Characterization of transcriptional cross-talk between the estrogen receptor and retinoic acid receptor in human breast cancer cells

Caroline Rousseau

Department of Medicine, Division of Experimental Medicine

McGill University, Montreal, Quebec, Canada

May 2004

A thesis submitted to McGill University, Faculty of Graduate Studies and Research, in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Ph.D.)

Your file Votre référence
ISBN: 0-612-98361-7
Our file Notre référence
ISBN: 0-612-98361-7

This thesis is dedicated to my mother and to my daughter.

TABLE OF CONTENTS

ABSTRACT

Retinoids are derivatives of vitamin A with demonstrated therapeutic potential for the treatment of breast cancer. The efficacy of retinoids *in vitro* and *in vivo* correlates with the expression of the estrogen receptor alpha (ERα). The role of ERα in mediating RA-induced sensitivity is not understood and is further complicated by the recent discovery of ERβ. This dissertation explores the transcriptional, as well as proliferative, response to RA in human breast cancer cells expressing ERα or ERβ. First, ER-negative breast cancer cells were stably transduced with ERα-deletion mutants using retroviral technology. We compared the effect of the ERα wild-type, ERα-deletion mutants or the parental ER-negative cells on transcriptional activity from the RARβ2 promoter, a gene regulated by retinoids and potentially involved in retinoid-mediated growth inhibition. We observed that expression of ERα suppressed basal expression of the RA-responsive gene RARβ2, while allowing it to be strongly induced by RA. Repression of basal RARβ2 transcription was confirmed by transient expression of a reporter plasmid containing the RARβ2 minimal promoter. We further determined that RARβ2 induction required the N-terminal AF-1 containing region of ERα, including the DNA-binding domain, but was independent of the C-terminal ligand-binding domain. The effect of ERα was specific for RAR-mediated transcription and did not alter transcription from vitamin D or thyroid hormone response elements. Moreover, the cross-talk between ERα and RAR was not mediated by sequestration of a number of common co-regulators. To characterize the growth and transcription effect of ERβ on retinoid-mediated pathways, we generated stable transfectants using this isoform. Significant RA-mediated growth inhibition was observed in the ERβ-positive

cells and not in the parental ER-negative cells. Furthermore, RA altered ERβ-mediated pathways, as shown by its inhibitory effects on the expression of the endogenous pS2 gene. Similar to ERα-positive breast cancer cells, ERβ-expressing cells exhibited altered RARβ expression, resulting in greater induction of RARβ gene expression upon RA treatment as compared to the parental ER-negative cells. In conclusion, we have characterized transcriptional cross-talk between ERα or ERβ and RAR in human breast cancer cells. Understanding the mechanism of action of retinoids in this disease will prove important to the targeted design of retinoid-based therapies.

RÉSUMÉ

Les rétinoïdes, dérivés de la vitamine A, démontrent des effets thérapeutiques prometteurs pour traiter les cancers mammaires. L'effet inhibiteur de l'acide rétinoïque (AR) sur la prolifération des cellules cancéreuses correspond avec l'expression du récepteur de l'oestradiol alpha (ROα). Par contre, la fonction du ROα dans la médiation de l'inhibition à la prolifération induite par l'AR est peu connue, et est compliquée par la nouvelle découverte d'un deuxième RO, ROβ. Nous avions pour but de caractériser le mécanisme d'interaction, aux niveaux transcriptionnel et prolifératif, entre le RO (α et β) et le récepteur de l'acide rétinoïque (RAR) dans des lignées de cellules cancéreuses mammaires. En première partie, nous avons utilisé un vecteur viral pour construire des lignées stables de cellules mammaires exprimant des mutants de délétions du ROα. L'activité transcriptionelle du RAR sur le promoteur RARβ2, un gène régulé par l'AR et potentiellement important à l'inhibition de la croissance cellulaire, a été comparé entre les cellules souches parentales qui ne contenaient pas de ROα, celles exprimant le ROα sauvage, et les cellules exprimant les mutants de délétions du ROα. L'effet du ROα était de supprimer l'expression basal du gène RARβ2, tout en lui permettant d'être fortement induit par l'AR. Nous avons constaté, par transfection transitoire d'un gène rapporteur comprenant le promoteur minime du RARβ2, que l'effet du RO sur l'expression de ce gène, est due à l'amino terminus du ROα, incluant le domaine de liaison à l'ADN. Le domaine de liaison au ligand n'est pas nécessaire pour cette interaction transcriptionelle entre le ROα et le RAR. L'effet transcriptionel du ROα est spécifique pour le promoteur comprenant un élément de réponse à l'AR (RARE) et ne change pas la transcription par les éléments de réponse à

la vitamine D3 (VDRE) ou aux hormones thyroïdiennes (TRE). De plus, l'interaction transcriptionnelle entre le ROα et le RAR n'est pas liée à la séquestration d'un nombre de co-régulateurs communs à ces deux voies de signalisation. Pour caractériser l'effet du ROβ sur l'inhibition de prolifération et la transcription dû à l'AR, nous avons généré des transfectants stables, par technique rétrovirale, en utilisant cet isoforme. Nous avons observé que l'AR inhibait la croissance des cellules exprimant le ROβ, tandis que les cellules souches parentales étaient résistantes à l'inhibition produite par l'AR. De plus, l'AR modifie la transcription induite par le ROβ, tel qu'observé sur l'expression du gène pS2. Semblable aux cellules cancéreuses mammaires exprimant le ROα, celles qui expriment le ROβ, avaient une plus grande induction de l'expression du RARβ2, causé par une réduction d'expression basal de ce gène. En conclusion, nous avons démontré une région d'interaction transcriptionnelle entre la signalisation induite par le RO et le RAR. Les mécanismes d'interaction entre ces deux récepteurs nucléaires sont à élucider pour améliorer ou mieux cibler l'effet de l'AR au niveau du contrôle de la croissance des tumeurs.

ACKNOWLEDGEMENTS

I wish to express thanks to Dr. Wilson Miller, my supervisor, for his guidance and much needed support at times when I thought I had lost faith in my project. I also thank my thesis committee members Dr. John White, Dr. Jacques Galipeau, Dr. Moulay Alaoui-Jamali and my advisor Dr. Lorraine Chalifour for their input on my project. The work presented herein was funded by several student awards. I wish to thank the McGill Majors Awards, FCAR, and the US Army Medical Research program, for their financial help.

This work could not have been accomplished without the help of many laboratory members. The days and evenings would have been long without the company of my friend and colleague, Kelly Davison. Thanks also to Angelika Roseneaur, Wenlin Shao, Sonia del Rincon, Micheal Witcher, April Colossimo, Marie-Claude Couture, Filippa Pettersson, Sylvie Cote, and Koren Mann for their companionship, knowledge and expertise. I wish to especially recognize some wonderful undergraduate students, Tyler Colburn and Jessica Nichol, for their drive and contribution to this project. To all the members of the Clinical Research Unit, I wish to thank you for your patience, understanding and for allowing me the freedom and time to complete this thesis.

Most importantly, I would like to thank my mother, whose strength and dedication to our family I remember fondly. Also thanks to my father, who always encourages me to pursue my interests, allows me to see the light at the end of the tunnel and whose financial support I have greatly appreciated for so many years. As well, I would like to thank Pascal Gad for his love and support on the many weekends that he spent taking care of our daughter alone. I am also grateful to Pascal's wide-ranging

technical skills during the preparation of manuscripts and of this thesis. Lastly, I would like to thank my daughter, Nikita, for being her wonderful self and for dissolving the stress of a bad day by her hug and exclamations of "maman...maman!"

PREFACE

In accordance with the "Guidelines for Thesis Preparations", the candidate has chosen to present the results of her research in manuscript-based format.

The texts of two original papers, one of which has been published and the other which has been submitted for publication, are included as chapters of this thesis. A General Introduction and a General Discussion are also included, as well as connecting texts that provide bridges between the different papers. The manuscripts presented in this thesis are listed below.

Chapter 2 **Rousseau, C**, Pettersson, F, Couture, M, Paquin, A, Galipeau, J, Mader S., Miller WH Jr. The N-terminal of the estrogen receptor (ERα) mediates transcriptional cross-talk with the retinoic acid receptor in human breast cancer cells. Journal of Steroid Biochemistry and Molecular Biology 86: 1-14, 2003.

Chapter 3. **Rousseau, C.**, Nichol, J., Pettersson, F., Miller W.H. Jr. ERβ sensitizes breast cancer cells to retinoic acid and contributes to altered RAR beta expression. Submitted to Molecular Cancer Research.

Two published papers that the candidate has co-authored, but has not included in this thesis are listed below:

(1) del Rincon SV, **Rousseau C**, Samanta R, Miller WH Jr. Retinoic acid-induced growth arrest of MCF-7 cells involves the selective regulation of the IRS-1/PI 3-kinase/AKT pathway.Oncogene. 22: 3353-60, 2003.

(2) Witcher M, Ross DT, **Rousseau C**, Deluca L, Miller WH Jr. Synergy between all-trans retinoic acid and tumor necrosis factor pathways in acute leukemia cells. Blood. 102: 237-45, 2003.

CONTRIBUTIONS OF AUTHORS

All of the research presented in Chapters 2 and 3 was performed by the candidate under the supervision of Dr. W.H. Miller Jr.. Others that have contributed to the work are listed below:

Franca Sicilia performed the flow cytometric analysis of the retrovirally transduced MDA-MB-231 cells in Chapter 2.

Filippa Pettersson performed the RT-PCR analysis of RARβ2 and the chromatin immunoprecipitation analysis demonstrated in Chapter 2.

Andre Paquin, under the supervision of Dr. J. Galipeau, provided the retrovirus vector, HC2, and valuable help in designing cloning strategies.

LIST OF ABBREVIATIONS

9-cis RA	9-*cis* retinoic acid
ACTR	activator of thyroid and RA receptor
ADH	alcohol dehydrogenase
AF-1	activation function-1
AF-2	activation function-2
AR	androgen receptor
ARC	activator-recruited factor
ATRA	all-*trans* retinoic acid
AzA-CdR	5-aza-2'-deoxycytidine
bp	base pair
brg-1	brahma-related gene 1
brm	brahma
CBP	CREB-binding protein
CREB	c-AMP response element binding protein
DBD	DNA binding domain
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DR	direct repeat
DRIP	vitamin D receptor interacting protein
E2	17β-estradiol
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein

EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERE	estrogen response element
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GR	glucocorticoid receptor
HAT	Histone acetyl transferase
HDAC	histone deacetylase
HSP	heat shock protein
IGF1	insulin-like growth factor-1
IRES	internal ribosomal entry site
IGF1R	insulin-like growth factor-1 receptor
kb	kilobase
kDa	kilodalton
LBD	ligand binding domain
MeCP	methyl CpG-binding protein
MCS	multiple cloning site
MR	mineralcorticoid receptor
mRNA	messenger ribonucleic acid
NCoR	nuclear hormone receptor corepressor
NR	nuclear receptor
OHT	tamoxifen

p/CAF	p300/CBP associated factor
PBS	phosphate buffered saline
PPAR	peroxisome proliferating associated receptor
PR	progesterone receptor
RAR	retinoic acid receptor
RNA	ribonucleic acid
RXR	retinoic X receptor
SERM	selective estrogen receptor modulator
SMRT	silencing mediator of retinoid and thyroid receptors
Sp-1	specificity protein 1
SWI/SNF	mating type switching / sucrose fermentation
TGFα	transforming growth factor alpha
TR	thyroid hormone receptor
TRAP	thyroid hormone receptor-associated protein
VDR	vitamin D receptor

LIST OF FIGURES and TABLES

CHAPTER 1

Literature Review

INTRODUCTION

The regulation of transcription is of paramount importance in the development of cancer. Aberrant expression of tumor promoting and tumor suppressing factors can lead to a cascade of events, culminating in de-regulation of the cell cycle. Regulation of transcription involves a complex set of events. The promoter of target genes must be accessed by several molecules, whose role it is to open the chromatin and render the transcriptional start site available to the basic RNA transcriptional machinery.

In the case of breast cancer, an enhanced transcriptional property of the estrogen receptor (ER) is believed to be one major cause of aberrant cellular proliferation. It is becoming increasingly evident that understanding the transcriptional regulation of target genes will aid in the design of targeted cancer therapies for breast, as well as other cancers.

1.1. TRANSCRIPTIONAL REGULATION

1.1.1. Transcription by Nuclear Receptors

Nuclear receptors are ligand-inducible transcription factors that modulate transcriptional activity by binding to DNA and recruiting accessory molecules. The nuclear receptor superfamily includes the Type I steroid receptors (ER, GR, AR, PR and MR) that bind to specific promoters as homodimers, the Type II non-steroid receptors (RAR, VDR, TR and PPAR) that form heterodimers with RXR, and the Type III orphan nuclear receptors, for which the ligands are yet unknown (1).

Nuclear receptors share a common basic structure that is defined by autonomous functional domains. Generally, nuclear receptors include an N-terminal region A/B, a

DNA-binding domain (DBD) located within region C, a linker region D, and a C-terminal region E containing the ligand binding domain (LBD). Some receptors also contain a C-terminal F region of unknown function (Fig. 1.1). Transcriptional activity is regulated by two transactivation functions (AF-1 and AF-2), located in the A/B and E regions respectively. The N-terminal AF-1 domain provides basal activity in the absence of ligand, whereas the C-terminal AF-2 domain is dependent upon binding of ligand to the receptor. (2-5). While these two functions have independent transactivation potential, they usually act together to modulate transcription from response elements.

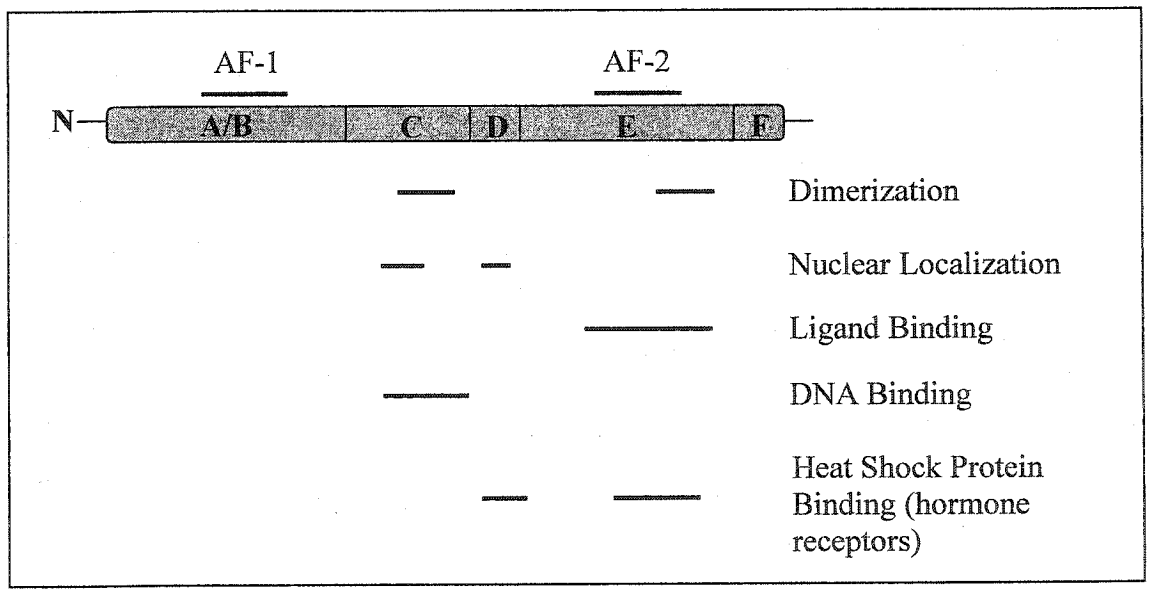

Figure 1.1. Structural domains of a typical nuclear receptor.

The A/B domain

The N-terminal A/B region is quite variable between receptor isoforms, and it is believed that this region is responsible for cell- and promoter-specificity (5). The N-

terminal region contains many residues that can be phosphorylated by different signaling pathways to alter transcription independently of ligand. For example, activation of transcription from specific response elements can occur upon phosphorylation of threonine and serine residues in the N-terminal region of the estrogen receptor by MAPK pathways (6-8). RAR can also be phosphorylated by cyclin-dependent kinases and this can alter the transcriptional activity of this receptor (9, 10). Phosphorylation in this region may enhance the binding of some of the general transcriptional factors, including TFIIB, TBP and TAF30, to the N-terminal region of the nuclear receptor (11-13).

The role of AF-1 in the N-terminal region is not as well characterized as that of the AF-2. Although the AF-1 region has been shown to bind coactivators, it does so with less affinity than AF-2. It has been proposed that AF-1 shows little independent activity at a simple promoter, but with a complex promoter, containing other transcription factor binding sites, the AF-1 may be important in aiding in the stability and recruitment of cofactors required for transcription (14).

The C domain

The C region of nuclear receptors contains a DNA-binding and dimerization domain. The DNA-binding domain consists of nine cysteines that form two zinc fingers involved in DNA interaction. At the base of the first zinc finger is the P-box. This region contains amino acids that are involved in recognition of specific DNA sequences. The residues in the second zinc finger, forming the D-box, confer specificity for half-site spacing and function as a DNA-dependent dimerization domain (15, 16).

Nuclear receptors regulate transcription from enhancer elements that may be several kilobases (kb) from their target promoters. In general, nuclear receptor families recognize DNA response elements with a core recognition motif of 6 bp. The idealized consensus sequence of [5'-AGAACA-3'] is preferentially recognized by the steroid receptors. The exception to this is the estrogen receptor, that recognizes the [5'-(A/G)GGTCA-3'] consensus half-site motif (5). The non-steroid receptors preferentially bind to the [5'-AG(G/T)TCA-3'] half-site recognition motif (5). These half-sites can be configured as palindromes, inverted palindromes or direct repeats (DRs). While some nuclear receptors can bind to DNA as monomers (ex. NGF1-B, SF-1), most bind as homo- or heterodimers (5).

As a rule, the steroid hormone receptors bind as homodimers to consensus sequences organized as palindromes. Non-steroid receptors bind as heterodimers with the promiscuous binding partner RXR, and recognize preferentially direct repeats (Fig. 1.2). Response elements that involve DRs spaced by 3, 4 and 5 bp mediate preferential regulation by VDR, TR and RAR respectively (5). In this case, the promiscuous partner RXR occupies the 5' end of the response element, with the heterodimeric partner occupying the downstream motif. In addition, a DR1 can serve as a response element for RXR or PPAR, and a DR2 can also be recognized by RARs (Fig. 1.2). However, these are general guidelines for binding of nuclear receptors to response elements and widely spaced DRs can act as promiscuous response elements for different nonsteroid receptors and even for ER (5). In addition to spacing, differences in half-site sequence and sequences flanking the response elements may also be important parameters in determining nuclear receptor binding.

The D domain

The D domain serves as a link between the DBD and the LBD. It is involved in binding to heat shock proteins (for Type I nuclear receptors) and also harbors a nuclear localization signal (17). Some groups have hypothesized that this domain is involved in maintaining the structural features required for corepressor interactions in the C-terminal domain (5, 18, 19).

The E domain

In addition to binding ligand, this domain mediates dimerization between nuclear receptors, interaction with heat-shock proteins (20), nuclear localization (21) and ligand-dependent activation via the AF-2 (5). The crystal structure of the LBD has been established for many nuclear receptors. In general, the LBD of nuclear receptors possesses twelve conserved α-helical regions, numbered H1 to H12. The helices are organized to form a mainly hydrophobic ligand-binding pocket. Upon binding of ligand, the receptor undergoes a conformational change that involves the folding of H12 over the ligand, generating a hydrophobic cleft (22).

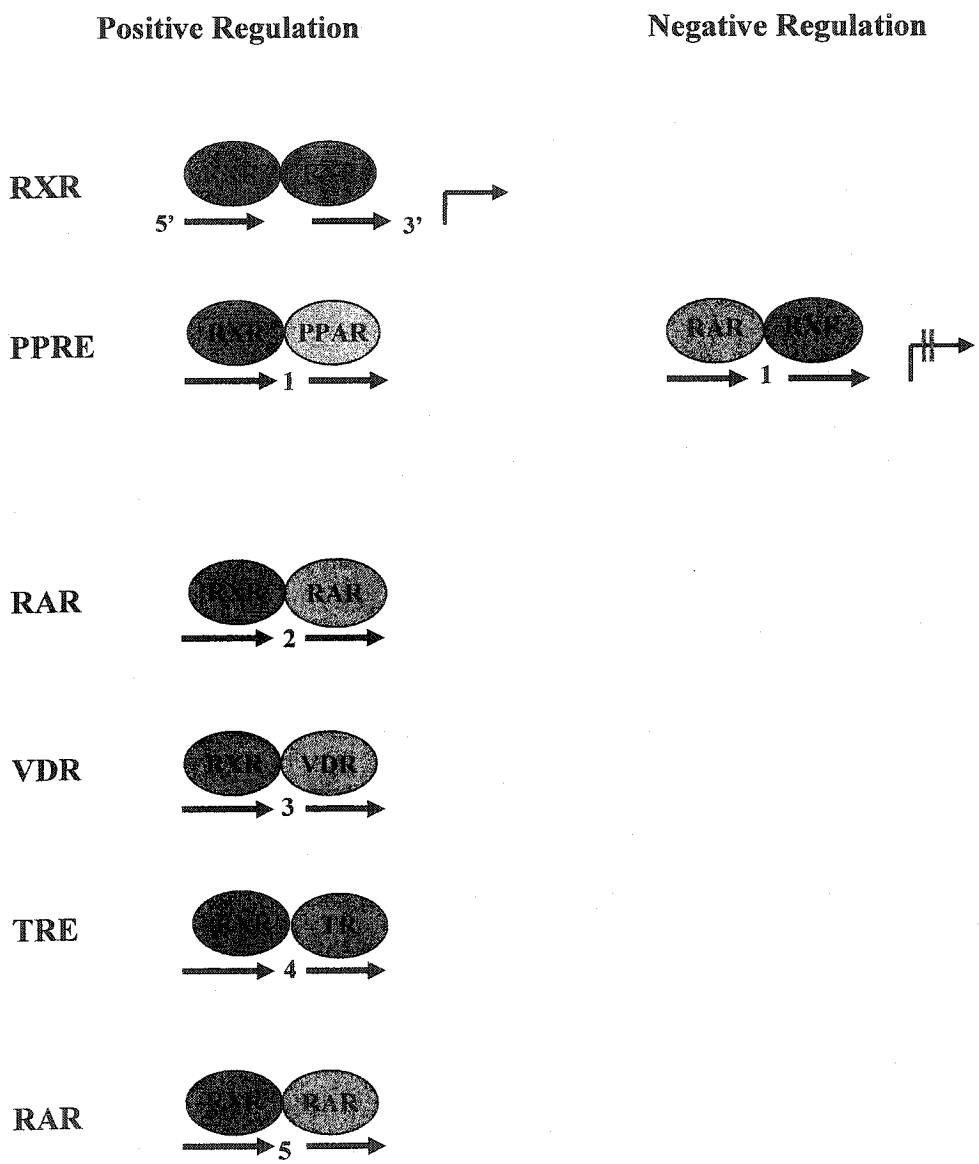

Figure 1.2. Response elements recognized by RXR heterodimers

1.1.2. Chromatin Remodeling

The activation of transcription is believed to be a two-step process, whereby chromatin remodeling occurs prior to recruitment of coactivators and the RNA Pol II machinery to the promoter (23, 24). Chromosomes are organized into repeating protein-DNA units called the nucleosome. The hormone responsive elements of genes are organized within the nucleosomes. Nucleosomes consist of two molecules of histone H2A, H2B, H3 and H4. This core, with the addition of linker DNA and histone H1, constitutes the fundamental repeating unit of chromatin. Almost two turns of DNA superhelix (146 bp) are wrapped around this octameric core. To allow the transcriptional machinery access to the transcriptional start site, the chromatin must be disrupted. There are two mechanisms for this: (1) ATP-dependent nucleosome remodeling complexes, and (2) covalent modification of histones via acetylation, phosphorylation, or methylation.

ATP-dependent chromatin remodeling

Nuclear receptors mediate the recruitment of several large ATP-driven machines, essential for exposing nucleosomal DNA. One such multiprotein complex, SWI/SNF, was originally identified in yeast. The mammalian homologs, hBRM and BRG-1, found in large complexes associated with selective BRG-1-associated factors (BAFs), were subsequently discovered (22, 25). Upon binding of ligand, subunits of this large complex are recruited to the AF-2 region of the nuclear receptors (22). These multiprotein chromatin remodeling complexes require ATP to catalyze nucleosome

mobilization in order to facilitate access to the DNA by the transcriptional machinery (5).

It is believed that the SWI/SNF complex acts before the modification of histones to activate transcription (26). However, this is not true of all promoters, and it has recently been shown that chromatin-remodeling enzymes can regulate key steps in transcription before, during and after assembly of the pre-initiation complex (27-29). The SWI/SNF complex is also thought to be expressed in limiting amounts and as such, nuclear receptor sequestration for interaction with its associated factor results in gene-specific chromatin remodeling (25).

Covalent Histone Modifications

Histone tails are the target of extensive post-translational modifications, including acetylation, phosphorylation and methylation. By far the most characterized histone modification is the acetylation of lysine residues in the histone tails. Histone acetylation is a highly dynamic, specific and reversible modification, recognized to be a regulatory switch for transcription (30).

The N-terminal tails of histones are highly positively charged and maintain a tight affinity with the negatively charged phosphate groups of DNA (30). Acetylation of the histone N-terminal, primarily on histone H3 and H4, neutralizes the positive charge of histones and leads to the disruption of a higher order chromatin, thereby increasing the access of transcription factors to the promoter.

It has further been suggested that the acetylation pattern at certain promoters serves as a unique recognition surface for specific chromatin-associated proteins (31).

P/CAF is one protein that is able to bind to specifically acetylated lysine residues (32), indicating that the acetylation of a distinct set of histones may direct a distinct transcriptional outcome (33).

Like acetylation, histone methylation can result in changes in chromatin accessibility. For example, tri-methylation of H3 at lysine 4 (H3-meK4) is usually associated with lysine 9 acetylation and results in active chromatin. Methylation of H3 at lysine 9 (H3-meK9) is associated with repressed chromatin (34, 35).

DNA Methylation

In addition to the control of transcription by nuclear receptors, there exist other mechanisms of transcriptional regulation. Methylation of C-residues in CpG islands near the promoter, and even in the coding region of target genes, has traditionally been associated with transcriptional repression. Many human cancers show aberrant regulation of DNA methylation (34), particularly at tumor suppressor genes. Furthermore, DNA hypermethylation has been found to be associated with drug resistance acquired during chemotherapy (36, 37).

DNA methylation is carried out by at least three DNA methyltransferase (DNMT) enzymes (38). Once methylated, the DNA is bound by specific proteins known as methyl CpG-binding proteins (MeCP) proteins (39). MeCPs can interact with histone deacetylating proteins (HDACs) via the corepressor Sin3. In addition to MeCPs, DNMTs themselves can recruit HDAC complexes (40). This connection, between MeCPs, DNMTs and HDAC-containing protein complexes, results in a restrictive chromatin structure that is inaccessible to transcriptional factors (41).

1.1.3. Coactivators

Nuclear receptors, when bound by an agonistic ligand, have the ability to recruit several coactivator proteins. To date, a large number of cofactors have been identified, whose expressions differ from cell to cell (42). The role of coactivators is to aid in remodeling chromatin by destabilizing the binding of nucleosomes and stabilizing the formation of the core-initiation transcription complex. Coactivators bind directly to nuclear receptors and recruit other accessory proteins such as p300, CBP and p/CAF. These accessory proteins contain histone acetyl transferase (HAT) activity, necessary for chromatin decondensation. Some coactivators (SRC-1, SRC-3) also exhibit HAT activity, thereby further contributing to increasing the accessibility of the DNA to the RNA Pol II machinery. The coactivators and associated accessory proteins form what is called a HAT complex.

Of the coactivators, the p160 family is the most characterized. These coactivators have leucine-rich motifs of the consensus sequence LXXLL, where L represents leucine and X any amino acid (43). These motifs, otherwise known as the NR boxes, are common to other proteins that are also involved in ligand-dependent nuclear receptor binding (43).

