS961 while not affected or down-regulated by AtRA and Am80, respectively. However, the effects observed in CDK1 expression are relatively weak so that further studies assessing the actual weight (if any) on breast cancer growth of this molecular player should be confirmed. Altogether, we propose that the weaker effects of RAR β - and RAR γ -selective ligands on RAR α -induced anti-proliferative genes may partially explain their lower efficacy as anticancer drugs.

The effects of retinoids have been shown to be highly dependent on the coexistence of ERs [2,9]. Indeed, multiple findings describing crosstalk between RAR and ER have been reported [8,9]. In line with this, our study showed a high number of retinoid-driven DEGs related to the ER pathway. For some genes such as the pro-proliferative IL17RB [61] and CXCL12 [62], a clear distinction between the anti-proliferative AtRA and Am80 and the ineffective CD2314 and BMS961 was observed. In general terms though, as represented by the pro-proliferative genes MYC [63] and GREB1 [64], no significant differences between the retinoids were observed. This work acquired further knowledge on the retinoid-driven effects on ER signaling by testing their specific effects in the pro-proliferative ER α and anti-proliferative ER β activities [65] using reporter gene cell lines. Interestingly, all compounds induced an increase of the ER α -mediated luciferase expression and this effect was significant for Am80, CD2314 and BMS961. Contrarily, only AtRA was able of up-regulating the expression of luciferase driven by ER β . It must be noted, as limitation, that the possible cell- (different RAR levels) and promoter-specific differences between the different cell lines may affect the results since RARs can bind specific estrogen responsive elements (gene/promoter-dependent effects) [8]. Furthermore, the high ratio between ER α and ER β levels (9:1) found in wild type T47D cells [65] reduces the probability of the observed differential effects on the ER subtypes as a crucial factor in the AtRA-driven decrease of proliferation. Nevertheless, the remarkable increase on ER β activity could point to

another mechanism that may partially underlie the strong AtRA-driven decrease of breast cancer cell proliferation so that further research should study this newly described retinoid-driven effect on ER β -mediated transcription.

Apart from the effects of retinoids on RAR and ER signaling, the current study revealed an evident interference with other NR pathways like VDR and GR. Concerning the GR pathway in breast cancer, there is a current controversy where studies have shown both pro- and anti-proliferative roles depending on the cellular context and ligand involved [66–69]. Although highly variable for pro-proliferative genes such as TNS4 [70] or EDN2 [71], the results showed that many anti-proliferative GR-related transcripts such as FOXO3 [72] or PLK2 [73], were strongly up-regulated by AtRA and Am80 while lower or no response was found for CD2314 and BMS961. Interplay between GR and RAR signaling has been described in other cellular types (e.g. T-cells) where liganded RAR α /RXR dimers have been shown to bind and enhance transcriptional activity of GR increasing its induced apoptotic processes [74] so that similar process may happen in T47D cells. Unlike the GR pathway, it is more generally agreed that VDR signaling can lead to inhibition of breast cancer growth [75]. Thus, activation of VDR has been shown to inhibit cell proliferation via expression of genes such as STS [76], CLMN or SERPINB1 [77]. We found that the expression of such genes and VDR itself, was highly enhanced upon treatment with AtRA and Am80 while no or lower effects were observed after exposure to CD2314 and BMS961. RARs and VDR have been described to interact being able to drive transcription [78,79]. Therefore, molecular mechanisms such as retinoid activation (ligand-receptor binding) of the VDR-RXR heterodimer, or transcriptionally active RAR-VDR dimers may possibly explain the observed effects and must be investigated in future studies.

In summary, the outcomes of the present study suggested that no single mechanism of action or biological process seem to completely explain the differential effects of retinoids on the proliferation of breast cancer cells. Instead, small and subtle differences on multiple pathways involved in proliferation, differentiation and apoptosis such as the RARs, ER, VDR and GR pathways, among

others, may sum up to determine the final response in terms of breast cancer growth. Thus, future studies should be carried out to acquire further insights on each of these pathways to eventually develop effective retinoid-based treatment strategies.

Conflicts of interests

The authors of the present study declare no conflicts of interest.

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Appendix. Supplementary Material

Supplementary material can be found in the end of this chapter.

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Appendix. Supplementary Figure A. RNA-seq evaluation of the GO terms and pathways over-represented upon analysis of the DEGs for each retinoid treatment. Figure A displays the biological pathways (GO) highlighted for each compound, clustering them by semantic similarity (axis X/Y represent semantic spaces) by means of the web-resource Revigo tool. Figure B focus on the p-values presented in biological processes known to play an important role in breast cancer growth. Down-regulated genes did not generate any GO term according to the criteria used (results not shown). Figure C summarizes important Nuclear Receptor pathways involved in breast cancer cell proliferation which were highly affected upon exposure to the retinoids.

Appendix. Supplementary Table A. List containing the biological processes (p-value < 0.005) over-represented upon GO analysis of all DEGs, up-regulated DEGs, and down-regulated DEGs for the treatments with ATRA, Am80, CD2314 and BMS961

AtRA GO (All DEGs)			
term_goid	p-value	term_name	size
GO:0022008	5.22E-13	neurogenesis	1433
GO:0030182	2.12E-12	neuron differentiation	1231
GO:0051960	9.00E-11	regulation of nervous system development	767
GO:0045597	1.16E-10	positive regulation of cell differentiation	830
GO:0031344	3.00E-10	regulation of cell projection organization	575
GO:0060284	1.22E-09	regulation of cell development	782
GO:0043067	8.01E-09	regulation of programmed cell death	1409
GO:0006915	1.98E-08	apoptotic process	1817
GO:0000904	9.44E-08	cell morphogenesis involved in differentiation	638
GO:0010942	5.30E-07	positive regulation of cell death	612
GO:0051046	1.25E-06	regulation of secretion	691
GO:0015711	1.90E-06	organic anion transport	425
GO:0051130	2.15E-06	positive regulation of cellular component organization	1142
GO:0051272	4.04E-06	positive regulation of cellular component movement	416
GO:0051962	4.23E-06	positive regulation of nervous system development	447
GO:0051174	4.86E-06	regulation of phosphorus metabolic process	1632
GO:0045834	5.46E-06	positive regulation of lipid metabolic process	130
GO:1902531	5.66E-06	regulation of intracellular signal transduction	1683
GO:0070371	7.05E-06	ERK1 and ERK2 cascade	258
GO:0042886	7.06E-06	amide transport	295
GO:0010647	7.67E-06	positive regulation of cell communication	1525
GO:0033500	8.67E-06	carbohydrate homeostasis	221
GO:0010631	8.67E-06	epithelial cell migration	222
GO:0006721	8.78E-06	terpenoid metabolic process	110
GO:0019216	8.87E-06	regulation of lipid metabolic process	288
GO:0061564	1.11E-05	axon development	460
GO:0022604	1.23E-05	regulation of cell morphogenesis	443
GO:0071300	1.62E-05	cellular response to retinoic acid	67
GO:0010648	1.81E-05	negative regulation of cell communication	1207
GO:0006939	1.87E-05	smooth muscle contraction	98
GO:0015833	2.27E-05	peptide transport	270
GO:0014706	2.68E-05	striated muscle tissue development	341
GO:0006820	3.02E-05	anion transport	561
GO:0050801	3.36E-05	ion homeostasis	683
GO:0045596	4.11E-05	negative regulation of cell differentiation	624
GO:0072073	4.20E-05	kidney epithelium development	137
GO:0009967	4.33E-05	positive regulation of signal transduction	1396
GO:0046879	4.55E-05	hormone secretion	308
GO:0008285	4.57E-05	negative regulation of cell proliferation	653
GO:0051271	4.90E-05	negative regulation of cellular component movement	249
GO:0034110	5.18E-05	regulation of homotypic cell-cell adhesion	26
GO:0071396	5.32E-05	cellular response to lipid	528
GO:0001657	5.44E-05	ureteric bud development	97
GO:0043010	5.68E-05	camera-type eye development	290
GO:1902284	5.76E-05	neuron projection extension involved in neuron projection guidance	45
GO:0006023	5.84E-05	aminoglycan biosynthetic process	114
GO:0090087	5.99E-05	regulation of peptide transport	213
GO:0016310	6.35E-05	phosphorylation	2230
GO:0002790	6.87E-05	peptide secretion	254
GO:0090257	7.10E-05	regulation of muscle system process	196
GO:0050678	7.16E-05	regulation of epithelial cell proliferation	293
GO:0034284	7.81E-05	response to monosaccharide	197
GO:0071345	8.51E-05	cellular response to cytokine stimulus	692
GO:0002521	0.0001002	leukocyte differentiation	466
GO:0007169	0.00010058	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0009968	0.00011135	negative regulation of signal transduction	1102
GO:0071322	0.00012986	cellular response to carbohydrate stimulus	129
GO:0030855	0.00013343	epithelial cell differentiation	567
GO:0007411	0.00014486	axon guidance	223
GO:0042692	0.00014518	muscle cell differentiation	355
GO:0032570	0.00017389	response to progesterone	50
GO:0001894	0.00017694	tissue homeostasis	206
GO:0045995	0.00019672	regulation of embryonic development	116
GO:0034762	0.00019797	regulation of transmembrane transport	402
GO:0031032	0.00020237	actomyosin structure organization	151
GO:0022408	0.00020483	negative regulation of cell-cell adhesion	133
GO:0051961	0.00022224	negative regulation of nervous system development	268
GO:1901342	0.00023962	regulation of vasculature development	239
GO:0030038	0.00025016	contractile actin filament bundle assembly	83

GO:0072088	0.00025402	nephron epithelium morphogenesis	75
GO:0061572	0.00025419	actin filament bundle organization	127
GO:0071634	0.00025421	regulation of transforming growth factor beta production	25
GO:0071407	0.00025866	cellular response to organic cyclic compound	527
GO:0050807	0.00030793	regulation of synapse organization	111
GO:0051592	0.00031509	response to calcium ion	120
GO:0060600	0.00035786	dichotomous subdivision of an epithelial terminal unit	10
GO:0043587	0.00035786	tongue morphogenesis	10
GO:0045444	0.0003701	fat cell differentiation	205
GO:0045778	0.00038128	positive regulation of ossification	86
GO:0032268	0.00038315	regulation of cellular protein metabolic process	2327
GO:0071248	0.00038855	cellular response to metal ion	140
GO:1902903	0.0004223	regulation of supramolecular fiber organization	278
GO:0048048	0.00051493	embryonic eye morphogenesis	34
GO:0030203	0.00064165	glycosaminoglycan metabolic process	163
GO:1901862	0.00064932	negative regulation of muscle tissue development	35
GO:0048635	0.00064932	negative regulation of muscle organ development	35
GO:0051246	0.00083067	regulation of protein metabolic process	2476
GO:0010811	0.00084751	positive regulation of cell-substrate adhesion	101
GO:0034220	0.00085947	ion transmembrane transport	1007
GO:0043269	0.00087349	regulation of ion transport	599
GO:0045765	0.00094269	regulation of angiogenesis	217
GO:0051345	0.00099156	positive regulation of hydrolase activity	916
GO:0007351	0.00099537	tripartite regional subdivision	12
GO:0001678	0.00100896	cellular glucose homeostasis	130
GO:0007044	0.00101765	cell-substrate junction assembly	85
GO:0001649	0.00110048	osteoblast differentiation	209
GO:1903035	0.00115794	negative regulation of response to wounding	69
GO:0030279	0.00115794	negative regulation of ossification	70
GO:0032956	0.00115923	regulation of actin cytoskeleton organization	282
GO:0051965	0.00119606	positive regulation of synapse assembly	62
GO:0034114	0.0012091	regulation of heterotypic cell-cell adhesion	18
GO:0006941	0.00121675	striated muscle contraction	151
GO:0032102	0.00123242	negative regulation of response to external stimulus	262
GO:2000736	0.00125806	regulation of stem cell differentiation	31
GO:0035282	0.00132447	segmentation	96
GO:0031325	0.00134884	positive regulation of cellular metabolic process	2789
GO:0050880	0.00140411	regulation of blood vessel size	143
GO:0010594	0.00144333	regulation of endothelial cell migration	115
GO:0040036	0.00150695	regulation of fibroblast growth factor receptor signaling pathway	25
GO:1902285	0.00152191	semaphorin-plexin signaling pathway involved in neuron projection guidance	13
GO:0060548	0.00156757	negative regulation of cell death	895
GO:0016311	0.00158784	dephosphorylation	440
GO:0035296	0.00161811	regulation of tube diameter	135
GO:0007265	0.00163437	Ras protein signal transduction	309
GO:0006687	0.00173787	glycosphingolipid metabolic process	72
GO:0031324	0.00193	negative regulation of cellular metabolic process	2323
GO:1903053	0.00193535	regulation of extracellular matrix organization	26
GO:1903729	0.00197768	regulation of plasma membrane organization	73
GO:0090184	0.00217943	positive regulation of kidney development	41
GO:0002718	0.00222092	regulation of cytokine production involved in immune response	57
GO:0034308	0.0022542	primary alcohol metabolic process	49
GO:0032233	0.0022542	positive regulation of actin filament bundle assembly	49
GO:0070555	0.00234955	response to interleukin-1	121
GO:0007435	0.00238729	salivary gland morphogenesis	34
GO:0006464	0.00238768	cellular protein modification process	3772
GO:1901203	0.00252184	positive regulation of extracellular matrix assembly	9
GO:0043087	0.00253733	regulation of GTPase activity	683
GO:0046890	0.00267339	regulation of lipid biosynthetic process	132
GO:0016202	0.00279669	regulation of striated muscle tissue development	103
GO:0099623	0.00293194	regulation of cardiac muscle cell membrane repolarization	21
GO:0045667	0.00296738	regulation of osteoblast differentiation	113
GO:1900024	0.00308105	regulation of substrate adhesion-dependent cell spreading	43
GO:0010634	0.00308933	positive regulation of epithelial cell migration	104
GO:1903522	0.0031288	regulation of blood circulation	299
GO:0099537	0.0031612	trans-synaptic signaling	607
GO:0060538	0.00318344	skeletal muscle organ development	183
GO:0010605	0.00324931	negative regulation of macromolecule metabolic process	2297
GO:0043435	0.00334446	response to corticotropin-releasing hormone	6
GO:0008582	0.00334446	regulation of synaptic growth at neuromuscular junction	5
GO:0051402	0.0034009	neuron apoptotic process	216
GO:0048634	0.0034068	regulation of muscle organ development	105
GO:0048592	0.00350424	eye morphogenesis	144
GO:0009792	0.00353718	embryo development ending in birth or egg hatching	574
GO:0051492	0.00355667	regulation of stress fiber assembly	69
GO:0050994	0.00356594	regulation of lipid catabolic process	52
GO:0000271	0.0036207	polysaccharide biosynthetic process	78

GO:0060445	0.00378876	branching involved in salivary gland morphogenesis	22
GO:0021561	0.0039645	facial nerve development	10
GO:0048640	0.0040075	negative regulation of developmental growth	88
GO:0043255	0.0040075	regulation of carbohydrate biosynthetic process	89
GO:0008284	0.00406703	positive regulation of cell proliferation	839
GO:1903034	0.00410939	regulation of response to wounding	146
GO:0060443	0.00425339	mammary gland morphogenesis	45
GO:1900115	0.00430601	extracellular regulation of signal transduction	16
GO:0030148	0.00492666	sphingolipid biosynthetic process	90
AtRA GO (Up-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0045597	5.43E-08	positive regulation of cell differentiation	830
GO:0006915	2.70E-07	apoptotic process	1817
GO:0051046	3.31E-07	regulation of secretion	691
GO:0043067	5.58E-07	regulation of programmed cell death	1409
GO:0022008	1.21E-06	neurogenesis	1433
GO:0031344	1.42E-06	regulation of cell projection organization	575
GO:0072073	2.31E-06	kidney epithelium development	137
GO:0060284	2.31E-06	regulation of cell development	782
GO:0045834	4.04E-06	positive regulation of lipid metabolic process	130
GO:0001657	4.72E-06	ureteric bud development	97
GO:0071300	5.83E-06	cellular response to retinoic acid	67
GO:0008285	9.20E-06	negative regulation of cell proliferation	653
GO:0030855	1.12E-05	epithelial cell differentiation	567
GO:0001894	1.33E-05	tissue homeostasis	206
GO:0033500	1.48E-05	carbohydrate homeostasis	221
GO:0050678	1.58E-05	regulation of epithelial cell proliferation	293
GO:0051960	1.95E-05	regulation of nervous system development	767
GO:0019216	2.78E-05	regulation of lipid metabolic process	288
GO:0071345	3.08E-05	cellular response to cytokine stimulus	692
GO:0030182	3.32E-05	neuron differentiation	1231
GO:0043587	3.39E-05	tongue morphogenesis	10
GO:1901342	5.55E-05	regulation of vasculature development	239
GO:0090184	5.77E-05	positive regulation of kidney development	41
GO:0006687	6.48E-05	glycosphingolipid metabolic process	72
GO:0051130	6.56E-05	positive regulation of cellular component organization	1142
GO:0022408	7.45E-05	negative regulation of cell-cell adhesion	133
GO:0010942	7.45E-05	positive regulation of cell death	612
GO:0045765	8.88E-05	regulation of angiogenesis	217
GO:0034110	9.75E-05	regulation of homotypic cell-cell adhesion	26
GO:0072088	9.77E-05	nephron epithelium morphogenesis	75
GO:0042886	0.00010733	amide transport	295
GO:0090183	0.0001233	regulation of kidney development	55
GO:0060443	0.00012468	mammary gland morphogenesis	45
GO:0051962	0.00016828	positive regulation of nervous system development	447
GO:0002521	0.00017945	leukocyte differentiation	466
GO:1903035	0.00019553	negative regulation of response to wounding	69
GO:0010648	0.0002205	negative regulation of cell communication	1207
GO:0014706	0.00024012	striated muscle tissue development	341
GO:0007169	0.00025276	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0006721	0.00029816	terpenoid metabolic process	110
GO:0010631	0.00030783	epithelial cell migration	222
GO:0071396	0.00031182	cellular response to lipid	528
GO:2000736	0.00032161	regulation of stem cell differentiation	31
GO:0019752	0.00034903	carboxylic acid metabolic process	961
GO:0015833	0.00035715	peptide transport	270
GO:0051272	0.000383	positive regulation of cellular component movement	416
GO:0070371	0.00040934	ERK1 and ERK2 cascade	258
GO:0010647	0.00045758	positive regulation of cell communication	1525
GO:0045995	0.00051947	regulation of embryonic development	116
GO:0051174	0.00054632	regulation of phosphorus metabolic process	1632
GO:0040036	0.00061079	regulation of fibroblast growth factor receptor signaling pathway	25
GO:0071634	0.00061079	regulation of transforming growth factor beta production	25
GO:0051271	0.00065788	negative regulation of cellular component movement	249
GO:0060548	0.00068959	negative regulation of cell death	895
GO:0097300	0.00070579	programmed necrotic cell death	35
GO:1902531	0.00070721	regulation of intracellular signal transduction	1683
GO:1902105	0.00075542	regulation of leukocyte differentiation	238
GO:0002790	0.00081874	peptide secretion	254
GO:0034114	0.00085981	regulation of heterotypic cell-cell adhesion	18
GO:0046394	0.00087514	carboxylic acid biosynthetic process	302
GO:0009108	0.0008805	coenzyme biosynthetic process	136
GO:0050801	0.00093834	ion homeostasis	683
GO:0031643	0.00095361	positive regulation of myelination	11
GO:0032268	0.00098157	regulation of cellular protein metabolic process	2327
GO:0051246	0.00108382	regulation of protein metabolic process	2476
GO:0046879	0.00111112	hormone secretion	308

GO:0097421	0.001125	liver regeneration	19
GO:0072074	0.001125	kidney mesenchyme development	19
GO:0034284	0.00112747	response to monosaccharide	197
GO:0009968	0.00114761	negative regulation of signal transduction	1102
GO:0090087	0.00117782	regulation of peptide transport	213
GO:0034308	0.00122398	primary alcohol metabolic process	49
GO:1903729	0.00128621	regulation of plasma membrane organization	73
GO:0001973	0.00138089	adenosine receptor signaling pathway	12
GO:0033003	0.001386	regulation of mast cell activation	39
GO:0030195	0.00140195	negative regulation of blood coagulation	50
GO:0060602	0.00144622	branch elongation of an epithelium	20
GO:0016310	0.00149097	phosphorylation	2230
GO:0002699	0.00152377	positive regulation of immune effector process	157
GO:0061550	0.00153062	cranial ganglion development	6
GO:0009792	0.00154527	embryo development ending in birth or egg hatching	574
GO:0050880	0.00158378	regulation of blood vessel size	143
GO:0010594	0.00162077	regulation of endothelial cell migration	115
GO:0006664	0.00175372	glycolipid metabolic process	116
GO:0045444	0.00179617	fat cell differentiation	205
GO:0050994	0.00181925	regulation of lipid catabolic process	52
GO:0009310	0.00183019	amine catabolic process	21
GO:0099623	0.00183019	regulation of cardiac muscle cell membrane repolarization	21
GO:0009967	0.00184445	positive regulation of signal transduction	1396
GO:0002221	0.00185502	pattern recognition receptor signaling pathway	160
GO:0010813	0.00192902	neuropeptide catabolic process	2
GO:0002386	0.00192902	immune response in mucosal-associated lymphoid tissue	2
GO:0031349	0.00194286	positive regulation of defense response	405
GO:0008284	0.00196539	positive regulation of cell proliferation	839
GO:0050819	0.0020617	negative regulation of coagulation	53
GO:0051092	0.00216349	positive regulation of NF-kappaB transcription factor activity	133
GO:0031347	0.00222437	regulation of defense response	769
GO:0043010	0.00229688	camera-type eye development	290
GO:0070555	0.0023789	response to interleukin-1	121
GO:0035296	0.00248746	regulation of tube diameter	135
GO:0070587	0.00259119	regulation of cell-cell adhesion involved in gastrulation	7
GO:0019441	0.00259119	tryptophan catabolic process to kynurenine	7
GO:2000027	0.00277157	regulation of organ morphogenesis	246
GO:0032102	0.00286912	negative regulation of response to external stimulus	262
GO:0030279	0.00312037	negative regulation of ossification	70
GO:1902284	0.0032559	neuron projection extension involved in neuron projection guidance	45
GO:0002718	0.00329566	regulation of cytokine production involved in immune response	57
GO:0033209	0.00347565	tumor necrosis factor-mediated signaling pathway	155
GO:0001819	0.00348446	positive regulation of cytokine production	386
GO:0034612	0.00377048	response to tumor necrosis factor	269
GO:0006939	0.00380307	smooth muscle contraction	98
GO:0002688	0.00380307	regulation of leukocyte chemotaxis	99
GO:0022604	0.00392244	regulation of cell morphogenesis	443
GO:0042692	0.00398891	muscle cell differentiation	355
GO:0008300	0.00401096	isoprenoid catabolic process	8
GO:0015711	0.00415538	organic anion transport	425
GO:0071322	0.00446537	cellular response to carbohydrate stimulus	129
GO:0002448	0.00471576	mast cell mediated immunity	48
GO:0001678	0.0047678	cellular glucose homeostasis	130
GO:0010811	0.00479352	positive regulation of cell-substrate adhesion	101
AtRA GO (Down-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0030182	5.16E-09	neuron differentiation	1231
GO:0022008	1.08E-07	neurogenesis	1433
GO:0000904	3.02E-07	cell morphogenesis involved in differentiation	638
GO:0051960	1.10E-06	regulation of nervous system development	767
GO:0061564	4.81E-05	axon development	460
GO:0051965	4.85E-05	positive regulation of synapse assembly	62
GO:0050807	6.23E-05	regulation of synapse organization	111
GO:0015711	7.66E-05	organic anion transport	425
GO:0031344	8.66E-05	regulation of cell projection organization	575
GO:0006820	0.00013181	anion transport	561
GO:0045778	0.00014823	positive regulation of ossification	86
GO:0060284	0.00019511	regulation of cell development	782
GO:0045667	0.00034359	regulation of osteoblast differentiation	113
GO:0006023	0.00037085	aminoglycan biosynthetic process	114
GO:0034762	0.00045334	regulation of transmembrane transport	402
GO:0051592	0.00053591	response to calcium ion	120
GO:0071248	0.00056652	cellular response to metal ion	140
GO:0051961	0.00061412	negative regulation of nervous system development	268
GO:0045597	0.00065744	positive regulation of cell differentiation	830
GO:0022604	0.00076564	regulation of cell morphogenesis	443
GO:0045596	0.00077535	negative regulation of cell differentiation	624

GO:1902359	0.00078959	Notch signaling pathway involved in somitogenesis	2
GO:0071608	0.00078959	macrophage inflammatory protein-1 alpha production	2
GO:0003365	0.00078959	establishment of cell polarity involved in amoeboid cell migration	2
GO:0090257	0.0013448	regulation of muscle system process	196
GO:0007411	0.00167467	axon guidance	223
GO:0006939	0.00176219	smooth muscle contraction	98
GO:0009890	0.00193948	negative regulation of biosynthetic process	1492
GO:0042475	0.00197557	odontogenesis of dentin-containing tooth	81
GO:1902531	0.00222765	regulation of intracellular signal transduction	1683
GO:0030203	0.00225381	glycosaminoglycan metabolic process	163
GO:0010942	0.00233723	positive regulation of cell death	612
GO:0034220	0.00247145	ion transmembrane transport	1007
GO:0051174	0.00252533	regulation of phosphorus metabolic process	1632
GO:0071398	0.00327143	cellular response to fatty acid	52
GO:0043255	0.00333001	regulation of carbohydrate biosynthetic process	89
GO:0043067	0.00336247	regulation of programmed cell death	1409
GO:0051272	0.00352464	positive regulation of cellular component movement	416
GO:0007156	0.00468931	homophilic cell adhesion via plasma membrane adhesion molecules	156
GO:0010647	0.00485684	positive regulation of cell communication	1525
Am80 GO (All DEGs)			
term_goid	p-value	term_name	size
GO:0022008	5.51E-13	neurogenesis	1433
GO:0030182	8.77E-13	neuron differentiation	1231
GO:0031344	2.62E-10	regulation of cell projection organization	575
GO:0050678	3.17E-10	regulation of epithelial cell proliferation	293
GO:0051960	2.48E-09	regulation of nervous system development	767
GO:0060284	3.03E-09	regulation of cell development	782
GO:0045597	3.24E-09	positive regulation of cell differentiation	830
GO:0006915	5.43E-09	apoptotic process	1817
GO:0043067	5.55E-09	regulation of programmed cell death	1409
GO:1902531	1.43E-08	regulation of intracellular signal transduction	1683
GO:0051272	1.73E-08	positive regulation of cellular component movement	416
GO:0008285	2.22E-08	negative regulation of cell proliferation	653
GO:0000904	2.84E-08	cell morphogenesis involved in differentiation	638
GO:0061564	4.59E-08	axon development	460
GO:0045596	4.66E-08	negative regulation of cell differentiation	624
GO:0030855	1.38E-07	epithelial cell differentiation	567
GO:0010648	1.42E-07	negative regulation of cell communication	1207
GO:0033500	1.85E-07	carbohydrate homeostasis	221
GO:0010631	1.85E-07	epithelial cell migration	222
GO:0072073	1.94E-07	kidney epithelium development	137
GO:0010647	2.35E-07	positive regulation of cell communication	1525
GO:0009967	2.91E-07	positive regulation of signal transduction	1396
GO:0001657	2.91E-07	ureteric bud development	97
GO:0010942	4.99E-07	positive regulation of cell death	612
GO:0090184	5.24E-07	positive regulation of kidney development	41
GO:0070371	8.65E-07	ERK1 and ERK2 cascade	258
GO:0051174	9.48E-07	regulation of phosphorus metabolic process	1632
GO:0051271	1.20E-06	negative regulation of cellular component movement	249
GO:1901342	1.24E-06	regulation of vasculature development	239
GO:0051046	1.25E-06	regulation of secretion	691
GO:0022604	1.69E-06	regulation of cell morphogenesis	443
GO:0007411	1.97E-06	axon guidance	223
GO:0042692	2.30E-06	muscle cell differentiation	355
GO:0090183	3.67E-06	regulation of kidney development	55
GO:0007169	4.20E-06	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0009968	7.19E-06	negative regulation of signal transduction	1102
GO:0051130	7.43E-06	positive regulation of cellular component organization	1142
GO:0045765	8.32E-06	regulation of angiogenesis	217
GO:0071300	9.24E-06	cellular response to retinoic acid	67
GO:0071396	9.47E-06	cellular response to lipid	528
GO:0001678	1.16E-05	cellular glucose homeostasis	130
GO:2000027	1.52E-05	regulation of organ morphogenesis	246
GO:0043010	1.64E-05	camera-type eye development	290
GO:0006023	1.66E-05	aminoglycan biosynthetic process	114
GO:0051223	2.23E-05	regulation of protein transport	746
GO:0006939	2.29E-05	smooth muscle contraction	98
GO:0007435	2.30E-05	salivary gland morphogenesis	34
GO:0019216	3.07E-05	regulation of lipid metabolic process	288
GO:0071322	3.33E-05	cellular response to carbohydrate stimulus	129
GO:0071345	3.42E-05	cellular response to cytokine stimulus	692
GO:0070201	3.44E-05	regulation of establishment of protein localization	821
GO:0009952	3.56E-05	anterior/posterior pattern specification	201
GO:0072088	3.85E-05	nephron epithelium morphogenesis	75
GO:0051962	4.57E-05	positive regulation of nervous system development	447
GO:0010634	5.22E-05	positive regulation of epithelial cell migration	104
GO:0060443	6.05E-05	mammary gland morphogenesis	45

GO:0002521	6.31E-05	leukocyte differentiation	466
GO:0010594	6.32E-05	regulation of endothelial cell migration	115
GO:0040036	7.84E-05	regulation of fibroblast growth factor receptor signaling pathway	25
GO:0072074	8.02E-05	kidney mesenchyme development	19
GO:0006721	9.83E-05	terpenoid metabolic process	110
GO:0031324	0.00010201	negative regulation of cellular metabolic process	2323
GO:0060602	0.00011692	branch elongation of an epithelium	20
GO:0031032	0.00012192	actomyosin structure organization	151
GO:0016310	0.00012204	phosphorylation	2230
GO:0030203	0.00014144	glycosaminoglycan metabolic process	163
GO:0022408	0.00015768	negative regulation of cell-cell adhesion	133
GO:0043587	0.00016149	tongue morphogenesis	10
GO:0060600	0.00016149	dichotomous subdivision of an epithelial terminal unit	10
GO:0034284	0.00016438	response to monosaccharide	197
GO:0009792	0.00017877	embryo development ending in birth or egg hatching	574
GO:0045995	0.00019815	regulation of embryonic development	116
GO:0051961	0.00020891	negative regulation of nervous system development	268
GO:0035282	0.00021083	segmentation	96
GO:0043087	0.00021186	regulation of GTPase activity	683
GO:0061572	0.00021969	actin filament bundle organization	127
GO:0014706	0.00022266	striated muscle tissue development	341
GO:0050801	0.0002299	ion homeostasis	683
GO:0030279	0.00023435	negative regulation of ossification	70
GO:0051345	0.00025842	positive regulation of hydrolase activity	916
GO:0046879	0.00026613	hormone secretion	308
GO:0071363	0.00026732	cellular response to growth factor stimulus	609
GO:0002688	0.00026915	regulation of leukocyte chemotaxis	99
GO:0043269	0.00027927	regulation of ion transport	599
GO:0010604	0.00028973	positive regulation of macromolecule metabolic process	2795
GO:1902284	0.00030331	neuron projection extension involved in neuron projection guidance	45
GO:0010605	0.00030575	negative regulation of macromolecule metabolic process	2297
GO:0061035	0.0003109	regulation of cartilage development	63
GO:1903707	0.00033095	negative regulation of hemopoiesis	132
GO:1903522	0.0003393	regulation of blood circulation	299
GO:0031325	0.00033984	positive regulation of cellular metabolic process	2789
GO:0070555	0.00034019	response to interleukin-1	121
GO:0006687	0.00036294	glycosphingolipid metabolic process	72
GO:0090257	0.0003694	regulation of muscle system process	196
GO:0032268	0.00039741	regulation of cellular protein metabolic process	2327
GO:0032534	0.00041236	regulation of microvillus assembly	7
GO:2000736	0.00041258	regulation of stem cell differentiation	31
GO:0048706	0.00041797	embryonic skeletal system development	122
GO:0050880	0.00042153	regulation of blood vessel size	143
GO:0030038	0.00045338	contractile actin filament bundle assembly	83
GO:0034612	0.00045374	response to tumor necrosis factor	269
GO:0001973	0.00045795	adenosine receptor signaling pathway	12
GO:0009855	0.00046232	determination of bilateral symmetry	123
GO:0033630	0.0004946	positive regulation of cell adhesion mediated by integrin	18
GO:0008284	0.00050351	positive regulation of cell proliferation	839
GO:0035296	0.0005366	regulation of tube diameter	135
GO:0061037	0.00055439	negative regulation of cartilage development	26
GO:1902105	0.00056015	regulation of leukocyte differentiation	238
GO:0002718	0.0005626	regulation of cytokine production involved in immune response	57
GO:0061053	0.00058161	somite development	85
GO:1904018	0.00058891	positive regulation of vasculature development	136
GO:0071407	0.00066298	cellular response to organic cyclic compound	527
GO:0097067	0.00070696	cellular response to thyroid hormone stimulus	13
GO:1902285	0.00070696	semaphorin-plexin signaling pathway involved in neuron projection guidance	13
GO:0032570	0.00074053	response to progesterone	50
GO:0014897	0.00076343	striated muscle hypertrophy	59
GO:1902903	0.00079349	regulation of supramolecular fiber organization	278
GO:2000177	0.00080486	regulation of neural precursor cell proliferation	78
GO:0048048	0.00080696	embryonic eye morphogenesis	34
GO:0034762	0.00081046	regulation of transmembrane transport	402
GO:0090002	0.00084455	establishment of protein localization to plasma membrane	140
GO:0001570	0.00085688	vasculogenesis	69
GO:0001562	0.00092969	response to protozoan	20
GO:0048745	0.00092969	smooth muscle tissue development	20
GO:0010464	0.00099091	regulation of mesenchymal cell proliferation	35
GO:0050807	0.00100017	regulation of synapse organization	111
GO:0072091	0.00102041	regulation of stem cell proliferation	61
GO:0009617	0.00117821	response to bacterium	559
GO:0060548	0.00119692	negative regulation of cell death	895
GO:0051246	0.00130105	regulation of protein metabolic process	2476
GO:1990778	0.00134444	protein localization to cell periphery	214
GO:0032330	0.00134965	regulation of chondrocyte differentiation	46
GO:0015711	0.00137422	organic anion transport	425