In the absence of ligand, the AF-2-containing helix 12 of nuclear receptors extends away from the ligand-binding domain (44). Upon binding of ligand, the AF-2 region undergoes a conformational change and helix 12 folds over to make tight contact with the ligand, exposing a hydrophobic site favorable to coactivator interaction. The LXXLL motif of coactivators makes direct contact with the hydrophobic cleft that is formed by helix 3, 5 and 12 of nuclear receptors (43).

Although the coactivators were initially characterized as molecules that bind the AF-2 domain of nuclear receptors, recent evidence suggests that coactivators can also bind the AF-1 domain (5). Indeed, it has been shown that the p160 coactivators can interact with the N-terminal AF-1-region of several steroid receptors. Binding of coactivators to the AF-1 domain involves a glutamine-rich region on the coactivators, and not an LXXLL motif (5). This is particularly important for transactivation via AR since most of this hormone receptors' transactivating activity is mediated by the A/B domain (45, 46). As an added example, interaction between the AF-1 and AF-2 domain of ERα, with distinct regions of SRC-1, appears to be required for full synergy between the AF-1 and AF-2 domains (47). Likewise, p300 has been shown to potentiate the AF-1 function of both human ERα and β. Direct interactions of p300 with a truncated ERα containing only the A/B domains have been demonstrated (48). Recently, p68 RNA helicase has been identified as a cofactor that is specific for the AF-1 domain of ERα. The association of p68, which then recruits CBP, occurs in the AF-1 region of ERα only upon phosphorylation in this domain (49).

Although the primary role of coactivators is to facilitate transcription, they can also be involved in transcriptional attenuation. Acetylation of lysine residues in the hinge region of ERα by the coactivator p300, has been shown to suppress ligand sensitivity (50). Acetylation may be therefore be involved in ligand-dependent transcriptional repression or attenuation (50). Furthermore, it has been suggested that coactivators themselves can be covalently modified to alter their function (51). As an example, p300 not only acetylates histones, but is able to acetylate SRC-3, resulting in uncoupling of the interaction between the ER and SRC-3 on target promoters (52).

Coactivators may also be involved in the recruitment of proteins involved in histone methylation. It has been shown that the argenine methyltransferase CARM1 can synergize with p160 proteins and p300 to enhance transcriptional activation through its ability to methylate histone H3 (53).

The specificity of coactivators for receptors and their regulation is poorly understood. There exists much redundancy between coactivators, and null deletion experiments in mice have shown that many coactivators have overlapping functions (11). It has been proposed that the function of a coactivator is determined by promoter context, receptor and ligand identity, and tissue specificity (51). To date, only CBP, p300 and TRAP220 are essential for viability (51).

1.1.4. Corepressors

The non-steroid nuclear receptors recruit corepressor proteins in the absence of ligand, while the steroid hormone receptors require binding of an antagonist to the LBD. Co-repressors form large multiprotein complexes with the nuclear receptors. These complexes include HDACs that associate with Sin3A and Sin3B (5). Sin3A is a large multidomain protein that acts as a scaffold for the corepressor complex.

The more commonly studied corepressors are NCoR and SMRT. These harbor a nuclear receptor interaction domain, referred to as the CoRNR box. This motif has the consensus sequence LXXI/HIXXXI/L, which interacts with specific residues in the same pocket required for coactivator binding (54). The CoRNR box contains an extended helix that cannot bind in the charged cleft of the nuclear receptor in the presence of agonistic ligand. In the presence of antagonists, or absence of ligand for

non-steroid nuclear receptors, helix H12 is repositioned, allowing corepressors to bind to the hydrophobic pocket (5, 55).

In addition to NcoR and SMRT, an ER-selective repressor of ER activity, REA, has also been identified. This corepressor enhances the potency of antiestrogens and is specific for ERα and ERβ (56).

Corepressors recruit a complex of proteins that exhibit HDAC activity. To date, 18 HDACs have been identified in humans (57). Many of these are ubiquitously expressed in cell lines and tissues, and are sensitive to HDAC inhibitors (57). HDACs have been shown to associate with SMRT and NCoR, and some HDAC members can also interact with DNMTI (58).

Recently, it has become known that direct interactions exist between corepressors and coactivators. Li et al have shown that the co-repressor NCoR can directly interact with ACTR, thus permitting the integration of nuclear receptor mediated repression and activation (59).

1.1.5. DRIP/TRAP proteins

In addition to the HAT and SWI/SNF complexes mentioned above, nuclear receptors can recruit a large complex of proteins called the DRIPS (TRAPS/Mediator/ARC) (60, 61). The DRIP complex consists of at least 9 proteins ranging in molecular weights from ~70 kDa to ~230 kDa (43). One subunit, DRIP205 (TRAP220), contains two LXXLL motifs that are critical for interactions with the C-terminal region of nuclear receptor in a ligand-dependent manner (62). Anchorage of this subunit to the receptor provides a scaffold for the binding of the rest of the DRIP

complex. The function of the DRIP complex is not well understood, but it is believed that it aids in enabling transcription by recruiting the basic transcriptional machinery. In addition to nuclear receptors, the DRIPS also interact with other transcription factors, such as the p65 subunit of NF-kB, and VP16 (63).

1.1.6. Biphasic Model of Transcriptional Regulation

The complexity of transcription, involving an orchestrated recruitment of components to the basal transcriptional machinery and including the integration of multiple signaling pathways, is essential to transcriptional regulation within chromatin structures important for development, differentiation and homeostasis. The presence of such large multiprotein complexes binding to nuclear receptors has led to a model of transcription that is multi-step in nature, where large protein complexes have temporally and spatially distinct functions (Fig. 1.3.). A possible sequence of events has been suggested, which begins with binding of ligand to the nuclear receptor, causing the release of corepressors, and the binding of the SWI/SNF/BRG-1 complex that acts to modify chromatin domains. Binding of coactivators then results in localized HAT activity and disruption of the nucleosomal structure.

The functional redundancy between transcription factors has lead to a model in which there is a combinatorial role for transcription factors in transcriptional regulation (24, 64). Chromatin immunoprecipitation assays have been useful in studying the interaction of cofactors with nuclear receptors at the promoter. Using this technique, it was determined that there exists a cyclic association and dissociation of coactivators. For example, p300 only appears to participate in the first cycle of ER cofactor

recruitment and this may catalyze chromatin modifications that prime the promoter for multiple rounds of transcription. This is consistent with p300 facilitating transcriptional initiation, but not re-initiation (65). Recruitment of p160s and DRIPs occur in opposite phases, suggesting an exchange between these coactivator complexes at the target promoter (66). A model has been proposed where initial recruitment of chromatin-modifying complexes such as p160s and CBP, is followed by displacement of the complex and interaction of the receptor with the DRIP complex to form a link with general transcriptional factors (11). Transcription would then occur in successive rounds (reviewed in (23) and (24)).

However, a general model of transcriptional activation has yet to be confirmed and its complexity is elegantly displayed by Metivier et al. who studied in great detail the transcriptional regulation from the pS2 promoter (64). They demonstrate that transcription from this promoter occurs in cycles and that silenced chromatin results when the NuRD complex, containing HDACs and remodeling activities is recruited to the promoter upon departure of TBP and TFIIA. It appears likely that the order of recruitment for chromatin-modifying complexes is promoter-specific, dependent upon the nature of the promoter, transcription factors available, and chromatin structure (67).

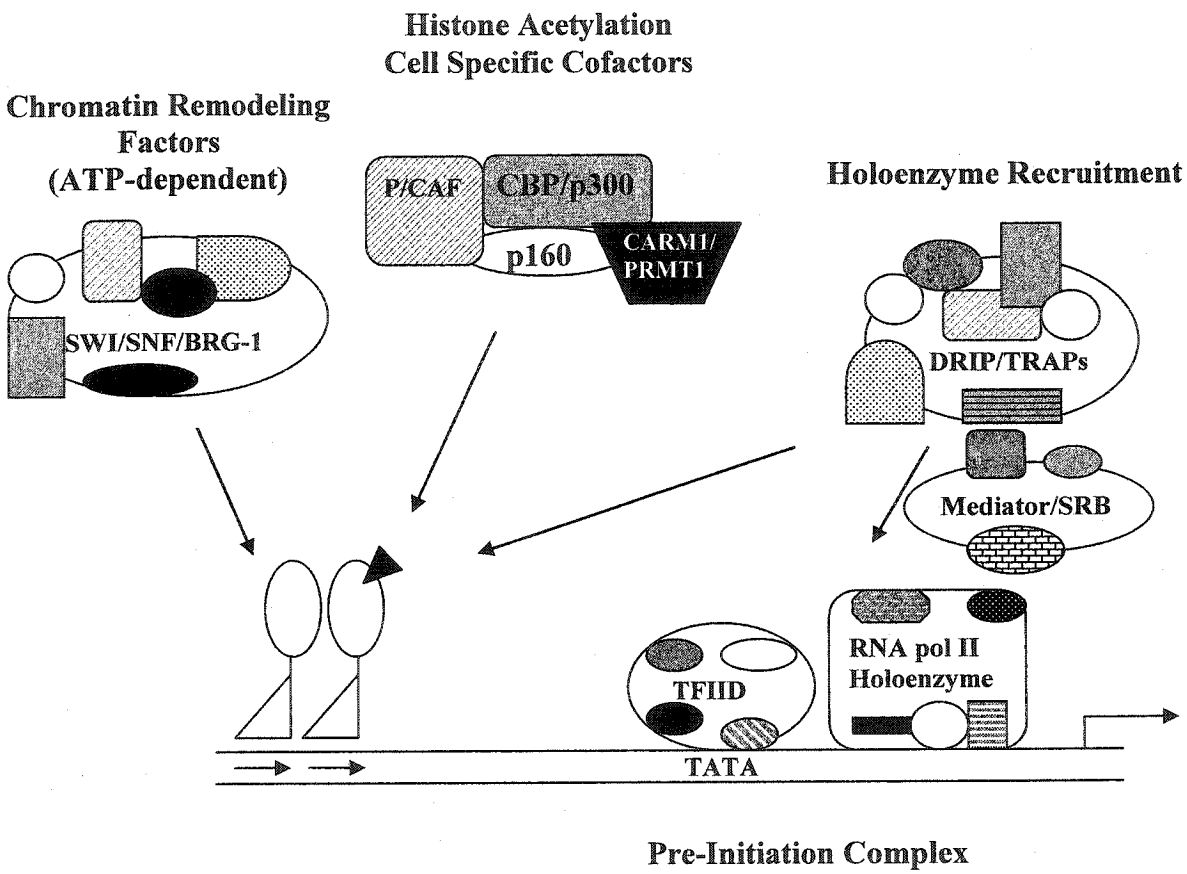

Figure 1.3. Ligand-dependent recruitment of multi-protein complexes to the promoter of target genes by nuclear receptors.

1.2. E2-MEDIATED SIGNALING

The natural estrogen, 17β-estradiol (E2) is a small lipophilic molecule that crosses the cell membrane and enters the nucleus where it binds to the estrogen receptor. In absence of hormone, it was initially thought that all steroid hormone receptors exist as inactive oligomers complexed with chaperone proteins (68). However, it has subsequently been shown, by immunocytochemistry and hormone binding assays, that the subcellular localization of ERα is the nucleus (69, 70).

1.2.1. ERα and ERβ

In 1996, it was discovered that the action of estrogen is mediated not only by estrogen receptor (ERα), but by a second estrogen receptor, ERβ (71-73). The two receptors are highly homologous in the DNA-binding domain (region C) and ligand-binding domain (region E) with 96% and 60 % homology respectively, but they differ significantly in the N-terminal A/B domain and hinge region (Figure 1.4.) (72). The ERβ protein is slightly smaller than ERα (530 aa vs 595 aa), is located on a different chromosome and has overlapping but distinctly different tissue distributions compared to ERα. ERα is most highly expressed in the pituitary, vagina, uterus and breast, while ERβ is most abundant in the ovary and prostate. However, ERβ is also present in mammary glands, bone, uterus, central nervous system and cardiovascular system. (74).

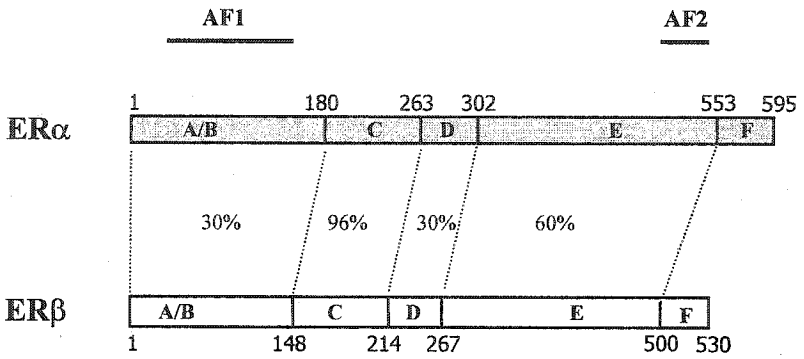

Figure 1.4. Schematic diagram comparing the structural domains of ERα and ERβ

Although ERα and ERβ recognize similar response elements, the transcriptional activity of the two receptors is gene- and cell-specific. For example, transcription of the pS2 gene, regulated by an ERE in its promoter region, is mediated by an active AF-1 and AF-2 domain of ER. Since ERβ has a weak AF-1 activity even in the presence of E2, it activates the pS2 promoter more weakly than ERα (75). Tamoxifen, which inhibits only the AF-2 activity of ERα and ERβ, is thus a more potent antagonist with ERβ, as opposed to its partial agonistic properties on an ERE with ERα (76).

In breast tumors, ERα is more highly expressed than ERβ, and there appears to be no correlation between ERα and ERβ expression (77). Although studies with ERα KO mice have demonstrated that ERβ does not mediate E2-dependent growth and development of the mammary gland, the role of ERβ in tumorigenesis remains to be determined (78). About 60-70% of breast epithelial cells express ERβ at all stages of

breast development, while ERα expression varies according to the developmental stage of the mammary gland (79). While most breast tumors express ERα alone or in combination with ERβ (80), there is some controversy as to the role of ERβ in breast cancer. Some groups have found that ERβ correlates with low biological aggressiveness of breast cancer and can even inhibit proliferation and invasion of breast cancer cells (79, 81). In support of this hypothesis, expression of ERβ in HeLa cells is sufficient to completely inhibit cyclin D1 gene activation by estrogen (82). In contrast, others have indicated that the ratio of ERα to ERβ alters in breast cancer progression, with increased expression of ERβ in relapsed patients exhibiting tamoxifen-resistant tumors (83). To complicate matters, several ERβ splice variants have been reported (74). Many of these differ in the region of the ligand-binding domain and have been identified in human breast tumors, as well as normal mammary tissue (84, 85).

1.2.2. Ligand-independent activation of ER

The transcriptional activity of ER can be modulated by signaling pathways, independently of ligand binding. Activation of MAPK pathways can lead to phosphorylation of serine residues in the N-terminal of the ERα and can lead to transcriptional activation. One of the best-characterized example of this is the activation of ERα by the epidermal growth factor (EGF). Several groups have shown that EGF can activate an ERE in a ligand-independent mechanism via serine phosphorylation in the AF-1 domain of ERα (76, 86). However, it has also been demonstrated that EGF can increase ERE-mediated reporter activity independently of ERα, indicating that the mechanism for cross-talk between ERα and EGF is not fully understood (87). Similarly,

increased cAMP levels and PKA activity have also been associated with ligand-independent ERα transactivation (88). Phosphorylation of serine residues by PKA in the N-terminal region of ERα causes an association with cyclin D1, leading to increased ligand-independent transactivation (89).

Modification of ERα-dependent transcription by extra-cellular ligands has also been observed with EGF, TGFα and IGF-1 (90). In addition, TPA, a PKC activator has also been shown to activate ERE-mediated transcription (90). This ligand-independent activity is mediated by the AF-1 region of ERα (90).

1.2.3. Classical signaling pathway of ER

ER recognizes consensus palindromic response elements of [5'(A/G)GGTCA-3'] motifs separated by 3 bp. The selectivity of the binding of ER to its response elements is due to contact of amino acids in the C-terminal part of the first zinc finger (P-box) with base pairs and phosphates. The A box, downstream of the second zinc finger, is involved in recognition of base pairs upstream of the ERE motif (91). Interestingly, the consensus sequence half site recognized by ER as a palindrome is identical to the half site motif comprising the direct repeat recognized by RAR/RXR.

In addition to promoter elements containing classical EREs, the ER can regulate transcription from promoters containing non-consensus EREs or low affinity half palindrome EREs (91, 92). While a few ER-regulated genes contain well-characterized EREs (Ex. pS2, lactoferrin, oxytocin, c-fos), many contain only a half-ERE and additional Sp1 sites (Ex. cathepsin D, RARα, c-myc, PR, hsp27) (reviewed in (91, 93)). Sp1 is a transcription factor that recognizes GC-rich motifs in the promoter of target

genes. Activation through $Sp1_{(N)}xERE_{1/2}$ requires interactions of both proteins with their cognate DNA elements (94). It has been demonstrated that ER and Sp1 physically interact with each other to allow the recruitment of cofactors required for transcription (94).

1.2.4. Non-classical signaling pathway of ER

In addition to its genomic action at classical EREs, ER can alter the regulation of target genes without directly binding to DNA. Several genes, containing only Sp1 or AP-1 binding sites in their promoters, can be altered by ER ligands. The mechanism for this appears to involve the tethering of ER to cofactors or directly to the AP-1 or Sp1 transcription factors (94, 95).

Even in absence of a known ERE in the promoter region, ER can modulate the transcription of the Sp1-regulated genes c-fos, TGF-α, RARα, bcl-2, IGFBP-4 and thymidylate synthase (94, 96, 97). This transcriptional modulation, which occurs by direct binding of ER to Sp1, appears to be ligand and cell context dependent. For example, ERα/Sp1 is activated by estrogen and antiestrogen in MCF-7 and MDA-MB-231 breast cancer cell lines, but not in HeLa cells. In contrast, ERβ/Sp1 exhibits minimal activity, regardless of the ligand (98). Although the mechanism and regulation of transcriptional activation at promoters containing both Sp1 sites and EREs is not well characterized, it has been suggested that the AF-1 domain of ERα is required for activation by Sp1 (98-101).

ER can also modulate the transcriptional activity of AP-1-mediated gene expression without binding to its response element. AP-1 is a transcription factor consisting of Jun-Jun homodimers or Jun-Fos heterodimers, whose activity is important for cellular differentiation, proliferation and transformation. Several groups have demonstrated that ER binds members of the Jun family (c-Jun and JunB), but not to the Fos family (102, 103).

The action of ER on AP-1 promoters varies depending upon the subtype of the receptor (α or β), ligand and cell-context (14, 95). When bound to estradiol, ERα activates transcription from an AP-1 element, whereas ERβ does not. However, in response to tamoxifen, both ERα and ERβ generally activate transcription (104). In addition to the effect of ligand and ER subtype, AP-1 activity is modulated in a cell-specific manner. In the breast, estrogens increase AP-1 activity whereas anti-estrogens inhibit this activity. However, in uterine cells, tamoxifen activates AP-1 activity, thereby contributing to pathways associated with cellular proliferation (105).

The cross-talk between ER and AP-1 is complex, and the mechanism for ER-mediated AP-1 activity is not fully understood. ERα enhances AP-1 mediated transcription in a manner that requires an active AF-1 and AF-2, but is independent of the DBD (14). It is generally thought that ERα aids in the formation and stability of a multiprotein complex, involving p160 coactivators, at AP-1 elements (14, 102). Alternatively, SERM-bound ER does not appear to be present in a complex of Jun/Fos. It has been proposed that SERM-bound ER recruits corepressors and HDACs away from the AP-1 site, allowing unopposed activity of coactivators recruited by Jun/Fos (14, 106).

Cyclin D1 is an important cell cycle-regulating protein that is differentially regulated by ERα and ERβ via an AP-1 site in its promoter. There is a strong correlation between enhanced expression of ERα, increased cyclin D1 expression, and a proliferative response (107). In HeLa cells, ERα enhances transactivation of cyclin D1 in response to estrogen, while ERβ decreases it (108). Furthermore, ERβ blocks ERα-mediated induction when both receptors are present, suggesting that ERβ may oppose the proliferative effect of ERα [Liu, 2002 #2132].

In addition to the effect of ER on AP-1 and SP-1 elements, ER cross-talk occurs with the NF-κB pathway. In the myocardium, estrogenic compounds can inhibit NF-κB-dependent gene expression of pro-inflammatory cytokines by competing for p300 [Pelzer, 2001 #2118]. In the bone, ER inhibits NF-κB-mediated transcription from the IL-6 promoter by binding to the Rel homology domain of NF-κB and the bZIP region of C/EBP beta (109). In another promoter context, NF-κB complexes cooperate with ERα to recruit cofactors. At the serotonin-1A receptor promoter, NF-κB and ERα synergize through non-classical EREs by a mechanism that does not involve direct receptor binding to DNA (110).

1.2.5. Non-genomic effects of estradiol

The existence of rapid effects elicited by estrogens suggested a non-transcriptional role for ER. Although the mechanisms leading to the rapid effects of estradiol are not fully understood, there is evidence for the presence of membrane-

bound ER (111) and transfection studies suggest that both ERα and ERβ can localize to the cell membrane (112).

Non-genomic actions of ER have been demonstrated at the vascular wall level, where estrogen triggers rapid vasodilation mainly due to increased nitric oxide (NO) release (113). In endothelial cells, the physical and functional coupling of ER to the lipid kinase phosphatidylinositol 3-OK kinase (PI3K) at the cell membrane leads to activation of the PI3K signaling cascade and ultimate release of NO (113).

In breast cancer cells, estradiol can increase cAMP levels independently of its genomic role. The increase in intracellular concentration of cAMP is sufficient to stimulate cAMP RE-mediated (CRE) gene transcription (114). In addition, membrane-associated ER may contribute to the proliferation of breast cancer cells by activating MAPK and Akt kinase signaling in the presence of estradiol (115). Furthermore, membrane-bound ER has been found to be associated with Her2/neu and may mediate resistance to tamoxifen-induced apoptosis (115). These non-genomic actions of ER involve the activation of important secondary messenger-stimulated genes.

1.3. BREAST CANCER

Despite advances in the treatment and understanding of breast cancer, this disease remains the leading cause of mortality for women in the industrialized world, second only to lung cancer (116). It is estimated that a woman's lifetime risk of being diagnosed with breast cancer is currently 1 in 8 (117).

The mammary gland only becomes fully differentiated at the time of pregnancy and subsequent lactation (118). It consists of 15 to 20 lobes, surrounded by adipose tissue.

Within each lobe are smaller lobules that end in dozens of secretory alveoli important for milk production. Linking the lobes, lobules and alveoli are the ducts. The ducts are composed of a luminal epithelial layer of cells and a basal myoepithelial layer, both of which are surrounded by a basement membrane. The majority of breast tumors arise from luminal epithelial cells that line the ductal and lobular structures of the mammary gland (116).

The maturation of the mammary gland is mediated by estradiol, which is synthesized by the ovaries under the control of the pituitary gonadotrophins (118). Estradiol is required for breast and uterine development, bone homeostasis, cardiovascular and central nervous system functions. In the breast, estradiol has a growth stimulatory effect that is linked to carcinogenesis. Susceptibility to breast cancer has been attributed to lifetime exposure to estrogen and as such, early menarche, late menopause, and nulliparity are correlated with an increased lifetime risk of developing breast cancer (116)

1.3.1 Molecular Mechanism of Breast Cancer

It is becoming evident that nuclear receptors serve as a point of convergence for multiple signal transduction pathways. Tumorigenesis can occur when disruption of these pathways leads to uncontrolled cellular proliferation. In the breast, cellular proliferation is guided by estradiol.

Upon binding of estradiol to the ER, the transcription of several genes is activated (Figure 1.5.). Some growth potentiating genes, like TGFα, are activated by E2 and secreted. In an autocrine fashion, TGFα binds the EGFR receptor located on the

surface of epithelial cells, resulting in a cascade of events supporting growth stimulation At the same time, E2-mediated transcription causes an increase in IGF1, which is released from the cell and activates growth stimulatory pathways in a paracrine fashion by binding to IGFR (119).

E2 also stimulates the transcription of c-Myc, a transcription factors thought to be a mediator of mitogenic stimulation (120, 121). Another gene thought to be involved in stimulation of cell growth is cyclin D1. Activation of cyclin D1 is associated with entry into the S phase of the cell cycle (122). The effect of estrogen is not limited to the activation of growth-stimulatory pathways. It also inhibits the transcription of the growth inhibitory molecule TGFβ (123).

Many growth factor signaling pathways modulated ER-mediated transcription via phosphorylation. For instance, the binding of IGF-1, EGF and TGFα to surface tyrosine kinase receptors leads to a MAPK cascade culminating in the phosphorylation of ERα and increased transcription from ERE-containing promoters (124).

Breast cancers often progress from hormone-dependent, antiestrogen-sensitive to hormone-independent, highly invasive phenotypes. Loss of the estrogen receptor leads to increased expression of several genes involved in invasion and metastasis. Increased expression of genes involved in the matrix metalloproteinase family (MMP) have been observed in ER-negative cells. These genes can be modulated by the AP-1 and NF-κB family of transcription factors. For example, NF-κB, which is usually maintained in an inactive state by protein-protein interaction with IκB, was found to be constitutively active in ER-negative breast cancer cells and primary breast tumors (125). NF-κB has been shown to protect cells from TNF-α, ionizing radiation and

chemotherapeutic agents and may thus contribute to the increased malignant phenotype of ER-negative tumors.

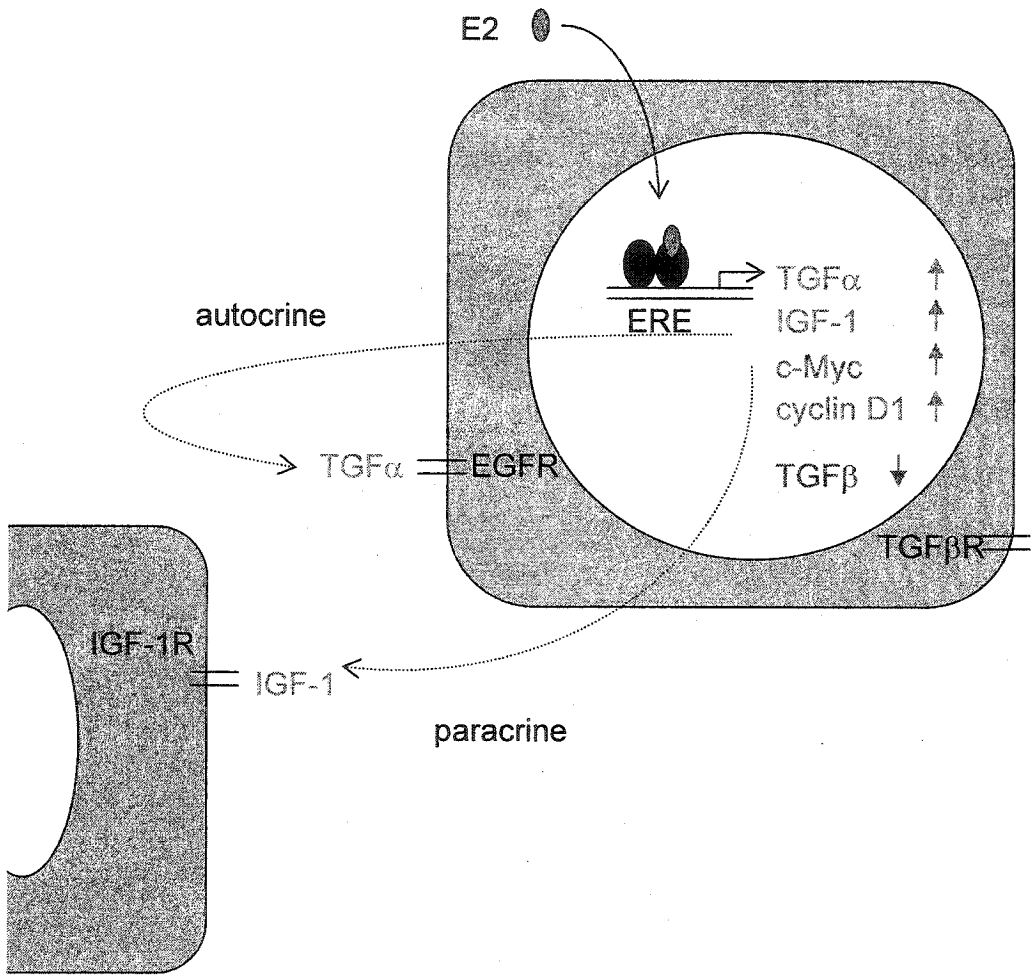

Figure 1.5. Activation of ER by its ligand, estradiol (E2), regulates genes involved in the proliferation of breast cancer cells.