GO:1903729	0.00142905	regulation of plasma membrane organization	73
GO:0010712	0.00145885	regulation of collagen metabolic process	37
GO:0006639	0.00147176	acylglycerol metabolic process	115
GO:0060445	0.00161161	branching involved in salivary gland morphogenesis	22
GO:0032092	0.00161301	positive regulation of protein binding	74
GO:0042471	0.00161521	ear morphogenesis	115
GO:0060047	0.00164041	heart contraction	253
GO:0001894	0.00172236	tissue homeostasis	206
GO:0043244	0.00181998	regulation of protein complex disassembly	86
GO:0086029	0.00205133	Purkinje myocyte to ventricular cardiac muscle cell signaling	5
GO:0008582	0.00205133	regulation of synaptic growth at neuromuscular junction	5
GO:0044253	0.00206933	positive regulation of multicellular organismal metabolic process	23
GO:0032956	0.00211595	regulation of actin cytoskeleton organization	282
GO:0002062	0.00217458	chondrocyte differentiation	98
GO:0010574	0.0022019	regulation of vascular endothelial growth factor production	31
GO:0030856	0.00231183	regulation of epithelial cell differentiation	119
GO:0045834	0.00231938	positive regulation of lipid metabolic process	130
GO:0002687	0.00248376	positive regulation of leukocyte migration	110
GO:0046890	0.00251788	regulation of lipid biosynthetic process	132
GO:0030033	0.00261874	microvillus assembly	24
GO:0032102	0.00273521	negative regulation of response to external stimulus	262
GO:0009891	0.0027717	positive regulation of biosynthetic process	1726
GO:0033209	0.00281484	tumor necrosis factor-mediated signaling pathway	155
GO:1903035	0.00285633	negative regulation of response to wounding	69
GO:0050953	0.00285803	sensory perception of light stimulus	216
GO:0048592	0.00289818	eye morphogenesis	144
GO:0051147	0.0030272	regulation of muscle cell differentiation	157
GO:0002526	0.00312565	acute inflammatory response	145
GO:0006897	0.00316848	endocytosis	678
GO:0031643	0.00319239	positive regulation of myelination	11
GO:0048875	0.00319239	chemical homeostasis within a tissue	11
GO:0019935	0.00320655	cyclic-nucleotide-mediated signaling	70
GO:0071634	0.00327064	regulation of transforming growth factor beta production	25
GO:0009890	0.00332702	negative regulation of biosynthetic process	1492
GO:0007178	0.00333602	transmembrane receptor protein serine/threonine kinase signaling pathway	316
GO:0035904	0.00340619	aorta development	42
GO:0045667	0.00353249	regulation of osteoblast differentiation	113
GO:0034114	0.00364669	regulation of heterotypic cell-cell adhesion	18
GO:0042832	0.00364669	defense response to protozoan	18
GO:0010813	0.00370398	neuropeptide catabolic process	2
GO:0002386	0.00370398	immune response in mucosal-associated lymphoid tissue	2
GO:1904426	0.00370398	positive regulation of GTP binding	2
GO:0016202	0.00381872	regulation of striated muscle tissue development	103
GO:0060538	0.00387277	skeletal muscle organ development	183
GO:1901343	0.00390032	negative regulation of vasculature development	82
GO:1903053	0.00403609	regulation of extracellular matrix organization	26
GO:0048704	0.00408618	embryonic skeletal system morphogenesis	93
GO:0010633	0.00436558	negative regulation of epithelial cell migration	53
GO:0030326	0.00446166	embryonic limb morphogenesis	127
GO:0048634	0.00455112	regulation of muscle organ development	105
GO:1901862	0.00455406	negative regulation of muscle tissue development	35
GO:0048635	0.00455406	negative regulation of muscle organ development	35
GO:0003197	0.00455406	endocardial cushion development	35
GO:0007351	0.00455961	tripartite regional subdivision	12
GO:1905330	0.00466012	regulation of morphogenesis of an epithelium	175
GO:0006941	0.00482359	striated muscle contraction	151
Am80 GO (Up-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0006915	4.56E-09	apoptotic process	1817
GO:0043067	1.53E-08	regulation of programmed cell death	1409
GO:0050678	1.85E-08	regulation of epithelial cell proliferation	293
GO:0008285	2.41E-08	negative regulation of cell proliferation	653
GO:0072073	4.70E-08	kidney epithelium development	137
GO:0001657	8.11E-08	ureteric bud development	97
GO:0031344	9.75E-08	regulation of cell projection organization	575
GO:0045597	1.22E-07	positive regulation of cell differentiation	830
GO:0022008	1.31E-07	neurogenesis	1433
GO:0030855	4.13E-07	epithelial cell differentiation	567
GO:0071345	5.76E-07	cellular response to cytokine stimulus	692
GO:1902531	7.87E-07	regulation of intracellular signal transduction	1683
GO:0010648	8.61E-07	negative regulation of cell communication	1207
GO:0030182	1.03E-06	neuron differentiation	1231
GO:0060284	1.38E-06	regulation of cell development	782
GO:0010942	1.63E-06	positive regulation of cell death	612
GO:0033500	1.66E-06	carbohydrate homeostasis	221
GO:0051272	1.70E-06	positive regulation of cellular component movement	416
GO:0010647	2.30E-06	positive regulation of cell communication	1525

GO:0051046	2.39E-06	regulation of secretion	691
GO:0090184	4.86E-06	positive regulation of kidney development	41
GO:0051174	4.95E-06	regulation of phosphorus metabolic process	1632
GO:0051271	5.05E-06	negative regulation of cellular component movement	249
GO:1901342	7.09E-06	regulation of vasculature development	239
GO:0009968	7.44E-06	negative regulation of signal transduction	1102
GO:0009967	8.67E-06	positive regulation of signal transduction	1396
GO:2000027	1.11E-05	regulation of organ morphogenesis	246
GO:0072088	1.12E-05	nephron epithelium morphogenesis	75
GO:0060443	1.19E-05	mammary gland morphogenesis	45
GO:0045765	1.20E-05	regulation of angiogenesis	217
GO:0090183	1.29E-05	regulation of kidney development	55
GO:0051960	1.31E-05	regulation of nervous system development	767
GO:0051130	1.48E-05	positive regulation of cellular component organization	1142
GO:0022604	1.72E-05	regulation of cell morphogenesis	443
GO:0070371	2.40E-05	ERK1 and ERK2 cascade	258
GO:0019216	2.48E-05	regulation of lipid metabolic process	288
GO:0001678	2.63E-05	cellular glucose homeostasis	130
GO:0032268	3.26E-05	regulation of cellular protein metabolic process	2327
GO:0006687	3.63E-05	glycosphingolipid metabolic process	72
GO:0010631	4.77E-05	epithelial cell migration	222
GO:0040036	5.06E-05	regulation of fibroblast growth factor receptor signaling pathway	25
GO:0045596	5.15E-05	negative regulation of cell differentiation	624
GO:0002521	5.22E-05	leukocyte differentiation	466
GO:0034612	5.25E-05	response to tumor necrosis factor	269
GO:0051246	7.72E-05	regulation of protein metabolic process	2476
GO:0045995	7.96E-05	regulation of embryonic development	116
GO:0072074	8.42E-05	kidney mesenchyme development	19
GO:0060602	0.00011608	branch elongation of an epithelium	20
GO:0007169	0.00011738	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0031325	0.0001291	positive regulation of cellular metabolic process	2789
GO:0006721	0.00016125	terpenoid metabolic process	110
GO:1904018	0.00016138	positive regulation of vasculature development	136
GO:0042692	0.00017324	muscle cell differentiation	355
GO:0031032	0.00017513	actomyosin structure organization	151
GO:0006939	0.00019762	smooth muscle contraction	98
GO:0034284	0.00020345	response to monosaccharide	197
GO:2000736	0.00022298	regulation of stem cell differentiation	31
GO:0061572	0.00022459	actin filament bundle organization	127
GO:0033209	0.00024019	tumor necrosis factor-mediated signaling pathway	155
GO:0060548	0.00025833	negative regulation of cell death	895
GO:0071322	0.00029112	cellular response to carbohydrate stimulus	129
GO:0010604	0.00030137	positive regulation of macromolecule metabolic process	2795
GO:0045834	0.00031678	positive regulation of lipid metabolic process	130
GO:1902105	0.00033879	regulation of leukocyte differentiation	238
GO:0000904	0.00034859	cell morphogenesis involved in differentiation	638
GO:0031324	0.00035175	negative regulation of cellular metabolic process	2323
GO:0061564	0.00041361	axon development	460
GO:0016310	0.00043154	phosphorylation	2230
GO:0009792	0.00048861	embryo development ending in birth or egg hatching	574
GO:0043587	0.00050068	tongue morphogenesis	10
GO:1903035	0.00051846	negative regulation of response to wounding	69
GO:0030279	0.00051846	negative regulation of ossification	70
GO:0048706	0.00052474	embryonic skeletal system development	122
GO:1990778	0.0005997	protein localization to cell periphery	214
GO:0030038	0.00060623	contractile actin filament bundle assembly	83
GO:0034114	0.00065497	regulation of heterotypic cell-cell adhesion	18
GO:0010605	0.00069034	negative regulation of macromolecule metabolic process	2297
GO:0071396	0.00069579	cellular response to lipid	528
GO:0090002	0.00070002	establishment of protein localization to plasma membrane	140
GO:0031643	0.00076107	positive regulation of myelination	11
GO:1903729	0.00081674	regulation of plasma membrane organization	73
GO:0071363	0.00084809	cellular response to growth factor stimulus	609
GO:0006023	0.00087036	aminoglycan biosynthetic process	114
GO:0050880	0.00087301	regulation of blood vessel size	143
GO:0070201	0.00091879	regulation of establishment of protein localization	821
GO:0010594	0.00094498	regulation of endothelial cell migration	115
GO:0051223	0.00098405	regulation of protein transport	746
GO:0046879	0.00103672	hormone secretion	308
GO:0001973	0.00110436	adenosine receptor signaling pathway	12
GO:0001562	0.0011064	response to protozoan	20
GO:0006897	0.00112925	endocytosis	678
GO:0022408	0.00123791	negative regulation of cell-cell adhesion	133
GO:0051345	0.00133903	positive regulation of hydrolase activity	916
GO:0032102	0.00136948	negative regulation of response to external stimulus	262
GO:0070555	0.00140317	response to interleukin-1	121
GO:0035296	0.00142992	regulation of tube diameter	135

GO:0071300	0.00169356	cellular response to retinoic acid	67
GO:0010813	0.00171095	neuropeptide catabolic process	2
GO:0002386	0.00171095	immune response in mucosal-associated lymphoid tissue	2
GO:0009855	0.00175747	determination of bilateral symmetry	123
GO:0090288	0.00175747	negative regulation of cellular response to growth factor stimulus	123
GO:0009952	0.00178534	anterior/posterior pattern specification	201
GO:0051962	0.00187087	positive regulation of nervous system development	447
GO:0014706	0.00203464	striated muscle tissue development	341
GO:0051492	0.0020862	regulation of stress fiber assembly	69
GO:0070587	0.00218133	regulation of cell-cell adhesion involved in gastrulation	7
GO:0043010	0.0022706	camera-type eye development	290
GO:0002718	0.00227862	regulation of cytokine production involved in immune response	57
GO:1902284	0.00232878	neuron projection extension involved in neuron projection guidance	45
GO:0001894	0.00234029	tissue homeostasis	206
GO:1905330	0.00239875	regulation of morphogenesis of an epithelium	175
GO:0002688	0.00240649	regulation of leukocyte chemotaxis	99
GO:0048048	0.00245806	embryonic eye morphogenesis	34
GO:1902903	0.00274876	regulation of supramolecular fiber organization	278
GO:0097300	0.00286447	programmed necrotic cell death	35
GO:1903707	0.00287771	negative regulation of hemopoiesis	132
GO:0046890	0.00307709	regulation of lipid biosynthetic process	132
GO:0006664	0.00310915	glycolipid metabolic process	116
GO:0030203	0.00320757	glycosaminoglycan metabolic process	163
GO:0090087	0.00335425	regulation of peptide transport	213
GO:0008300	0.0033831	isoprenoid catabolic process	8
GO:0032233	0.00382248	positive regulation of actin filament bundle assembly	49
GO:0010634	0.00383453	positive regulation of epithelial cell migration	104
GO:0008284	0.00385036	positive regulation of cell proliferation	839
GO:0019752	0.00392265	carboxylic acid metabolic process	961
GO:0060538	0.00395979	skeletal muscle organ development	183
GO:0030195	0.0042894	negative regulation of blood coagulation	50
GO:0060046	0.00450181	regulation of acrosome reaction	17
GO:1901203	0.00491936	positive regulation of extracellular matrix assembly	9
GO:0009617	0.00493245	response to bacterium	559
GO:0009896	0.00496253	positive regulation of catabolic process	328
GO:0019049	0.00499158	evasion or tolerance of host defenses by virus	3
GO:0032907	0.00499158	transforming growth factor beta3 production	3
GO:0042214	0.00499158	terpene metabolic process	3
Am80 GO (Down-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0030182	1.02E-07	neuron differentiation	1231
GO:0022008	9.24E-07	neurogenesis	1433
GO:0000904	8.40E-06	cell morphogenesis involved in differentiation	638
GO:0061564	1.33E-05	axon development	460
GO:0007411	3.40E-05	axon guidance	223
GO:0051960	4.41E-05	regulation of nervous system development	767
GO:0032412	5.45E-05	regulation of ion transmembrane transporter activity	181
GO:0086029	7.14E-05	Purkinje myocyte to ventricular cardiac muscle cell signaling	5
GO:0072091	0.00017842	regulation of stem cell proliferation	61
GO:0045596	0.00022789	negative regulation of cell differentiation	624
GO:0007156	0.00024935	homophilic cell adhesion via plasma membrane adhesion molecules	156
GO:0007435	0.00047889	salivary gland morphogenesis	34
GO:0060284	0.00070665	regulation of cell development	782
GO:0031344	0.00092058	regulation of cell projection organization	575
GO:0042475	0.00102013	odontogenesis of dentin-containing tooth	81
GO:0098773	0.00109703	skin epidermis development	82
GO:0061037	0.00130002	negative regulation of cartilage development	26
GO:0010631	0.00138053	epithelial cell migration	222
GO:0050807	0.00140914	regulation of synapse organization	111
GO:0060047	0.0014585	heart contraction	253
GO:0043269	0.00154203	regulation of ion transport	599
GO:0043010	0.001751	camera-type eye development	290
GO:0071300	0.00193362	cellular response to retinoic acid	67
GO:0042636	0.00221609	negative regulation of hair cycle	4
GO:0034762	0.00238402	regulation of transmembrane transport	402
GO:0051961	0.00242176	negative regulation of nervous system development	268
GO:0035282	0.00272924	segmentation	96
GO:0051797	0.00280939	regulation of hair follicle development	15
GO:0086001	0.00282778	cardiac muscle cell action potential	50
GO:0032570	0.00282778	response to progesterone	50
GO:0051272	0.00338398	positive regulation of cellular component movement	416
GO:0009953	0.00362701	dorsal/ventral pattern formation	101
GO:0045684	0.00372064	positive regulation of epidermis development	33
GO:0071396	0.0037708	cellular response to lipid	528
GO:0042692	0.00422406	muscle cell differentiation	355
GO:0010634	0.00426406	positive regulation of epithelial cell migration	104
GO:0019731	0.00429005	antibacterial humoral response	56

GO:0002066	0.00429005	columnar/cuboidal epithelial cell development	55
GO:0010464	0.00461778	regulation of mesenchymal cell proliferation	35
GO:0071675	0.00461778	regulation of mononuclear cell migration	35
GO:1902531	0.00470277	regulation of intracellular signal transduction	1683
CD2314 GO (All DEGs)			
term_goid	p-value	term_name	size
GO:0071363	9.34E-07	cellular response to growth factor stimulus	609
GO:0030182	7.52E-06	neuron differentiation	1231
GO:0030855	9.35E-06	epithelial cell differentiation	567
GO:0022008	2.40E-05	neurogenesis	1433
GO:0040036	3.06E-05	regulation of fibroblast growth factor receptor signaling pathway	25
GO:0010648	6.64E-05	negative regulation of cell communication	1207
GO:0060602	0.00011723	branch elongation of an epithelium	20
GO:0051272	0.00017007	positive regulation of cellular component movement	416
GO:0006915	0.00022228	apoptotic process	1817
GO:1902531	0.00024331	regulation of intracellular signal transduction	1683
GO:0042692	0.00030505	muscle cell differentiation	355
GO:0021562	0.00052869	vestibulocochlear nerve development	7
GO:0043067	0.00053588	regulation of programmed cell death	1409
GO:0090184	0.00055121	positive regulation of kidney development	41
GO:2000027	0.00055849	regulation of organ morphogenesis	246
GO:0009968	0.00062803	negative regulation of signal transduction	1102
GO:0010813	0.00064429	neuropeptide catabolic process	2
GO:0050801	0.00069276	ion homeostasis	683
GO:0045995	0.00070419	regulation of embryonic development	116
GO:1904018	0.00071398	positive regulation of vasculature development	136
GO:0071345	0.00084061	cellular response to cytokine stimulus	692
GO:2000736	0.00102061	regulation of stem cell differentiation	31
GO:0072074	0.00117784	kidney mesenchyme development	19
GO:0051960	0.00118063	regulation of nervous system development	767
GO:0071415	0.00122151	cellular response to purine-containing compound	9
GO:1901342	0.0012286	regulation of vasculature development	239
GO:0010634	0.00133179	positive regulation of epithelial cell migration	104
GO:0090257	0.0015602	regulation of muscle system process	196
GO:0071312	0.00157015	cellular response to alkaloid	34
GO:0010631	0.00161824	epithelial cell migration	222
GO:0006941	0.00167855	striated muscle contraction	151
GO:0060600	0.00171222	dichotomous subdivision of an epithelial terminal unit	10
GO:0021561	0.00171222	facial nerve development	10
GO:1903522	0.00175649	regulation of blood circulation	299
GO:0010464	0.00179412	regulation of mesenchymal cell proliferation	35
GO:0000904	0.00187388	cell morphogenesis involved in differentiation	638
GO:0042214	0.00190026	terpene metabolic process	3
GO:0009952	0.00195207	anterior/posterior pattern specification	201
GO:0050807	0.00197022	regulation of synapse organization	111
GO:0060445	0.00209287	branching involved in salivary gland morphogenesis	22
GO:0060047	0.00214719	heart contraction	253
GO:0051147	0.00217272	regulation of muscle cell differentiation	157
GO:0045597	0.00237428	positive regulation of cell differentiation	830
GO:0043087	0.00257633	regulation of GTPase activity	683
GO:0090183	0.00263629	regulation of kidney development	55
GO:0010594	0.00267097	regulation of endothelial cell migration	115
GO:0070252	0.0027043	actin-mediated cell contraction	94
GO:0055002	0.0029413	striated muscle cell development	139
GO:0015711	0.0030131	organic anion transport	425
GO:0044252	0.00302239	negative regulation of multicellular organismal metabolic process	12
GO:0007351	0.00302239	tripartite regional subdivision	12
GO:0036066	0.00302239	protein O-linked fucosylation	12
GO:0002718	0.00316274	regulation of cytokine production involved in immune response	57
GO:0009792	0.00330023	embryo development ending in birth or egg hatching	574
GO:0050678	0.00352453	regulation of epithelial cell proliferation	293
GO:0061564	0.00358867	axon development	460
GO:1990791	0.00373654	dorsal root ganglion development	4
GO:0014897	0.00376385	striated muscle hypertrophy	59
GO:0045765	0.00378533	regulation of angiogenesis	217
GO:0051965	0.00444575	positive regulation of synapse assembly	62
GO:0019722	0.00460117	calcium-mediated signaling	149
GO:0002700	0.00474413	regulation of production of molecular mediator of immune response	103
CD2314 GO (Up-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0030855	1.40E-05	epithelial cell differentiation	567
GO:0040036	9.64E-05	regulation of fibroblast growth factor receptor signaling pathway	25
GO:2000736	0.00028065	regulation of stem cell differentiation	31
GO:0010813	0.0003647	neuropeptide catabolic process	2
GO:0071345	0.00037877	cellular response to cytokine stimulus	692
GO:0072074	0.00040605	kidney mesenchyme development	19
GO:0060602	0.00049996	branch elongation of an epithelium	20