1.3.2. Chemoprevention

The treatment and management of breast cancer involves both chemopreventive and chemotherapeutic agents. A combination of treatments consisting of endocrine therapies (ovarian ablation, aromatase inhibitors, SERMs), cytotoxic chemotherapy or targeted intervention (Herceptin) offer the best outcome for breast cancer patients. The objective of chemoprevention therapy is to use noncytotoxic agents to protect against the development and progression of mutant cells to malignancies. Interesting chemopreventive agents currently in development are the selective estrogen receptor modulators (SERMs) and the retinoids (126).

SERMS

The management of breast cancer has focused on targeting the growth stimulatory pathways of E2 detailed above (Fig. 1.5.). SERMs are classes of drugs that antagonize the growth-stimulatory properties of estrogen in the breast by binding to the estrogen receptors and inhibiting its transcriptional activity. The ideal SERM would have antiestrogenic effects in the breast and endometrium, and have estrogenic effects on lipid profiles, the central nervous system, skeleton, vagina and cardiovascular system.

Tamoxifen is one SERM that is currently in use for the treatment of breast cancer. It is a partial antagonist exhibiting antagonistic properties in the breast and estrogenic activity in the uterus and bone. One of the limitations of tamoxifen, other than its lack of efficacy in ER-negative tumors, is that it causes an increased risk of uterine cancer (127). Additionally, the beneficial actions of endocrine therapy are often short-lived and acquired resistant to treatment invariably occurs. The development of

SERMS with greater specificity combined with an understanding of resistance will benefit the hormonal therapy of breast cancer.

Retinoids in Chemoprevention

Vitamin A (retinol) and its derivatives are required for several important physiological processes, including vision, reproduction, metabolism, differentiation, hematopoiesis, bone development and normal embryonic development (128). In animal models, vitamin A deficiency has been associated with a higher incidence of cancer (129). Much evidence supports the notion that retinoids can prevent cancer by inhibiting progression from premalignant to malignant stages (130-132). Retinoids have been shown to modulate normal rat mammary epithelial cell proliferation, morphogenesis and functional differentiation (133).

1.4. RETINOIDS

Retinoids comprise a large group of compounds that have the ability to bind to retinoic acid receptors. The most well characterized and biologically active retinoids are all-*trans* and 9-*cis* retinoic acid (ATRA and 9-*cis* RA respectively) (Fig. 1.6.).

1.4.1. Biosynthesis and Metabolism of Retinoic Acid

Vitamin A (retinol) is obtained from the diet, particularly from eggs, milk, butter and fish-liver oils. It can also be obtained from plant origins in the provitamin β-carotene form. Following digestion and the oxidative cleavage of carotenoids to retinal

and reduction of retinal to retinol, the retinol is then converted back to retinyl esters for storage in the liver (134) (Fig. 1.6.). When needed, liver retinyl esters are cleaved and retinol is released to be delivered to other parts of the body (134). In the blood, retinol, which is the most abundant circulating retinoid, is bound by retinol-binding protein. Retinol can cross the cell membrane, where it is converted to retinal by a reversible dehydrogenation reaction, involving alcohol dehydrogenase (ADH), short chain dehydrogenase (SDR) and Cyt P450 (134). Retinal is then converted to ATRA by a non-reversible reaction mediated by retinal dehydrogenases. Isomerization of ATRA into 13-cis-RA and 9-cis-RA can then occur spontaneously or via isomerases (134).

1.4.2. Retinoid Binding Proteins

In the cell, retinol and retinal are bound by cellular retinol binding protein (CRBPI and CRBPII). These shuttle proteins serve to protect the easily oxidized retinol from nonspecific dehydrogenases (135). The closely related cellular retinoic acid binding proteins (CRABPI and CRABPII) bind RA and some metabolites, but not retinol or retinal. These cellular retinoid binding proteins protect cells against excess RA levels and thus function to modulate the concentration of ATRA available within the cells (134). In the treatment of cancer by retinoids, decreased sensitivity to RA had been attributed in some cases to increased levels of CRABP (136, 137). CRABPs also function as shuttle proteins to deliver all-trans RA to other metabolic enzymes and to facilitate retinoid transport into the nucleus (138).

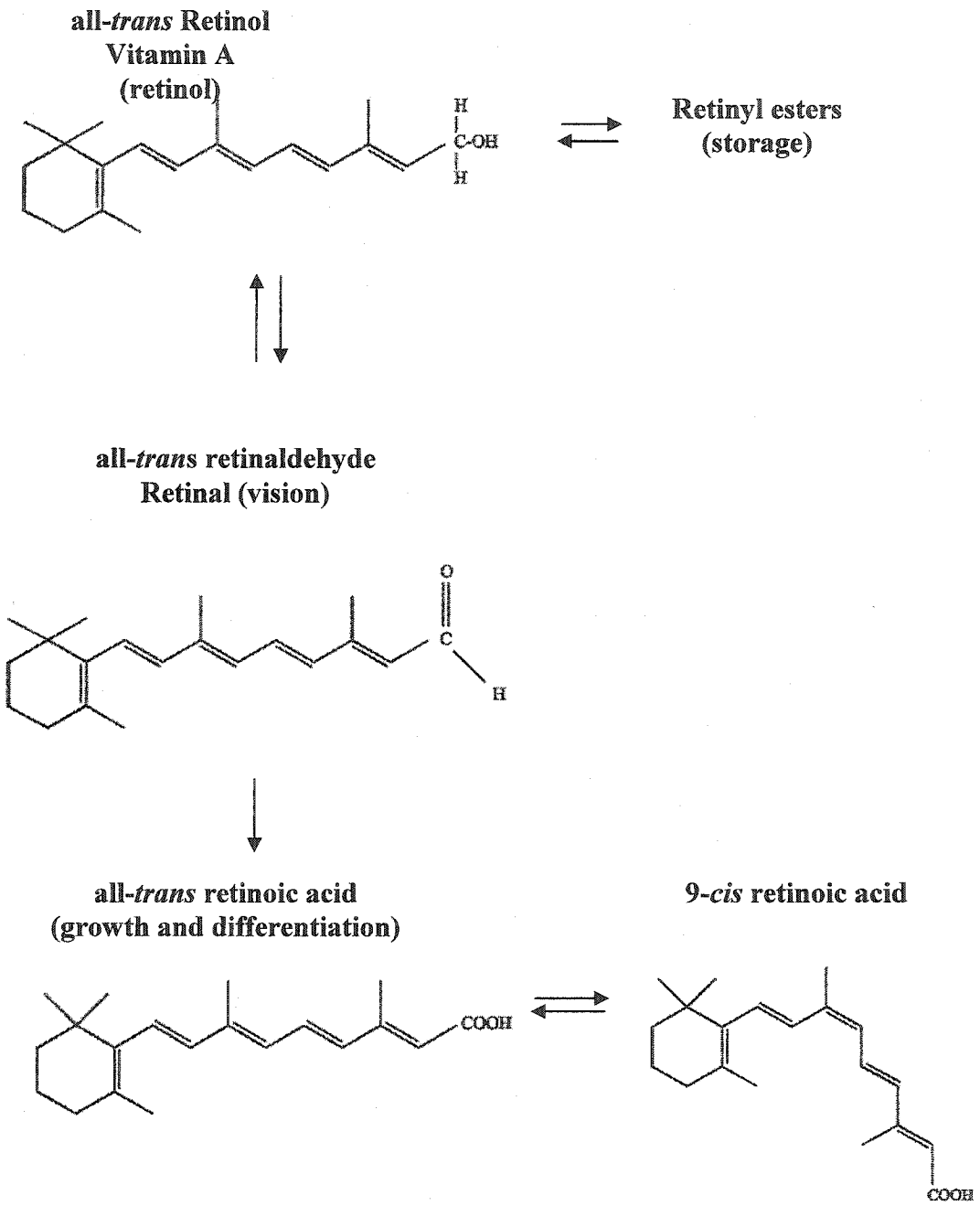

Figure 1.6. Biosynthesis of retinoic acid

1.4.3. Retinoic Acid Receptors – Direct regulation of transcription

Retinoids act by binding to members of the retinoic acid nuclear receptor family, RAR and RXR, each comprising of isotypes, α, β and γ, encoded by separate genes. Alternative splicing, or alternative use of distinct promoters, results in several isoforms differing in the N-terminal region. ATRA and its stereoisomer 9-*cis*-RA are the natural ligands for RARs, whereas RXRs bind only 9-*cis* RA (139). RAR and RXR bind as heterodimers to retinoic acid response elements (RAREs). In the absence of ligand (or in the presence of an antagonist), RAR/RXR heterodimers bind to corepressors (NcoR or SMRT) and recruit histone deacetylases (HDACs), resulting in a condensed chromatin, unavailable to the transcription machinery. When ligand binds to the nuclear receptor, it undergoes a conformational change that favors the release of corepressors and the binding of coactivators, rendering the chromatin more accessible to the transcription machinery, thereby activating transcription.

Retinoic acid receptors recognize response elements containing a minimal half site consensus sequence [5'-AGGTCA-3'] that can be configured into a variety of structured motifs, including direct repeats and palindromes (140). The classical response element for RAR/RXR heterodimers is a DR5, where direct repeats of [5'-AGGTCA-3'] are spaced by 5 nucleotides (Fig 1.2). Examples of genes containing this classical retinoic acid response element in their promoters include RARβ2 and alcohol dehydrogenase 3 (141). Direct repeat response elements spaced by 2 nucleotides (DR2) can also be found in the promoter region of retinoid-regulated genes, as is the case for CRBP1 (142).

RAR/RXR heterodimers can also recognize direct repeat response elements with a spacing of 1 (DR1), although this response element is less efficient than DR2s or DR5s (142). In the binding of RAR/RXR to a DR1 element, the RAR is in the 5' position, with RXR in the 3' position on the response element (142, 143). A DR1 element is also more promiscuous, allowing the binding of RXR heterodimers of COUP-TF and PPAR, in addition to RAR (Fig 1.2). Examples of retinoid-regulated genes that contain response elements with a DR1 motif include CRBPI and CRABPII (142, 144).

An overview of the regulation of transcription involving RXR heterodimers is illustrated in Figure 1.2. The dual function of RXR as a homodimer and heterodimer is complex. It has been proposed that the relative concentration of each receptor in the cell may affect the interaction of RXR with a particular response element (140). In addition, RXR heterodimers are more stable and have a higher affinity for DNA than the RXR homodimers (142). The availability of ligands may also influence that interaction of RXR with a particular partner. For example, RXR homodimers are favored by high concentrations of 9-cis RA ligand. Furthermore, the binding preference of a particular RXR heterodimer partner depends upon the nucleotides located at positions 1, 2 and 4 of the core motif, as well as the spacer nucleotide (145).

Differential display, subtractive hybridization and gene array experiments have been useful in identifying retinoid-regulated genes. Although as many as 532 genes have been proposed to be RA targets, most of these are believed to be indirectly induced by retinoids, via intermediate transcription factors or non-classical association of retinoic acid receptors with other proteins (146). Balmer and Blomhoff have compiled

published data from numerous researchers and have classified RA-regulated genes (146). Of the 532 genes analyzed, they identified 27 genes that are direct targets of RA, as mediated by liganded heterodimer of retinoid receptors to known RAREs. Of interest, two important subsets of genes emerged. The first includes genes that are involved in retinoid handling and metabolism. The second subset consists of genes containing homeobox domains, thereby underlining the importance of retinoids in development. Increasingly, gene array technologies have been exploited to find novel retinoid target genes. Since many newly identified genes do not contain recognizable retinoid response elements, their regulation is most likely indirectly mediated by retinoids (147).

1.4.4. Retinoic Acid Receptors - Indirect regulation of transcription

Like ER, the retinoid receptors can also mediate gene expression in an indirect manner, without binding to a cognate RARE. Importantly, retinoids antagonize the expression of genes with AP-1 enhancer elements in their promoters. Although AP-1 transrepression mediated by RARs is dependent on ligand, an active AF-2 is not required (148). The mechanism for this regulation is not known but may involve competition for limited amounts of cofactors, formation of abortive complexes with DNA-bound transcription factors, inhibition of phosphorylation of c-Jun by inactivation of Jun amino terminal kinase (JNK) signaling pathway, or disruption of Jun-Fos heterodimers (130). Cross-talk with AP-1 results in retinoid-mediated down-regulation of genes involved in proliferation and inflammation. Such genes include metalloproteases (stromelysin-1, collagenase, and gelatinase), proto-oncogenes (c-fos

and c-myc), growth factors and their receptors (TGF-β1, EGFR, IL-6 and IL-6R) (141). In addition, NF-κB-mediated transcription is also altered by retinoids, although both positive and negative regulations have been reported in different cell types (149, 150). In APL cells, synergy has been demonstrated between retinoids and TNF, leading to induction of NF-κB target genes and differentiation (150).

1.5. RETINOIC ACID RECEPTORS AND CANCER

Since retinoids are involved in regulation of cell growth and differentiation, it is not surprising that changes in expression or function of retinoid receptors have been found in malignant cells.

1.5.1. Acute Promyelocytic Leukemia

Perhaps the most extensively studied disease involving a deregulation of retinoic acid receptors is acute promyelocytic leukemia (APL). APL is characterized by increased promyelocytes that are unable differentiate into granulocytes. In greater than 95% of affected individuals, this disease is distinguished by a reciprocal translocation between a region on chromosome 17 and chromosome 15, resulting in a fusion between RARα and PML (140). Of the two resulting fusion gene products, RARα-PML and PML-RARα, it is believed that only PML-RARα contributes to the disease state by blocking promyelocytic differentiation (151). This fusion protein contains the DBD and LBD of RARα. PML-RARα has a higher affinity for HDACs, which is further enhanced by oligomerization of the fusion proteins, thus generating a super-repression of RARα signaling pathways in APL blasts. Treatment of APL with high doses of all-

trans RA results in the dissociation of the repressor complex and relieves the HDAC-dependent block of differentiation, resulting in clinical remission (141).

1.5.2. Aberrant expression of retinoic acid receptor β

As mentioned previously, the RARβ promoter contains a classical RARE and consequently, its expression is increased by RA. In a number of different human carcinomas, including breast, lung, head and neck, and prostate, the expression of RARβ is greatly reduced (152-156). Since the loss of RARβ expression is present in various malignancies, it has been proposed that loss of this gene is an important event in tumorigenesis, and that RA-induced growth suppression is in part mediated by restored RARβ expression (157). In support of this hypothesis, there exists a correlation in vivo between induction of RARβ and clinical response to retinoids in patients with oral premalignant lesions and patients with renal cell carcinoma (158, 159). Furthermore, it has been demonstrated that transfection of RARβ into RARβ-negative cervical, breast, and lung cancer cells increases cell responsiveness to retinoids (156, 160). Although this correlation has not yet been established in patients with breast cancer, introduction of RARβ into RA-insensitive breast cancer cell lines restores RA responsiveness to growth inhibition (161).

All three RAR subtypes, α, β, and γ, are expressed in normal mammary epithelial tissue (116). Loss of expression of RARβ seems to be an early event in breast cancer carcinogenesis (154, 155) and it is proposed that RARβ may be a tumor suppressor gene. In 48% of breast cancer specimens, loss of heterozygosity was

detected at chromosomal region 3p24, a locus that includes the region coding for RARβ (162). In breast cancer cells, there is a progressive decrease in RARβ expression level during carcinogenesis, and investigators have demonstrated that RARβ is consistently down-regulated or lost (154, 155, 163). In addition, the induction of RARβ by RA appears to correlate with the growth-inhibitory effect of retinoids (164, 165).

There are 3 isoforms of RARβ - β1, β2, and β4 that differ in the amino terminal A-domain resulting from alternative splicing and usage of alternative promoters (P1 or P2). Of these, RARβ2 is the isoform most important for mediating the growth inhibitory effects of RA (157).

RARβ and ER status

A clinical study of the retinol, fenretinide, found a beneficial effect in breast cancer chemoprevention only in premenopausal women, suggesting that there is a hormonal component to the retinoid response in vivo (166). (167)Several investigators have shown that in ER-positive but not in ER-negative breast cancer cell lines, RARβ downregulation can be reversed by RA (164, 168). Although some groups have found no relation between the decrease in RARβ expression in breast cancer development and ER status (154), others have shown that expression of RARβ can be upregulated by RA in ER+ but not in ER- breast cancer cells (163, 169, 170). When an RARβ expression vector was introduced into hormone-independent cells, these acquired sensitivity to RA (164). Similarly, when RARβ antisense or an RARβ antagonist was added to hormone-dependent cells, the RA-sensitivity was inhibited (164).

Although the mechanism of growth inhibition by RA in breast cancer cells is unknown, there is evidence for a two-way interactive regulatory pathway between the ER and RAR. As such, RA can downregulate ER (171) and can inhibit downstream E2-induced gene expression (172, 173). However, the mechanism of growth inhibition by retinoids extends beyond the inhibition of ER-mediated growth-proliferation, since retinoids and anti-estrogens exhibit additive effects in chemoprevention of breast cancer in animal models (174).

RARβ promoter

The biologically active RARβ2, is under the control of the P2 promoter, containing a high affinity RA-responsive element (RARE) (Fig 1.7). This sequence contains a direct repeat of the motif [5'-GTTCAC-3'], spaced by 5 nucleotides, that is recognized by RXR-RARα heterodimers (175). The expression of RARβ is believed to be regulated by RARα. This isotype is highly expressed in many cell types and has been shown to regulate RARβ expression upon ligand binding (176).

Transcription from the natural RARβ2 promoter is also regulated by sequences surrounding it. Sequences upstream from the RARE include a c1`1`AMP response element (CRE) and TRE-like sequences (for binding of AP-1 elements). Both have been found to contribute to RA-dependent activation of this promoter (177). In addition, the RARβ promoter contains a series of response elements for other known transcription factors. Located 3' to the TATA box is an Sp1 binding site and an Octabox. More recently, 2 AP-1 sites, an AP-2 site and a second Sp1 site have been identified in this

promoter (178). Also downstream from the βRARE is the TATA box and the INR. The INR is a pyrimidine-rich region that is located around the transcription start site and is believed to affect TFIID recruitment and/or function. In EC cells, in vivo footprinting experiments have identified an INR element which is occupied in an RA-dependent manner and contributes to promoter activity (179). Distinct differences in complexes binding to the 5' regulatory region of the RARβ promoter may account for the cell-type specificity in the expression of RARβ by RA (165).

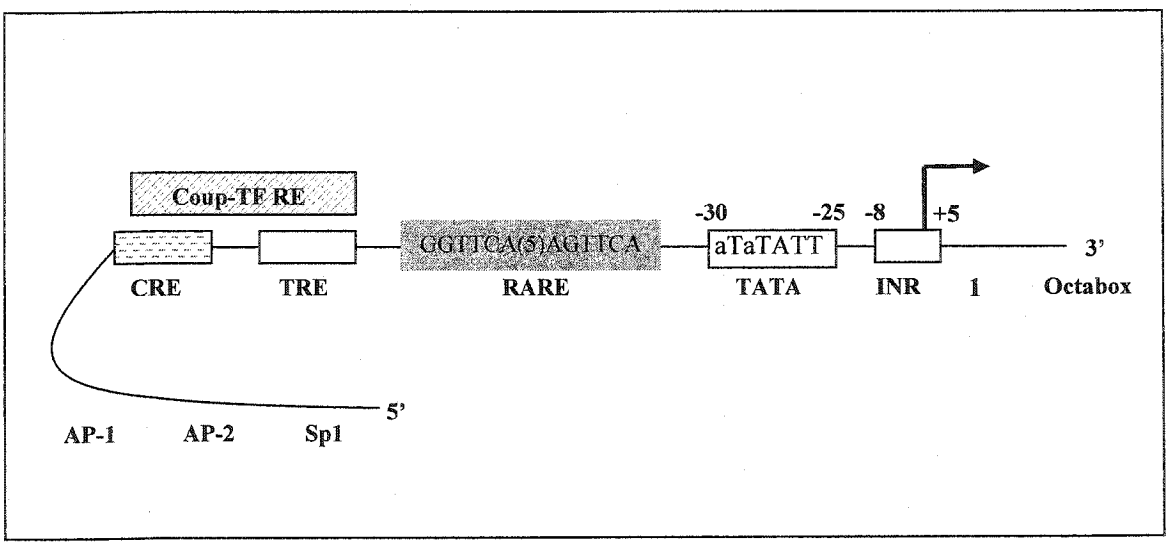

Figure 1.7. Schematic representation of the RARβ2 promoter (CRE– cyclic AMP response element, TRE- TPA response element, INR– initiator-like sequence, RARE- retinoic acid response element, Coup-TF RE- COUP-TF response element)

Regulation of RARβ expression

It has been suggested that the decreased expression of RARβ in breast cancer cells is a consequence of limiting amounts of cofactors available to enable transcription.

(169). Other possible mechanisms for the decrease of RARβ expression in breast cancer cells include availability of RA, RAR/RXR expression levels, promoter architecture and the presence or levels of orphan receptors (179). For example, studies have shown that the orphan receptor COUP-TF induces RARβ expression in a RA- and RARα-dependent manner by binding to a DR8 element in the RARβ promoter. This acts by enhancing the interaction of RARα with CBP and thus activating transcription. (180). Conversely, in lung cancer cells, overexpression of the orphan receptor nur77 has been found associated with a loss of RARβ inducibility and RA resistance (181). In lung cancer cells, COUP-TF is highly expressed in ATRA-sensitive cell lines while nur77 is associated with ATRA resistance (129).

Furthermore, epigenetic changes such as methylation of CpG islands in the promoter region and acetylation of chromosomal histones have been shown to alter the expression of RARβ (182). As discussed previously, methylation at the promoter results in a more repressive chromatin state via recruitment of a Sin3A/HDAC corepressor complex. However, the methylation status of the RARβ promoter in various cancer cell lines has yielded conflicting reports (181, 183-186).

Antitumor Activity of Retinoids in Breast Cancer Cells

The antineoplastic mechanism of retinoids is not well understood but there is evidence that RARβ plays an important role in the differentiation of many cell types (160, 168). Loss of RARβ is associated with tumor progression and inducibility of the RARβ2 promoter leading to expression of RARβ correlates with the growth inhibitory

effects of RA (130). In breast cancer cells, comparison of breast cancer biopsy specimens and non-neoplastic breast tissue indicated a correlation between RARβ2 silencing by methylation of the promoter, with tumor progression (183).

However, the question remains, what is the role of RARβ in the demonstrated antitumor activity of RA in breast cancer? One possibility could be the unique ability of RARβ to inhibit AP-1 activity and thus inhibit the activity of the oncogenes c-Jun and c-Fos on AP-1 containing genes (187). In tumor models involving chemical carcinogenesis of the skin, retinoids have been shown to block the promotion step by inhibiting TPA-induced AP-1 activity (188). In lung-tumor derived cell lines, transfection of RARβ2 leads to induction of several genes, as identified by array analysis, whose functions have demonstrated involvement in apoptosis or the host's immune response (189).

Mitogenic stimulation has been found linked to enhancement of AP-1 activity, while growth inhibition by RA is paralleled by decreased AP-1 activity. Cells that are unresponsive to RA have an increased AP-1 background activity that is not repressed by RA (190). Similarly, when the AP-1 activity of MCF7 cells was increased by stable transfection of cJun, the cell line became resistant to RA (191).

Other possible mechanisms to explain the antitumor effects of retinoids include induction of TGF-β, IGFBP-3, and reduced bcl-2 levels. Increased IGFBP-3 generally acts to counteract the growth-promoting effects of insulin-like growth factors (IGFs) (192).

Besides these changes, retinoids have also been shown to reduce the activity of aromatase, which catalyzes the rate-limiting step in estrogen biosynthesis (181).

Aromatase activity in tumors or surrounding tissue may play a role in promoting tumor growth due to the mitogenic effect of estrogen. An approach to breast cancer therapy has thus been to reduce estrogen production by aromatase (193).

The tumor-suppressive function of retinoids has been associated with apoptosis, growth arrest and differentiation, and recent studies have even linked retinoids to induction of senescence in some tumors (194). However, it is not clear to what extent, if any, the induced RARβ contributes to the response to growth inhibitory effects of RA in ER-positive cells, and the precise function of RARβ is still unknown.

1.6. TRANSCRIPTIONAL CROSS-TALK

The control of transcription involves an integration of signals stemming from signal transduction pathways to nuclear transcription factors such as the nuclear receptors. The end result of gene activation requires large protein complexes needed to remodel chromatin and render the promoter accessible to the pre-initiation complex. The complexity of transcriptional regulation, including common co-regulatory proteins and a myriad of regulatory switches involving phosphorylation and methylation, provide numerous possibilities of cross-talk between nuclear receptors.

Examples of cross-talk have already been discussed in other sections of this literature review. Clinical studies and experiments with cell lines have both provided evidence for a role of ERα in RA-mediated growth inhibition in breast cancer cells. There is evidence that RARα may be higher in ERα-positive cell lines (163, 195). Furthermore, an ERα-negative cell line stably transfected with ERα has a restored ability to respond to the growth inhibitory effects of RA, as well as increased RARα

expression (196). In addition, retinoids can act on estrogen pathways, thereby raising the possibility of cross-talk between these two growth-regulating pathways (171, 197, 198).

SPECIFIC AIMS

Retinoids have had recent clinical success in acute promyelocytic leukemia and in the chemoprevention of solid tumors. Although they have not been extensively tested clinically in breast cancer, retinoids can inhibit the growth of breast cancer cells in vitro and the development of breast cancer in animal models. While there is considerable evidence that the inhibition of human breast cancer cell growth by retinoic acid is modulated by expression of estrogen receptor, the mechanism of retinoid action in these cells has yet to be defined. The goal of this project was to explore the interaction between the expression of estrogen receptor in human breast cancer cells and their response to retinoids. The role of the estrogen receptor in RA response is further complicated by the recent discovery of another estrogen receptor, ERβ. As such, it was also my objective to characterize the contributions, if any, of ERβ in retinoid-mediated growth arrest.

For this purpose, the following specific aims were developed:

(1) Define the functional domain of ERα required to confer retinoid sensitivity to RA-resistant ER-negative breast cancer cells.

(2) Contrast retinoid-induced transcriptional activation and protein binding to retinoid response elements (RAREs) in ER-positive and ER-negative cells.

(3) Determine whether expression of ERα alters expression or function of transcriptional intermediates.

(4) Study the effects of ERα or ERα-deletion mutants on transcription from the RARβ promoter, a gene regulated by retinoids and potentially involved in retinoid-mediated growth inhibition.

(5) Generate ERβ-positive stable transfectants of the ER-negative breast cancer cell line MDA-MB-231 and characterize the growth and transcription effect of ERβ on retinoid-mediated pathways.

The purpose of characterizing the interaction of the signaling pathways between ER and retinoids in the suppression of cell proliferation is to provide a strategic approach to the development of innovative therapies for the treatment or prevention of breast cancer. For rational development of such novel therapies, it is important to clarify how retinoids exert their effects on breast cancer cells, how their activity is regulated by expression of ER, and what determines sensitivity vs. resistance to these compounds.

PREFACE TO CHAPTER 2

The chemopreventive properties of retinoids in breast cancer are intimately linked to expression of ERα. However, no previous studies have explored the region of ERα required to confer RA sensitivity. In this Chapter, we describe the stable transfection of ERα-deletion mutants into the parental ER-negative cells MDA-MB-231, and identify the functional domains necessary to restore responsiveness to retinoids. We focus our studies of transcriptional cross-talk on the expression and regulation of the RARβ2 gene in these cells. This gene is down-regulated in many types of cancers, including breast carcinoma, and its induction by RA may be necessary for RA-mediated growth suppression in some cell types. In this Chapter, we also examine whether ERα expression affects the transcriptional activity of other RXR-heterodimers and whether the observed crosstalk between ERα and RAR might be mediated by sequestration of several coregulators common to RAR and ER signaling.