GO:0006915	0.00061552	apoptotic process	1817
GO:0071363	0.00068999	cellular response to growth factor stimulus	609
GO:0010648	0.00089856	negative regulation of cell communication	1207
GO:0090184	0.0010587	positive regulation of kidney development	41
GO:0042214	0.00108022	terpene metabolic process	3
GO:0036066	0.00134198	protein O-linked fucosylation	12
GO:0043067	0.00152531	regulation of programmed cell death	1409
GO:0050801	0.00154756	ion homeostasis	683
GO:0009792	0.00162231	embryo development ending in birth or egg hatching	574
GO:0009952	0.00174529	anterior/posterior pattern specification	201
GO:0019935	0.0021975	cyclic-nucleotide-mediated signaling	70
GO:1903729	0.00272017	regulation of plasma membrane organization	73
GO:0009261	0.00275158	ribonucleotide catabolic process	31
GO:2000027	0.00277545	regulation of organ morphogenesis	246
GO:0072088	0.00311698	nephron epithelium morphogenesis	75
GO:1902531	0.0032306	regulation of intracellular signal transduction	1683
GO:0010815	0.00351022	bradykinin catabolic process	5
GO:0003100	0.00351022	regulation of systemic arterial blood pressure by endothelin	5
GO:0061370	0.00351022	testosterone biosynthetic process	5
GO:0060729	0.00351022	intestinal epithelial structure maintenance	5
GO:0060548	0.00387163	negative regulation of cell death	895
GO:0090183	0.00395359	regulation of kidney development	55
GO:0010464	0.00431265	regulation of mesenchymal cell proliferation	35
GO:0050678	0.00438694	regulation of epithelial cell proliferation	293
GO:0090257	0.00458272	regulation of muscle system process	196
GO:0002718	0.00461355	regulation of cytokine production involved in immune response	57
GO:0051173	0.00463354	positive regulation of nitrogen compound metabolic process	1721
GO:1904018	0.00467884	positive regulation of vasculature development	136
CD2314 GO (Down-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0030182	2.03E-06	neuron differentiation	1231
GO:0000904	2.40E-06	cell morphogenesis involved in differentiation	638
GO:0022008	7.79E-06	neurogenesis	1433
GO:0061564	3.31E-05	axon development	460
GO:0051965	4.05E-05	positive regulation of synapse assembly	62
GO:0050807	6.83E-05	regulation of synapse organization	111
GO:0007411	8.34E-05	axon guidance	223
GO:0051960	9.02E-05	regulation of nervous system development	767
GO:0071363	0.00011722	cellular response to growth factor stimulus	609
GO:0086029	0.00038694	Purkinje myocyte to ventricular cardiac muscle cell signaling	5
GO:0021562	0.00080591	vestibulocochlear nerve development	7
GO:0071415	0.00137023	cellular response to purine-containing compound	9
GO:0042475	0.00170002	odontogenesis of dentin-containing tooth	81
GO:0001649	0.00207725	osteoblast differentiation	209
GO:0045597	0.00213513	positive regulation of cell differentiation	830
GO:0043010	0.00239382	camera-type eye development	290
GO:0006941	0.00266005	striated muscle contraction	151
GO:1902285	0.00292041	semaphorin-plexin signaling pathway involved in neuron projection guidance	13
GO:0007169	0.00300172	transmembrane receptor protein tyrosine kinase signaling pathway	661
GO:0016055	0.00309091	Wnt signaling pathway	477
GO:0086001	0.00382672	cardiac muscle cell action potential	50
GO:0010634	0.00420752	positive regulation of epithelial cell migration	104
GO:0051284	0.00443795	positive regulation of sequestering of calcium ion	16
GO:0051272	0.00471306	positive regulation of cellular component movement	416
BMS961 GO (All DEGs)			
term_goid	p-value	term_name	size
GO:0030855	0.00016495	epithelial cell differentiation	567
GO:0010594	0.00037206	regulation of endothelial cell migration	115
GO:0090184	0.000373	positive regulation of kidney development	41
GO:0050678	0.00056949	regulation of epithelial cell proliferation	293
GO:0010631	0.00058456	epithelial cell migration	222
GO:0051272	0.00064263	positive regulation of cellular component movement	416
GO:0010648	0.0007361	negative regulation of cell communication	1207
GO:0010634	0.00104654	positive regulation of epithelial cell migration	104
GO:0051223	0.00107078	regulation of protein transport	746
GO:1904018	0.001127	positive regulation of vasculature development	136
GO:0071407	0.00116683	cellular response to organic cyclic compound	527
GO:2000736	0.00118471	regulation of stem cell differentiation	31
GO:0071396	0.00124352	cellular response to lipid	528
GO:0090092	0.0013857	regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	209
GO:0090183	0.00145695	regulation of kidney development	55
GO:0010464	0.00187993	regulation of mesenchymal cell proliferation	35
GO:0070120	0.0022283	ciliary neurotrophic factor-mediated signaling pathway	5
GO:0071504	0.0022283	cellular response to heparin	5
GO:0071363	0.00247431	cellular response to growth factor stimulus	609
GO:0090288	0.00274463	negative regulation of cellular response to growth factor stimulus	123
GO:0009968	0.00274565	negative regulation of signal transduction	1102

GO:0097421	0.00280176	liver regeneration	19
GO:2001022	0.0030597	positive regulation of response to DNA damage stimulus	65
GO:0060602	0.00325948	branch elongation of an epithelium	20
GO:0072298	0.00330898	regulation of metanephric glomerulus development	6
GO:0007178	0.00336995	transmembrane receptor protein serine/threonine kinase signaling pathway	316
GO:0006915	0.00342412	apoptotic process	1817
GO:0070201	0.00360972	regulation of establishment of protein localization	821
GO:0002062	0.00362086	chondrocyte differentiation	98
GO:0009952	0.00369573	anterior/posterior pattern specification	201
GO:0045597	0.00405927	positive regulation of cell differentiation	830
GO:2000027	0.00437122	regulation of organ morphogenesis	246
GO:0009896	0.004423	positive regulation of catabolic process	328
GO:0021954	0.00448149	central nervous system neuron development	71
GO:0071447	0.00458624	cellular response to hydroperoxide	7
GO:0021562	0.00458624	vestibulocochlear nerve development	7
GO:0043067	0.00476783	regulation of programmed cell death	1409
BMS961 GO (Up-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0090184	0.0001559	positive regulation of kidney development	41
GO:0030855	0.00031252	epithelial cell differentiation	567
GO:0050678	0.00035189	regulation of epithelial cell proliferation	293
GO:2000736	0.00058672	regulation of stem cell differentiation	31
GO:0090183	0.00062747	regulation of kidney development	55
GO:0010464	0.00093871	regulation of mesenchymal cell proliferation	35
GO:0090092	0.00135024	regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	209
GO:0071504	0.00153488	cellular response to heparin	5
GO:0097421	0.00163788	liver regeneration	19
GO:1904018	0.00168006	positive regulation of vasculature development	136
GO:0060602	0.00190914	branch elongation of an epithelium	20
GO:0021954	0.00199645	central nervous system neuron development	71
GO:0060548	0.00203962	negative regulation of cell death	895
GO:0007178	0.00227126	transmembrane receptor protein serine/threonine kinase signaling pathway	316
GO:0072298	0.00228324	regulation of metanephric glomerulus development	6
GO:0010648	0.00253375	negative regulation of cell communication	1207
GO:0051223	0.00287958	regulation of protein transport	746
GO:0071345	0.00291524	cellular response to cytokine stimulus	692
GO:0009896	0.00295396	positive regulation of catabolic process	328
GO:0071447	0.00317008	cellular response to hydroperoxide	7
GO:0003158	0.00363386	endothelium development	117
GO:0006915	0.00363917	apoptotic process	1817
GO:2000027	0.00393582	regulation of organ morphogenesis	246
GO:0009952	0.00399607	anterior/posterior pattern specification	201
GO:0030488	0.00411926	tRNA methylation	26
GO:0048706	0.00446033	embryonic skeletal system development	122
GO:0071363	0.00446179	cellular response to growth factor stimulus	609
GO:1901381	0.00459162	positive regulation of potassium ion transmembrane transport	27
GO:0090288	0.00464097	negative regulation of cellular response to growth factor stimulus	123
BMS961 GO (Down-regulated DEGs)			
term_goid	p-value	term_name	size
GO:0010634	0.00015547	positive regulation of epithelial cell migration	104
GO:0070828	0.0004332	heterochromatin organization	12
GO:0008593	0.00074333	regulation of Notch signaling pathway	68
GO:0045597	0.00126247	positive regulation of cell differentiation	830
GO:0002062	0.00207397	chondrocyte differentiation	98
GO:0071407	0.00234408	cellular response to organic cyclic compound	527
GO:0071396	0.00241174	cellular response to lipid	528
GO:0030947	0.0024158	regulation of vascular endothelial growth factor receptor signaling pathway	28
GO:0010631	0.00261597	epithelial cell migration	222
GO:0010594	0.00336093	regulation of endothelial cell migration	115
GO:0060612	0.00419321	adipose tissue development	37
GO:0051272	0.00442042	positive regulation of cellular component movement	416

Appendix. Supplementary Table B. List containing the signaling pathways (p-value < 0.005) over-represented upon analysis of all DEGs, up- and down-regulated DEGs for the treatments with AtRA, Am80, CD2314 and BMS961

Pathway Analysis AtRA (All DEGs)			
Pathway	p-value	Source	Size
Vitamin D Receptor Pathway	3,66E-07	Wikipathways	184
Axon guidance	2,93E-05	Reactome	487
Non-integrin membrane-ECM interactions	3,09E-05	Reactome	46
Vitamin A and Carotenoid Metabolism	4,00E-05	Wikipathways	43
PPAR signaling pathway - Homo sapiens (human)	4,93E-05	KEGG	72
Extracellular matrix organization	6,73E-05	Reactome	295
Adipogenesis	7,01E-05	Wikipathways	131
Axon guidance - Homo sapiens (human)	7,53E-05	KEGG	177
ECM proteoglycans	0,000127262	Reactome	57
Developmental Biology	0,000134216	Reactome	748
Metabolism of lipids and lipoproteins	0,000143609	Reactome	728
Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)	0,00015698	Wikipathways	42
Pathways in cancer - Homo sapiens (human)	0,000201855	KEGG	397
Metabolism	0,000208405	Reactome	2035
Signaling by Retinoic Acid	0,000245755	Reactome	44
PPAR signaling pathway	0,000337252	Wikipathways	68
Rap1 signaling pathway - Homo sapiens (human)	0,000360863	KEGG	212
Phase 1 - Functionalization of compounds	0,000631307	Reactome	110
Nucleotide GPCRs	0,000658554	Wikipathways	11
NHR	0,000666814	Signalink	49
Beta1 integrin cell surface interactions	0,000983142	PID	70
Integrin cell surface interactions	0,000983142	Reactome	68
Pre-NOTCH Processing in Golgi	0,00106125	Reactome	12
Import of palmitoyl-CoA into the mitochondrial matrix	0,00106125	Reactome	12
Pathway_PA165986194 -need delete	0,001254317	PharmGKB	24
Acetaminophen Pathway, Pharmacokinetics	0,001254317	PharmGKB	24
Adenosine P1 receptors	0,001474588	Reactome	4
Fosphenytoin (Antiarrhythmic) Metabolism Pathway	0,001474588	SMPDB	4
Neurophilin interactions with VEGF and VEGFR	0,001474588	Reactome	4
Spinal Cord Injury	0,002034495	Wikipathways	117
VEGFA-VEGFR2 Signaling Pathway	0,002096082	Wikipathways	236
Vitamin A (retinol) metabolism	0,002096177	EHMN	26
RXR and RAR heterodimerization with other nuclear receptor	0,002096177	PID	26
A tetrasaccharide linker sequence is required for GAG synthesis	0,002096177	Reactome	26
Alpha 6 Beta 4 signaling pathway	0,002124419	Wikipathways	33
Sialic acid metabolism	0,002124419	Reactome	33
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0,002131459	KEGG	56
ECM-receptor interaction - Homo sapiens (human)	0,002317444	KEGG	82
Validated nuclear estrogen receptor alpha network	0,002371623	PID	65
Syndecan interactions	0,00239323	Reactome	21
Visual phototransduction	0,002603667	Reactome	102
Nuclear Receptors Meta-Pathway	0,002810991	Wikipathways	316
Transmembrane transport of small molecules	0,003112599	Reactome	628
Synthesis of IP3 and IP4 in the cytosol	0,003318808	Reactome	28
Fatty Acid Omega Oxidation	0,003345048	Wikipathways	15
Negative regulation of TCF-dependent signaling by WNT ligand antagonists	0,003345048	Reactome	15
Aromatase Inhibitor Pathway (Multiple Tissues), Pharmacodynamics	0,003485645	PharmGKB	5
Hippo signaling pathway - Homo sapiens (human)	0,003783138	KEGG	154
Integrin	0,003883399	INOH	124
EPH-Ephrin signaling	0,004060299	Reactome	78
Transcription factor regulation in adipogenesis	0,004062006	Wikipathways	22
The canonical retinoid cycle in rods (twilight vision)	0,004062006	Reactome	22
EV release from cardiac cells and their functional effects	0,004174833	Wikipathways	10
Bicarbonate transporters	0,004174833	Reactome	10
Leukocyte transendothelial migration - Homo sapiens (human)	0,004470534	KEGG	116
Nucleotide-like (purinergic) receptors	0,004576067	Reactome	16
Pathway analysis AtRA (Up-regulated DEGs)			
Pathway	p-value	Source	Size
Vitamin A and Carotenoid Metabolism	2,47E-05	Wikipathways	43
Adipogenesis	4,51E-05	Wikipathways	131
Vitamin D Receptor Pathway	5,51E-05	Wikipathways	184
Metabolism	5,63E-05	Reactome	2035
Metabolism of lipids and lipoproteins	8,28E-05	Reactome	728
Adenosine P1 receptors	0,000409583	Reactome	4
Neurophilin interactions with VEGF and VEGFR	0,000409583	Reactome	4
The canonical retinoid cycle in rods (twilight vision)	0,000431205	Reactome	22
Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)	0,000689127	Wikipathways	42
Sialic acid metabolism	0,00075661	Reactome	33
Validated nuclear estrogen receptor alpha network	0,000903314	PID	65

Signaling by Retinoic Acid	0,000951999	Reactome	44
Aromatase Inhibitor Pathway (Multiple Tissues), Pharmacodynamics	0,000987798	PharmGKB	5
Nucleotide GPCRs	0,001265066	WikiPathways	11
Phase 1 - Functionalization of compounds	0,001485639	Reactome	110
Type II interferon signaling (IFNG)	0,001547016	WikiPathways	37
Caspase activation via extrinsic apoptotic signalling pathway	0,001708416	Reactome	28
antigen processing and presentation	0,001826872	BioCarta	12
Vitamins	0,001906	Reactome	6
PPAR signaling pathway - Homo sapiens (human)	0,002011123	KEGG	72
Nuclear Receptors Meta-Pathway	0,002158666	WikiPathways	316
Axon guidance	0,002540122	Reactome	487
Tryptophan catabolism	0,002540626	Reactome	13
Type II diabetes mellitus	0,00317011	WikiPathways	22
tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde	0,003218282	HumanCyc	7
Non-integrin membrane-ECM interactions	0,003303352	Reactome	46
Activation of IRF3/IRF7 mediated by TBK1/IKK epsilon	0,003424753	Reactome	14
Spinal Cord Injury	0,003599698	WikiPathways	117
Folate Metabolism	0,003771861	WikiPathways	66
RA biosynthesis pathway	0,003895077	Reactome	23
Copper homeostasis	0,004142672	WikiPathways	55
Beta1 integrin cell surface interactions	0,004178768	PID	70
Integrin cell surface interactions	0,004178768	Reactome	68
VEGFA-VEGFR2 Signaling Pathway	0,004183357	WikiPathways	236
Thiamine metabolism - Homo sapiens (human)	0,00449694	KEGG	16
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0,004637407	KEGG	56
Signaling by VEGF	0,00484328	Reactome	290
Nifedipine Activity	0,004968739	WikiPathways	8
Axonal growth inhibition (RHOA activation)	0,004968739	Reactome	10
Glial Cell Differentiation	0,004968739	WikiPathways	8
NAD Biosynthesis II (from tryptophan)	0,004968739	WikiPathways	8
Pathway Analysis AtRA (Down-regulated DEGs)			
Pathway	p-value	Source	Size
Hippo signaling pathway - Homo sapiens (human)	3,98E-05	KEGG	154
Ectoderm Differentiation	7,45E-05	WikiPathways	142
Pathways in cancer - Homo sapiens (human)	9,33E-05	KEGG	397
Ca2+ pathway	0,000489106	Reactome	55
Synthesis of IP3 and IP4 in the cytosol	0,000657632	Reactome	28
Thyroid hormone signaling pathway - Homo sapiens (human)	0,000918703	KEGG	118
Phosphatidylinositol signaling system - Homo sapiens (human)	0,000997774	KEGG	99
Inflammatory mediator regulation of TRP channels - Homo sapiens (human)	0,000997774	KEGG	99
Signaling pathways regulating pluripotency of stem cells - Homo sapiens (human)	0,001092023	KEGG	142
Rap1 signaling pathway - Homo sapiens (human)	0,001173489	KEGG	212
Prostanoid ligand receptors	0,001260231	Reactome	9
Elastic fibre formation	0,001875317	Reactome	36
G alpha (12/13) signalling events	0,001887001	Reactome	90
Axon guidance - Homo sapiens (human)	0,001983494	KEGG	177
Inositol phosphate metabolism - Homo sapiens (human)	0,002259666	KEGG	71
Phosphatidylinositol phosphate metabolism	0,002335199	EHMN	91
cxc4 signaling pathway	0,002382519	BioCarta	11
Adrenaline,noradrenaline inhibits insulin secretion	0,002579116	Reactome	23
Vitamin D Receptor Pathway	0,002742832	WikiPathways	184
ECM proteoglycans	0,00276828	Reactome	57
Pathway_PA165986194 -need delete	0,003033055	PharmGKB	24
Acetaminophen Pathway, Pharmacokinetics	0,003033055	PharmGKB	24
phospholipases	0,003058975	HumanCyc	39
Pre-NOTCH Processing in Golgi	0,003116606	Reactome	12
Axon guidance	0,003192113	Reactome	487
Extracellular matrix organization	0,003321536	Reactome	295
Wnt Canonical	0,003374472	INOH	120
Wnt Mammals	0,003374472	INOH	120
Glycosaminoglycan metabolism	0,003775006	Reactome	122
HCN channels	0,003820173	Reactome	4
Imipramine/Desipramine Pathway, Pharmacokinetics	0,003820173	PharmGKB	4
Clomipramine Pathway, Pharmacokinetics	0,003820173	PharmGKB	4
Clomipramine Metabolism Pathway	0,003820173	SMPDB	4
D-Glutamine and D-glutamate metabolism - Homo sapiens (human)	0,003820173	KEGG	4
Serotonin and anxiety-related events	0,003975054	WikiPathways	13
ID	0,004098563	NetPath	26
A tetrasaccharide linker sequence is required for GAG synthesis	0,004098563	Reactome	26
Non-integrin membrane-ECM interactions	0,00424522	Reactome	46
NRAGE signals death through JNK	0,004296209	Reactome	62
Developmental Biology	0,004913148	Reactome	748
Pathway Analysis Am80 (All DEGs)			
Pathway	p-value	Source	Size
Axon guidance - Homo sapiens (human)	4,90E-10	KEGG	177
Developmental Biology	1,04E-05	Reactome	748
Axon guidance	1,21E-05	Reactome	487