CHAPTER 2

The N-terminal of the Estrogen Receptor (ERα) Mediates Transcriptional Cross-Talk with the Retinoic Acid Receptor in Human Breast Cancer Cells

Journal of Steroid Biochemistry and Molecular Biology 86:1-14, 2003

2.1. ABSTRACT

Transcriptional cross-talk exists between the estrogen receptor (ERα) and retinoic acid receptor (RAR) pathways in human breast cancer cells. We have previously shown that re-expression of ERα in ER-negative cells stimulates the transcriptional and growth inhibitory effects of all-*trans*-retinoic acid (tRA) by a mechanism that is independent of the ER-ligands estradiol and tamoxifen. In this study, we generated cell lines stably expressing ERα deletion mutants to elucidate the mechanism whereby ERα modulates RAR transcriptional activity. Using RT-PCR and RNase protection assays, we observed that expression of ERα suppresses basal expression of the RA-responsive gene RARβ2, while allowing it to be strongly induced by tRA. Repression of basal RARβ2 transcription was confirmed by transient expression of the reporter plasmid βRE-TKCAT, containing the RARβ2 promoter. In the ERα-negative cells, on the other hand, transcription was only weakly induced by RA. We further determined that this effect of ERα on RARβ induction required the N-terminal AF-1 containing region, including the DNA-binding domain, but was independent of the C-terminal ligand-binding domain. Consistent with these results, the ER-agonist estradiol and the AF-2 antagonist 4-hydroxytamoxifen had no significant effect on βRARE activity. Conversely, the full ER-antagonist ICI 182,780, which blocks ERα AF-1 activity, was able to completely relieve repression of basal βRARE activity. The effect of ERα is specific for RAR-mediated transcription and does not occur on promoters containing typical response elements for the vitamin D or thyroid hormone receptors. Moreover, the cross-talk between ERα and RAR does not seem to be mediated by sequestration of a number of common co-regulators, suggesting a novel

mechanism whereby the N-terminal region of ERα modulates the transcriptional activity of RAR.

2.2. INTRODUCTION

Nuclear receptors modulate transcriptional activity by binding to DNA and recruiting accessory molecules to activate or inhibit transcription. Nuclear receptors include the steroid receptors (ER, GR, AR, PR and MR) that bind to specific promoters as homodimers, the non-steroid receptors (RAR, VDR, TR and PPAR) that form heterodimers with RXR, and the orphan nuclear receptors, for which the ligands are yet unknown. Nuclear receptors share a common basic structure and contain two independent transactivation functions (AFs). The N-terminal AF-1 domain, in the A/B region of the receptor, provides basal activity in the absence of ligand, whereas the C-terminal AF-2 domain, located in region E, is dependent on binding of ligand to the receptor. In order to activate transcription via the AF-1 and AF-2 domains, the receptor also requires binding to a response element in the promoter of specific genes, via its C-domain (2-4).

The study of nuclear receptors has mainly focused on the activation or repression of transcription via the AF-2 domain. In the presence of ligand, co-activator proteins, such as the p160 family members, are known to associate with the nuclear receptors. Those proteins recruit other coregulators that act to acetylate histones, thereby opening the chromatin and activating transcription. In the absence of ligand, or in the presence of antagonists, nuclear receptors recruit the corepressors SMRT and NCoR and histone deacetylases, which inhibit transcription (11, 199, 200).

The ligand-independent AF-1 region of nuclear receptors is less well characterized. However, many studies have reported that phosphorylation of the receptors in the A/B domain modulate their transcriptional activity in absence of ligand (7, 201). For example, the estrogen receptor α (ERα) AF-1 domain is known to be phosphorylated by the MAPK pathway, the phosphatidyl-inositol 3-kinase (PI3-K)/AKT pathway and cyclin dependent kinases (CDKs), leading to hormone-independent activation of the receptor (8, 86, 202, 203). Moreover, the AF-1 domain of ERα recruits several coactivator proteins, some that bind uniquely to this domain (49, 204), and others that also bind to the AF-2 region (48, 205).

In addition, nuclear receptors can regulate transcription without binding to their cognate response elements, through the interaction with other transcription factors like AP1 and Sp1 (94, 103, 206). The mechanism of nuclear receptor signaling is thus becoming increasingly complex and the role of the nuclear receptors extends far beyond that of simple ligand-induced transcriptional activators.

Retinoids, such as retinoic acid (RA), are derivatives of vitamin A that induce differentiation in the treatment of acute promyelocytic leukemia (APL) and cause growth inhibition in a variety of other cell types, including breast cancer cells (171, 207-209). Several natural and synthetic retinoids can inhibit the development of mammary tumors and cause regression of established tumors in rats (210-212). There is also clinical evidence that retinoids may be beneficial in breast cancer prevention (166, 213). Retinoids mediate their effects by binding to the nuclear receptors RAR and RXR, which form heterodimers that activate transcription of genes containing retinoic acid response elements (RAREs) in their promoter regions (214).

Cross-talk between the RAR and ERα pathways in human breast cancer cells has been suggested by evidence that inhibition of growth by RA in human breast cancer cells correlates with expression of ERα. In general, ERα-positive cells are growth inhibited in response to retinoids, whereas ERα-negative cells are usually resistant (171, 196, 197, 215-217). We have previously demonstrated that re-expression of ERα in an ERα-negative human breast cancer cell line modulates RAR signaling (197). We found that stable expression of ERα in the ERα-negative human breast cancer cell line MDA-MB-231 led to increased induction by retinoic acid (RA) of an RARE reporter construct and sensitized the cells to growth inhibition by RA. This growth inhibitory effect was independent of the ER ligands estradiol and tamoxifen, suggesting an original mechanism of cross-talk between the RAR and ERα pathways (197, 218). In particular, growth inhibition by RA was observed in ERα-positive cells that are refractory to tamoxifen, thus suggesting a possibility for combination therapy in certain breast cancers (197, 219, 220).

To further analyze the role of ERα in modulation of RAR transcriptional activity, we generated a panel of stably transduced cell lines expressing ERα-deletion mutants and studied the expression and regulation of the RARβ2 gene in these cells. Expression of RARβ2 is regulated in part by the presence of an RARE characterized by two direct repeats of the motif GTTCAC, spaced by 5 nucleotides (221). This gene is down-regulated in many types of cancers, including breast carcinoma, and its induction by RA may be necessary for RA-mediated growth suppression in some cell types (161, 169, 181, 222, 223).

The effects of the stable transfection of ERα or ERα-deletion mutants on the pattern of RARβ2 expression were evaluated. We determined that the RARβ2 expression levels were altered when the N-terminal domain of ERα, including the DBD, was expressed. We also examined whether ERα expression affected the transcriptional activity of other RXR-heterodimers and whether the observed crosstalk between ERα and RAR might be mediated by sequestration of several coregulators common to RAR and ER signaling.

2.3. MATERIALS AND METHODS

Cells. MDA-MB-231 (clone 10A) and the ERα-positive subclone S30 were obtained courtesy of Dr. V C Jordan. MCF-7 cells were purchased from the ATCC. The cells were maintained in phenol red free α-MEM (Invitrogen) supplemented with 5% charcoal-stripped serum. S30 were cultured in the presence of 0.5 μg/ml G418 (GibcoBRL). The 293GPG retroviral packaging cells, derived from human embryonic kidney cells (224, 225) expressing viral structural genes under the control of a tetracycline-regulated promoter, were routinely maintained in DMEM (Life Technologies) supplemented with 5% FBS, 1 μg/ml tetracycline (Sigma Chemical Co), 2 μg/ml puromycin (Sigma Chemical Co), and 0.3 μg/ml G418 (GibcoBRL). All cells were maintained in 5%CO_2 at 37 °C in a humidified atmosphere.

Construction of stable cell lines. ERα wild-type (pSG5-HEGO) and deletion mutants (pSG5-HEG19, pSG5-HEG11, pKCR2-HE15) were obtained from Sylvie Mader. Retroviral vectors were constructed by cloning the above cDNA in the multiple cloning

site (MCS) of the murine stem-cell virus retroviral vector (HC2) (226). The HC2 vector was designed to coexpress an inserted cDNA upstream of an IRES and the eGFP gene. Using this bicistronic vector, the eGFP serves as a reporter of provirus transfer and expression in target cells. The HC2 vector that we used for retroviral transfection is a modified version of the AP2 retroviral vector (224). While the AP2 vector contains a 5' cytomegalovirus promoter element (CMV), the HC2 vector contains the LTR promoter. However, after transfection in the 293GPG producer cells, the budding viral particles carrying the RNA products contain neither the LTR nor CMV at the 5'end. In the MDA-MB-231 target cell line, after reverse transcription, both the AP2 and HC2 retroviral vectors give rise to the cloned cDNA of interest upstream of an IRES and eGFP, flanked by two LTRs. Once integrated into the genome of the target cells, the AP2 and HC2 vectors are thus indistinguishable.

Stable expression in the 293gpg packaging cells was achieved using lipofectamine cotransfection of the retroviral vector with a zeocin resistant plasmid (pJGr) for selection in a ratio of 25:1. Individual colonies were selected using cloning rings (approximately 20 for each stable cell line) and were subjected to flow cytometry to detect green fluorescence from expression of the eGFP. Positive clones were then screened by northern blot using a full length ERα probe for expression of a bicistronic ERα-eGFP mRNA product having the expected size (data not shown). This was a necessary step prior to generation of the virus, as some of the producer clones exhibited rearrangement of the integrated plasmid. Those clones expressing the correct RNA products were then selected and viral particles were collected, as described (226), for infection of the MDA-MB-231 ERα-negative breast cancer cell lines. To achieve

transduction, MDA-MB-231 target cells in were seeded at 10 000 cells/well in 24 well plates and allowed to adhere overnight. Each day, for three consecutive days, 50 μL of concentrated virus was added to the media. The efficiency of gene transfer for MDA-MB-231 cells was subsequently assessed by flow cytometric analysis of eGFP expression. Analysis was performed with an Epics XL-MCL device (Coulter/Beckman). To achieve a transduction efficiency of 100%, cell sorting was performed on the basis of green fluorescence using a FACStar Sorter (Beckon Dickson) and mixed populations of transduced cells were used for all subsequent experiments. Pooled populations were routinely analyzed by flow cytometry for eGFP expression, thus confirming expression of the bicistronic RNA and the stable expression of ERα.

Western Blots. Whole cell extracts were isolated from confluent 150mm plates of transduced cells with a lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors, while nuclear extracts were isolated according as previously described (227). Protein lysates (50 μg) were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (BioRad). The B1 ERα Ab from Dr. P. Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSRM, France) was utilized for detection of C-terminal deletion mutants and the AER311 ERα antibody (Upstate Biotechnology) was used for detection of N-terminal deletion mutants. Membranes were exposed to anti-β-actin antibody (Sigma Chemical Co) to control for loading. Whole cell extracts were also isolated from cells treated with 10^{-6} M tRA (Sigma Chemical Co), 10^{-7} M OHT (kindly provided by Dr.

A.E. Wakeling (Zeneca, United Kingdom)), 10^{-7} M ICI 182,780 (Sigma Chemical Co) and 10^{-7} M estradiol (Sigma Chemical Co) treated for 24 h. Protein lysates (50 μg) were subjected to gel electrophoresis as above. The membrane was incubated with Aer611-Ab15 anti-ERα antibody (Neomarkers) and incubated with 2° antibody at a 4000 fold dilution prior to analysis by chemiluminescence.

Growth Curves. All cell lines were routinely cultured in α-MEM phenol red-free medium (Life Technologies, Inc, Ontario, Canada) supplemented with 5% charcoal stripped serum. Growth experiments were performed by culture of 1 x 10^3 cells per well in 24 well plates. In the treated cells, the above media containing a final concentration of 10^{-5} M tRA (Sigma Chemical Co) was replenished one day after seeding, and subsequently on days 3 and 5. Controls for treated cells were treated with identical concentrations of vehicle alone. Quadruplicate samples were counted on days 3, 5, and 7 by hemocytometer and trypan blue cells were excluded from the count.

Analysis of RARβ expression. Expression of RARβ was assessed using RT-PCR and RNAse protection assay (RPA). Total RNA was isolated by the guanidine thiocyanate method as described (197). cDNA was prepared from 1 μg RNA in a reaction containing 20 pmol random hexamer primers, 5mM DTT, 0.5mM dNTPs, 1U/μl RNase inhibitor, 10U/μl MMLV reverse transcriptase and First Strand Buffer (Gibco BRL). PCR amplification of the RARβ2 cDNA was performed using the following primers; 5'-AGAGTTTGATGGAGTT-3' and 5'- CATTCGGCCTGGGTGAATCCACTG-3', in a reaction containing 1 μl cDNA, 0.2mM dNTPs, 0.2 μM of each primer, 8% glycerol,

1.5 mM $MgCl_2$ and 1.25 U AmpliTaq DNA polymerase (Perkin Elmer). The 227 bp fragment was amplified by 35 cycles of 94 °C for 30s, 57°C for 30s and 72°C for 2 min., followed by a 5 min. extension period. The amplified products were visualized on 2.5 % agarose gels containing ethidium bromide.

RPA was performed as previously described (228). The human RARβ riboprobe containing bases 1545-1721 cloned into pGEM4Z (Promega) and the control GAPDH riboprobe were a kind gift from Dr. W.W. Lamph (Ligand Pharmaceuticals, San Diego, CA). The probes were linearized with *EcoRI* and radiolabelled using T7 polymerase and [^{32}P]-CTP (Amersham). Hybridization of riboprobes was carried out at 50 °C overnight, and RNAse digestion was performed at 30 °C for 1 h with ribonuclease T1. The RNAse-resistant fragments were resolved by gel electrophoresis on a 6% urea-polyacrylamide sequencing gel.

Transient transfections and CAT assays. MCF-7 (4 x 10^5), S30 (3 x 10^5) and 231-derived cells (3 x 10^5) cells were plated in 6 well plates and allowed to adhere overnight in phenol red free α-MEM media supplemented with 5% charcoal-stripped FBS. Transfections were performed using Fugene (Boehringer Mannheim) according to the manufacturer's guidelines. Briefly, 1 μg of βREtkCAT (a gift from Dr. H. Sucov, Institute for Genetic Medicine, University of Southern California School of Medicine, Los Angeles, CA) that carries the sequence 5'-AGCTTAAGGTTCACCGAAAGTTCACTCGCATAGCTGCT-3' was cotransfected with 1 μg of pCMVβGAL plasmid (Clontech Laboratories) as an internal control using a ratio of 2:1 Fugene to DNA. After 5 hours, tRA was added to each well at a final

concentration of 10^{-6} M without changing the transfection media. Cells were harvested 48 h post-transfection and the β-gal activity was assayed as described (197). The CAT activity was measured according to a modified protocol of the organic diffusion method (197). For the VDRE experiments, the VDRE-LacZ reporter containing the HSP68 minimal promoter, a VDR-pSG5 plasmid (both were generous gifts from Dr. J.H. White, McGill University, Montreal, Canada), and the TK-CAT reporter (from Dr. H. Sucov), for control of transfection efficiency, were transfected in a ratio of 1:0.2:1. Cells were treated with 10^{-8} M 1,25-dihydroxyvitamin D3 (Vit D3) and harvested 48 h later, as described above. Transfections with a T_3RE response element, the pBLTK80 promoter (a kind gift from Dr. E. Silva, Lady Davis Institute, Montreal, Canada) were performed in a similar manner with cotransfection of T_3Rβ1-pSG5 (also from Dr. E. Silva) and the pCMVβGAL plasmid. Cells were then treated with 10^{-7} M 3,3',5-Triiodo-$_L$-Thyronine (T_3) (Sigma Chemical Co) and harvested as above.

Studies testing the activity of the ER deletion mutants on an ERE were performed by co-transfecting 1μg ERE(3)-tk-CAT, 1μg CMV-βgal and 1μg of the receptor. 4 hours later, vehicle or estradiol (10^{-7}M) was added, and the cells were harvested after 24 h. For co-transfections with the co-regulatory molecules, 1 μg of reporter construct (βRE-tk-CAT) was cotransfected with 1 μg of pCMV-βGAL vector and increasing amounts (0.01-1 μg) of SRC-1-pBKCMV (a kind gift from Dr. S.A. Onate, Baylor College of Medicine, Houston, Texas) CBP-pBKCMV, p300-pBKCMV (both generous gift from Dr. M. Brown, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts). The amount of transfected cDNAs in

the dose-response transfections was kept constant at 3 μg by adjusting the concentration of the corresponding empty CMV expression vector.

Chromatin Immunopreciptation (ChIP). 10^6 cells were seeded one day before treatment with 1 μM RA for one hour. To measure histone acetylation levels, formaldehyde-cross-linked and sonicated chromatin was immunoprecipitated overnight with 5 μl of an antibody raised against acetylated form of histone H4 N-terminal tail (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. 1 μl out of 20 μl extracted DNA was used for PCR amplification with the FastStart Taq DNA Polymerase kit (Roche Molecular Biochemicals, Laval, Quebec, Canada) by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min. Primers for PCR detection of the RARβ2 promoter were: sense 5'-TCC TGG GAG TTG GTG ATG TCA G-3' and anti-sense 5'-AAA CCC TGC TCG GAT CGC TC-3'.

2.4. RESULTS

Retrovirus mediated stable expression of ERα deletion mutants. To extend our previous results that expression of ERα in a subclone of the ERα-negative MDA-MB-231 cell line restores retinoid response, we sought to determine which functional domain of the ERα protein is required to confer retinoid sensitivity. We used retroviral technology to obtain stable expression of a series of ERα-deletion mutants in the ERα-negative parental MDA-MB-231 human breast cancer cell line (**Fig 2.1.A**). The cDNA of wild-type ERα and ERα-deletion mutants were cloned into the bicistronic murine stem cell virus-based retroviral vector (MSCV-HC2). After infection, cells were sorted

by FACS to obtain a polyclonal population of MDA-MB-231 cells that were 100% transduced. A shift in fluorescence intensity was evident for all the transduced cell lines (**Fig 2.1.B**). Although transduction efficiencies of 50-80% were achieved for each of the ERα deletion mutants, prior to cell sorting, we were unsuccessful in generating MDA-MB-231 cells expressing wild-type ERα. In one attempt, we detected only a truncated product by western blot. Sequencing of this clone revealed that there was a stop codon at amino acid 345, and we labeled this clone HE345 (**Fig 2.1.A**). In all further attempts of generating a wild-type ERα clone, the cells expressed the bicistronic mRNA for approximately 2 weeks before reverting to an ER-negative phenotype. Even after cell sorting, the cells lost expression of ERα despite being maintained in estrogen-depleted media. We therefore utilized the S30 cell line (an ERα-positive clone derived from MDA-MB-231) as a control for full length ERα (229).

We had no difficulties, however, generating viable cell lines stably expressing HE15, lacking domains E and F, HEG19 lacking domain A/B and HEG11 lacking domain C, the DNA-binding domain (Fig 2.1.A). Transduced polyclonal stable cell lines were tested for their expression of ERα by western blots performed on nuclear extracts. Using an N-terminal ERα specific antibody, we were able to detect a signal at approximately 67 kDA for the wild-type ERα in MCF-7 and also confirmed expression of HE345, HE15 and HEG11 at approximately 39, 32 and 59 kDa respectively (Fig 2.2.A). Using a C-terminal ERα specific antibody, we were able to detect the presence of HEG19 at approximately 47 kDa, as well as HEG11 and the wild-type ERα in S30 and MCF-7 (Fig 2.2.B). As expected, the empty retroviral vector, HC2, did not express the estrogen receptor.

Growth response of the stable transfectants to RA. As mentioned above, previous studies by our lab and others have shown that RA inhibits mainly ERα-positive breast cancer cells, whereas ERα-negative cells are usually resistant to retinoids. We engineered the stable cell lines expressing various ERα-deletion mutants to have a system in which to study the interactions between the ERα and RAR pathways. When the stable cell lines were initially established, we immediately tested their sensitivity to RA. We were unable to detect significant RA-mediated growth inhibition in the cell lines stably expressing the ERα N-terminal deletion (HEG19) and the DBD-deletion (HEG11). However, cells expressing the C-terminal deletion mutant HE345 were approximately 50% growth-inhibited after 7 days of treatment with 10^{-5} M tRA (Fig 2.3.). The growth inhibition was similar to that of the S30 and MCF-7 cell lines expressing wild-type ERα. However, after multiple passages, the stable cell lines expressing the C-terminal deletions lost their sensitivity to RA.

Suppression of RARβ2 expression by ERα. We have previously shown that responsiveness to RA is restored in S30 cells without a change in RARα expression. Since the induction of RARβ2 has been associated with response to retinoids in certain types of cancer, we assessed the expression of this gene in MCF-7, MDA-MB-231 and MDA-MB-231 stably expressing full-length ERα (S30) and ERα deletion mutants, using an RNase protection assay (RPA). In contrast to RARα, we observed significant differences in the level of expression of RARβ2. In fact, in some of the stable cell lines, expression of RARβ2 was strongly suppressed by ERα in the absence of RA (Fig

2.4.A). MCF-7, S30 and MDA-MB-231 expressing C-terminal deletion mutants of ERα (HE15, HE345) all expressed significantly lower levels of RARβ2 than parental MDA-MB-231 and the mock-transduced HC2 ($p<0.05$), as shown by densitometry analysis of at least three different RPA experiments. On the other hand, expression of the N-terminal deletion mutant HEG19 or the DBD-deletion mutant HEG11 did not affect expression of RARβ2, indicating that the N-terminal region of ERα, including the DBD, is necessary for this effect.

Importantly, expression of RARβ2 was strongly induced in response to RA in S30, HE15 and HE345, but only very weakly in the other cells (Fig 2.4.B). Using the chromatin immunoprecipitation assay (ChIP), we also showed that the decrease in basal expression of RARβ2 in ER-positive S30 cells, compared to MDA-MB-231, is associated with a decreased level of promoter acetylation. However, RA induced a rapid increase in histone acetylation in S30, but only a minor increase in the parental cells (Fig 2.4.C).

Effect of ERα-deletion mutants on the transcriptional activity of a transiently expressed RARE. To further characterize the effect of ERα on RARβ2 expression, we transiently expressed a reporter driven by the RARE of the RARβ2 promoter in the stably transduced cell lines and assessed transcriptional activity in the absence and presence of 10^{-6} M tRA (Fig 2.5.). In agreement with the results above, we found that MCF-7, S30, HE15 and HE345 displayed significantly lower basal activity from this promoter in the absence of RA than MDA-MB-231, HC2, HEG19 and HEG11 ($p<0.001$) (Fig 2.5.A). The effect of ERα was due to the βRARE and not any part of the

thymidine kinase promoter since we observed no differences in transcriptional activity between the different cell lines when we transiently transfected the TK-CAT reporter alone (data not shown). We also transiently transfected wild-type ERα into MDA-MB-231 and observed the response on the basal activity of the βRE-tk-CAT to ensure that the transcriptional background of the S30 cell line, which was derived by clonal selection and not by retroviral technology, was comparable to that of the mutant stables (data not shown). We detected an 8 fold decrease in basal activity from the βRE-tk-CAT in the presence of wild-type ERα, similar to that observed with the ERα-positive S30 cells. Upon addition of RA, there was a strong induction of transcriptional activity in the cells that had a suppressed baseline, and only a very weak induction in the parental cells and in HC2, HEG19 and HEG11 (Fig 2.5.B). Thus, expression of the N-terminal region of ERα converts RARβ2 expression from constitutive to ligand inducible.

The transcriptional effect of ERα is specific for the βRARE. RAR heterodimerizes with RXR and recognizes specific sequences in the promoters of various genes. In Figure 2.5 we demonstrated that ERα alters the transcriptional activity from a DR5 element located in the promoter region of the RARβ2 gene. VDR and TR also heterodimerize with RXR, but recognize primarily DR3- and DR4-containing promoters, respectively (230). To determine if the transcriptional effects of ERα expression are specific for RAR-RXR mediated transcription, we studied the activity of a typical VDRE and TRE in the different ERα-transduced cell lines. Transcription from the VDRE-LacZ reporter construct, containing a DR3 response element, was unaffected

by the presence of ERα or its deletion mutants (Fig 2.6.A). Likewise, transcription from the DR4-containing TRE reporter, pBLTK80, did not significantly differ between ERα-negative and ERα-positive cell lines (Fig 2.6.B). These results indicate that suppression of baseline transcription by ERα is specific for the RAR-RXR heterodimer and that ERα does not alter the transcriptional activity of other RXR heterodimers.

Inhibition of ERα AF-1 activity restores basal activity from the RARβ2 promoter. Given that the deletion of the N-terminal, AF-1 containing, but not the C-terminal, AF-2 containing region of ERα abolished its regulation of RARβ transcription (see Fig 2.4 and 2.5), we went on to study the effect of pharmacological inhibition of the AF-1 and AF-2 activities. 4-hydroxytamoxifen (OHT) has been shown to inhibit the AF-2-mediated transcriptional activity of ERα while still allowing activity from the AF-1 (231-233). In contrast, the pure antagonist ICI 182,780, which binds to the C-terminal region of the estrogen receptor, prevents the binding of coactivators, and inhibits both the AF-1 and AF-2 activities of ERα by inducing rapid degradation and decreased nuclear localization (234-236). We tested the activity of the βRARE reporter in the presence of these antagonists and the agonist estradiol in MCF-7 and in the stably transduced MDA-MB-231 cell lines (Fig 2.7.A). As expected, OHT, ICI 182,780 and estradiol had no effect on the HC2 null-transfected cell line, which behaves like parental MDA-MB-231. Similarly, these ligands had no effect on βRARE-activity in HEG11, which lacks the DNA-binding region, or HE345, which lacks the ligand-binding region. Likewise, the elevated basal βRARE activity observed in HEG19 was unaffected by the presence of these ligands. In the MCF-7 cell line, expressing wild-type ERα, OHT had

a minimal effect on basal transcription from the βRARE. On the other hand, ICI 182,780 completely released inhibition of this RAR-mediated transcription and restored baseline activity to the same level as seen in the ERα-negative cell line HC2. Addition of estradiol, which activates the ERα AF-2 domain, did not significantly affect the βRARE activity in these cells. To verify that a decrease of ERα protein expression was not responsible for the altered βRARE activity caused by ICI 182,780, we assessed the level of ERα in MCF-7 treated with estradiol and ICI 182,780 (Fig 2.7.B). As previously reported, estradiol and ICI 182,780 both down-regulate expression of ERα (237). Although the expression of ERα is similar in the estradiol- and ICI-treated cells, only ICI has an effect on βRARE activity, indicating that inhibition of the ERα AF-1 activity does indeed block the effect of ERα on RAR-mediated transcription.

To confirm that the ERα AF-1 domain is active in MDA-MB-231 cells, we transiently transfected an ERE-containing reporter construct, ERE(3)-tk-CAT, and assessed transcriptional activity in the presence of estradiol, tamoxifen, and ICI 182,780 (Fig 2.8.). We found that the S30 cell line expressing wild-type ERα displayed significant activity in the absence of added ligand, and its activity was further induced by the addition of estradiol. The ligand-independent activity in S30 cells may be attributed to an active AF-1 and AF-2 domain. Transient transfection of MDA-MB-231 with the ERα expression vector and the ERE(3)-tk-CAT promoter also resulted in high basal activation (data not shown), supporting the notion that the ligand-unoccupied ERα is not transcriptionally different in S30 and in the MDA-MB-231 background. The HE345- and HE15-stably expressing cell lines also displayed ligand-independent activity, and this was unaltered by estradiol, tamoxifen and ICI 182,780 (Fig 2.8).

HEG19, which lacks the AF-1 domain, showed no activity in the absence of ligand, but was strongly induced by E2 and repressed by the antagonists tamoxifen and ICI 182,780. In agreement with these results, transient transfection of the ERα deletion mutants in MDA-MB-231 (data not shown) resulted in similar activity from the ERE(3)-tk-CAT as the stable transfectants. Together, these results demonstrate that ERα has significant AF-1 activity in MDA-MB-231 cells.

ERα does not alter the expression of several known receptor cofactors. We examined whether the N-terminal domain of ERα may have a squelching effect on RAR-mediated transcription by altering the availability of co-activators or co-repressors common to the RAR and ERα pathways. We first selected a panel of p160 co-activators (SRC-1, GRIP1 and AIB1), co-integrators (p300 and CBP) and co-repressors (NCoR and SMRT), and examined their expression levels by Northern blot. The expression of these common co-factors was not significantly altered between these cell lines (data not shown). The only marked difference was the previously reported increase in AIB1 expression levels in MCF-7 cells (238). However, this was not observed in any of the MDA-MB-231-derived ERα-positive cell lines. Thus, expression of ERα did not directly affect the levels of any of these co-factors. We further investigated whether the N-terminal domain of ERα may be titrating co-factors away from the RAR-RXR, since those may be present in limiting amounts. Given that SRC-1, CBP and p300 have been shown to bind to and activate the AF-1 domain of ERα, and are involved in cross-talk between other nuclear receptors (205, 239, 240), we chose to study the effect of overexpressing these proteins in our cells. We transiently transfected increasing

amounts of the coactivators into MDA-MB-231, MCF-7 and HE345 cells, in an attempt to relieve the ERα-mediated suppression of basal βRARE activity. However, forced expression of these proteins did not alter the basal βRARE activity in MCF-7 and HE345 (Fig 2.9.). Likewise, transient expression of increasing amounts of the co-repressors SMRT and N-CoR had no significant effect on basal βRARE activity in any of the cells (data not shown).

Since co-regulators are known to regulate transcription at least in part by altering histone acetylation, we also assessed the effect of the HDAC inhibitor trichostatin A (TSA) on basal βRARE activity. TSA has been shown to facilitate transcription on transiently transfected reporters, indicating that transfected reporter constructs can be organized in nucleoprotein structures sensitive to histone acetylation (241, 242). Furthermore, chromatin deacetylation is a mechanism for repression of transcription from the βRARE promoter (243). However, TSA did not cause an increase in the activity of the βRARE reporter, nor did it increase the expression of endogenous RARβ2 in ERα-expressing cells (data not shown).

2.5. DISCUSSION

Numerous preclinical studies have shown that retinoids can inhibit proliferation of some, but not all, breast cancer cells *in vitro* (171, 209). In animal models, several synthetic retinoids are able to inhibit the development of mammary tumors, and can even cause complete regression of established tumors in rats (210, 211). Since there is significant evidence that the response to retinoids in human breast cancer cell lines

correlates with expression of ERα, we sought to characterize the transcriptional cross-talk that exists between the ERα and RAR pathways.

We generated human breast cancer cell lines stably expressing ERα deletion mutants by retroviral technology. Retroviral transduction of the ERα-negative cell line MDA-MB-231 generated a very high percentage of positive clones with ERα cDNA integrated at random sites in the genome, thereby avoiding clonal artifact. The ERα-deletion mutants HE15, HE19 and HE11 have been previously characterized for their localization, binding to estradiol, to EREs and activation of ERE-containing constructs (244). We confirmed the expression of all of the ERα deletion mutants by western blot using nuclear extracts (Fig 2.2.). Although the expression level of HEG19 is less than that of the other stable mutants, it is comparable to that of ERα in S30 and MCF7. Furthermore, we showed that the ERα mutants lacking in N-terminal (HEG19) and C-terminal (HE15 and HE345) were able to bind an ERE and activate transcription in MDA-MB-231 cells (Fig 2.8.).

While we confirmed expression of the ERα deletion mutants, we were unable to generate a stably transduced cell line expressing wild-type ERα. Stable transfection of ERα into ER-negative breast cancer cells has been reported to be difficult (245), and ERα transfected cells often do not exhibit a typical response to ligand stimulation (229, 246). We used the S30 cell line, an ERα-positive clone derived from MDA-MB-231, as a positive control for full-length ERα (229). This cell line differs from the ERα-positive cell line MCF-7 in that it is growth inhibited by estradiol and unaffected by ICI and OHT (229). However, we have previously shown that its response to retinoids is similar

to that of other ERα-positive cells (197). Furthermore, transient transfection of MDA-MB-231 with wild-type ERα yielded identical results on the βRE-tk-CAT and ERE(3)-tk-CAT as that observed in the S30 cells, suggesting that the S30 cell line could effectively be compared to the mutant stables.

To elucidate how ERα expression may alter RA signaling, we studied the expression and regulation of the RA-inducible gene RARβ2. Induction of RARβ2 expression has been associated with retinoid response in a variety of cancer cell types (222, 223, 247). Interestingly, we found that the basal expression of RARβ2 RNA was significantly lower in cells expressing full-length ERα or the C-terminal deletion mutants HE15 and HE345 than in the parental cells or cells expressing HEG19 and HEG11 (Fig 2.4.). Likewise, using a βRARE reporter construct, we found significantly decreased basal activity in MCF-7, S30, HE15 and HE345, although this activity was strongly induced by the addition of RA. In MDA-MB-231, HC2, HEG19 and HEG11, baseline βRARE activity was elevated, but was only weakly induced by RA (Fig 2.5.). Thus, the expression of ERα, with or without its C-terminal LBD, causes a loss of basal RARβ transcription and expression, but confers inducibility by RA.

Cells expressing the HEG19 and HEG11 deletion mutants have the same phenotype as the ERα-negative parental cells, confirming that the effect of ERα on baseline βRARE activity is mediated by the N-terminal half of ERα, including the DBD. This represents a novel interaction between nuclear receptor families, whereby the N-terminal, AF-1 containing domain of ERα can function independently of AF-2 to alter the transcriptional activity of a different nuclear receptor, RAR, on its response element.

To investigate the importance of the ERα AF-1 domain in repression of basal RARβ expression, we tested the effects of specific ER ligands on transcriptional activity from the βRARE. The effect of these ER ligands on βRARE activity (Fig 2.7.A) supports an important role for the AF-1 domain of ERα in mediating cross talk between RAR and ERα, since only ICI 182,780 significantly affected activity from the βRARE promoter. Inhibition of the AF-2 activity using tamoxifen did not significantly alter βRARE activity. We confirmed that the effect of ICI 182,780 was not simply mediated by a decrease in the ERα expression level, since estradiol similarly down-regulated the receptor and yet had no effect on βRARE activity (Fig 2.7.B). These data suggest that the function of ERα in altering RAR-mediated transcription takes precedence over its expression levels. In addition, it lends support to the notion that HEG19, although more weakly expressed, can still be effectively compared to the other mutant stables.

Since the activity of the ERα AF-1 domain is generally weak, and exhibits cell type specificity (248), we tested whether there is constitutive activity from unliganded ERα in MDA-MB-231 cells. Transfection of an ERE-containing reporter construct into the stably-transfected MDA-MB-231 cell lines confirmed that the full-length ERα as well as the C-terminal deletion mutants (HE345 and HE15) display constitutive AF-1-driven activity, which may be responsible for regulation of RAR-activity in these cells (Fig 2.8.).

The regulation of RARβ is not well understood. It has been proposed that methylation and deacetylation of the RARE promoter can alter the expression of RARβ

(155, 184, 185). However, the methylation sites often are not consistent from one cell line to another, and it has been shown that even when the promoters are methylated in the same manner, they can differentially respond to RA (186). It has also been proposed that different levels of coregulatory factors may affect the efficiency of transcription from the βRARE (186). The ERα AF-1 activity has been shown by several groups to be regulated by recruitment of some of the same cofactors that bind and activate the AF-2 (48, 205, 239). We reasoned that if ERα attenuates the activity of ligand-free RAR/RXR heterodimers by sequestering known co-activators, as suggested by the fact that the RARβ promoter is more highly acetylated in untreated S30 than in MDA-MB-231, overexpression of these factors would restore basal activity from the βRARE promoter. However, transfection of increasing amounts of SRC-1, CBP or p300 did not alter the activity of the βRARE. This suggests that ERα is not acting by sequestering these specific factors, but does not exclude the possibility for the involvement of other co-factors. Since repression of RARβ2 by ERα is unaffected by the presence of E2, such a factor would have to bind to the N-terminal region of both the unliganded (yet transcriptionally active via AF-1) and agonist-bound ERα, as well as to RAR. Moreover, since treatment of ERα-positive cells with the HDAC inhibitor TSA did not increase transcription from the βRARE, nor did it increase RARβ2 expression (not shown), it seems unlikely that the observed repression is due to increased binding of a corepressor or HDACs.

In addition to regulation by co-activators or co-repressors, the ligand-independent activity of nuclear receptors is strongly regulated via cross-talk with other signaling pathways, including growth factor and stress-induced pathways (7, 86, 203,

249, 250). It is thus possible that enhanced kinase activity, which is a characteristic of ERα-negative breast cancer cells (251, 252), may play a role in altering the activity of the nuclear receptors. Since it has previously been shown that the transcriptional activity of RAR (253, 254) and the N-terminal region of ERα can be modulated by phosphorylation (8, 86, 203, 255) it is possible that these receptors may compete as substrates for a kinase that positively regulates their basal activity. To explore this possibility, we are currently studying if ERα alters the activity of RAR/RXR and/or transcriptional coregulators by changing their phosphorylation status. Given that ERα seems to specifically regulate RAR/RXR signaling, while not affecting VDR/RXR or TR/RXR, it is likely that this kind of regulation would affect RAR and not RXR.

The role of RARβ in mediating RA responsiveness to breast cancer cells has been the subject of some discussion. Regulation of RARβ2 by RA has been linked to retinoid response in a variety of cancer cell types and there is evidence to suggest that RARβ can act as a tumor suppressor (222, 223, 247). The stable cell lines that we constructed using various ERα-deletion mutants provided a means to study the regulation of RARβ2 by ERα and the role of these proteins in conferring sensitivity to the growth inhibitory effect of RA. Our data support the link between induction of RARβ2 and growth inhibition by RA (Fig 2.3.). However, after the cells were continually passaged, they became resistant to RA-mediated growth inhibition, even though they retained their ability to induce RARβ2. Only the MCF-7 and S30 cells, expressing wild-type ERα, exhibited significant and persistent growth inhibition, as previously reported (197). This suggests that induction of RARβ2 alone is not enough to regulate growth inhibition by retinoids in MDA-MB-231 cells. There are perhaps

other areas of cross-talk between the ERα and RAR in breast cancer cells that require the expression of the wild-type ERα to mediate growth inhibition by RA.

In conclusion, we have provided experimental evidence in support of a novel interaction between nuclear receptors, whereby the N-terminal region of ERα suppresses the baseline activity of RAR/RXR-mediated transcription from the RARβ2 promoter. This suppression can be overcome by addition of RA, leading to a greater induction of RARβ2. In support of the importance of the ERα pathway in the mediating the growth inhibitory effects of RA, a clinical study of the retinol, fenretinide, found a beneficial effect in breast cancer chemoprevention only in premenopausal women (167). Further studies need to be undertaken to understand the role of ERα in the cross-talk with RAR in order to optimize the design of retinoid-based therapies targeted to the prevention or treatment of breast cancer.

2.6. ACKNOWLEDGEMENTS

We thank Angelika Rosenauer for technical assistance, and Franca Sicilia for analyses of the retrovirally transduced cell lines by flow cytometry. This work was supported by the Canadian Breast Cancer Research Initiative (CBCRI), the Canadian Institute for Health Research (CIHR, MT-13147), the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR), and the US Army Medical Research and Materiel Command (USAMRMC) Breast Cancer Research Program pre-doctoral award number DAMD17-97-1-7167

Figure 2.1. – Retroviral-mediated expression of ERα-deletion mutants in MDA-MB-231 ERα-negative breast cancer cells. **(A)** The cDNA of wild-type ERα (HEGO), ERα-deletion mutants HE15, HE345, HEG19 and HEG11 were cloned into the bicistronic murine stem cell virus-based retroviral vector (MSCV-HC2). This vector contains a packaging signal (ψ), multiple cloning site (MCS), internal ribosomal entry site (IRES) and enhanced green fluorescence protein (eGFP) flanked by two long terminal repeats (LTRs). **(B)** Flow cytometric analysis of retrovirally transduced MDA-MB-231. Subsequent to cell sorting, the stably transduced polyclonal cell populations were analyzed by flow cytometry for green fluorescence. The non-transduced MDA-MB-231 control cell line is represented by the hatched line in each sample. The solid line indicates the transduced cell line. HC2 represents the retroviral vector expressing eGFP only. The percentage of stably transduced cells was calculated according to the selected gates shown in each panel.

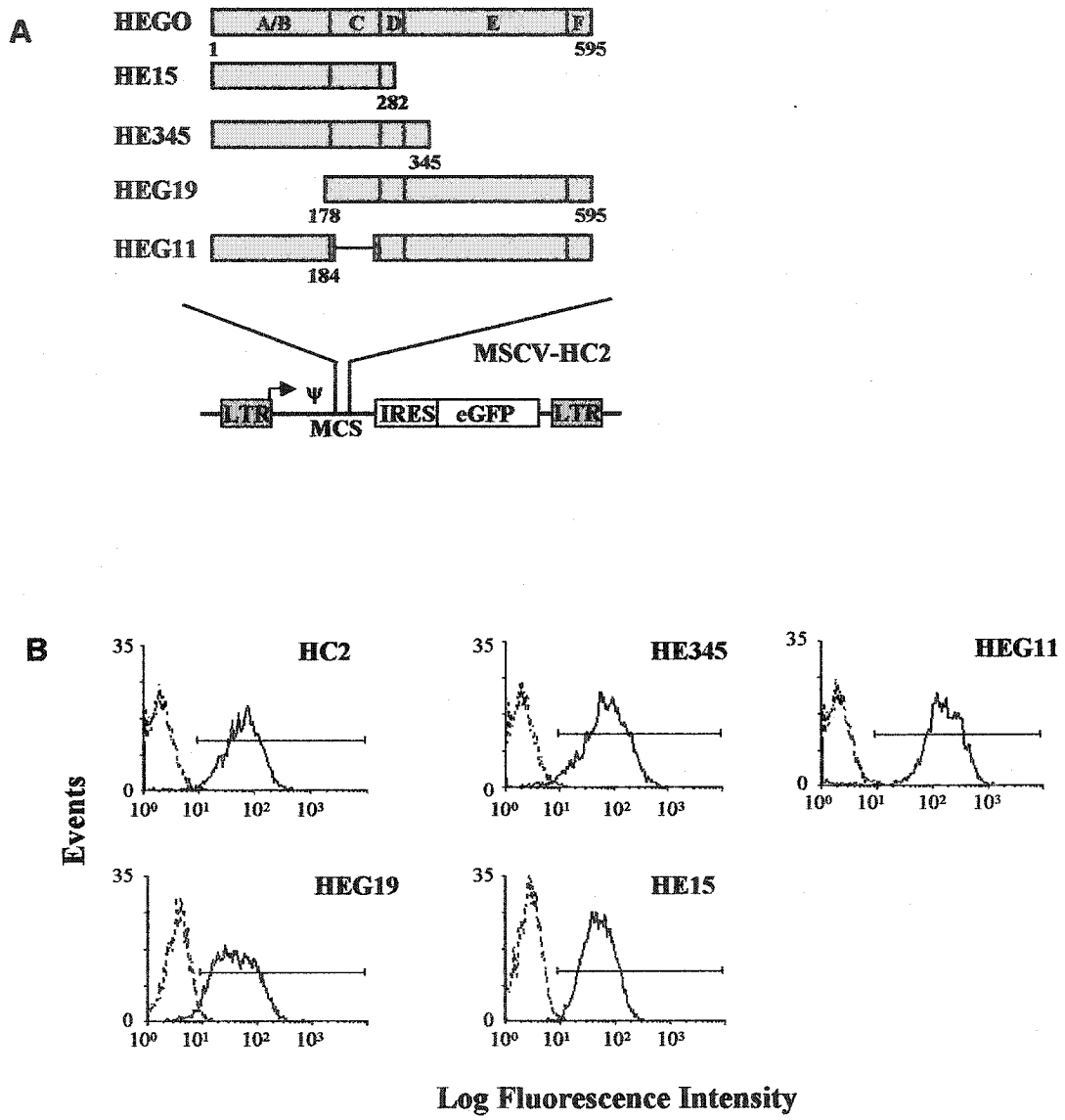

Figure 2.1. Retroviral-mediated expression of ERα-deletion mutants in MDA-MB-231 ERα-negative breast cancer cells.

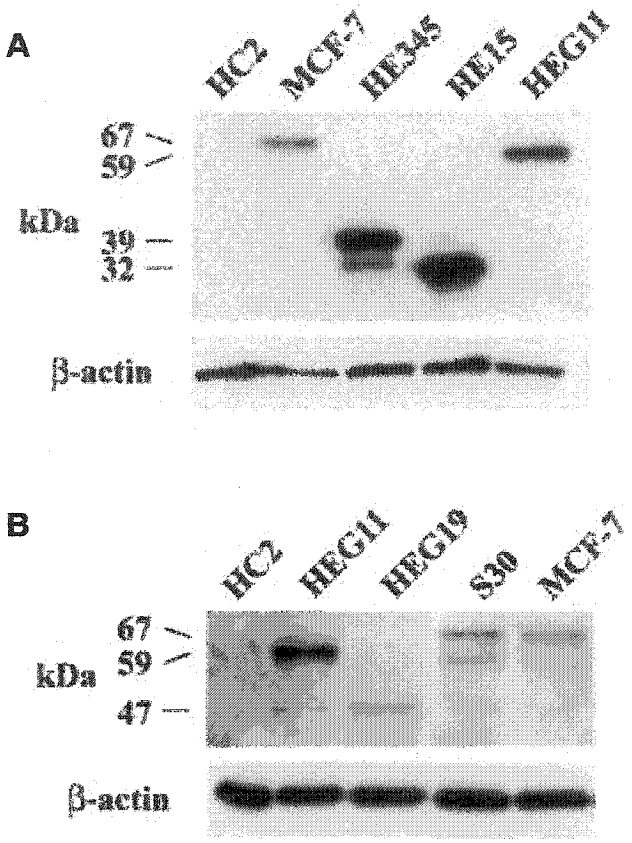

Figure 2.2. Protein expression of the transduced cells. Immunoblots were performed using 50 μg of nuclear extracts for the **(A)** ERα-C-terminal deletion mutants using the B1 antibody **(B)** ERα-N-terminal deletion mutants using the AER311 antibody. The approximate molecular weights are indicated in the left side of each blot and β-actin, which was used as a loading control, is also shown.

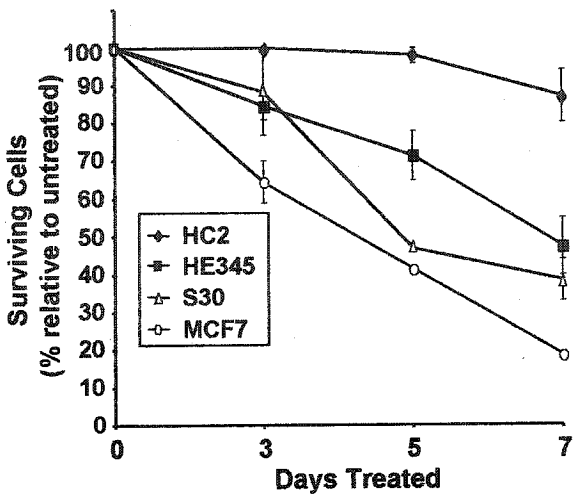

Figure 2.3. Growth response of ERα-positive breast cancer cells (S30 and MCF-7), the ERα C-terminal deletion mutant stable transfectant (HE345) and the empty retroviral vector (HC2) after continuous exposure to RA.

Figure 2.4. Basal RARβ2 expression is suppressed but inducible by RA in cells expressing the N-terminal of ERα. **(A)** RNAse protection analysis, confirming that ERα suppresses the basal expression of RARβ. The RNAse-protected band corresponding to RARβ is identified, and GAPDH expression was used as a quantitative loading control. Densitometric results of the RNAse protection analysis (*n=3*) were obtained by phosphoimaging analysis of the gels. Data are represented in arbitrary units and the value for each sample was normalized for RNA loading by using the GAPDH signal. Significant differences ($P < 0.05$) compared to the HC2 negative control are indicated with asterisks (*), as determined by ANOVA. **(B)** RT-PCR analysis of RARβ2 in cells treated with RA (10^{-6} M) or vehicle for 24 h, showing strong induction of RARβ2 expression by RA in S30, HE345 and HE15. **(C)** Chromatin Immunoprecipitation analysis show a decreased level of acetylated histone H4 associated with the RARβ promoter in S30 cells, compared to parental MDA-MB-231, but a rapid increase in histone acetylation in response to RA. The cells were treated with RA (10^{-6} M) or vehicle for 1 hr.

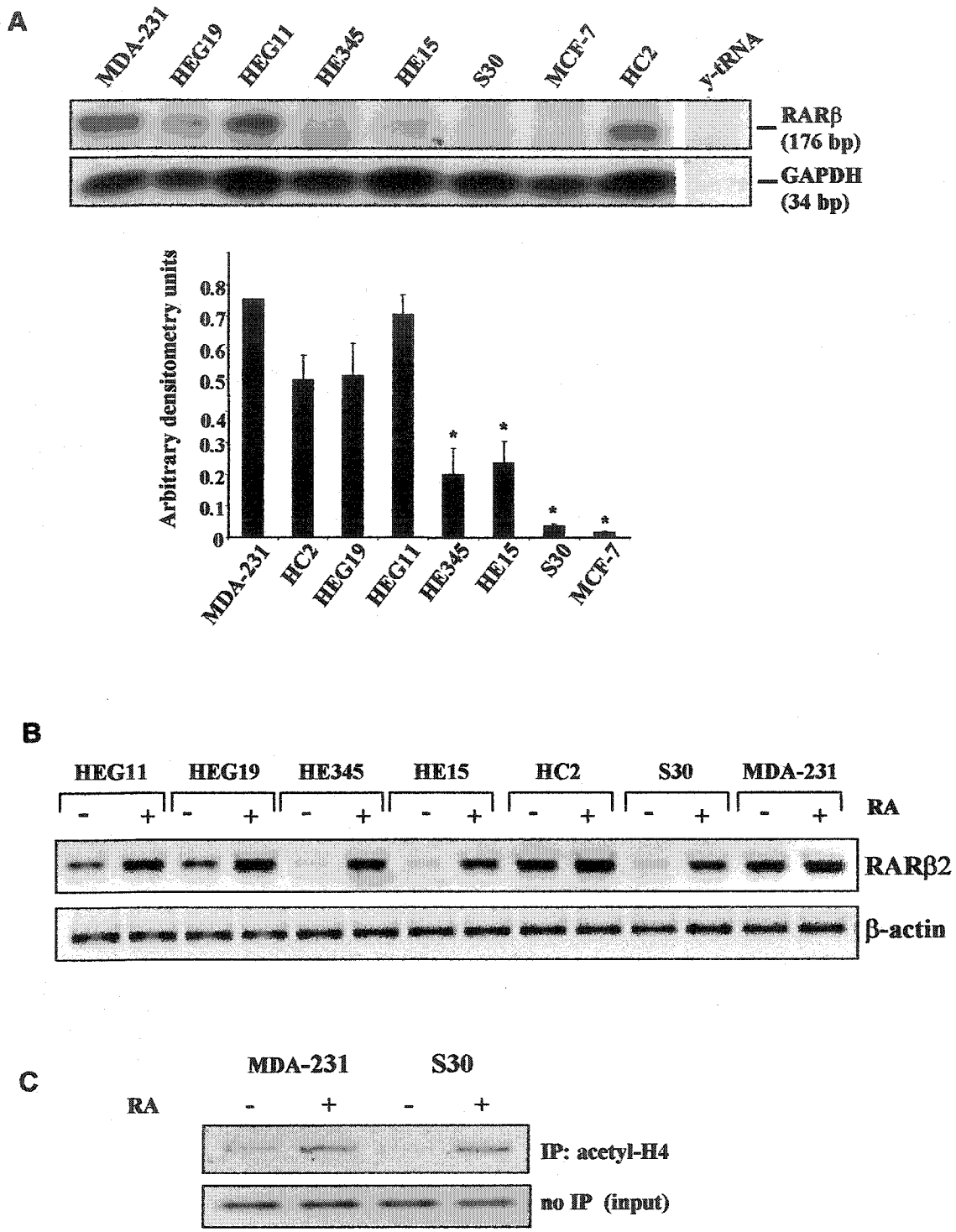

Figure 2.4. Basal RARβ2 expression is suppressed but inducible by RA in cells expressing the N-terminal of ERα.

Figure 2.5. Expression of ERα increases the fold induction of transcriptional activity from the βRARE promoter by suppressing basal activity in the absence of RA. **(A)** Transcriptional activity from the βRE-tk-CAT in the absence of ligand. Results are expressed as CAT activity relative to the MDA-MB-231 cell line **(B)** Retinoid-induced transcriptional activity in ERα-negative MDA-MB-231, ERα-positive MCF7 and S30, and MDA-MB-231 stably transduced to express ERα deletion mutants. Cell were transfected with 1 μg βRE-tk-CAT and 1μg CMV-βGal expression vector and treated with 10^{-6} M tRA or vehicle. The fold induction for each cell line is indicated in the box below. The transcriptional activity is shown as the mean +/- SEM of at least three independent experiments preformed in triplicate. Statistical significance was calculated using ANOVA, comparing each cell line to the HC2 negative control and results that differ significantly ($P < 0.001$) are indicates by asterisks (*).

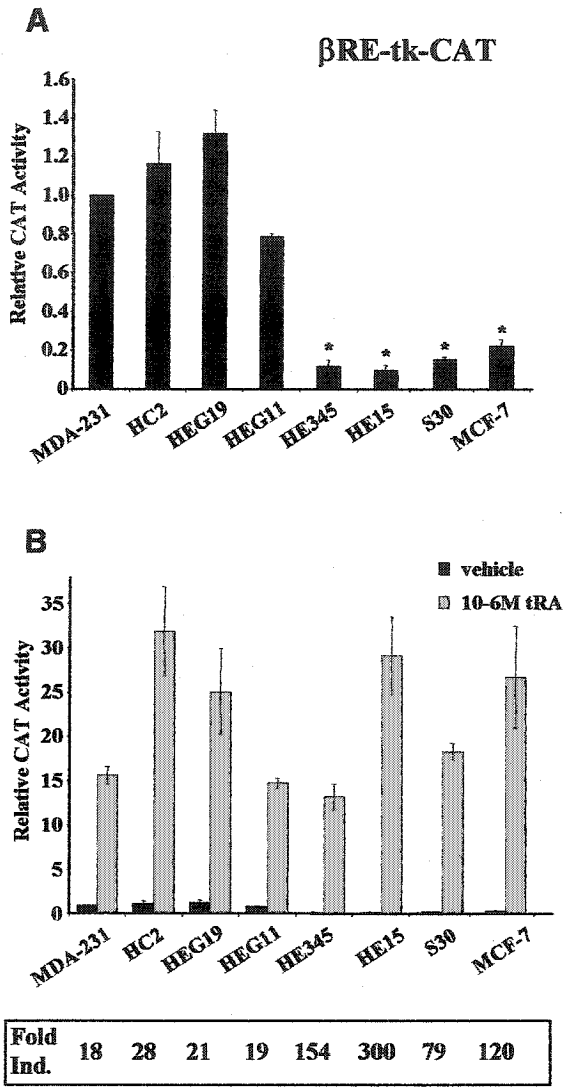

Figure 2.5. Expression of ERα increases the fold induction of transcriptional activity from the βRARE promoter by suppressing basal activity in the absence of RA

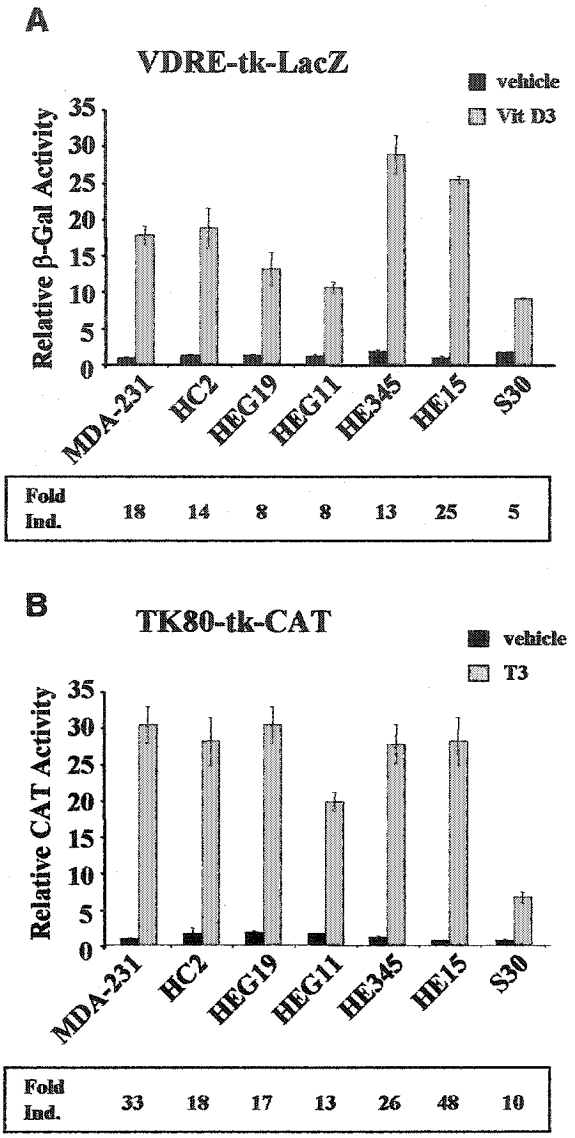

Figure 2.6. Effect of ERα expression on transcriptional activity from a VDRE and TRE. **(A)** The DR3-containing VDRE-tk-LacZ reporter was transfected in the absence or presence of $1,25(OH)_2$-D3 and **(B)** the DR4-containing TK80-tk-CAT reporter was transfected in the absence or presence of T3. The transcriptional activity is shown as the mean of at least three separate experiments performed in triplicate. The fold induction for each cell line is indicated in the box below.