Vitamin D Receptor Pathway	1,98E-05	Wikipathways	184
Pathways in cancer - Homo sapiens (human)	2,44E-05	KEGG	397
Spinal Cord Injury	2,78E-05	Wikipathways	117
NHR	3,03E-05	Signalink	49
Non-integrin membrane-ECM interactions	3,29E-05	Reactome	46
Rap1 signaling pathway - Homo sapiens (human)	4,21E-05	KEGG	212
Ectoderm Differentiation	5,40E-05	Wikipathways	142
VEGFA-VEGFR2 Signaling Pathway	0,000123037	Wikipathways	236
Adipogenesis	0,000143157	Wikipathways	131
HIF-1-alpha transcription factor network	0,000188716	PID	67
Vitamin A and Carotenoid Metabolism	0,000220175	Wikipathways	43
Nuclear Receptors Meta-Pathway	0,000240992	Wikipathways	316
cxcr4 signaling pathway	0,000295045	BioCarta	11
Rho GTPase cycle	0,000390902	Reactome	145
Pathway_PA165986194 -need delete	0,000447422	PharmGKB	24
Acetaminophen Pathway, Pharmacokinetics	0,000447422	PharmGKB	24
Ephrin B reverse signaling	0,000447422	PID	24
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0,00052023	KEGG	56
Validated transcriptional targets of TAp63 isoforms	0,00052023	PID	57
Malaria - Homo sapiens (human)	0,000675029	KEGG	49
Sialic acid metabolism	0,000695448	Reactome	33
Sudden Infant Death Syndrome (SIDS) Susceptibility Pathways	0,000744813	Wikipathways	159
Vitamin A (retinol) metabolism	0,000763324	EHMN	26
Adenosine P1 receptors	0,000886852	Reactome	4
Nuclear Receptor transcription pathway	0,000938942	Reactome	51
Syndecan interactions	0,00098053	Reactome	21
Metabolism of lipids and lipoproteins	0,001026895	Reactome	728
Signaling pathways regulating pluripotency of stem cells - Homo sapiens (human)	0,00111587	KEGG	142
Signaling by Retinoic Acid	0,001221907	Reactome	44
Caspase activation via extrinsic apoptotic signalling pathway	0,001233625	Reactome	28
Hemostasis	0,001295342	Reactome	693
Macrophage markers	0,001398496	Wikipathways	9
Signaling by VEGF	0,001522681	Reactome	290
Basal cell carcinoma - Homo sapiens (human)	0,001720756	KEGG	55
Extracellular matrix organization	0,001797359	Reactome	295
Nuclear Receptors	0,00186468	Wikipathways	38
EPH-ephrin mediated repulsion of cells	0,001904818	Reactome	30
Leukocyte transendothelial migration - Homo sapiens (human)	0,0019446	KEGG	116
MAPK signaling pathway - Homo sapiens (human)	0,002083282	KEGG	255
Aromatase Inhibitor Pathway (Multiple Tissues), Pharmacodynamics	0,002115465	PharmGKB	5
Glycerophospholipid biosynthesis	0,002160026	Reactome	131
Signaling events mediated by the Hedgehog family	0,002179419	PID	23
RA biosynthesis pathway	0,002179419	Reactome	23
Platelet activation, signaling and aggregation	0,002271615	Reactome	283
Ovarian Infertility Genes	0,002331809	Wikipathways	31
Celecoxib Pathway, Pharmacodynamics	0,002599285	PharmGKB	58
Wnt signaling pathway - Homo sapiens (human)	0,002740705	KEGG	143
IL1 and megakaryocytes in obesity	0,002756751	Wikipathways	24
Glycosaminoglycan metabolism	0,002764406	Reactome	122
Factors involved in megakaryocyte development and platelet production	0,002769674	Reactome	133
Nitrogen Metabolism - Homo sapiens (human)	0,002899075	KEGG	17
GPCR signaling-G alpha q	0,003027357	INOH	274
Nucleotide GPCRs	0,003316547	Wikipathways	11
Alpha 6 Beta 4 signaling pathway	0,003403293	Wikipathways	33
Endothelin Pathways	0,003403293	Wikipathways	33
Glucocorticoid Receptor Pathway	0,003454015	Wikipathways	71
VEGFA-VEGFR2 Pathway	0,003733385	Reactome	282
thyroid hormone biosynthesis	0,003784638	HumanCyc	2
Transport of connexons to the plasma membrane	0,003784638	Reactome	2
Estrogen metabolism	0,003813282	Wikipathways	18
Proteoglycans in cancer - Homo sapiens (human)	0,003831823	KEGG	205
Alpha6Beta4Integrin	0,004315867	NetPath	74
mechanism of gene regulation by peroxisome proliferators via ppara	0,004658738	BioCarta	53
Signaling by NOTCH1	0,004806614	Reactome	74
Tyrosine metabolism - Homo sapiens (human)	0,004813773	KEGG	35
Endochondral Ossification	0,004828455	Wikipathways	64
Pathway Analysis Am80 (Up-regulated DEGs)			
Pathway	p-value	Source	Size
Axon guidance - Homo sapiens (human)	2,21E-05	KEGG	177
Vitamin A and Carotenoid Metabolism	7,20E-05	Wikipathways	43
Axon guidance	0,00010534	Reactome	487
Vitamin D Receptor Pathway	0,00011955	Wikipathways	184
Caspase activation via extrinsic apoptotic signalling pathway	0,00014131	Reactome	28
Spinal Cord Injury	0,00014475	Wikipathways	117
Metabolism of lipids and lipoproteins	0,00014841	Reactome	728
Developmental Biology	0,00016377	Reactome	748
NHR	0,00020842	Signalink	49

Nuclear Receptors Meta-Pathway	0,00022558	Wikipathways	316
Adenosine P1 receptors	0,00030691	Reactome	4
Sialic acid metabolism	0,00042296	Reactome	33
Metabolism	0,00042687	Reactome	2035
Ephrin B reverse signaling	0,00042812	PID	24
Signaling by Retinoic Acid	0,00050266	Reactome	44
Adipogenesis	0,0005037	Wikipathways	131
VEGFA-VEGFR2 Signaling Pathway	0,00054713	Wikipathways	236
Validated transcriptional targets of TAp63 isoforms	0,00058822	PID	57
Aromatase Inhibitor Pathway (Multiple Tissues), Pharmacodynamics	0,00074269	PharmGKB	5
Nucleotide GPCRs	0,00087871	Wikipathways	11
Rho GTPase cycle	0,00108903	Reactome	145
Glycerophospholipid biosynthesis	0,00120641	Reactome	131
Factors involved in megakaryocyte development and platelet production	0,00150989	Reactome	133
EPH-ephrin mediated repulsion of cells	0,00151657	Reactome	30
Acyl chain remodelling of PE	0,00151657	Reactome	30
Bladder cancer - Homo sapiens (human)	0,00165287	KEGG	41
Bladder Cancer	0,00181322	Wikipathways	31
Non-integrin membrane-ECM interactions	0,00191124	Reactome	46
CDP-diacylglycerol biosynthesis	0,00207434	HumanCyc	23
The canonical retinoid cycle in rods (twilight vision)	0,00207434	Reactome	22
Tyrosine metabolism	0,00253602	INOH	34
RA biosynthesis pathway	0,00255802	Reactome	23
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0,0025645	KEGG	56
tumor suppressor arf inhibits ribosomal biogenesis	0,00311852	BioCarta	24
Celecoxib Pathway, Pharmacodynamics	0,00321058	PharmGKB	58
Aryl Hydrocarbon Receptor	0,00327004	Wikipathways	46
Glycosphingolipid metabolism	0,00327004	Reactome	46
Tyrosine metabolism - Homo sapiens (human)	0,00345603	KEGG	35
Sudden Infant Death Syndrome (SIDS) Susceptibility Pathways	0,00356595	Wikipathways	159
Nifedipine Activity	0,00377364	Wikipathways	8
Axonal growth inhibition (RHOA activation)	0,00377364	Reactome	10
Glial Cell Differentiation	0,00377364	Wikipathways	8
Nucleotide-like (purinergic) receptors	0,00408213	Reactome	16
Leukocyte transendothelial migration - Homo sapiens (human)	0,00418313	KEGG	116
Surfactant metabolism	0,00449615	Reactome	26
Vitamin A (retinol) metabolism	0,00449615	EHMN	26
Type II interferon signaling (IFNG)	0,00460354	Wikipathways	37
IL2 signaling events mediated by PI3K	0,00460354	PID	37
Pathways Analysis Am80 (Down-regulated DEGs)			
Pathway	p-value	Source	Size
Ectoderm Differentiation	2,27E-06	Wikipathways	142
Axon guidance - Homo sapiens (human)	4,91E-06	KEGG	177
TCF dependent signaling in response to WNT	4,75E-05	Reactome	195
Signaling pathways regulating pluripotency of stem cells - Homo sapiens (human)	6,58E-05	KEGG	142
Pathways in cancer - Homo sapiens (human)	0,000149634	KEGG	397
Signaling by Wnt	0,000219119	Reactome	286
Lidocaine (Local Anaesthetic) Action Pathway	0,000239439	SMPDB	31
Imipramine Action Pathway	0,000324528	SMPDB	33
Calcium Regulation in the Cardiac Cell	0,000452328	Wikipathways	149
Antiarrhythmic Pathway, Pharmacodynamics	0,000514125	PharmGKB	55
Rap1 signaling pathway - Homo sapiens (human)	0,000591359	KEGG	212
Pathway_PA165986194 -need delete	0,000910655	PharmGKB	24
Acetaminophen Pathway, Pharmacokinetics	0,000910655	PharmGKB	24
IL1 and megakaryocytes in obesity	0,000910655	Wikipathways	24
TGF-beta signaling pathway - Homo sapiens (human)	0,000922726	KEGG	84
cxcr4 signaling pathway	0,000927355	BioCarta	11
ABC transporters - Homo sapiens (human)	0,001258766	KEGG	45
Wnt signaling pathway - Homo sapiens (human)	0,001300848	KEGG	143
Formation of the beta-catenin:TCF transactivating complex	0,001677594	Reactome	95
GPCR signaling-G alpha q	0,001798753	INOH	274
Levomethadyl Acetate Action Pathway	0,001893236	SMPDB	29
Levallophan Action Pathway	0,001893236	SMPDB	29
Dimethylthiambutene Action Pathway	0,001893236	SMPDB	29
Ethylmorphine Action Pathway	0,001893236	SMPDB	29
Pentazocine Action Pathway	0,001893236	SMPDB	29
Naltrexone Action Pathway	0,001893236	SMPDB	29
Buprenorphine Action Pathway	0,001893236	SMPDB	29
Alvimopan Action Pathway	0,001893236	SMPDB	29
Naloxone Action Pathway	0,001893236	SMPDB	29
Dihydromorphine Action Pathway	0,001893236	SMPDB	29
Ketobemidone Action Pathway	0,001893236	SMPDB	29
Levorphanol Action Pathway	0,001893236	SMPDB	29
Propoxyphene Action Pathway	0,001893236	SMPDB	29
Tramadol Action Pathway	0,001893236	SMPDB	29
Diphenoxylate Action Pathway	0,001893236	SMPDB	29

Anileridine Action Pathway	0,001893236	SMPDB	29
Oxycodone Action Pathway	0,001893236	SMPDB	29
Alfentanil Action Pathway	0,001893236	SMPDB	29
Oxymorphone Action Pathway	0,001893236	SMPDB	29
Hydrocodone Action Pathway	0,001893236	SMPDB	29
Hydromorphone Action Pathway	0,001893236	SMPDB	29
Sufentanil Action Pathway	0,001893236	SMPDB	29
Remifentanil Action Pathway	0,001893236	SMPDB	29
Fentanyl Action Pathway	0,001893236	SMPDB	29
Carfentanil Action Pathway	0,001893236	SMPDB	29
3-Methylthiofentanyl Action Pathway	0,001893236	SMPDB	29
Methadyl Acetate Action Pathway	0,001893236	SMPDB	29
Dezocine Action Pathway	0,001893236	SMPDB	29
Mepivacaine Action Pathway	0,001893236	SMPDB	29
Chloroprocaine Action Pathway	0,001893236	SMPDB	29
Dibucaine Action Pathway	0,001893236	SMPDB	29
Levobupivacaine Action Pathway	0,001893236	SMPDB	29
Benzocaine Action Pathway	0,001893236	SMPDB	29
Bupivacaine Action Pathway	0,001893236	SMPDB	29
Oxybuprocaine Action Pathway	0,001893236	SMPDB	29
Prilocaine Action Pathway	0,001893236	SMPDB	29
Procaine Action Pathway	0,001893236	SMPDB	29
Proparacaine Action Pathway	0,001893236	SMPDB	29
Ropivacaine Action Pathway	0,001893236	SMPDB	29
Cocaine Action Pathway	0,001893236	SMPDB	29
Escitalopram Action Pathway	0,001893236	SMPDB	29
Lidocaine (Local Anaesthetic) Metabolism Pathway	0,002000035	SMPDB	4
D-Glutamine and D-glutamate metabolism - Homo sapiens (human)	0,002000035	KEGG	4
Nalbuphine Action Pathway	0,002153138	SMPDB	30
Acetaminophen Metabolism Pathway	0,002153138	SMPDB	31
Molecules associated with elastic fibres	0,002153138	Reactome	31
fmlp induced chemokine gene expression in hmc-1 cells	0,002153138	BioCarta	30
Desipramine Action Pathway	0,002153138	SMPDB	30
Hippo signaling pathway - Homo sapiens (human)	0,002178902	KEGG	154
Inflammatory mediator regulation of TRP channels - Homo sapiens (human)	0,002401501	KEGG	99
Ovarian Infertility Genes	0,002436649	Wikipathways	31
Melanogenesis - Homo sapiens (human)	0,002689734	KEGG	101
Heroin Action Pathway	0,002744808	SMPDB	32
Codeine Action Pathway	0,002744808	SMPDB	32
Initiation of transcription and translation elongation at the HIV-1 LTR	0,002744808	Wikipathways	32
Extracellular matrix organization	0,002864082	Reactome	295
Endothelin Pathways	0,003078639	Wikipathways	33
phospholipids as signalling intermediaries	0,003078639	BioCarta	33
Cardiac Hypertrophic Response	0,003156406	Wikipathways	54
Cardiac conduction	0,003309768	Reactome	135
Basal cell carcinoma - Homo sapiens (human)	0,003420634	KEGG	55
Fluoxetine Action Pathway	0,003439152	SMPDB	34
Muscle contraction	0,003439205	Reactome	198
aspirin blocks signaling pathway involved in platelet activation	0,003520608	BioCarta	17
BMP2 signaling TGF-beta MV	0,003700399	INOH	56
Elastic fibre formation	0,003827336	Reactome	36
Nicotine Action Pathway	0,004244161	SMPDB	36
Methadone Action Pathway	0,004244161	SMPDB	36
Citalopram Action Pathway	0,004244161	SMPDB	36
Imipramine Metabolism Pathway	0,004878846	SMPDB	6
Mitochondrial Gene Expression	0,004881931	Wikipathways	19
Pathways Analysis CD2314 (All DEGs)			
Pathway	p-value	Source	Size
Organic cation transport	8,16E-05	Reactome	11
Organic cation/anion/zwitterion transport	0,0004787	Reactome	16
Vitamin A and Carotenoid Metabolism	0,00077795	Wikipathways	43
Wnt Signaling Pathway and Pluripotency	0,00120194	Wikipathways	101
Rap1 signaling pathway - Homo sapiens (human)	0,00124467	KEGG	212
Hematopoietic Stem Cell Differentiation	0,00125657	Wikipathways	47
EV release from cardiac cells and their functional effects	0,00179788	Wikipathways	10
VEGF and VEGFR signaling network	0,00179788	PID	10
Activation of anterior HOX genes in hindbrain development during early embryogenesis	0,00242498	Wikipathways	11
ESC Pluripotency Pathways	0,00295666	Wikipathways	115
Validated transcriptional targets of TAp63 isoforms	0,00312919	PID	57
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0,00312919	KEGG	56
Ectoderm Differentiation	0,00381596	Wikipathways	142
Neurophilin interactions with VEGF and VEGFR	0,00386701	Reactome	4
Dopamine metabolism	0,00404493	Wikipathways	13
Phenylalanine degradation	0,00404493	INOH	13
Visual phototransduction	0,00462354	Reactome	102

Pathway Analysis CD2314 (Up-regulated DEGs)			
Pathway	p-value	Source	Size
Validated transcriptional targets of Tap63 isoforms	0,000787599	PID	57
EV release from cardiac cells and their functional effects	0,000820578	Wikipathways	10
Organic cation transport	0,000820578	Reactome	11
Activation of anterior HOX genes in hindbrain development during early embryogenesis	0,001111914	Wikipathways	11
Vitamin A and Carotenoid Metabolism	0,001495572	Wikipathways	43
Dopamine metabolism	0,001871839	Wikipathways	13
Glucocorticoid Receptor Pathway	0,002530199	Wikipathways	71
Organic cation/anion/zwitterion transport	0,00289237	Reactome	16
GPCRs, Class C Metabotropic glutamate, pheromone	0,00289237	Wikipathways	15
Rap1 signalling	0,003508405	Reactome	16
Estrogen biosynthesis	0,003724207	Reactome	5
protein citrullination	0,003724207	HumanCyc	5
Adipogenesis	0,004405002	Wikipathways	131
Tyrosine metabolism - Homo sapiens (human)	0,004781619	KEGG	35
Regulation of lipolysis in adipocytes - Homo sapiens (human)	0,004825066	KEGG	56
Norepinephrine Neurotransmitter Release Cycle	0,004965799	Reactome	18
Pathway Analysis CD2314 (Down-regulated DEGs)			
Pathway	p-value	Source	Size
Mesodermal Commitment Pathway	0,00034961	Wikipathways	153
ESC Pluripotency Pathways	0,00069803	Wikipathways	115
NrCAM interactions	0,00076793	Reactome	7
Deactivation of the beta-catenin transactivating complex	0,00080489	Reactome	31
VEGF and VEGFR signaling network	0,00162599	PID	10
Hippo signaling pathway - Homo sapiens (human)	0,00248307	KEGG	154
Repression of WNT target genes	0,00323613	Reactome	14
Wnt Signaling Pathway and Pluripotency	0,00343573	Wikipathways	101
BMP2 signaling TAK1	0,00371917	INOH	15
RANKL-RANK (Receptor activator of NFKB (ligand)) Signaling Pathway	0,00465924	Wikipathways	55
Nitrogen metabolism - Homo sapiens (human)	0,00477905	KEGG	17
Regulation of Wnt-B-catenin Signaling by Small Molecule Compounds	0,00477905	Wikipathways	17
Pathway Analysis BMS961 (ALL DEGs)			
Pathway	p-value	Source	Size
Activation of anterior HOX genes in hindbrain development during early embryogenesis	0,000502514	Wikipathways	11
HIF-2-alpha transcription factor network	0,001989394	PID	36
Estrogen biosynthesis	0,002175349	Reactome	5
Validated nuclear estrogen receptor alpha network	0,0028805	PID	65
Adipogenesis	0,003588268	Wikipathways	131
Vitamin A and Carotenoid Metabolism	0,003841865	Wikipathways	43
The canonical retinoid cycle in rods (twilight vision)	0,004153749	Reactome	22
AP-1 transcription factor network	0,004224752	PID	71
RAF-independent MAPK1/3 activation	0,004724588	Reactome	23
Aryl Hydrocarbon Receptor	0,004906231	Wikipathways	46
Pathway Analysis BMS961 (Up-regulated DEGs)			
Pathway	p-value	Source	Size
Activation of anterior HOX genes in hindbrain development during early embryogenesis	0,00033067	Wikipathways	11
HIF-2-alpha transcription factor network	0,00117711	PID	36
Estrogen biosynthesis	0,00164051	Reactome	5
AP-1 transcription factor network	0,00228859	PID	71
Vitamin A and Carotenoid Metabolism	0,00229819	Wikipathways	43
The canonical retinoid cycle in rods (twilight vision)	0,00277835	Reactome	22
Aryl Hydrocarbon Receptor	0,00294861	Wikipathways	46
Spinal Cord Injury	0,00422543	Wikipathways	117
Nifedipine Activity	0,0044768	Wikipathways	8
Glial Cell Differentiation	0,0044768	Wikipathways	8
Pathway Analysis BMS961 (Down-regulated DEGs)			
Pathway	p-value	Source	Size
Mesodermal Commitment Pathway	0,00337508	Wikipathways	153

Chapter 8

General Discussion

The term retinoid is applied to any chemical presenting structural or functional similarity to vitamin A. Despite their “promiscuous” character which allows retinoids to affect an enormous amount of signaling pathways within a cell, retinoids principally exert their genomic or non-genomic actions via their cognate nuclear receptors (NRs), namely the Retinoic Acid Receptor alpha (RAR α), Retinoic Acid Receptor beta (RAR β) and Retinoic Acid Receptor gamma (RAR γ). Resulting from their broad effects on multiple signaling pathways, retinoids are known to exert a plethora of physiological, toxicological and pharmacological effects related to many different areas such as vision, development, or cancer, respectively [1]. In the context of breast cancer, different *in vitro* studies showed several decades ago that retinoids are capable of decreasing proliferation while increasing differentiation and apoptotic rates [1]. As a result, high interest in retinoids as anticancer agents was raised and plenty of research focussed on these chemicals for treatment of breast cancer. However, the effects seen in clinical trials were inconclusive and resistance to the retinoid treatment was often observed [1,2]. In order to understand the causes leading to the sensitivity or resistance to retinoids, their toxicological risk and, in turn, develop effective breast cancer treatment strategies, knowledge on the retinoid-driven molecular mechanisms is required. There are multiple pathways by which retinoids can inhibit breast cancer growth, within which, the classical RAR genomic signaling via heterodimerization with Retinoid X Receptors (RXRs) and the crosstalk with other nuclear receptor signaling (e.g. estrogen receptor, ER) pathways have been highlighted [1]. Despite the obvious need of a complete knowledge of these two modes of action to better understand the variable response to retinoids observed in breast cancer, these processes are still far from being completely characterized.

Previous research has shown that RAR α , RAR β and RAR γ can lead to different and even opposite responses on breast cancer cell proliferation [1,3,4]. Thus, changes in the related molecular pathways, expression or activation status of the different RAR subtypes may lead to pro-proliferative and/or anti-proliferative biological responses [1–5]. In consequence, studies evaluating the specific roles and modes of action of these receptors will bring important insights to better understand the differential

effects of retinoids in NR cell signaling and breast cancer growth. Apart from the distinct effects on breast cancer cell proliferation, directed by the respective RAR subtypes and their ligands, the effectiveness of retinoids has also been shown to highly depend on the presence of ER [6]. In this matter, it has been shown that the majority of ER+ breast cancer cells are often responsive to retinoid-based treatments whilst most ER- cells display no retinoid-mediated inhibition of proliferation [6]. Thus, crosstalk between ER and RAR signaling has been previously reported [7,8], however, the molecular events taking place on this interplay are still not completely understood requiring further research.

Due to the scientific gap in the comprehension of the mechanisms involved in the retinoid-driven modulation of cell proliferation, the aim of the present thesis was to investigate different molecular events elicited after retinoid treatment, in order to better understand the observed effects on breast cancer growth (see **Chapter 1**). To do so, multiple biochemical and cell-based *in vitro* studies assessing effects of general and subtype-selective RAR ligands in the absence or presence of 17 β -estradiol (E₂) were carried out. Thus, all along the present thesis the effects of general and subtype-selective RAR agonists on the proliferation of the ER+ MCF7/BUS and T47D cell lines were evaluated. In addition, the specific effects in breast cancer cell signaling, including up-stream mechanisms of action such as possible differential effects on the classical RAR genomic pathway (**Chapters 2, 3 and 7**) and the potential interference between retinoid and other NR signaling pathways such as the ER pathway were thoroughly evaluated (**Chapters 4, 5, 6 and 7**).

General overview of the principal results of this thesis

All along the present thesis, proliferation studies evaluating the effects of general and selective retinoids on breast cancer cell proliferation in the absence and presence of E₂ were carried out. The results clearly showed in two ER+ breast cancer cell lines (MCF7/BUS and T47D) that general and RAR α -selective ligands have a high anti-proliferative impact on basal breast cancer growth, while

RAR β - and RAR γ -agonists display much lower efficiency as anticancer agents. Moreover, in the presence of E₂ the general RAR agonist *all-trans* retinoic acid (AtRA) showed to antagonize the proliferative effects of E₂ in the breast cancer cell models studied. To evaluate possible molecular mechanisms underlying the distinct proliferative responses to these retinoids, effects on the classical RAR genomic signaling (gene expression and coregulator binding) but also the retinoid-driven interference with other signaling pathways (e.g. ER) were investigated.

Concerning RAR genomic signaling this thesis mainly focused on the characterization of RAR-related gene expression and the comparison of the coregulator binding signatures of the three RARs (α , β and γ) subtypes with the hypothesis that the distinct observed RAR-subtype-mediated gene expression and concomitant effects on proliferation could be partially driven by differential interactions of coregulators with the respective RARs. As preliminary study, **chapter 2** first confirmed the suitability of the use of ligand binding domains (LBDs) of RARs for this kind of research since no remarkable differences were found between the full length RAR α and its isolated LBD. Next, **chapter 3** compared coregulator binding profiles of all RAR-subtypes (LBDs) upon incubation with distinct general and selective ligands. The results of **chapter 3** showed that, despite a generally high similarity, several coactivator and corepressor motifs selectively associated with a specific RAR subtype. These results are deemed as a striking starting point and they merit further confirmation in a more physiological scenario. Therefore, studies testing these interactions within a cellular context (e.g. ChIP studies on breast cancer cells) should be carried out in the near future to bring further insights in this matter.

Regarding the repercussions on other NR pathways regulating breast cancer growth, this thesis also investigated the effects of retinoids on ER-related molecular events promoting cell proliferation. **Chapter 4** acquired knowledge on the possible AtRA-mediated interference on different basal and E₂-induced ER α -related molecular events using the ER⁺ breast cancer cell line MCF7/BUS and the U2OS-ER α -Luc reporter gene cell line. The results of **chapter 4** showed that while AtRA decreased E₂-induced ER-mediated transcription and proliferation, this compound did not significantly affect E₂-

driven ER α coregulator binding and subcellular localization. Thus, this study does not support the possibility of these specific molecular mechanisms as underlying causes of the inhibitory effects of AtRA on the E₂-induced ER-mediated transcription. Strikingly, despite the high inhibition of proliferation elicited by AtRA alone, this chemical increased the basal ER α -driven gene expression, the coactivator binding and the nuclear accumulation of ER α by processes different from the direct binding as cognate ligand to ER α . **Chapter 5** assessed the effects on the same ER α -related events and cell lines of the RAR subtype-selective agonists Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ) to get insights on the molecular players possibly driving this ATRA-induced phenomenon and with a new hypothesis stating that the divergent effects observed in the basal breast cancer cell proliferation upon treatment with distinct RAR-subtype-selective agonists may be partially explained by differential effects on ER signaling. Although none of these retinoids showed to be a cognate ligand of ER α , these chemicals elicited other multiple but variable (compound-, endpoint- and cell-dependent) effects on ER α signaling. Thus, the results of **chapter 5** did not evidence the involvement of a specific RAR subtype but rather pointed at other AtRA-related pathways or players as main drivers of the AtRA-mediated effects on the basal ER α signaling. Altogether, **chapters 4 and 5** revealed several unknown effects of retinoids on ER α signaling, however, the specific molecular players and mechanisms of action possibly leading to these newly found retinoid-driven changes on ER α -related events still need to be further investigated.

In **chapters 6 and 7**, to acquire further insights in the interference of retinoid treatment with ER and other signaling pathways playing a role in breast cancer growth, we performed wide studies on gene expression (RNA-seq) on the ER⁺ breast cancer cell line T47D. **Chapter 6**, focused on the effects of the general agonist AtRA on the E₂-induced transcriptome showing that despite the inhibitory effects of this retinoid on the E₂-driven cell proliferation, AtRA triggered both counteracting and additive effects on the gene expression patterns generated by E₂. However, deeper analysis showed more frequent inhibitory/counteracting effects by AtRA on the E₂-induced responses of genes known to

regulate breast cancer growth. In addition, **Chapter 6** indicated that AtRA alone can affect multiple NR pathways such as ER or VDR pathways which play vital roles in breast cancer growth. This finding, led us to hypothesize that differential effects on many signaling pathways (e.g. NR pathways) may explain the differences observed upon exposure to general and selective RAR ligands. Thus, **chapter 7** assessed and compared the effects of these retinoids on the proliferation and transcriptomic signatures of T47D cells. Interestingly, while the general agonist AtRA and the RAR α -selective agonist highly inhibited cell proliferation, the RAR β -agonist CD2314 and the RAR γ -agonist BMS961 showed to be ineffective anticancer agents which even exerted slight pro-proliferative effects. Next, **chapter 7** showed several differences on the gene expression profiles generated by the effective (AtRA and Am80) and ineffective (CD2314 and BMS961) anticancer treatments. In fact, apart from the observed differential retinoid-driven effects on RAR signaling, other NR pathways such as the ER, VDR or GR pathways were distinctly affected when comparing effective and ineffective retinoid treatments. Thus, **chapter 7** suggested that small differences on many other pathways involved in breast cancer growth may add up to eventually dictate the final effectiveness of a specific retinoid-based breast cancer therapy. Thus, these findings merit further research on the molecular mechanisms by which retinoids can regulate these pathways.

In the next sections of this chapter, we will discuss in depth the results obtained all along the present thesis, their implications and the recommended studies for the future. To do so, the outcomes generated by the present thesis are organized by the different endpoints tested. Thus, a detailed analysis of the effects of retinoids on 1) cell proliferation, 2) basal ER signaling, 3) E₂-induced ER signaling and 4) other pathways possibly affecting the proliferation of the breast cancer cell lines MCF7/BUS and T47D is provided. Furthermore, an additional reflection generated by this thesis related to the need of a multi-endpoint approach for the assessment of the potential modulation of NR signaling by chemicals such as AtRA is included. Finally, at the end of this chapter, the principal conclusions drawn upon the completion of the present thesis are briefly highlighted.

Proliferation studies in ER+ breast cancer cell lines

In the whole thesis, looking for a link between the effects at the molecular level of the different test compounds (estrogens and retinoids) and their repercussions on breast cancer growth, proliferation studies in the ER+ breast cancer cell models MCF7/BUS and T47D were performed. Estrogens like estradiol (E₂) and retinoids such as *all-trans* retinoic acid (AtRA) are known to modulate proliferation and progression of ER+ breast cancer cells [1,6,9]. Most of the studies evaluating effects of E₂ and retinoids on breast cancer growth and the related molecular events have used the ER+ wild type MCF7 cell line as an E₂-dependent breast cancer cell model [7,8]. However, MCF7 itself is a highly heterogeneous polyclonal cell line and plenty of research has described distinct levels of estrogen and anti-estrogen sensitivity/resistance when comparing different MCF7 clones [10–12]. In addition, research on the molecular events leading to the retinoid effects (e.g. gene expression) in different ER+ breast cancer cell lines such as T47D is limited. Thus, within the present thesis, both estrogen- and retinoid-mediated effects on the proliferation of the highly E₂-responsive MCF7/BUS subline [13] (**chapter 4 and 5**) and the wild type T47D (**chapter 6 and 7**) were investigated. For this purpose, the ER-agonist E₂, the general RAR agonist AtRA and the subtype selective Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ) were utilized.

The results of the present thesis firstly showed that E₂ exerted significant pro-proliferative effects in both MCF7/BUS and T47D breast cancer cell lines (**chapters 4 and 6**), corroborating the responsiveness of these ER+ breast cancer cell lines to E₂ (positive control) and therefore, the suitability of these cell models [13,14]. Secondly, the general agonist AtRA showed to decrease both basal and E₂-induced proliferation of MCF7/BUS (**chapter 4**) and T47D (**chapter 6**) cells as previously reported for other ER+ breast cancer cell lines [6,8,15,16]. Finally, subtype-selective ligands showed cell-, concentration- and time-dependent differential effects. The results of this thesis indicated that while the RAR α agonist Am80 remarkably inhibited proliferation of the two breast cancer cell lines studied even at low nanomolar concentrations, RAR β (CD2314) and RAR γ (BMS961)

agonists showed lower potential as anti-proliferative agents. When comparing both cell lines, a discrepancy could be observed between the results obtained after treatment with CD2314 and BMS961 (**chapters 5 and 7**). Although these differences were yet small, the weak anti-proliferative/pro-proliferative effects of these compounds in MCF7/BUS and T47D cells, respectively, may suggest possible cell-dependent differential effects that can affect the final response to retinoids in breast cancer therapy. Supporting this concept, previous studies have investigated these cellular differences at the translational level using proteomics analysis as a readout and it was demonstrated that the proteomes of MCF7 and other ER+ breast cancer cell lines such as T47D highly differ showing different expression patterns in proteins known to play fundamental roles in cancer growth and progression [17]. Highlighting these differences between proliferative responses of distinct breast cancer cell lines, our study additionally showed that MCF7/BUS displayed a rather bi-phasic pattern when analyzing different time points. In fact, while long-term exposure to retinoids at micromolar concentrations inhibited MCF7/BUS proliferation to a higher or lower extent, some of them slightly favoured pro-proliferative behaviour at the initial stage post-treatment. Altogether, it suggests that extrapolating results from a specific ER+ breast cancer cell line should be done cautiously.

In summary, apart from the few cell-dependent differences, the proliferation results obtained in the present thesis mainly pointed at general RAR agonists and RAR α -selective agonists as strong inhibitors of proliferation while reporting RAR β - and RAR γ -selective ligands as more ineffective breast cancer treatment agents. Aiming at a better understanding of the molecular events driving these differential effects of RAR α , RAR β and RAR γ signaling, the present thesis evaluated possible modes of action related to both the RAR genomic pathway and the interplay with other NR pathways (e.g. ER) which will be addressed in subsequent sections.

Novel insights in the Classical RAR-RXR Genomic Pathway. Assessment of RAR subtype-specific gene expression and a possible underlying role of RAR-coregulator interactions.

One of the main molecular mechanisms by which retinoids can modulate breast cancer cell proliferation is the classical RAR-RXR Genomic Signaling [1]. By this pathway, RARs (α , β and γ) form heterodimers with RXRs (α , β and γ) and bind to specific regions in the DNA named retinoic acid responsive elements (RAREs) and to coregulators, thereby regulating transcription of RAR-mediated genes known to modulate breast cancer growth [18–20]. Based on the hypothesis stating that differential RAR-subtype-mediated gene expression and coregulator binding could partially drive the distinct observed effects on breast cancer growth exerted by retinoids, this thesis evaluated and compared the effects of general and subtype-selective RAR agonists on both coregulatory binding (**Chapters 2, 3**) and gene expression (**Chapter 7**). In **Chapter 7** this work investigated potential RAR subtype-specific regulation of gene expression in breast cancer cells (T47D) upon exposure to the different retinoids. Although relatively similar transcriptomic patterns occurred for all compounds tested, differences in the magnitudes of response between effective (anti-proliferative) and ineffective (weakly pro-proliferative) retinoids were observed. Thus, known direct RAR-target genes hampering proliferation such as *RAR β* [6] or *BTG2* [20] showed to be highly up-regulated upon exposure to the general RAR agonist AtRA and the *RAR α* -selective Am80 while remarkably weaker responses were found for the *RAR β* - (CD2314) and *RAR γ* -selective (BMS961) agonists. It has to be noted that the selectivity of the different retinoids should not be confounded with specificity. This implies that, all selective retinoids at the high concentrations tested are able to residually activate the other RAR subtypes. In addition, lower expression levels of a certain RAR (e.g. *RAR β*) will also influence the final outcome. In any case though, the results shown in our proliferation and gene expression studies point at *RAR α* as a principal driver of the anti-proliferative and RAR-related genomic response to retinoids.

The present thesis revealed multiple RAR-mediated genes differentially modulated by the different RAR subtypes. However, chromatin immunoprecipitation studies have reported that the distinct RAR subtypes can be found at remarkably coincident regions in the genome suggesting functional redundancy [1,7]. Among the possible underlying mechanisms that could explain both the similar localization of the RARs along the genome and the differential effects on gene expression observed, the present thesis hypothesized that upstream in the process, potential subtype-specific coregulator interactions could underlie the distinct subtype-driven gene expression and proliferative behaviour (**chapters 2 and 3**). Binding of RARs to coregulators (apo-form-bound corepressors and ligand-dependent coactivators or corepressors) is a key intermediate regulatory step in RAR-RARE mediated gene expression since it regulates chromatin structure and accessibility for transcription leading to activation or blocking of gene expression [18]. Thus, depending on the specific interactions with the RAR subtypes and the cellular levels of these different types of coregulators, genomic RAR signaling and the corresponding biological response to retinoids may differ [1,21,22]. To study NR-coregulator interactions, most research has only used the functional part of interest of NRs, namely their Ligand Binding Domains (LBDs). This is due to the fact that RARs, as all NRs, are proteins with three principal domains viz, the AF-1 domain, the DNA Binding Domain (DBD) and the Ligand Binding Domain (LBD) which can exert their functions independently (in isolation) [23]. However, inter-domain communication within the full length receptor has been described to play a role in this process [24–31] and the use of a full length receptor or its isolated LBD may influence the final response [32]. In consequence, prior to studying differences between the commercially available RAR-subtype LBDs (**chapter 3**), comparison of the coregulator binding profiles of a full length RAR and its isolated LBD was required (**chapter 2**). For the studies assessing RAR-coregulator interactions as a possible novel dimension underlying the differential subtype-driven gene expression and proliferation patterns, this thesis used the MARCoNI Coregulator Binding assay, which allows the testing of RAR interactions with 154 motifs belonging to 65 coregulatory proteins.