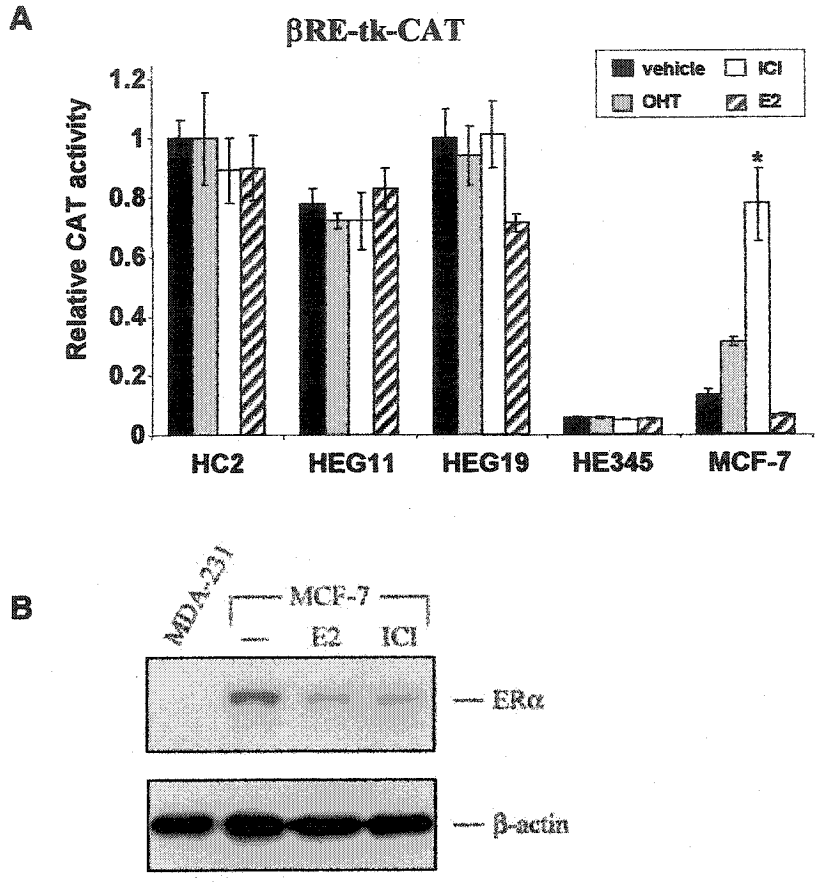

Figure 2.7. Suppression of ERα AF-1 activity restores basal activity from the βRARE to the level seen in ERα-negative cells. **(A)** Baseline transcriptional activity of the βRE-tk-CAT was evaluated after treating the cells one hour post-transfection with vehicle, 10^{-7}M tamoxifen, 10^{-7}M ICI 182,780, or 10^{-7}M estradiol. Transcriptional activity is shown as relative CAT activity compared to HC2 and significant differences between the vehicle and treated samples ($P < 0.05$) are indicated by asterisks (*). **(B)** Immunoblot of 50 μg of MDA-MB-231 (negative control) and MCF-7 whole cell extracts depicting the effect of 10^{-7}M estradiol and 10^{-7}M ICI 182,780 on ERα protein expression after 24 hours. Cells were also immunoblotted for β-actin as a loading control.

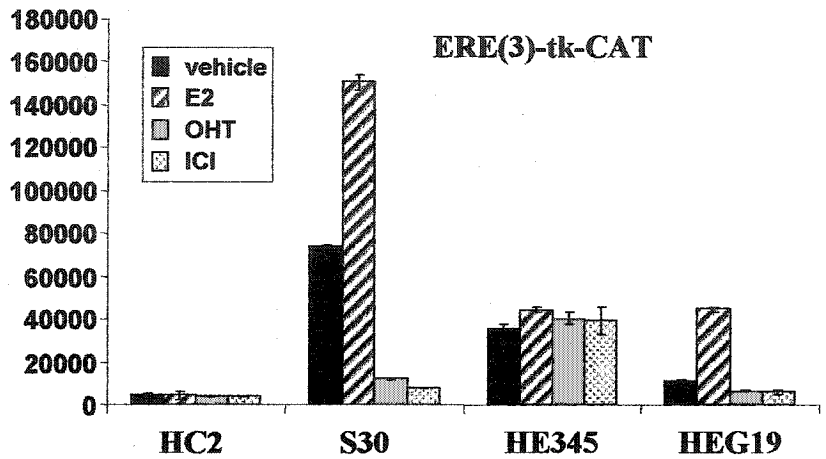

Figure 2.8. Full-length ER (S30) and the C-terminal deletion mutant (HE345) display ligand independent activity on an ERE. Cells were cotransfected with ERE(3)-tk-CAT and CMV-βgal and treated with vehicle, 10^{-7} M E2, 10^{-7} M OHT or 10^{-7} M ICI for 48 hrs

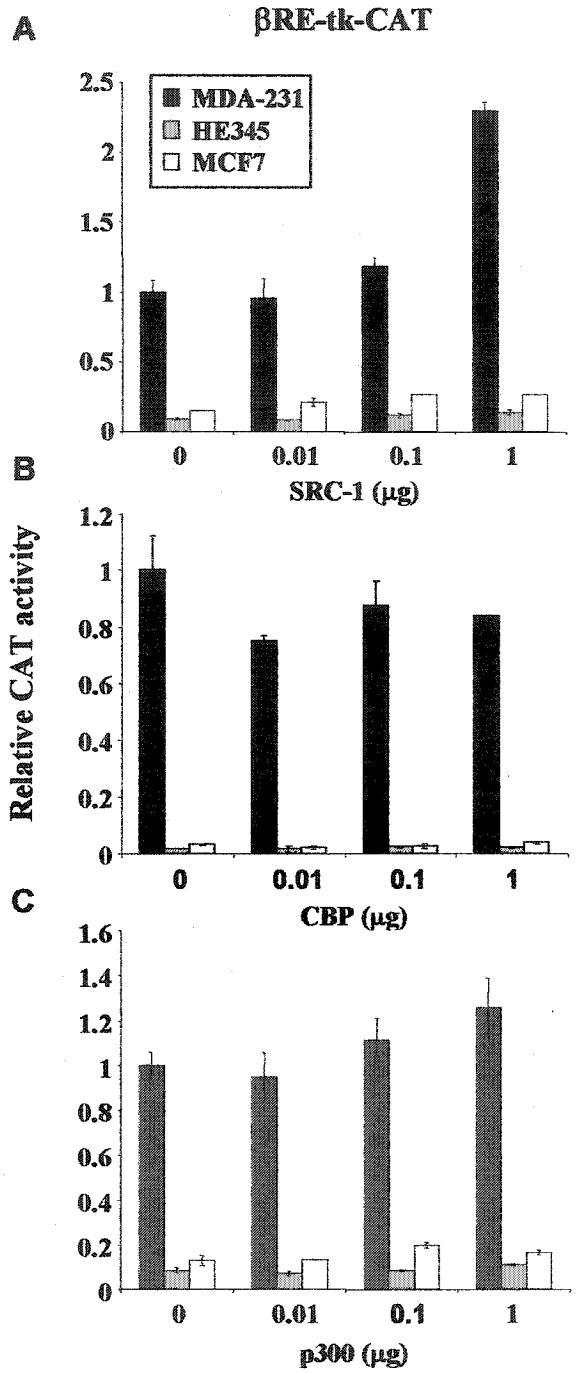

Figure 2.9. Transient overexpression of **(A)** SRC-1, **(B)** p300 or **(C)** CBP does not alter basal βRARE activity in MDA-MB-231, HE345 or MCF-7. Cells were cotransfected with βRE-tk-CAT, CMV-βgal and increasing amounts of expression vectors encoding the indicated coactivator. The total amount of DNA was kept constant by transfecting with various amounts of corresponding empty expression vector and CAT activity was assessed after 24 hrs

PREFACE TO CHAPTER 3

The discovery of a second estrogen receptor in 1996 (ERβ), increased the complexity of defining the role of ER (now ERα) in breast cancer. Since breast tumors can express both isoforms of the estrogen receptor, there currently exists the potential for ER signaling to be mediated by ERα, ERβ or both. However, some controversy has arisen regarding the role of ERβ as a good or bad prognostic marker in breast tumorigenesis. Given the high homology between ERα and ERβ in the DNA-binding domain and the ligand binding domain, and the presence of ERβ in breast cancer cells, it became of interest to study the growth and transcriptional effect of retinoids in ERβ-expressing breast cancer cells. In this Chapter, we describe how ERβ-positive stable transfectants were generated and how they respond to RA at the RARβ2 promoter. We also examine the transcriptional cross-talk between ERβ and RAR using an estrogen response element (ERE) and the endogenous estradiol-responsive pS2 gene.

CHAPTER 3

ERβ sensitizes breast cancer cells to retinoic acid and contributes to altered RAR beta expression.

Molecular Cancer Research (submitted)

3.1. ABSTRACT

The ability of retinoids to inhibit breast cancer cell growth correlates with ERα status, as demonstrated by the anti-proliferative effects of retinoids in ERα-positive breast cancer cells, and their use as chemopreventive agents in pre-menopausal women. The discovery of ERβ, also present in breast cancer cells, has added a new level of complexity to this malignancy. To determine the retinoid response in ERβ-expressing breast cancer cells, we used retroviral transduction of ERβ in ER-negative MDA-MB-231 cells. Western blot and immunofluorescence confirmed expression and nuclear localization of ERβ, while functionality was demonstrated using an ERE-containing reporter. Significant RA-mediated growth inhibition was observed in the transfected ERβ-positive cells. Addition of estradiol, tamoxifen or ICI, had no effect on cell growth and did not alter RA sensitivity. ERE-mediated transcriptional activity was inhibited by retinoids, as shown by decreased expression of the pS2 gene. ERβ-positive cells exhibited increased AP-1 activity in response to estradiol, but this was decreased by addition of RA. Although we noted no change in the level of RARα, ERβ-expressing cells had altered RARβ expression, resulting in greater induction of RARβ gene expression upon RA treatment. These data were similar to that observed in ERα-positive breast cancer cells, and indicate that retinoids are effective in inhibiting breast cancer cell growth in a sub-population of breast cancer expressing exclusively ERβ.

3.2. INTRODUCTION

Estrogens are potent mitogens in the mammary gland that are required for normal development but are also involved in the progression of mammary carcinoma. The action of estrogen is mediated by binding to the estrogen receptor (ER), a ligand-activated transcriptional factor. Although it was initially believed that the action of estrogen was mediated by a single estrogen receptor (ERα), it was subsequently determined that a second estrogen receptor (ERβ) exists (71, 72). The two receptors are highly homologous in the DNA-binding domain (region C) and ligand-binding domain (region E). However, they greatly differ in the N-terminal A/B domain and hinge region (72). The tissue distribution of ERα and ERβ is equally divergent, with ERα being most highly expressed in the pituitary, vagina, uterus and breast, and ERβ in the ovary, prostate, and lung. The presence of ERβ in the breast, albeit in lower concentration than ERα, has led to deliberation regarding its role in mammary development and tumorigenesis (74). About 60-70% of breast epithelial cells express ERβ at all stages of breast development, while ERα expression varies according to the developmental stage of the mammary gland (79). Studies with ERα KO mice have demonstrated that ERβ does not mediate E2-dependent growth and development of the mammary gland (78). Since many breast tumors express ERα alone or in combination with ERβ (80), there is interest in determining the role of ERβ in breast cancer. Some groups have found that ERβ correlates with low biological aggressiveness of breast cancer and can even inhibit proliferation and invasion of breast cancer cells (79, 81). In contrast, others have indicated that the ratio of ERα to ERβ alters in breast cancer progression, with increased expression of ERβ in relapsed patients exhibiting tamoxifen-resistance (256).

The clinical approaches to controlling hormonally responsive breast cancer have primarily focused on ERα and its target genes. In patients with hormonally responsive breast cancer, current treatment involves blocking the action of ERα using antiestrogen therapies. However, hormonal treatment is limited by the development of resistance to tamoxifen and alternative therapies targeting other signaling pathways need therefore be explored.

Retinoids are derivatives of vitamin A that induce differentiation in the treatment of acute promyelocytic leukemia (APL) and can cause growth inhibition in a variety of other cell types, including breast cancer cells (171, 207-209). Several natural and synthetic retinoids can inhibit the development of mammary tumors and cause regression of established tumors in rats (210-212). Furthermore, clinical evidence supports the benefit of retinoids for breast cancer prevention in pre-menopausal women (166, 213).

Retinoids mediate their effects by binding to a group of nuclear receptors (RAR and RXR) belonging to the superfamily of nuclear receptors that includes ER. The activated receptors are transcription factors that heterodimerize to bind to retinoic acid response elements (RAREs) present in the promoter regions of target genes (214). Interestingly, the response to retinoids in breast cancer cell lines appears to correlate with the expression of ERα, suggesting a possible cross-talk between the retinoic acid and estrogen receptor pathways (197, 257). We have previously reported that the expression of ERα in ERα-negative human breast cancer cells modulates RAR signaling. We found that stable expression of ERα in the ER-negative human breast cancer cell line MDA-MB-231 led to increased activity of an RARE reporter construct

and sensitized the cells to growth inhibition by retinoic acid (RA) (197). Since breast tumors can express both the isoforms of the estrogen receptor, there exists the potential for ER signaling to be mediated by ERα, ERβ or both.

To evaluate the effects of ERβ on retinoid-mediated growth inhibition, we engineered hERβ-stably-transduced cells from the ERα-negative breast cancer cell line, MDA-MB-231, using retroviral technology. We observed several similarities between the stable ERβ expressing cell line and ERα-expressing breast cancer cells with regards to the growth and transcriptional response to retinoids. Our results suggest that retinoids could be used to target subpopulations of breast cancer cells that express functional ERβ.

3.3. MATERIALS AND METHODS

Cells. MDA-MB-231 (clone 10A) and the ERα-positive subclone (S30) were obtained courtesy of Dr. V.C. Jordan. All cell lines were routinely cultured in α-MEM phenol red-free medium (Life Technologies, Inc, Ontario, Canada) supplemented with 5% charcoal stripped serum. For the culture of S30 cells, 0.5 μg/ml G418 (GibcoBRL) was added to the above media. All cells were maintained in 5% CO_2 at 37 °C in a humidified atmosphere.

Construction of stable cell lines. The hERβ expression vector (1590 bp) was kindly provided by Dr. S. Mader. Retroviral vectors were constructed by cloning the above cDNA in the multiple cloning site (MCS) of the murine stem-cell virus retroviral vector (HC2). This technique has been previously described in detail (258). Concentrated virus

was used to infect MDA-MB-231 cells. Pooled populations of transduced cells were routinely analyzed by flow cytometry for eGFP expression, thus confirming expression of the bicistronic RNA and the stable expression of ERβ.

Western Blots. Whole cell extracts were isolated from confluent 150mm plates of transduced cells as previously described (258). Whole cell extracts were also isolated from cells treated with 10^{-6} M tRA (Sigma Chemical Co), 10^{-7} M tamoxifen (kindly provided by Dr. A.E. Wakeling (Zeneca, United Kingdom), 10^{-7} M ICI 182,780 (Sigma Chemical Co) and 10^{-7} M estradiol (Sigma Chemical Co) treated for 24 h. Protein lysates (50 μg) were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (BioRad). Membranes were probed using the ERβ Ab (QED Biosciences) and incubated with 2° antibody at a 4000 fold dilution prior to analysis by chemiluminescence. Membranes were exposed to anti-β-actin antibody (Sigma Chemical Co) to control for loading

Northern Blots. Expression of RARα and pS2 were analyzed by northern blot as previously described (171). Briefly, 20 μg of total RNA was isolated 24 h post-treatment with indicated ligands and electrophoresed on a 1% formaldehyde/agarose gel and blotted onto nitrocellulose filter. The filters were hybridized to radiolabeled probes of RARα (PstI fragment) or pS2, washed and autoradiographed.

Immunofluorescence. Cells were grown on cover slips until semi-confluent monolayers were obtained and then fixed with 4% cold paraformaldehyde in PBS.

Coverslips were washed with PBS-0.5% Triton X-100 containing 10% fetal calf serum (Gibco) for 5 minutes at room temperature. Incubation with 1:50 anti-ERβ (N-19 from Santa Cruz) diluted in PBS-0.1% Triton X-100 was performed for 3 hrs in a humid chamber. Cells were washed extensively with PBS-0.01% Triton X-100 and staining was performed using Alexa Fluor® 546-conjugated anti-goat secondary antibody (Molecular Probes) diluted 1:1000 in PBS-0.01% Triton X-100 for 30 minutes. Cells were washed again and 2 μg/ml DAPI (Molecular Probes) solution was added for 5 minutes to visualize nuclear staining. Coverslips were mounted onto glass slides using Immuno-Mount (Shandon Inc.) and cells were visualized with an Olympus BX51 fluorescence microscope. An oil immersion, 100x objective was selected for the observations.

Cell proliferation studies. Cells were seeded in 24-well plates at a density of 2000 cells/well. In the treated cells, a final concentration of 10^{-5} M tRA, 10^{-7} M E2, 10^{-7} M OHT or 10^{-7} M ICI 182,780 was replenished one day after seeding, and subsequently on days 3 and 5. Controls for treated cells contained identical concentrations of vehicle alone. After 6 days of treatment, cells were fixed in 10% trichloroacetic acid and subsequently stained with sulforhodamine B (SRB, Sigma). SRB is an aminoxanthene dye that binds to basic amino acid residues and gives an index of culture cell protein that is linear with cell number (259). Bound SRB was solubilized in 10mM unbuffered Tris and optical density was measured at 570 nm in a microplate reader for quadruplicate samples.

Analysis of RARβ expression. Expression of RARβ was assessed by RT-PCR as previously described (258). Briefly, cDNA was prepared from 1 μg RNA and PCR amplification was performed using RARβ2 primers. The 227 bp fragment was amplified using 35 cycles with a melting temperature of 57°C and was visualized on a 2.5 % agarose gel.

Transient transfections and CAT assays. Cells were plated at 2 x 10^5 cells/well in 6 well plates and allowed to adhere overnight in phenol red free α-MEM media supplemented with 5% charcoal-stripped FBS. Transfections were performed using Fugene (Boehringer Mannheim) according to the manufacturer's guidelines. Transfections of the βRE-tk-CAT and ERE(3)-tk-CAT have previously been described (258). Briefly, 1 μg of reporter plasmid was transfected with 1 μg of pCMV-βGal plasmid as an internal control using a ratio of 2:1 Fugene (Boehringer Mannheim) to DNA. Cells were treated with the indicated ligands after 5 hours and harvested 48 hours post-transfection. For transfection of an AP-1 response element, the above methodology was followed with the Coll73-Luc reporter, except that cells were treated with the indicated ligands for 24 h. The luciferase assay was performed in accordance with the manufacturer's guidelines (Promega) and measured using a Lumat LB-9507 luminometer (PerkinElmer Instruments, Germany). 12-O-tetra-decanoyl-phorbol-13-acetate (TPA, 100ng/ml) was used as a positive control.

Statistical Analysis. Results from representative experiments are shown as means of the number of replicates. Statistical analysis was performed using the Student's t-test where statistical significance was noted for $P<0.05$.

3.4. RESULTS

Stable expression of ERβ. To determine if ERβ can alter retinoid-mediated growth inhibition and transcription, we generated stable expression of hERβ in the ERα-negative parental MDA-MB-231 human breast cancer cell line (Fig 3.1.A). The ERβ cDNA was inserted upstream of an IRES and the eGFP gene in the HC2 retroviral vector, thereby allowing us to use flow cytometric analysis to monitor transduction efficiency. MDA-MB-231 cells were transduced with either the empty HC2 retroviral vector containing only eGFP (Ctrl), or retroviral vector expressing both ERβ and eGFP (ERβ). To achieve the highest transduction efficiency, cell sorting was performed on the basis of green fluorescence and a shift in fluorescence intensity was evident for both the retrovirus control and ERβ-transduced cell lines (Fig 3.1.B). In all experiments described, both the parental cell line (MDA-MB-231) and the empty retroviral transduced cell line (Ctrl) were utilized as ER-negative controls. However, to avoid superfluous data, only the empty retroviral transduced cell line (Ctrl) will be shown herein. Transduced polyclonal stable cell lines were tested for their expression of ERβ by western blots performed on whole cell extracts. Protein expression of ERβ at approximately 55 kDA was evident in the transduced cell line (ERβ) and, as expected, was absent in the cell line expressing only the empty retroviral vector (Ctrl) (Fig 3.1.C).

Localization of ERβ to the nucleus was further confirmed using immunofluorescence (Fig 3.1.D).

Growth and transcriptional response to ERβ ligands. Several groups have previously shown that re-introducing ERα into an ERα-negative cell line alters the proliferative response to the ERα agonist, estradiol (246, 260). In the ERβ-stably transduced cell line (ERβ), treatment with estradiol, tamoxifen, or ICI resulted in no significant change in proliferation (Fig 3.2.A). This is in agreement with a previous study in which the growth rate of ERβ-positive breast cancer cells was unaffected by estradiol or ICI (79). To validate the functionality of the expressed ERβ, we investigated the transcriptional activity from a reporter construct containing 3 tandem EREs, in the presence of known ERβ ligands. We observed an induction of transcription in response to estradiol, and complete inhibition of transcription from this reporter in the presence of tamoxifen and ICI in the ERβ-positive cells. As expected, these ligands had no effect in the cell line expressing only the retroviral vector (Ctrl). Increased baseline transcription in the ERβ cells may be attributed to residual estrogen in media or to ligand-independent receptor activity. These data confirm the functionality of ERβ in the stably transduced cell line.

Growth response of the ERβ stable transfectant to RA. We compared the growth inhibitory effect of RA in ERα-positive (ERα) and ERβ-positive cells (ERβ). The ERα stable cells are an ERα-positive subclone (S30) of MDA-MB-231 and were not derived from retroviral transduction (229). However, both cell lines were derived from the

identical parental cell line, MDA-MD-231. After 6 days of treatment with RA, we observed significant growth inhibition only in the cells expressing ERα or ERβ (Fig 3.3.A). To determine if the growth-inhibitory action of RA is altered by ERβ ligands, we compared the effect of RA in the presence of the ERβ agonist (E2) and antagonist (ICI). As seen in Figure 3.3.B, 6 days of treatment with RA alone resulted in approximately 50% growth reduction for ERβ-expressing breast cancer cells, and this remained unchanged regardless of the presence of ER agonistic or antagonistic ligand.

RA alters ERβ-mediated gene expression. The expression of ERα-regulated genes can be altered by RA (261). To determine if similar transcriptional effects would be observed in ERβ-positive cells, we studied the expression of pS2, a known ER-responsive gene containing an ERE in its promoter region. In ERβ-positive cells, pS2 is induced by 24 hours of treatment with estradiol, while RA entirely inhibits the expression of this E2-regulated gene (Fig 3.4.A). In addition, on a synthetic promoter containing 3 tandem EREs, RA inhibits transcription, and this inhibition is maintained even in the presence of estradiol (Fig 3.4.B). These data indicate that ER-mediated transcription is inhibited by RA, and that an ER agonist cannot rescue the transcriptional inhibitory effect of RA.

RA decreases estradiol-induced AP-1 activity in ERβ-positive breast cancer cells. The growth-inhibitory effect of retinoids in breast cancer cells has often been attributed to its inhibitory action on AP-1-mediated transcription. Using a reporter construct from the collagenase promoter containing an AP-1 response element (Coll73-Luc), we

observed increase in AP-1 activity upon E2 treatment in the ERβ stable transfectants, while both OHT and ICI treatment did not alter AP-1 transcription (Fig 3.5.). These results were unexpected since E2 has previously been characterized as an inhibitor of ERβ-mediated AP-1 activity, and OHT an activator. The Ctrl cell line was unaffected by ER ligands or RA (data not shown). When compared to vehicle-treated cells, treatment with RA alone did not significantly alter AP-1 mediated transcription. However, when ERβ expressing cells are co-treated with RA and E2, the AP-1-stimulatory actions of estradiol are blocked by RA.

ERβ alters the expression of RARβ2. Since we demonstrated that RA alters ERβ-mediated gene transcription, we evaluated the effect of ERβ on RAR-mediated genes. Both RARα and RARβ2 are retinoid regulated genes that have been shown to be important for RA-mediated growth inhibition. While some groups have shown that an increased level of RARα correlates with RA-mediated growth inhibition, others have attributed the inhibition of growth by RA to induction of RARβ2. To determine the level of these nuclear receptors in our stably transfected cell lines, we assessed the expression of RARα and RARβ2 by Northern blot and semi-quantitative RT-PCR respectively (Fig 3.6.). We noted no significant change in expression level of RARα in the Ctrl cell line, the ERα-positive or ERβ-positive stable transfectants, before or after RA treatment (Fig 3.6.A). Since the induction of RARβ2 has been associated with response to retinoids in certain types of cancer, we also assessed the expression of this gene in our system by semi-quantitative RT-PCR. It is well documented that RARβ2 is present at much lower levels than RARα in breast cancer cells, rendering it difficult to

perform a simple northern or western blot. In contrast to RARα, we observed significant differences in the level of expression of RARβ2. In the ERα or ERβ stable transfectants, expression of RARβ2 was suppressed in the absence of RA and, unlike the ER-negative cells, RARβ2 expression levels were strongly induced in response to RA (Fig 3.6.B).

Effect of ERβ on the transcriptional activity of a transiently expressed RARE. To further characterize the effect of ERβ on RARβ2 expression, we transiently expressed a reporter driven by the RARE of the RARβ2 promoter in the stably transduced cell lines and assessed transcriptional activity in the absence and presence of 10^{-6} M tRA (Fig 3.7.). We observed that cells expressing the ERβ receptor, as compared to the ER-negative cells, displayed significantly lower basal activity from this promoter in the absence of RA (Fig 3.7.B). Transient transfection of only the TK-CAT part of the reporter did not differ between the cell lines, indicating that the effect of ERα or ERβ on transcriptional activity was due to the βRARE and not any part of the thymidine kinase promoter (data not shown). We detected an approximately 10-fold decrease in basal activity from the βRE-tk-CAT in the presence of ERα or ERβ (Fig 3.7.B). Upon addition of RA, there was a strong induction of transcriptional activity in the cells that had a suppressed baseline, and a weaker induction in the parental cells (Fig 3.7.A). This transcriptional effect was only observed on an RARE, since reporter constructs containing VDRE, TRE or PPRE were unaffected by the expression of ERα or ERβ (data not shown). Thus, expression of ERβ converts RARβ2 expression from constitutive to ligand inducible.