As preliminary step, **chapter 2** evaluated, using functional RAR α full length (FL) and its truncated ligand binding domain (LBD) whether their coregulator binding signatures upon agonistic (AtRA) and antagonistic (Ro415253) actions were comparable. The results of this study showed high concordance between both RAR variants in both qualitative terms (type of coregulators binding to RAR α) as well as quantitative parameters such as EC50s and efficacy (modulation indexes at saturating concentrations of the ligands). In addition, the coregulator binding assay clearly discriminated the agonist AtRA from the antagonist Ro415253 both when tested with the RAR α -FL and the RAR α -LBD. Thus, agonist binding to RAR α (FL and LBD) decreased interactions with apo-form-bound corepressors while increasing binding of known and unknown ligand-dependent coregulators [18,33]. On the contrary, Ro415253 acted as a neutral antagonist decreasing all coregulator interactions with RAR α and generating the so-called “naked” receptor [18,33]. In summary, chapter 2 showed that the microarray coregulator binding assay used can accurately reflect RAR-coregulator interactions and that the more easily available RAR-LBDs can be used as starting point to predict the behaviour of their full length counterparts especially as far as direct (ant)agonist activities are concerned.

Once shown that LBDs are a useful mean to get insights in RAR-coregulator binding, a study characterizing and comparing the coregulator binding profiles of the LBDs belonging to the distinct RAR subtypes was performed (**chapter 3**). To do so, general and subtype-selective ligands were utilized. This work showed the capability of the coregulatory binding assay to discriminate between subtype-selective ligands and between agonistic/antagonistic actions of retinoids for all RARs as evidenced by the respective EC50s and modulation indexes. Next, **chapter 3** further revealed a large number of motifs belonging to coregulators never described before as RAR interactors. Next, the previously suggested high ligand-independent activity of RAR β [34] was confirmed as shown by its higher basal coregulator binding and lower response to agonist actions. Strikingly, despite the high similarity between the AtRA-induced coregulator binding profiles presented by the different RAR subtypes, several coregulator motifs showing selectivity for a certain RAR variant (especially for

RAR α) were found. This finding requires further attention since the specific binding of the different RAR subtypes to ligand-induced coactivators or ligand-induced corepressors could activate or block expression of RAR-mediated genes potentially modulating the final response to retinoids in terms of breast cancer growth [21,22]. In consequence, future studies should investigate the role played by the newly found RAR coregulators (**chapter 3**) by testing their cellular levels and especially their actual physiological presence and actions in the promoter regions of the anti-proliferative genes found to be differentially modulated by the distinct RAR-subtypes.

Crosstalk of retinoids with basal (hormone-independent) ER signaling

Apart from their classical genomic RAR-RARE signaling, the different RAR subtypes have been shown to exert multiple non-genomic actions on other pathways [8,35,36]. For instance, previous studies have shown that apo-RAR α can regulate the activity of apo-ER α signaling affecting the basal expression of ER-mediated genes involved in the maintenance of breast cancer growth [36]. Nevertheless, similar effects upon general and subtype-selective retinoid treatment have not been described so far. Within the present thesis it was hypothesized that the distinct breast cancer cell response to general and selective retinoids may partially occur due to differential effects on the basal pro-proliferative ER α signaling. To test such a hypothesis, effects of the different retinoids on basal ER-related molecular events such as gene transcription, coregulator binding or cytoplasmic/nuclear localization were studied (**chapters 4-7**). Regarding gene expression, both evaluation of the whole transcriptome (in T47D cells) and of specific genes (in both T47D and MCF7/BUS cells) were carried out. In addition, complementary studies investigating the retinoid-mediated effects on ER α - and ER β -mediated transcription were performed by using the reporter gene (luciferase-based) cell lines T47D-ER α -Luc, U2OS-ER α -Luc and U2OS-ER β -Luc. **Chapter 6 and 7** showed that the lists of Differentially Expressed Genes (DEGs) in the wild type T47D cell line indicated effects of retinoids on ER-related signaling as one of the highly over-represented pathways. Thus, multiple estrogen-responsive genes known to modulate breast cancer cell proliferation such as CXCL12 [37,38] were

affected upon retinoid treatment. Further analysis of the RNA-seq, RT-qPCR and reporter gene data (**chapters 5 and 7**) showed differential responses depending on the cell line, the compound and the concentration tested. MCF7/BUS cells (**chapter 5**) showed up-regulation of specific ER-target genes such as the pro-proliferative TFF1 [39] upon exposure to retinoids, which was more pronounced for the more ineffective anticancer agents CD2314 and BMS961. In line with this pattern, the reporter gene studies also suggested the potential of retinoids to increase ER α -mediated transcription. However, comparison of the different reporter gene cell lines, namely, T47D-ER α -Luc and U2OS-ER α -Luc, showed some discrepancies. Thus, while CD2314 and to a lower extent AtRA and Am80 showed to increase the activity of ER α -mediated gene transcription in both systems, BMS961 only up-regulated the expression of luciferase in the breast cancer reporter gene cell line T47D-ER α -Luc. This difference in behaviour between reporter cell lines together with the different regulation of other ER-mediated genes such as PKIB in MCF7BUS cells where only CD2314 showed to elicit significant up-regulation, pointed at gene- (promoter-) and cell-specific gene expression modulation. Indeed, factors such as different cellular levels of the RARs or other transcription factors and their known capacity to differently bind to specific ERE sequences may also help to explain the results obtained [8,40,41]. Supporting the idea of gene-specific effects on ER α -driven transcription, the overview provided by the RNA-seq study in T47D (**chapter 7**) showed a rather variable regulation of ER-mediated gene expression by the different retinoids with no clear ligand-related trend to be concluded. Thus, while the expression of some pro-proliferative ER target genes like CXCL12 [37,38] was more down-regulated by the anti-proliferative AtRA and Am80, other retinoid-induced ER-mediated genes also crucial in breast cancer cell proliferation such as GREB1, showed no significant differences among the different retinoid treatments.

After confirming the retinoid-mediated effects of retinoids on the basal ER α -related transcription, the present thesis tested several molecular events possibly leading to such modulation such as the effects on the expression of the ER α gene itself, ER α coregulator binding and subcellular localization. First,

it was evaluated whether the retinoid-driven effects on ER-mediated signaling may be partially driven by the mere up-regulation of the expression of *ERα* in both MCF7/BUS cells (**chapters 4-5**) and T47D cells (**chapters 6**). However, none of the retinoids was able of significantly affecting the transcript levels of *ERα* rejecting this hypothesis as underlying cause. Next, the effects of retinoids on the coregulator binding patterns using both purified *ERα*-LBDs and full length *ERα* contained in lysates of MCF7/BUS cells were assessed (**chapters 4-5**). Based on the results obtained with the isolated receptor (*ERα*-LBD), it was concluded that none of the retinoids can act as cognate ligands of *ERα*. However, when using cell lysates, full length *ERα* coactivator binding was increased upon exposure to AtRA suggesting a possible activation in the context of a whole *ERα* transcriptional complex. In addition, **chapters 4 and 5** found that AtRA could increase nuclear localization of *ERα* while decreasing its cytoplasmic levels. Altogether, our coregulator binding assays and our studies on *ERα* subcellular localization suggest that *ERα* interactions with proteins whose coregulator binding patterns and nuclear translocation is modulated by AtRA (e.g. CRABP II) [34,42,43] could play a principal role in these phenomena and research focussed on these points must be performed.

Once shown that retinoids can exert crucial actions on the basal functioning of *ERα*, the effects of these chemicals on the other type of ER, namely *ERβ*, were tested. *ERβ* unlike *ERα*, has been reported to elicit anti-proliferative effects in breast cancer cells [44]. Thus, the effects of retinoids on *ERβ* activity as another potential mechanism of crosstalk with ERs were assessed. To do so, the reporter cell line U2OS-*ERβ*-Luc known to possess only the *ERβ* subtype [41] was used (**chapters 6-7**). Strikingly, the results showed that, unlike for *ERα*, AtRA was the only retinoid able to increase basal *ERβ*-mediated transcription. Previous evidence of crosstalk between *ERβ* and RARs has been described where retroviral transduction of *ERβ* in ER- breast cancer cells increased RAR-related signaling upon AtRA treatment [45]. In consequence, despite the rather low levels of *ERβ* usually present in ER⁺ breast cancer cells [44], one could speculate that activation of the *ERβ*-mediated gene

expression may be one among many other modes of action by which AtRA leads to the observed high inhibition of breast cancer growth.

Altogether, this thesis demonstrated the potential of retinoids to affect the E₂-independent activity of ER α and ER β at different molecular levels and in a compound-, concentration, time- and cell-specific manner. The molecular players and events driving the observed modulation patterns on the basal ER signaling pathway and their physiological relevance in the final sensitivity/resistance to retinoids remain to be further identified in future research.

Crosstalk of retinoid with E₂-induced ER signaling

Once it was shown how retinoids can affect the activity of ERs in the absence of any estrogenic stimulation (**chapters 5 and 7**), the present thesis additionally evaluated the modulation by these chemicals on the functioning and effects of activated/liganded ER α (**chapters 4 and 6**). In accordance with previous literature [46], the proliferation studies of this thesis confirmed in the cell models used, the potential of retinoids to counteract the E₂-induced breast cancer cell proliferation [46]. To better comprehend the mechanisms of action involved in such retinoid-mediated antagonism, the present thesis evaluated different molecular endpoints related to the estrogen-activated ER α signaling and the potential effects of co-exposure with the general retinoid AtRA on them. Thus, multiple endpoints such as ER-driven gene expression, ER α coregulator binding and ER α subcellular levels and localization were assessed using the breast cancer cell lines MCF7/BUS and T47D in combination with the reporter cell lines T47D-ER α -Luc, U2OS-ER α -Luc and U2OS-ER β -Luc.

In order to evaluate ER α -mediated gene transcription, the present thesis made use of different methods including reporter gene assays, RT-qPCR and RNA-seq. To investigate the effects of a certain chemical on E₂-induced specific ER α and ER β genomic signaling, reporter gene (luciferase) studies represent a simple and suitable methodology to acquire an initial overview. The results of our study showed that AtRA to a higher or lower extent can decrease both E₂-induced ER α - and ER β -mediated

luciferase expression. In accordance with our data, previous reporter gene studies using different reporter gene cell lines have shown the potential inhibition exerted by retinoids on E₂-induced ER-mediated transcription [47]. Apart from the previously reported competition between ER and RAR for close/overlapping DNA binding regions [7], the AtRA-mediated effects found in the reporter gene studies may be driven by different mechanisms of action such as ligand-receptor binding (AtRA-ER) or effects on other molecular pathways or processes related to ER signaling (e.g. coregulator binding or subcellular levels). This work showed no direct AtRA-mediated antagonistic actions by means of assays comparing ER α coregulator binding profiles upon exposure to E₂ alone and in combination with AtRA, where AtRA did not modify the E₂ induced coregulatory binding patterns. In consequence, the idea of a competition between E₂ and AtRA for direct binding to the ligand binding pocket (LBP) of the LBD seems therefore not so likely. Unliganded RARs have also been reported to be capable of directly interacting with ERs and be present in active ER transcriptional complexes playing a crucial role in the E₂-induced ER-mediated transcription [8]. In this aspect, the coregulator binding studies using cell lysates did not show any effect of liganded RARs on the E₂-induced coactivator interactions of ER α upon co-incubation with AtRA. Altogether, the coregulator binding studies support that the possibilities of AtRA as a cognate ligand (antagonist/partial agonist) or liganded RAR as suppressor of the E₂-induced ER α coactivator binding via protein-protein interactions are not likely to occur. Next, it was evaluated whether AtRA may affect the E₂-induced nuclear translocation to or retention in the nucleus of ER α . However, results showed again no significant changes on the E₂-driven nuclear translocation and/or accumulation of ER α . When looking at individual genes in the MCF7/BUS RT-qPCR studies (**chapter 4**), it was observed that the E₂-driven up-regulation of ER target genes such as *TFF1* was hampered upon co-treatment with AtRA in line with studies assessing effects of retinoids on expression of E₂-induced ER target genes in other breast cancer cell lines [7,8]. However, our genome-wide transcriptomic study in T47D cells (**Chapter 6**), showed variable AtRA-driven modulation of many of the estrogen-responsive genes. Thus, co-exposure with AtRA exerted both

inhibitory/counteracting and additive/enhancing effects on the initial E₂-induced responses in T47D. These results led us to state that the interplay between estrogen and retinoid signaling can act in different directions in a gene-dependent manner. In agreement with this conclusion, other transcriptomic studies in the wild type MCF7 cell line also reported retinoid-driven gene-dependent modulation on the expression of estrogen responsive genes [7,8]. However, a closer look to the transcriptomic data (**chapter 6**) showed that co-treatment with AtRA more often generated inhibiting/counteracting effects on the E₂-driven responses of genes known to affect breast cancer growth.

In summary, retinoids such as AtRA showed to affect the final transcriptomic response to E₂ in different ways depending on the gene considered. However, this modulation seems not to be exerted by direct (ligand-receptor) binding, direct protein-protein interactions between the trinomial ER-RAR-coregulators or a change of the E₂-induced subcellular localization of ER α (as shown in this thesis). However, future studies should further study these and other endpoints at the gene (promoter) level to completely unravel the molecular processes leading to the observed effects of AtRA on the E₂-induced ER-mediated gene expression and proliferation.

Interplay of retinoid signaling with other pathways

The results obtained all along the present thesis suggested that the retinoid-driven modulation of breast cancer growth is partially mediated by many different signaling pathways. Thus, apart from the classical RAR genomic pathway and the extensive crosstalk with ER signaling, interplay with other molecular pathways was observed or deduced from the outcomes generated in the **chapters 6 and 7** with the T47D breast cancer cell line.

A general conclusion drawn in this thesis, is that, despite the fact that retinoids can exert remarkable effects on ER α signaling (e.g. AtRA), these responses seem not to be provoked by direct retinoid binding to ER α . Thus, other factors playing a role in the ER α -related proliferation, transcription,

coregulator binding and subcellular localization must be taken into account as well. In fact, retinoids have been reported to affect many pathways which, in turn, can influence, in one way or another, the ER-related pro-proliferative effects in breast cancer cells such as kinase or AP-1 pathways [2,48–50]. Thus, a potential retinoid-induced activation of ER α via kinase-mediated phosphorylation of the receptor may explain the observed effects on ER-mediated gene expression, and nuclear localization of ER α [49,50]. In addition,, specific effects on other transcription factors involved in breast cancer growth and known to relate with ER α signaling such as AP-1 could also explain the obtained responses [49,51]. Altogether, despite no clear and direct evidence of modulation of ER α via indirect signaling was provided in this work, one could speculate that these molecular players (AP-1 or kinases) may be involved in the observed ER α -related phenomena so that future research should assess their possible roles in the retinoid-driven effects on ER α signaling.

Regarding retinoid crosstalk with other NR pathways of importance in breast cancer growth, interference with the anti-proliferative VDR or the dual (pro-/anti-proliferative) GR was highlighted by the retinoid-driven changes in the transcriptome of T47D cells (**Chapter 7**). In fact, multiple genes related to these NR pathways and known to modulate breast cancer cell proliferation, differentiation and apoptosis, were differentially expressed upon exposure to the respective retinoids. In addition, this modulation was often significantly different when comparing cancer treatments with effective (AtRA and Am80) and ineffective (CD2314 and BMS961) retinoids. Thus, despite the fact that crosstalk between Retinoid/RAR signaling and the VDR and the GR pathway has been previously described at certain molecular levels [52–54], to the best of our knowledge, this is the first work that suggests that the different responses to retinoid-based breast cancer therapies could be partially driven by parallel effects on these NR pathways. However, the gene expression studies included in the present thesis must be accompanied by further research to get more detailed evidence on this indication. Thus, new studies should be carried out to assess all possible actions of retinoids on the VDR and the GR pathways such as the assessment of direct ligand-receptor binding and activation, chromatin

immunoprecipitation (DNA binding) studies or the evaluation of the effects of knocking out such receptors on the retinoid-mediated response in breast cancer cell proliferation, among others.

Revealing the need for a battery of assays (multi-endpoint approach) to fully assess chemical-driven modulation of NR activity. The representative example of AtRA.

The results obtained all along this thesis clearly showed the limitations of studying single endpoints when investigating the potential of a compound to affect NR signaling. Chemicals affecting multiple signaling pathways could modulate the activity or events linked to a specific NR via mechanisms different to the classical ligand-receptor binding. This fact supports the need, in our view, to always include a battery of different assays for a complete evaluation of all NR-related endpoints to better understand cell signaling from the ligand exposure to the transcriptional and translational events. In fact, our work showed that the natural retinoid AtRA, without being a cognate ligand, was able to differently affect the ER α -mediated gene expression, the ER α coregulator binding and the ER α subcellular localization. Consequently, as evidenced by the AtRA illustrative example, the present thesis supported the notion that the testing strategies evaluating the potential of a chemical to modulate NR signaling should be based on a complete battery of methods assessing different endpoints which should include: biochemical ligand binding assays, reporter gene assays, coregulator binding assays, NR phosphorylation studies, gene expression studies (RT-qPCR and RNA-seq) and assessments on subcellular (cytoplasm/nucleus) and DNA localization (e.g. ChIP assays), among others.

Final conclusions and highlights of the present thesis

This thesis investigated molecular events possibly elicited after treatment with retinoids to better understand their distinct effects on breast cancer growth. Thus, this work assessed different molecular mechanisms which may be involved in the differential effects of retinoids on breast cancer growth with emphasis on the classical RAR-RARE Genomic Pathway and the interference of retinoid (RAR) signaling with other NR pathways such as the pro-proliferative ER α pathway. To do so, biochemical

and cell-based studies assessing effects of general and subtype-selective RAR ligands on NR-related endpoints such as proliferation, gene expression, coregulator binding or subcellular localization, were performed. Altogether, the outcomes of this thesis can be summarized in a few take-home messages. Firstly, the general and selective retinoids tested exerted differential effects on the proliferation and RAR-mediated gene expression of ER+ breast cancer cells. These distinct effects were accompanied by differential subtype-specific coregulator binding which may play a role in this process. For all these molecular processes, the results do support a main role of RAR α pointing at this subtype for follow-up studies to further understand the distinct retinoid-mediated effects on the classical RAR genomic signaling and related effects on ER+ breast cancer growth. Secondly, the results of this work highlighted the “promiscuous” character of retinoids which affected many signaling pathways (RAR, ER, VDR, GR...). This multifaceted character makes chemicals such as retinoids be able to affect the functioning of NRs (e.g. ER) by mechanisms other than only the classical ligand-receptor binding, as seen in this thesis. In consequence, retinoids represent an example where a battery of assays should be applied to completely characterize all effects on a specific NR pathway. Finally, the distinct effects on breast cancer cell proliferation of the general and RAR subtype-selective agonists seem not to be based on a single factor but on many differential molecular events like the different RAR subtype coregulator binding and transcription or the distinct effects of retinoids on other signaling pathways (e.g. other NR pathways) of importance in breast cancer growth.

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Chapter 9

Summary

In **chapter 1** of this thesis, background information on the molecular actions and effects elicited by retinoids in the context of nuclear receptor (NR) signaling and breast cancer treatment was given. In addition, the state of the art and the knowledge gap still remaining in this area was highlighted. The principal objective of the thesis was defined which was to investigate different molecular events possibly elicited after retinoid treatment to better understand the observed effects on breast cancer cell proliferation. Finally, the general outline of the present thesis was provided.

Chapter 2 assessed whether the ligand binding domain (LBD) of the Retinoic Acid Receptor (RAR) can be used to study RAR-coregulator binding events as an alternative to the use of the full length (FL) RAR. Thus, this work aimed to characterize and compare ligand-driven coregulator interactions of both the RAR α -LBD and the RAR α -FL. We studied the coregulator motif interactions of RAR α -FL and RAR α -LBD upon incubation with the RAR agonist *all-trans* retinoic acid (AtRA) and the RAR antagonist Ro415253 using the Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI). The results obtained showed that the coregulator binding profiles are highly similar for the RAR α -FL and the RAR α -LBD regarding both qualitative aspects like the type of coregulators that bind or dissociate upon (ant)agonist action and quantitative parameters such as EC50s and the maximum responses (modulation indexes at saturating ligand concentration). Moreover, based on the coregulator binding signatures, the RAR agonist AtRA could be clearly discriminated from the RAR antagonist Ro415253 using both the RAR α -FL and the RAR α -LBD.

Once established that coregulator binding patterns could be adequately characterised using LBDs, **Chapter 3** assessed the coregulator binding profiles of the LBDs belonging to the RAR alpha (RAR α), beta (RAR β) and gamma (RAR γ) focusing on possible differential outcomes between the different RARs. To this end, this study used the MARCoNI coregulator binding assay to characterize coregulator motif binding patterns of the distinct RAR subtypes upon incubation with the general agonist *all-trans*-Retinoic Acid (AtRA); the subtype-selective agonists Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ); and the antagonist Ro415253. **Chapter 3** showed multiple ligand-

dependent RAR-coregulator interactions for all RARs, including binding events not described before. It also confirmed a greater ligand-independent functioning of RAR β based on both higher basal and lower ligand-induced coregulator binding. In addition, despite the generally high similarity, various coregulator motifs showed subtype-selective binding, providing a basis for a potential role of coregulator binding in the receptor subtype-specific responses. Finally, **Chapter 3** also proved that subtype-selective agonists and agonistic/antagonistic actions of ligands for all the RAR variants can be easily discriminated by the MARCoNI coregulator binding assay.

After characterizing a possible role of coregulator binding in RAR subtype-specific responses, in **chapter 4** and **chapter 5** of this thesis, the crosstalk between retinoid and estrogen signaling in the estrogen receptor positive (ER+) breast cancer MCF7/BUS cell line was investigated. To this end, in **chapter 4**, the effects of AtRA on the basal and E₂-induced estrogen receptor alpha (ER α) signaling were characterised using the MCF7/BUS and the U2OS-ER α -Luc cell lines. The effects of AtRA on different ER α -related events such as ER α -mediated cell proliferation and gene expression, ER α -coregulator binding and ER α subcellular localization were evaluated. While AtRA-driven inhibition of E₂-induced signalling was found in the proliferation and gene expression studies, no significant effects on the E₂-directed coregulator binding and subcellular distribution of ER α were observed. Strikingly, **chapter 4** showed that in the absence of E₂, basal ER α -mediated gene expression, ER α -coregulator binding (in lysate samples) and ER α nuclear localization were increased when exposing the cells to micromolar concentrations of AtRA. However, further coregulator binding assays making use of isolated ER α -LBDs suggested that direct binding of AtRA to ER α does not take place so that other molecular mechanisms must be involved in this phenomenon, which must be studied in the future.

In **Chapter 5** the studies performed in chapter 4 using AtRA were extended to the RAR subtype-selective ligands. Thus, it was investigated whether the distinct effects of RAR subtype-selective agonists on breast cancer cell proliferation could partially be driven by differential effects on the ER α

pathway. To test that hypothesis, evaluation of the effects driven by the general agonist AtRA and the subtype-selective agonists Am80 (RAR α), CD2314 (RAR β) and BMS961 (RAR γ) on basal ER α -related events was carried out using the MCF7/BUS and the U2OS-ER α -Luc cell lines. The results obtained indicated that proliferation, ER α -mediated gene expression, ER α -coregulator binding and ER α subcellular localization can be affected by some of the retinoids in a compound-specific manner. Nonetheless, the responses did not reveal a clear distinction between the different effective (anti-proliferative) AtRA and Am80 and the more ineffective retinoids CD2314 and BMS961. In addition, the coregulator binding studies with the isolated ER α -LBD showed that none of the compounds modulated ER α by direct binding (ligand-receptor) suggesting other pathways as underlying causal factors of the distinct effects of RAR subtype-selective ligands on ER α signaling. From all retinoids tested, the general RAR-agonist AtRA and the RAR β agonist CD2314 displayed the most remarkable effects on ER α signaling. Upon AtRA and CD2314 treatment at micromolar concentrations up-regulation of ER-mediated transcription was observed. However these retinoids differed when comparing other ER α -related molecular events. Thus, while AtRA increased ER α nuclear localization and coregulator binding, CD2314 exerted no significant effects on these molecular endpoints.

To obtain further insight in the crosstalk between retinoid and estrogen signaling in breast cancer, in **chapter 6** of the present thesis, the effects of the general RAR agonist AtRA on the cell proliferation and the related gene expression profiles upon treatment with E₂ were studied in the ER⁺ breast cancer cell line T47D. For this purpose, different assays determining the effects on cell proliferation and the transcriptomic pattern (RNA-seq) were used. In addition, the effects of AtRA on the specific ER α - and ER β -mediated gene expression were assessed by means of reporter gene assays with the T47D-ER α -Luc and U2OS-ER β -Luc cell lines. The results obtained from the proliferation and reporter gene studies pointed at AtRA as a repressor of the E₂-induced breast cancer growth and as inhibitor of the E₂-driven expression mediated by ER α and ER β . To further understand the underlying modes of action of RAR-ER crosstalk, in a next step **chapter 6** acquired further insights on the possible interplay

between estrogen and retinoid signalling by means of a transcriptomic analysis. A high proportion of estrogen-responsive genes were found to be modulated by a single treatment with AtRA. In addition, RNA-seq data showed that AtRA can exert a variable modulation of the E₂-induced transcriptomic responses. In other words, AtRA both counteracted and enhanced gene expression responses induced by E₂. Further analysis showed that the estrogen-responsive genes whose E₂-driven expression was counteracted upon co-exposure to AtRA were more often related to biological processes involved in breast cancer cell proliferation, differentiation or apoptosis. Altogether, the results of **chapter 6** supported the existence of genomic crosstalk between estrogen and retinoid signaling eliciting distinct responses depending on the gene considered. However, co-treatment with AtRA more frequently presented inhibiting/counteracting effects on E₂-induced genes involved in breast cancer growth.

In **Chapter 7**, the studies assessing the effects of the general agonist AtRA on the proliferation and related transcriptomic patterns in the T47D breast cancer cell line were extended to the RAR subtype-selective agonists. The effects of the different retinoids on RAR or other NR pathways of importance in breast cancer cell proliferation (e.g. ER pathway) were assessed by analyzing gene expression patterns. This study showed that the general AtRA and the RAR α agonist Am80 highly hampered T47D breast cancer cell proliferation while the RAR β agonist CD2314 and the RAR γ agonist BMS961 showed to be ineffective anticancer agents which even slightly increased proliferative rates. The transcriptomic profiles clearly differentiated between the effective and ineffective retinoids showing multiple differences in gene expression. **Chapter 7** also suggested RAR α as the main RAR subtype involved in the effects of retinoids on gene expression as shown by the higher and relatively similar effects observed on the transcriptomic signature elicited by AtRA and Am80. Differences between the anti-proliferative retinoids (AtRA and Am80) and the ineffective retinoids (CD2314 and BMS961) mainly occurred in terms of magnitude of expression. Thus, relevant biological processes such as proliferation, differentiation and apoptosis and pathways such as the RAR, the ER, the VDR and the GR pathways were differentially modulated by the effective and ineffective retinoids. Altogether, the

main conclusion drawn in this chapter was that small differences in effects on multiple pathways regulating breast cancer cell proliferation may sum up leading to the distinct responses elicited by the different retinoids in T47D breast cancer cells.

Finally, in **Chapter 8** we first provided an overview on the principal results generated throughout the present thesis. Next, we went in detail with a discussion of all the findings of the thesis. In this analysis, comparison with previous literature, implications of the results obtained and follow-up studies to be performed in the future, were incorporated.

Appendix

Acknowledgements

List of Publications

Curriculum Vitae

Overview of Completed Training Activities

Acknowledgements

Let's start with the big boss, Ivonne. Firstly, thank you for giving me the chance to do a PhD at the Department of Toxicology. I still remember when you told me about that possibility coming into the roof room for students of the old building of Toxicology. Secondly, thank you for your wise advice and supervision in the worse moments of my PhD stage. I know I am sometimes a bit stubborn and difficult to deal with but in the end, you manage to guide me in the correct direction. Finally, I would like to say thanks because apart from toxicology I learn from you many other things such as efficiency, decision-making, leadership and resilience.

Now PamGene people! John, Thank you for giving me the opportunity to work in this challenging project. I am thankful for your constructive feedback, which helped a lot to improve the research. Thank you also for your availability to receive a phone call at any time. Rene, thanks for your expert opinion! I enjoyed our scientific talks since you are kind of a walking encyclopaedia of molecular biology. Apart from science, it is a pity we did not do much because you seem a good guy to have as a friend. Diana, I know I drove you a bit crazy! I really appreciate your help in the lab and I was amazed with your speed and accuracy. My offer to teach Spanish or guitar to your son is still there!

Now time to thank our TOX/WUR stuff. Jochem, thanks for your support all along this period. I really appreciate your valuable feedback and your continuous willingness to help. I think all people from TOX misses you, since your mix of high knowledge and kindness is unique! Laura, thanks so much for all your technical and psychological support. Thanks also for being such an effective buffer (zen) when the meetings were getting a bit nasty. Guido, thanks for your help in the difficult world of omics. Hans, thanks for teaching me about good little things such as birding (Linnaeus was a mere beginner compared to Johannes H.J. van den Berg). I still remember when you gave me the first lesson of cell culture and we already had our first laughs...hehehehe... Alberto (Bert), thanks for being so nice every time I asked you a favour, even being extremely busy. I think you should be more selfish and think in yourself instead of helping everyone all the time. I loved our conversations about history and music. Sebas, thanks for all your jokes and for being an inspirational example on how to deal happily and successfully with difficulties. Lidy, thanks for all your help to fix all kind of administrative issues I got during the PhD, I really appreciate it! Thanks also for organizing so nice musical-social events which brought happiness to everyone. Nico, thanks for your scientific sense of humour. Hans B., thanks for giving me the opportunity to work in nanotoxicology again understanding and giving me support to finalize my thesis as well. Karsten, thanks for teaching me the sensory science of beer. Letty & Henk, you are just cool! Irene and Gré, thanks for your help in my 1st stage as a PhD.

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To thank the other PhDs I will be brief and say the 1st thing that comes to my mind. Agatha (my polish/latina smiling friend); Wasma (The Singer); Reiko (kawui-ne); Samantha (heavy metal); Justine (catwoman); Sunday (smoothies); Hequn (big bird & chicken); Nina & Si (also get MARCoNI); Barae (biking to Den Bosch); Alexandros (“coffee” spots), Arif (1st TOX teacher), Abdul & Amer (so much laughs); Ashraf (reggaeton); Anna (the boat); Felicia (I want your samples); Shuo, Miaoying, Ixchel and Tien (lovely); Diana (Mande?); Artem, Bohan, Qianrui, Danlei, Jia and Lu (unpredictable); Mebra (Hala Madrid!); (Menno (WUR song); Mengying (Dobby-Socks = Destiny); Suparmi (RasaSayange);

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Now my family! Sometimes the people you love the most are those that never hear these words from you. I think, that is my case, so I would like to say to my sisters and parents, I love you! Thanks for all your help in the good and bad moments, thanks for all your advices but also for letting me make hundreds of mistakes and learn from them, thanks for being an example on how to treat well and respect others and, last but not least, thanks for being always there supporting me without asking much because you know I do not like to speak about things that make me sad. So, 1000 thanks for everything! “Merci beaucoup” to my “suegros” (parents in law) as well! The defence lunch is gonna be even funnier with you meeting my parents for the 1st time! It will be kind of a Tower of Babel moment! :D

The last words are for my girl (Cecile). I think that 50% of the success of a scientist belongs to the partner so you got ½ of a PhD... Congratulations! During these years I was almost always able to work hard, smile and joke from 9:00 to 21:00, no matter how bad my situation was (ruining the mood of people is not my cup of tea). However, in the end of the day, when you were coming home, I was often not able to keep that pace. You were the one next to me when I was exhausted, when I was sad, when I was just annoying. I have no words to describe how much I thank you for being always there standing the often boring and worried Nacho. Thanks for being with me in the Netherlands, for listening to me every night and for making me massage when “I had pain everywhere”. Je t'aime à l'infini et au-delà! Last thanks to Boniface & Luna that brought puppies in the last stage of my PhD to make me stronger!

List of Publications

I. Miro Estruch, D. Melchers, R. Houtman, L.H.J. de Haan, J.P. Groten, J. Louisse, I.M.C.M Rietjens. Characterization of the differential coregulator binding signatures of the Retinoic Acid Receptor subtypes upon (ant)agonist action, *Biochim. Biophys. Acta - Proteins Proteomics*. 1865 (2017) 1195–1206.

I. Miro Estruch, L.H.J. de Haan, D. Melchers, R. Houtman, J. Louisse, J.P. Groten, I.M.C.M Rietjens. The effects of all-trans retinoic acid on estrogen receptor signaling in the estrogen-sensitive MCF/BUS subline, *J. Recept. Signal Transduct.* (2018) 1–10.

I. Miro Estruch, Guido J.E.J. Hooiveld, Laura H.J. de Haan, Jochem Louisse, John P. Groten, Ivonne M.C.M. Rietjens. Insights in the interference exerted by retinoic acid signaling on the estradiol-induced proliferation and transcriptomic signatures of the T47D breast cancer cell line. *In preparation*.

I. Miro Estruch, Guido J.E.J. Hooiveld, Aziza H.B. Adam, Laura H.J. de Haan, Jochem Louisse, John P. Groten, Ivonne M.C.M. Rietjens. Relating transcriptomic profiles induced by general and subtype-selective retinoic acid receptor agonists to their divergent proliferative effects in the T47D cell line. *Submitted for publication*.

Curriculum vitae

Ignacio Miro Estruch was born the 5th of March of 1984 in Alcoy (Spain). In 2007, Ignacio obtained a Degree in Veterinary Science (Universidad Cardenal Herrera) acquiring additional clinical expertise by means of a 2-year internship at the University Veterinary Hospital. Next, Ignacio followed a MSc programme in Public Health and Health Management (2009-2010) with a MSc thesis related to food allergens. In addition, he worked as a public health (governmental) officer in the field of food safety for 1 year. In 2011, Ignacio moved to the Netherlands and followed the MSc Programme in Food safety at Wageningen University. During these

studies, Ignacio performed his MSc thesis in the Department of Toxicology under the supervision of Ivonne Rietjens, where he investigated embryotoxic and neurotoxic effects of polystyrene nanoparticles. In addition, Ignacio conducted an internship at Rikilt Institute of Food Safety where he evaluated the intestinal translocation of silver nanoparticles using *in vitro* models. After obtaining the MSc in Food Safety in 2013, Ignacio initiated his research on the present PhD thesis entitled “Novel insights in the molecular mechanisms of action of retinoids and their potential repercussions on breast cancer cell proliferation”. During this PhD, Ignacio has followed multiple courses integrated in the postgraduate education programme on toxicology (PET) in order to be registered as European Toxicologist in the near future. In addition, Ignacio has acquired knowledge on aspects such as didactics and leadership since he has lectured, guided and supervised several MSc students. Ignacio is currently working at the Department of Toxicology in a project entitled BIORIMA (Biomaterial Risk Management) which main objective is to develop an integrated risk management framework for nanobiomaterials used in advanced therapeutic medicinal products and medical devices.

Overview of completed training activities

Conferences

Nuclear Receptor Research Network (Annual Meeting, Amsterdam 2016)

Society of Toxicology (Annual meeting, Baltimore, USA. 2017)

Discipline specific courses

Pathobiology (Postgraduate Education in Toxicology (PET), Utrecht, 2014)

Cell Toxicology (Postgraduate Education in Toxicology (PET), Leiden, 2014)

Laboratory aAnimal Science (Postgraduate Education in Toxicology (PET), Utrecht, 2014)

Risk Assessment (Postgraduate Education in Toxicology (PET), Wageningen, 2014)

Molecular Toxicology (Postgraduate Education in Toxicology (PET), Amsterdam, 2015)

Toxicogenomics (Postgraduate Education in Toxicology (PET), Maastricht, 2015)

Mutagenesis & Carcinogenesis (Postgraduate Education in Toxicology (PET), Leiden, 2015)

General courses

VLAG PhD week (VLAG, Baarlo, 2014)

Scientific Writing (WGS, Wageningen, 2014)

Project and Time Management (WGS, Wageningen, 2014)

Philosophy and ethics of Food Science and Technology (VLAG, Wageningen, 2016)

Other activities and optional courses

Thesis Proposal

Scientific presentations TOX-WUR

General Toxicology (WUR, Wageningen, 2014)

Environmental Toxicology (WUR, Wageningen, 2015)

Concepts and methods in Epidemiology (WUR, Wageningen, 2015-2016)

***Approved by Graduate School VLAG**

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