An active ERβ is required to maintain RARβ2 inducibility. The ligands OHT and ICI 182,780 both inhibit the transcriptional activity of ERβ. We tested the activity of the βRARE reporter in the presence of these antagonists, and the agonistic ligand estradiol in the ERβ-positive cells (Fig 3.8.A). As expected, OHT, ICI 182,780 and estradiol had no effect on the Ctrl empty retroviral-transfected cell line. In cells stably expressing ERβ, OHT and ICI 182,780 completely released inhibition of the RAR-mediated transcription and restored baseline activity to the same level as seen in the ERα-negative cell line (Ctrl). Addition of estradiol, which activates ERβ via the AF-2 domain, further decreases βRARE activity in these cells. Since it is known that these ligands can alter the expression levels of ERα, we verified their effects on the expression of the stably transduced ERβ cells after 24 hours of treatment. Although there were no changes in mRNA expression (data not shown), the protein levels were significantly altered in the presence of the various ligands (Fig 3.8.B). As previously reported with ERα, estradiol and ICI 182,780 both down-regulate expression of ERβ. Although the expression of ERβ is similarly down-regulated by both of these ligands, only the antagonist ICI 182,870 increases the transcriptional activity from the βRARE. These data indicate that inhibition of ERβ transcriptional activity allows transcription from the βRARE. Conversely, activation of ERβ transcription with estradiol inhibits βRARE-mediated transcription.

3.5. DISCUSSION

Breast cancer is a hormone-dependent malignancy, with standard therapy directed at regulating ERα-mediated signaling. In pre-malignant, as well as malignant breast lesions, ER expression is significantly increased (262). The discovery of the ERβ in both normal and malignant breast tissue has added a new level of complexity, although the expression of ERβ in the breast is less abundant than ERα and there is controversy regarding its role in breast physiology and tumorigenesis (263).

Retinoids have demonstrated some therapeutic potential for the treatment of breast cancer (166. Although they do not target ER directly, there is considerable evidence correlating the presence of ER? with RA sensitivity [Rubin, 1994 #1416, 197, 213, 215-217). Importantly, clinical trials using a retinoid derivative have demonstrated efficacy in preventing contralateral breast cancer in pre-menopausal women exclusively, further suggesting a role for ERα in RA-mediated growth inhibition (167). Since ERβ can also be detected in breast cancer cells, we wished to determine its prognostic implications in the management of breast cancer with retinoids. For this purpose, we engineered ERβ-positive breast cancer cells using retroviral transfection of the ERα-negative MDA-MB-231 cell line. Although several ERβ isoforms have been identified in human breast cancer tissue and cell lines, we used the wild-type ERβ of 530 aa (80).

Expression of ERβ was confirmed by western blot and immunofluorescence. Functionality of the transduced ERβ was then demonstrated by transient transfection, using a synthetic ERE-containing reporter construct, and by estradiol-mediated activation of the pS2 gene. Although stable expression of ERβ restored ligand-dependent transcription, the growth properties of the stable cell lines were unaffected by

the ERβ ligands estradiol, tamoxifen or ICI. These results are in agreement with those previously reported in which ER ligands did not alter the proliferation of ERβ-expressing stable cell lines (79, 264). These data suggest that the estradiol-driven neoplastic process of the breast that has been described for ERα may not be pertinent to ERβ. While ER ligands did not alter the proliferation of ERβ-transduced cells, we observed that retinoids retained their ability to inhibit proliferation in ERβ-positive, as well as ERα- positive breast cancer cells, and that cell growth was inhibited regardless of co-treatment with ER ligands.

The mechanism for retinoid-mediated growth inhibition is not well understood. Retinoids suppress estradiol-mediated proliferation and transcriptional activity, and can antagonize the proliferative effects of AP-1 (265, 266). In the ERβ-transduced cells, we demonstrate that RA can suppress ERE-mediated transcription and represses estradiol-activated endogenous gene expression (pS2). However, repression of ERβ activity alone cannot explain the growth inhibitory properties of retinoids since ER ligands do not alter the proliferation of ERβ-positive breast cancer cells.

Increased AP-1 activity generally leads to activation of cell proliferation signals (267, 268). Since the growth inhibitory mechanisms of retinoids have, in part been attributed to the antagonism of this activity, we explored the possibility that ERβ-expressing cells may have altered AP-1 activity in response to RA. Several groups have demonstrated that, unlike ERα, antiestrogens activate ERβ-mediated AP-1 activity, while E2 is antagonistic (14, 95). In contrast, using a reporter construct from the collagenase promoter containing an AP-1 response element (Coll73-Luc), we noted that E2 increased AP-1 activity in these cells. Furthermore, the antiestrogens OHT and ICI

did not alter AP-1 mediated transcription in our stably transduced ERβ cells. These results, which contradict those observed in transient transfection of ERβ, are in accordance with those reported in another ERβ stably transfected MDA-MB-231 cell line in which antiestrogens were shown to be incapable of activating AP-1 response elements (264). We also report that addition of RA decreased estradiol-activated AP-1 activity, but did not significantly alter AP-1-mediated transcription.

The growth inhibitory action of retinoids has often been attributed to increased expression of RARα and to RARβ2 induction (196, 223, 247). We found that the expression of RARα was unchanged in response to RA, but that the basal expression of RARβ2 RNA was significantly lower in cells expressing ERα or ERβ than in parental ER-negative cells. Induction of RARβ2 expression has been associated with retinoid response in a variety of cancer cell types and provides another example of the cross talk between the ER and RAR mediated pathways in human breast cancer cells (222, 223, 247). Expression of ERβ alone is sufficient to alter the basal transcriptional properties of the RARβ2 promoter, as demonstrated by CHIP analysis showing increased acetylation at the promoter in absence of any ligand. Using ER ligands, we determined that the function of ERβ in altering RAR-mediated transcription takes precedence over its expression levels. Although both ICI and E2 decrease ERβ protein expression, these ligands oppose each other in their action on the RARβ promoter. We have previously shown that the N-terminal region of ERα, including the DBD, was important for mediating transcriptional cross-talk with RAR. Although ERβ varies greatly from ERα in the N-terminal region, there are some similarities. For example, it has been reported that both receptors can bind p300 at the N-terminal in absence of ligand (48). Therefore,

it remains a possibility that ERβ or ERα interaction with RAR transcription pathway may involve squelching for limited known or unknown cofactors. Stable cell lines using ERβ deletion mutants or ERβ variants will provide greater insight into this transcriptional interaction. It would be of special interest to determine the effect of ERβcx expression on retinoid activity in breast cancer cells. This isoform has been detected in human breast cancer, shows preferential dimerization with ERα and has a dominant negative effect on ligand-dependent ERα reporter gene transactivation (269).

The complexity of nuclear receptor signaling is becoming increasingly evident. While the ligand-dependent (via AF-2) and independent (via AF-1) functions require the DBD for their activity, nuclear receptors can also regulate transcription without binding to their cognate response elements. The role of nuclear receptors therefore extends far beyond that of simple ligand-induced transcriptional activators. Given the promiscuity of coactivators and corepressors with different nuclear receptors, it is not surprising that cross-talk exists between the different nuclear receptor families.

In conclusion, we provide evidence of nuclear receptor signaling cross-talk between ERβ and RAR in human breast cancer cells. We demonstrate that RA can significantly decrease the growth of ERβ-positive breast cancer cells in the presence or absence of ER ligands, thereby supporting the use of retinoids for the management of ERβ-positive breast cancer.

3.6. ACKNOWLEDGEMENTS

This study was supported in part by the Canadian Breast Cancer Research Initiative (CBCRI) and the Canadian Institute for Health Research (CIHR, MT-13147). We wish to thank Daniel Larocque for his technical expertise in the immunofluorescence studies.

Figure 3.1. Retroviral-mediated expression of hERβ in MDA-MB-231 ERα-negative breast cancer cells. **(A)** The cDNA of wild-type hERβ was cloned into the bicistronic murine stem cell virus-based retroviral vector. This vector contains a packaging signal (ψ), multiple cloning site (MCS), internal ribosomal entry site (IRES) and enhanced green fluorescence protein (eGFP) flanked by two long terminal repeats (LTRs). **(B)** Flow cytometric analysis of transduced MDA-MB-231 cells subsequent to cell sorting. The solid lines represent eGFP expression in the empty vector transduced cell (Ctrl) and ERβ-transduced cells (ERβ), while the dashed lines represent untransduced MDA-MB-231 cells. **(C)** ERβ protein expression of the transduced cells was analyzed by immunoblots using 50 μg of cell extracts and the QED anti-ERβ antibody. β-actin, which was used as a loading control, is also shown. **(D)** Immunostaining of transduced cells using an ERβ antibody (right panels) and visualized by fluorescence microscopy. DAPI staining was included to visualize the nucleus (left panels).

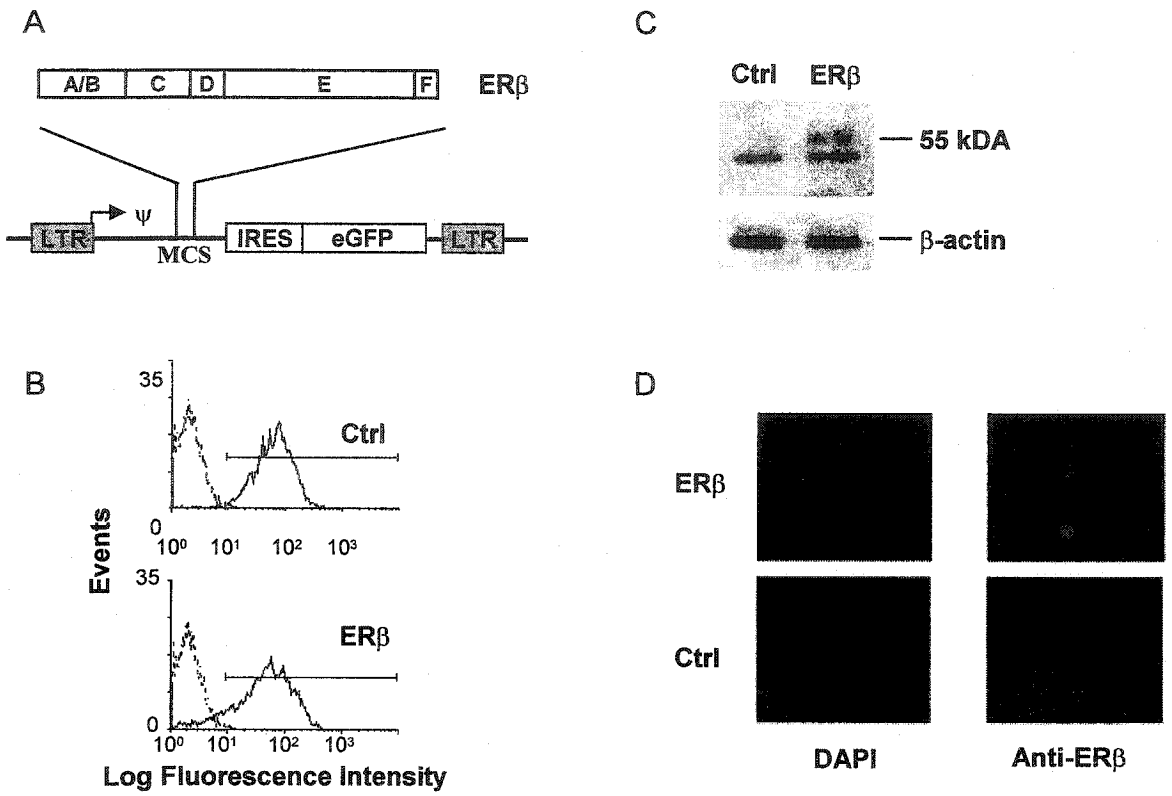

Figure 3.1. Retroviral-mediated expression of hERβ in MDA-MB-231 ERα-negative breast cancer cells.

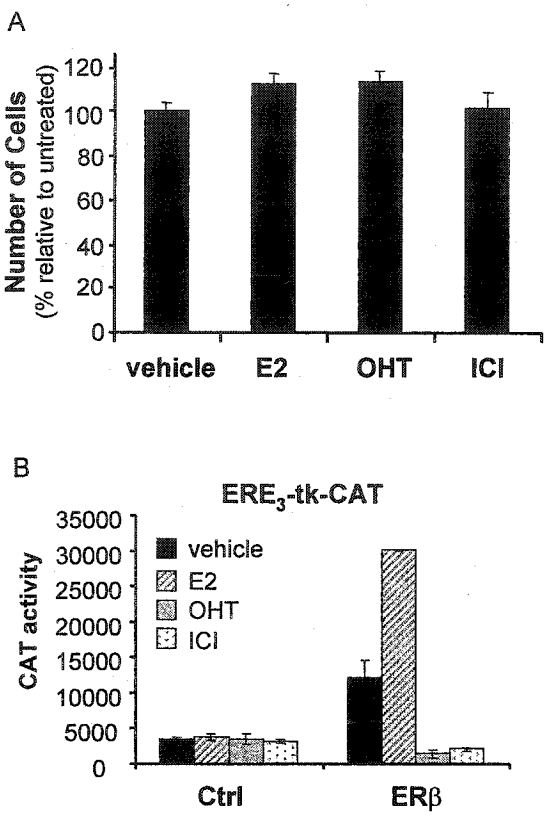

Figure 3.2. Effect of ERβ ligands on the growth and transcriptional properties of ERβ-transduced cells. **(A)** After 6 days of treatment with 10^{-7} M estradiol (E2), 10^{-7} M tamoxifen (OHT) or 10^{-7} M ICI 182,780, cell number was assessed using SRB staining as described in Materials and Methods. Data is representative of two separate experiments performed in quadruplicate. **(B)** ERβ-transduced cells exhibit ligand-dependent transcriptional activity from an ERE_3-tk-CAT reporter. Data was normalized with βGAL and represents the average of two independent experiments performed in triplicate.

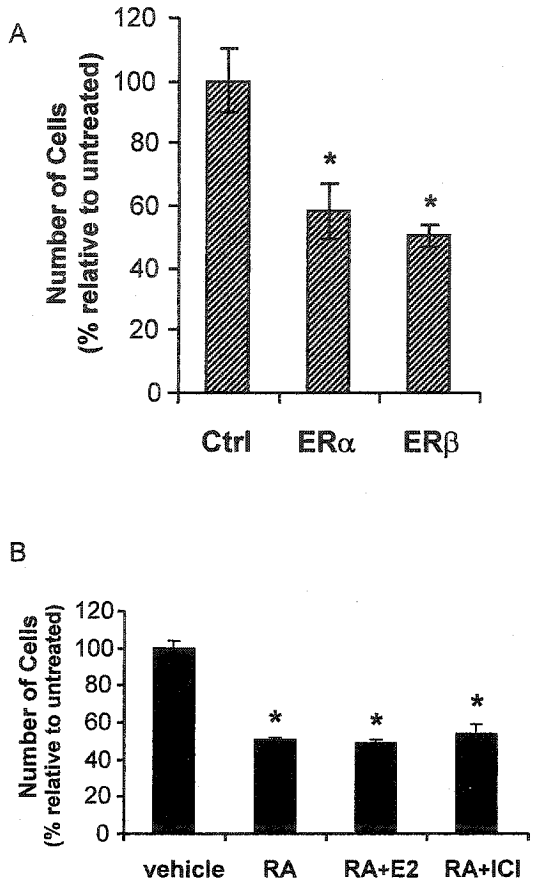

Figure 3.3. ERβ-transduced cells are growth inhibited by retinoic acid. **(A)** The number of viable cells stably expressing either the empty retroviral vector (Ctrl), ERα, or ERβ was assessed using SRB staining as described in Materials and Methods after 6 days of 10^{-5} M RA treatment and compared to untreated cells. **(B)** Retinoic acid-mediated growth inhibition in ERβ-transduced cells is unaltered by the E2 (10^{-7} M) or ICI 182,870 (10^{-7} M) after 6 days. Data represents an average of at least 3 different experiments, performed in triplicate. Statistical significance was calculated using Dunnett's test. Results that differ significantly from Ctrl (A) or treatment with vehicle (B) are indicated with an asterisk ($P < 0.05$).

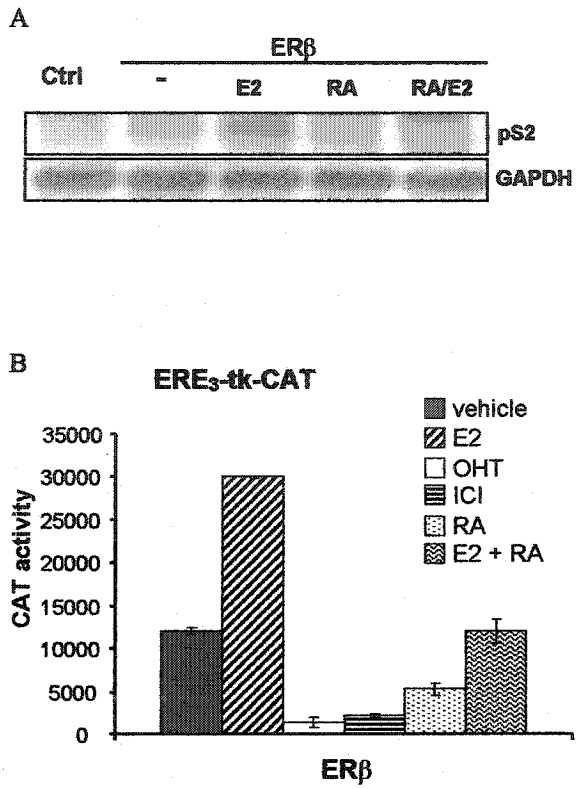

Figure 3.4. Analysis of gene regulation in ERβ-transduced breast cancer cells. **(A)** Northern blot analysis of RA-induced regulation of pS2 expression. Cells were treated with 10^{-5} M RA, 10^{-7} M E2 or a combination of both ligands for 24 hours. Twenty μg of total cellular RNA was loaded in each lane and the corresponding GAPDH expression is also represented. **(B)** RA inhibits transcription from an ERE. Cells were co-transfected with ERE_3-tk-CAT and CMV-βGAL and treated with the indicated ligands for 48 hours.

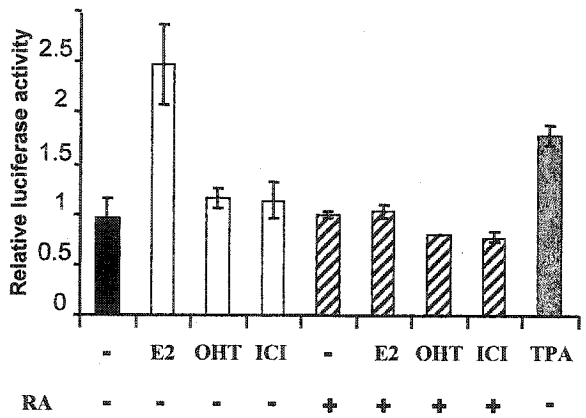

Figure 3.5. Analysis of AP-1-mediated transcriptional activity in ERβ-transduced breast cancer cells. ERβ-transduced cells were transfected with 1 μg collagenase promoter containing an AP-1 response element (Coll73-Luc) and 1 μg CMV-βGAL expression vector and treated with vehicle, 10^{-7} M estradiol, 10^{-7} M tamoxifen, 10^{-7} M ICI 182,780, 10^{-6} M RA or 100 ng/ml TPA for 24 h. Transcriptional activity is shown as the mean ± S.E.M. of at least two independent experiments performed in triplicate.

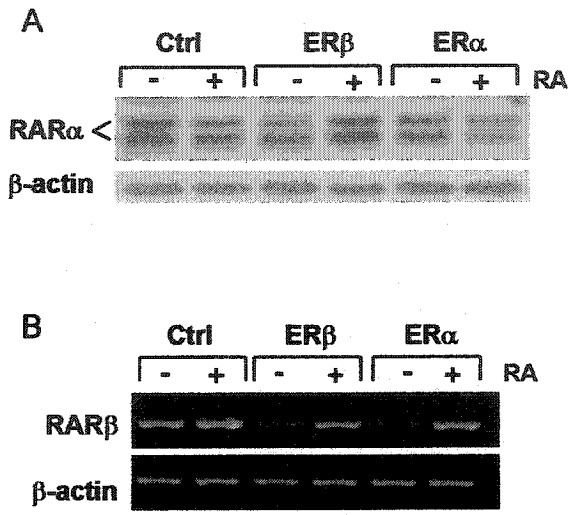

Figure 3.6. Retinoid receptor expression and regulation by RA in ER-positive cells. **(A)** RARα northern blot analysis of twenty μg of total cellular RNA isolated after 24 hours of 10^{-5} M RA (or vehicle) treatment. The corresponding GAPDH expression is shown to control for loading. **(B)** Basal RARβ2 expression is suppressed but inducible by RA in ER-expressing cells. RT-PCR analysis of RARβ2 in cells treated with RA (10^{-6} M) or vehicle for 24 h, using β-actin to control for variability in cDNA.

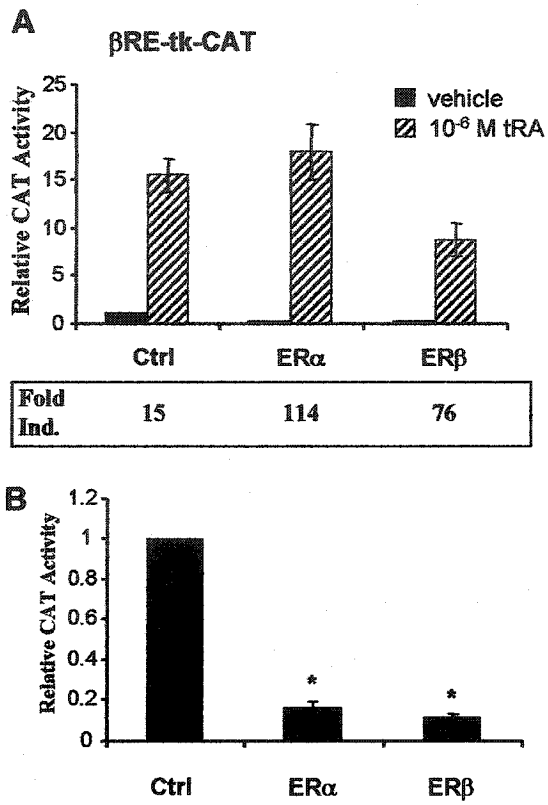

Figure 3.7. Expression of ERα or ERβ increases the fold induction of transcriptional activity from the βRARE promoter by suppressing basal activity in the absence of RA. **(A)** Retinoid-induced transcriptional activity in the absence (Ctrl) or presence of ERα or ERβ. Cells were transfected with 1 μg βRE-tk-CAT and 1 μg CMV-βGAL expression vector and treated with 10^{-6} M RA or vehicle for 48 h. The fold induction for each cell line is indicated in the box below. Transcriptional activity is shown as the mean ± S.E.M. of at least three independent experiments performed in triplicate. **(B)** Transcriptional activity from the βRE-tk-CAT in absence of ligand. Results are expressed as CAT activity relative to the ER-negative Ctrl cell line. Statistical significance was calculated using Dunnett's test, comparing each cell line to Ctrl. Results that differ significantly from Ctrl are indicated with an asterisk ($P < 0.05$).

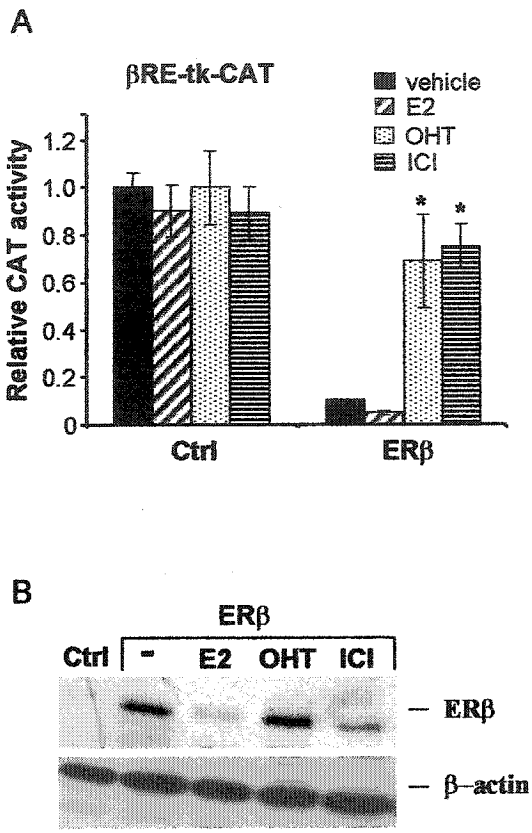

Figure 3.8. ERβ ligands alter transcriptional activity from the βRE-tk-CAT. **(A)** Baseline transcriptional activity of the βRE-tk-CAT was evaluated after treating the cells 1 h post-transfection with vehicle, 10^{-7} M tamoxifen, 10^{-7} M ICI 182,780, or 10^{-7} M estradiol. Transcriptional activity is shown as relative CAT activity compared to Ctrl and significant differences between the vehicle and treated samples ($P < 0.05$) are indicated with an asterisk. **(B)** Immunoblot of 50 μg of whole cell extracts depicting the effect of the ERβ ligands mentioned above on ERβ protein expression after 24 h. Cells were also immunoblotted for β-actin as a loading control.

CHAPTER 4

GENERAL DISCUSSION

Discussion

This thesis project began with the observation, from our lab and others, that the presence of ERα renders human breast cancer cells more sensitive to the growth inhibitory properties of retinoids. The clinical relevance of this project was later substantiated by results from clinical trials, indicating that the potential benefit of retinoids in preventing contralateral breast cancer occurred primarily in pre-menopausal women (166). I began this project with the intent to study the mechanism by which growth of ERα-positive human breast cancer cells are inhibited by retinoids. My objective was to understand the role of ERα in retinoid-mediated growth inhibition, in hopes of providing more strategic approaches to the development of therapies for the treatment or prevention of breast cancer.

The first aim of this project was to identify the domain of ERα important for conferring sensitivity to retinoids. To realize this objective, the laborious task of generating stable cell lines of ERα-wild type and of several ERα-deletion mutants was undertaken, using the parental ER-negative MDA-MB-231 cell line (described in Chapter 2.3), . We reasoned that using a retroviral method to generate stable expression of ERα-deletion mutants and ERβ in ER-negative breast cancer cells would be advantageous over the stable transfection of a single expanded clone. The difficulty in generating stable transfection of ERα into ER-negative breast cancer cells is well documented (229, 245, 246) and, while I confirmed expression of the ERα deletion mutants, I was unable to generate a stably transduced cell line expressing wild-type ERα. I therefore obtained the previously characterized S30 cell line, generated by

isolation of a single clone stably expressing ERα in the parental cell line MDA-MB-231, as a positive control (229).

One of the major hurdles of this project was the manipulations of the stable cell lines. After a few weeks in culture, some the transduced cells lost expression of GFP and presumably of ERα expression. As such, it was necessary to frequently verify GFP expression of the transduced cells by flow cytometry. In order to study exclusively positive cells, cultured transduced cells were regularly sorted to obtain maximal expression of ERα or of the ERα-deletion mutants. This was an extensive process since cells continually required to be expanded and stocks frozen prior to performing experiments. In addition, it was often necessary to confirm the expression and functionality of the transduced ERα-deletion mutant.

Despite these difficulties, the panel of stably-transduced cell lines expressing various ERα-deletion mutants was utilized to observe the effect of ERα on both RA-mediated growth and RA-mediated gene expression. Although RA has been shown to activate several genes involved in growth inhibition, only a few have defined RAREs and are immediate targets of RA (146)]. I elected to study the expression and regulation of the RA-inducible gene RARβ2. Induction of RARβ2 expression has been associated with retinoid response in a variety of cancer cell types (222, 223, 247). Within the promoter region of the RARβ2 gene is the βRARE with a DR5 configuration. Interestingly, only the breast cancer cells expressing ERα exhibit an increase in fold induction using a βRARE reporter construct, and similar results are observed with the endogenous RARβ2 gene. However, the increase in fold activation did not result in increased activity when treated with RA, but rather by a significant decrease in baseline

activity from the promoter in the absence of any ligand. These results indicate that ERα modifies the transcriptional response from the βRARE in absence of RA, and is suggestive of a transcriptional cross-talk between ERα and RAR in human breast cancer cells. Interestingly, the basal expression of RARβ2 RNA was significantly lower in cells expressing full-length ERα or in cells expressing only the N-terminal of ERα (including the DBD). In MDA-MB-231 (the parental ER-negative cell line), HC2 (empty retroviral vector), HEG19 (N-terminal ERα deletion mutant) and HEG11 (lacking the DBD), baseline βRARE activity was elevated and was only weakly induced by RA. I therefore concluded that the N-terminal region of ERα results in decreased basal RARβ expression, but confers its inducibility by RA.

Interestingly, there exist other genes with similar expression patterns in retinoid sensitive breast cancer cells. SOX9, a member of the high mobility group (HMG) box gene family of transcription factors, exhibits decreased basal expression but is induced by RA in retinoid sensitive cell lines. Cell lines that are unresponsive to RA do not induce SOX9 expression, and in ERα-negative cells, constitutively high expression of this gene has been demonstrated (270). In addition, RA has been shown to induce IGFBP-3 mRNA levels in retinoid sensitive breast cancer cells (271). In the ERα-positive breast cancer cell line MCF-7, IGFBP-3 is not expressed at baseline but is induced by treatment with RA (192). In contrast, ERα-negative, retinoid unresponsive breast cancer cell lines exhibit high basal expression of IGFBP-3 that is not significantly increased by RA (272). Our lab has also discovered that another gene, UBE1L, is regulated in a similar manner by RA in human breast cancer cell lines (Sonia del Rincon et al, unpublished results). However, the regulation of the above genes has

not yet been studied in the stable cell lines expressing the ERα-deletion mutants. Induction of target genes, from low baseline levels, may represent a possible mechanism of growth inhibition by retinoids.

When the effect of the ERα-deletion mutants on RA sensitivity was initially studied, the cells expressing either wild-type ERα or ERα with the LBD deletion were significantly growth inhibited by RA (discussed in Chapter 2). This provided preliminary evidence of the significance of the N-terminal region of ERα in mediating the growth inhibitory effects of RA. Since the growth inhibition correlated with induction of RARβ2, we hypothesized that induction of this gene may be important for RA-mediated growth inhibition. However, after the cells were continually passaged, they became resistant to RA, even though they retained their ability to induce RARβ2. This data suggests that induction of RARβ2 alone is not enough to generate an anti-proliferative response to retinoids in MDA-MB-231 cells. In support of this notion, recent evidence has surfaced demonstrating that RARβ2 induction is not observed in several breast cancer cells sensitive to retinoids (273).

The role of RARβ in mediating RA responsiveness to breast cancer cells has been the subject of some controversy. Regulation of RARβ2 by RA has been linked to retinoid response in a variety of cancer cell types and there is evidence to suggest that RARβ can act as a tumor suppressor (222, 223, 247). The stable cell lines that I constructed using various ERα-deletion mutants provided a means to study the regulation of RARβ2 by ERα and the role of ERα proteins in conferring sensitivity to the growth inhibitory effect of RA. While I provide experimental evidence in support of a novel interaction between nuclear receptors, whereby the N-terminal region of ERα

suppresses the baseline activity of RAR/RXR-mediated transcription from the RARβ2 promoter, it can also be concluded that induction of RARβ2 is insufficient to explain the anti-proliferative effects of RA. Other areas of cross-talk between ERα and RAR need therefore to be explored.

The mechanism by which retinoids inhibit growth in breast cancer cells is not well understood. In fact, both anti-proliferative and anti-apoptotic effects have been observed in retinoid-treated cells (273). Several retinoid-responsive gene products have been identified that are central to both the anti-proliferative and anti-apoptotic processes, including down-regulation of cyclin D1, cdk2 and the antiapoptotic protein Bcl-2 (273). Retinoids have also been shown to decrease the inhibitor of apoptosis family member, survivin (273). As an attempt to identify a mechanism for retinoid induced growth inhibition, several groups have suggested that individual factors, such as induction of RARβ2, inhibition of AP-1 and activation of RARα, are key mediators of this activity (164, 187, 274, 275). However, neither the extent of cell cycle protein regulation, nor AP-1 regulation fully predicts the antiproliferative effect of retinoids, suggesting that growth inhibition requires regulation of a spectrum of RAR-regulated gene products (273).

Although not discussed in the manuscripts included in Chapters 2 and 3 of this dissertation, the search for retinoid altered genes involved in RA-mediated sensitivity was a complementary part of this research project. Using the Clontech Atlas™ Human Cancer cDNA expression array specific for 588 cancer-related genes, differentially regulated genes in the ERα-positive and retinoid sensitive S30 cell line were identified. Using this technique, 10 genes were selected that were differentially expressed

following 24 h RA treatment (Appendix A, Figure A.1 and Table A.1). However, when I compared the regulation of these genes in ERα-positive versus ERα-negative cell lines, no significant differences were noted. Although an in depth analysis of gene regulation by retinoids was beyond the scope of this thesis project, it would certainly be of clinical value to identify retinoid target genes important for growth inhibition that differ in their regulation in the presence of ERα. This experiment was performed early in the course of this research project and warrants further investigation using a more extensive gene array, and more reproducible cell lines.

The identification of RARβ2 as an RA-regulated gene whose induction was increased by expression of ERα, provided our lab with a model in which to study transcriptional cross-talk. To understand how ERα alters RARβ expression, I explored the hypothesis that ERα may modulate the expression or activity of transcriptional intermediates. Although it is not known whether cofactor levels are clinically related to carcinogenesis, it seems likely that changes in the levels or activity of cofactors could have profound effect on target gene expression. Indeed, increased expression of some cofactors, notably of AIB-1 in breast and prostate cancer, has been reported (238, 276). However, I did not observe significant differences in gene or protein expression of the coactivators p-300, CBP, SRC-1, TIF-2, AIB-1 or of the corepessors SMRT and NCoR, in ERα-positive versus ERα-negative cells (data not shown). In addition, the possibility of squelching by the N-terminal domain of ERα was discussed in Chapter 2. Several nuclear receptors have been shown to modulate each others' activities by squelching, or recruiting, common cofactors (277-279). However, transfecting increasing amounts of the common cofactors CBP, p-300 and SRC-1 did not alter the inhibitory effects of

ERα on baseline transcription from the βRARE. These cofactors were selected based upon their demonstrated ability to bind both RAR and the N-terminal region of ERα (5, 47, 48) Although my data indicate that squelching of these common cofactors does not clarify the mechanism of this transcriptional cross-talk, I cannot exclude the possibility that the N-terminal region of ERα squelches other known or novel cofactors.

The role of the estrogen receptor in RA response is further complicated by the recent discovery of another estrogen receptor, ERβ. ERβ can heterodimerize with ERα, interact with the same ligands and shares greater than 90% homology with the DNA-binding domain. Since ERβ is also present in human breast tumors, I wished to determine if ERβ positive cells would be potential therapeutic targets for retinoids. I reported transcriptional cross-talk with ERβ and retinoids, and showed that ERβ, like ERα, exhibits a reduction in expression of RARβ leading to a greater induction of RARβ after RA treatment. Using the ERβ stably transduced cells, retinoids inhibit ERβ-mediated pathways and, like ERα-positive cells, exhibit increased induction of RARβ2. However, inhibition of E2-mediated transcription is not sufficient to mediate growth inhibition by RA since E2 alone did not increase the proliferation rate of the ERβ positive cells and E2 antagonists had no effect on the growth of ERβ-transduced MDA-MB-231 cells.

The Future of Retinoids in Breast cancer

Interestingly, preclinical studies have shown that retinoids can increase the activity of ER-antagonists such as tamoxifen, which is widely used in prevention and treatment of breast cancer (174, 280). Moreover, a phase I/II trial of tRA and tamoxifen

observed some objective responses in patients who had previously progressed on tamoxifen alone (218). The mechanism of action of retinoids and traditional ER inhibitors such as tamoxifen are already known to be different and thus potentially additive or synergistic (281). This encourages further studies of the potential use of retinoids as part of novel combination therapies of breast cancer. For rational development of such novel therapies, it is important to clarify how retinoids exert their effects on breast cancer cells, how their activity is regulated by expression of ER, and what determines sensitivity vs. resistance to these compounds.

Unfortunately, in the clinic, resistance to retinoids rapidly occurs due to pharmacokinetic or cellular mechanisms (282). As an example, studies with Cyt P450 inhibitors suggest that cytochome P450 induction may be involved in RA resistance by increasing the clearance and metabolism of RA (134) In the cell, resistance to RA has been attributed to increased CYP26 or CRABP expression, increased excretion of RA by the P-glycoprotein drug-efflux pump, or by point mutations in the ligand-binding domain of the RAR receptor (134).

In addition to the drawback of acquired resistance, the toxicity of natural derivatives of vitamin A limits their therapeutic use. Synthetic compounds that are selective for the different retinoid receptor isotypes have been generated to circumvent retinoid toxicities. The generation of specific retinoids or rexinoids targeted to a particular isotype of RARs or RXRs is opening new avenues for cancer therapy and chemoprevention.

The future of retinoids is almost certainly dependent upon their efficacy in combination with other chemotherapeutic or chemopreventive therapies. Combination

therapy using retinoids and demethylating agents or histone deacetylase inhibitors has displayed promising antitumor effects in vitro (283, 284). Aza-CdR and sodium-phenylbutyrate are potent inducers of growth arrest, differentiation, or apoptosis of transformed and tumor cells *in vitro* and *in vivo* (285, 286, 287). Combinations of these substances with retinoic acid warrant further evaluation in breast cancer models. Retinoids have also shown synergy with certain chemotherapeutic agents (288, 289). Wang et al have demonstrated that pre-treatment with RA lowers the threshold for cell killing by the chemotherapeutic agents Taxol and Adriamycin. As an added example, the combination of heregulin and retinoids was more effective in inducing branching morphogenesis of breast cancer cells cultivated in 3D collagen gels, than either agent alone (290).

Understanding the cross-talk of nuclear receptor with other signaling transduction pathways will no doubt be important to the design of targeted combination therapies. In breast cancer cells, determining the mechanism of retinoid induced growth inhibition may improve the possibility of developing non-retinoid drugs able to mimic the effects of retinoids on signal transduction pathways responsible for the tumor-suppressive effect. Evidently, this strategy requires identifying the downstream gene targets regulated by the antiproliferative effect of retinoids (194).

Concluding Remarks

Although I was unable to uncover the mechanism for RA sensitivity in ER-positive cell, the results presented in this thesis have unearthed several important observations. First, a novel cross-talk between ERα and RAR, involving the N-terminal

region of ERα was reported. This interaction is not observed with other members of the nuclear receptor family that heterodimerize with RXR. Second, I define two models, one involving transient transfection of the minimal βRARE promoter, and the other involving expression of the endogenous RARβ2 gene, to study this interaction. Also, I exclude the possibility of squelching of the common cofactors, p300, SRC-1 and CBP, by ER. Third, I report that transcriptional cross talk exists between ERβ and RAR, and that RA inhibits activation of ERβ-mediated gene activation. Finally, I demonstrate that RA can be used to inhibit the growth of ERβ-positive breast cancer cells. Together, the conclusions derived from this research project have contributed to enhanced understanding of the effect of retinoids in human breast cancer cells.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

The work presented in this doctoral thesis has provided several original contributions to the existing body of scientific knowledge in the field of nuclear receptors.

In Chapter 2, we show that there exists transcriptional cross-talk between the ERα and RAR at the RA-responsive element located in the RARβ2 promoter. This is the first study demonstrating that the N-terminal region of ERα, including the DBD, alters the transcriptional activity of another nuclear receptor, RAR, in absence of any ligand. We further demonstrated that this cross-talk is specific for RAR since other promoters that utilize RXR as a partner (ex. VDR, TR and PPAR) are unaffected by the presence of ER.

In Chapter 3, we investigate the effect of introducing full-length ERβ into the ER-negative cell line MDA-MB-231. This is the first report describing the effect of retinoids on an ERβ-positive breast cancer cell line. We report significant RA-mediated growth inhibition in ERβ-positive cells, indicating that retinoids are effective in inhibiting breast cancer cell growth in a sub-population of breast cancer expressing exclusively ERβ. In addition, our studies demonstrate that ERβ ligands do not alter the response to retinoids, thereby validating the use of retinoids, regardless of the presence of ERβ agonist or antagonist. We further show that RA alters ERβ-mediated pathways, as shown by its inhibitory effects on the expression of the endogenous pS2 gene.

Our findings demonstrate the presence of cross-talk between ER and RAR and underscore the importance of characterizing this cross-talk in order to understand the role of ER in sensitizing breast cancer cells to retinoids.

REFERENCES

1. Keaveney, M., and Stunnenberg, H. G. (1995) Retinoic acid receptors. In *Inducible gene exression* (Baeuerle, P. A., ed) pp. 187-241, Birkh,,user Press, Boston
2. Mangelsdorf, D. J., and Evans, R. M. (1995) The RXR heterodimers and orphan receptors. *Cell* 83, 841-850.
3. Ribeiro, R. C., Kushner, P. J., and Baxter, J. D. (1995) The nuclear hormone receptor gene superfamily. *Annu Rev Med* 46, 443-453.
4. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and et al. (1995) The nuclear receptor superfamily: the second decade. *Cell* 83, 835-839.
5. Aranda, A., and Pascual, A. (2001) Nuclear hormone receptors and gene expression. *Physiol Rev* 81, 1269-1304.
6. Joel, P. B., Smith, J., Sturgill, T. W., Fisher, T. L., Blenis, J., and Lannigan, D. A. (1998) pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol Cell Biol* 18, 1978-1984.
7. Kato, S., Masuhiro, Y., Watanabe, M., Kobayashi, Y., Takeyama, K. I., Endoh, H., and Yanagisawa, J. (2000) Molecular mechanism of a cross-talk between oestrogen and growth factor signalling pathways. *Genes Cells* 5, 593-601.
8. Rogatsky, I., Trowbridge, J. M., and Garabedian, M. J. (1999) Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A-CDK2 complex. *J Biol Chem* 274, 22296-22302
9. Crowe, D. L., and Kim, R. (2002) A phosphorylation defective retinoic acid receptor mutant mimics the effects of retinoic acid on EGFR mediated AP-1 expression and cancer cell proliferation. *Cancer Cell Int* 2, 15.
10. Bastien, J., Adam-Stitah, S., Riedl, T., Egly, J. M., Chambon, P., and Rochette-Egly, C. (2000) TFIIH interacts with the retinoic acid receptor gamma and phosphorylates its AF-1-activating domain through cdk7. *J Biol Chem* 275, 21896-21904.

11. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 20, 321-344.

12. Kumar, R., and Thompson, E. B. (2003) Transactivation functions of the N-terminal domains of nuclear hormone receptors: protein folding and coactivator interactions. *Mol Endocrinol* 17, 1-10.

13. Sadovsky, Y., Webb, P., Lopez, G., Baxter, J. D., Fitzpatrick, P. M., Gizang-Ginsberg, E., Cavailles, V., Parker, M. G., and Kushner, P. J. (1995) Transcriptional activators differ in their responses to overexpression of TATA-box-binding protein. *Mol Cell Biol* 15, 1554-1563.

14. Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M., and Webb, P. (2000) Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol* 74, 311-317.

15. Beato, M., Herrlich, P., and Schutz, G. (1995) Steroid hormone receptors: many actors in search of a plot. *Cell* 83, 851-857.

16. Keaveney, M., and Stunnenberg, H. (1995) Retinoic acid receptors. In *Inducible Gene Expression* (Baeuerle, P. A., ed) Vol. 2 pp. 187-241, Birkhauser Press, Boston

17. Chambraud, B., Berry, M., Redeuilh, G., Chambon, P., and Baulieu, E. E. (1990) Several regions of human estrogen receptor are involved in the formation of receptor-heat shock protein 90 complexes. *J Biol Chem* 265, 20686-20691.

18. Chen, J. D., and Evans, R. M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* 377, 454-457

19. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and et al. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* 377, 397-404.

20. Housley, P. R., Sanchez, E. R., Danielsen, M., Ringold, G. M., and Pratt, W. B. (1990) Evidence that the conserved region in the steroid binding domain of the glucocorticoid receptor is required for both optimal binding of hsp90 and protection from proteolytic cleavage. A two-site model for hsp90 binding to the steroid binding domain. *J Biol Chem* 265, 12778-12781.

21. Picard, D., and Yamamoto, K. R. (1987) Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *Embo J* 6, 3333-3340.

22. Feng, W., Ribeiro, R. C., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998) Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* 280, 1747-1749.

23. Dilworth, F. J., and Chambon, P. (2001) Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. *Oncogene* 20, 3047-3054.

24. McKenna, N. J., and O'Malley, B. W. (2002) Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 108, 465-474.

25. Hsiao, P. W., Deroo, B. J., and Archer, T. K. (2002) Chromatin remodeling and tissue-selective responses of nuclear hormone receptors. *Biochem Cell Biol* 80, 343-351.

26. Cosma, M. P., Tanaka, T., and Nasmyth, K. (1999) Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. *Cell* 97, 299-311.

27. Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000) Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. *Cell* 103, 667-678.

28. Fry, C. J., and Peterson, C. L. (2002) Transcription. Unlocking the gates to gene expression. *Science* 295, 1847-1848.

29. Soutoglou, E., and Talianidis, I. (2002) Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. *Science* 295, 1901-1904.

30. Gregory, P. D., Wagner, K., and Horz, W. (2001) Histone acetylation and chromatin remodeling. *Exp Cell Res* 265, 195-202.

31. Strahl, B. D., and Allis, C. D. (2000) The language of covalent histone modifications. *Nature* 403, 41-45.

32. Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K., and Zhou, M. M. (1999) Structure and ligand of a histone acetyltransferase bromodomain. *Nature* 399, 491-496.

33. Kraus, W. L., and Wong, J. (2002) Nuclear receptor-dependent transcription with chromatin. Is it all about enzymes? *Eur J Biochem* 269, 2275-2283.

34. Schneider, R., Bannister, A. J., and Kouzarides, T. (2002) Unsafe SETs: histone lysine methyltransferases and cancer. *Trends Biochem Sci* 27, 396-402.

35. Santos-Rosa, H., Schneider, R., Bernstein, B. E., Karabetsou, N., Morillon, A., Weise, C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2003) Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin. *Mol Cell* 12, 1325-1332.

36. Nyce, J. W. (1997) Drug-induced DNA hypermethylation: a potential mediator of acquired drug resistance during cancer chemotherapy. *Mutat Res* 386, 153-161.

37. Strathdee, G., MacKean, M. J., Illand, M., and Brown, R. (1999) A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. *Oncogene* 18, 2335-2341.

38. Geiman, T. M., and Robertson, K. D. (2002) Chromatin remodeling, histone modifications, and DNA methylation-how does it all fit together? *J Cell Biochem* 87, 117-125.

39. Kalebic, T. (2003) Epigenetic changes: potential therapeutic targets. *Ann N Y Acad Sci* 983, 278-285.

40. Fuks, F., Burgers, W. A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. (2000) DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat Genet* 24, 88-91.

41. Futscher, B. W., Oshiro, M. M., Wozniak, R. J., Holtan, N., Hanigan, C. L., Duan, H., and Domann, F. E. (2002) Role for DNA methylation in the control of cell type specific maspin expression. *Nat Genet* 31, 175-179.

42. Diel, P. (2002) Tissue-specific estrogenic response and molecular mechanisms. *Toxicol Lett* 127, 217-224.

43. Glass, C. K., and Rosenfeld, M. G. (2000) The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 14, 121-141.

44. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature* 375, 377-382.

45. Palvimo, J. J., Kallio, P. J., Ikonen, T., Mehto, M., and Janne, O. A. (1993) Dominant negative regulation of trans-activation by the rat androgen receptor: roles of the N-terminal domain and heterodimer formation. *Mol Endocrinol* 7, 1399-1407.

46. Alen, P., Claessens, F., Verhoeven, G., Rombauts, W., and Peeters, B. (1999) The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. *Mol Cell Biol* 19, 6085-6097.

47. McInerney, E. M., Tsai, M. J., O'Malley, B. W., and Katzenellenbogen, B. S. (1996) Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. *Proc Natl Acad Sci U S A* 93, 10069-10073.

48. Kobayashi, Y., Kitamoto, T., Masuhiro, Y., Watanabe, M., Kase, T., Metzger, D., Yanagisawa, J., and Kato, S. (2000) p300 mediates functional synergism between AF-1 and AF-2 of estrogen receptor alpha and beta by interacting directly with the N-terminal A/B domains. *J Biol Chem* 275, 15645-15651.

49. Endoh, H., Maruyama, K., Masuhiro, Y., Kobayashi, Y., Goto, M., Tai, H., Yanagisawa, J., Metzger, D., Hashimoto, S., and Kato, S. (1999) Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. *Mol Cell Biol* 19, 5363-5372.

50. Wang, C., Fu, M., Angeletti, R. H., Siconolfi-Baez, L., Reutens, A. T., Albanese, C., Lisanti, M. P., Katzenellenbogen, B. S., Kato, S., Hopp, T., Fuqua, S. A., Lopez, G. N., Kushner, P. J., and Pestell, R. G. (2001) Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. *J Biol Chem* 276, 18375-18383.

51. McKenna, N. J., and O'Malley, B. W. (2002) Minireview: nuclear receptor coactivators--an update. *Endocrinology* 143, 2461-2465.

52. Chen, H., Lin, R. J., Xie, W., Wilpitz, D., and Evans, R. M. (1999) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* 98, 675-686.

53. Chen, D., Huang, S. M., and Stallcup, M. R. (2000) Synergistic, p160 coactivator-dependent enhancement of estrogen receptor function by CARM1 and p300. *J Biol Chem* 275, 40810-40816.

54. Sohn, Y. C., Kim, S. W., Lee, S., Kong, Y. Y., Na, D. S., Lee, S. K., and Lee, J. W. (2003) Dynamic inhibition of nuclear receptor activation by corepressor binding. *Mol Endocrinol* 17, 366-372.

55. Smith, C. L., Nawaz, Z., and O'Malley, B. W. (1997) Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol Endocrinol* 11, 657-666.

56. Montano, M. M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P., and Katzenellenbogen, B. S. (1999) An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. *Proc Natl Acad Sci U S A* 96, 6947-6952.

57. Thiagalingam, S., Cheng, K. H., Lee, H. J., Mineva, N., Thiagalingam, A., and Ponte, J. F. (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. *Ann N Y Acad Sci* 983, 84-100.

58. Robertson, K. D., Ait-Si-Ali, S., Yokochi, T., Wade, P. A., Jones, P. L., and Wolffe, A. P. (2000) DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet* 25, 338-342.

59. Li, X., Kimbrel, E. A., Kenan, D. J., and McDonnell, D. P. (2002) Direct interactions between corepressors and coactivators permit the integration of nuclear receptor-mediated repression and activation. *Mol Endocrinol* 16, 1482-1491.

60. Fondell, J. D., Ge, H., and Roeder, R. G. (1996) Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc Natl Acad Sci U S A* 93, 8329-8333.

61. Rachez, C., Suldan, Z., Ward, J., Chang, C. P., Burakov, D., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1998) A novel protein complex

that interacts with the vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system. *Genes Dev* 12, 1787-1800.

62. Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* 398, 824-828.

63. Naar, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W., and Tjian, R. (1999) Composite co-activator ARC mediates chromatin-directed transcriptional activation. *Nature* 398, 828-832

64. Metivier, R., Penot, G., Hubner, M. R., Reid, G., Brand, H., Kos, M., and Gannon, F. (2003) Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 115, 751-763.

65. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. (2000) Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* 103, 843-852.

66. Burakov, D., Crofts, L. A., Chang, C. P., and Freedman, L. P. (2002) Reciprocal recruitment of DRIP/mediator and p160 coactivator complexes in vivo by estrogen receptor. *J Biol Chem* 277, 14359-14362.

67. Narlikar, G. J., Fan, H. Y., and Kingston, R. E. (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell* 108, 475-487.

68. Weigel, N. L. (1996) Steroid hormone receptors and their regulation by phosphorylation. *Biochemical Journal* 319, 657-667

69. Matsuda, K., Ochiai, I., Nishi, M., and Kawata, M. (2002) Colocalization and ligand-dependent discrete distribution of the estrogen receptor (ER)alpha and ERbeta. *Mol Endocrinol* 16, 2215-2230.

70. King, W. J., and Greene, G. L. (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* 307, 745-747.

71. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* 93, 5925-5930.

72. Mosselman, S., Polman, J., and Dijkema, R. (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* 392, 49-53.

73. Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F., and Giguere, V. (1997) Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol* 11, 353-365.

74. Dechering, K., Boersma, C., and Mosselman, S. (2000) Estrogen receptors alpha and beta: two receptors of a kind? *Curr Med Chem* 7, 561-576.

75. Cowley, S. M., and Parker, M. G. (1999) A comparison of transcriptional activation by ER alpha and ER beta. *J Steroid Biochem Mol Biol* 69, 165-175.

76. Hall, J. M., and McDonnell, D. P. (1999) The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 140, 5566-5578.

77. Murphy, L. C., Leygue, E., Dotzlaw, H., Coutts, A., Lu, B., Huang, A., and Watson, P. H. (2000) Multiple Facets of Estrogen Receptor in Human Breast Cancer. In *Endocrine Oncology* (Ethier, S. P., ed) Vol. 2 pp. 17-34, Humana Press Inc., Totowa

78. Couse, J. F., and Korach, K. S. (1999) Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* 20, 358-417.

79. Lazennec, G., Bresson, D., Lucas, A., Chauveau, C., and Vignon, F. (2001) ER beta inhibits proliferation and invasion of breast cancer cells. *Endocrinology* 142, 4120-4130.

80. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J. A. (2001) Mechanisms of estrogen action. *Physiol Rev* 81, 1535-1565.

81. Jarvinen, T. A., Pelto-Huikko, M., Holli, K., and Isola, J. (2000) Estrogen receptor beta is coexpressed with ERalpha and PR and associated with nodal status, grade, and proliferation rate in breast cancer. *Am J Pathol* 156, 29-35.

82. Liu, M. M., Albanese, C., Anderson, C. M., Hilty, K., Webb, P., Uht, R. M., Price, R. H., Jr., Pestell, R. G., and Kushner, P. J. (2002) Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. *J Biol Chem* 277, 24353-24360.

83. Speirs, V., and Kerin, M. J. (2000) Prognostic significance of oestrogen receptor beta in breast cancer. *Br J Surg* 87, 405-409.

84. Lu, B., Leygue, E., Dotzlaw, H., Murphy, L. J., Murphy, L. C., and Watson, P. H. (1998) Estrogen receptor-beta mRNA variants in human and murine tissues. *Mol Cell Endocrinol* 138, 199-203.

85. Tong, D., Schuster, E., Seifert, M., Czerwenka, K., Leodolte, S., and Zeillinger, R. (2002) Expression of estrogen receptor beta isoforms in human breast cancer tissues and cell lines. *Breast Cancer Res Treat* 71, 249-255.

86. Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *Embo J* 15, 2174-2183.

87. Gehm, B. D., McAndrews, J. M., Jordan, V. C., and Jameson, J. L. (2000) EGF activates highly selective estrogen-responsive reporter plasmids by an ER-independent pathway. *Mol Cell Endocrinol* 159, 53-62.

88. El-Tanani, M. K., and Green, C. D. (1997) Two separate mechanisms for ligand-independent activation of the estrogen receptor. *Mol Endocrinol* 11, 928-937.

89. Lamb, J., Ladha, M. H., McMahon, C., Sutherland, R. L., and Ewen, M. E. (2000) Regulation of the functional interaction between cyclin D1 and the estrogen receptor. *Mol Cell Biol* 20, 8667-8675.

90. Ignar-Trowbridge, D. M., Pimentel, M., Parker, M. G., McLachlan, J. A., and Korach, K. S. (1996) Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. *Endocrinology* 137, 1735-1744.

91. Sanchez, R., Nguyen, D., Rocha, W., White, J. H., and Mader, S. (2002) Diversity in the mechanisms of gene regulation by estrogen receptors. *Bioessays* 24, 244-254.

92. Harrington, W. R., Sheng, S., Barnett, D. H., Petz, L. N., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (2003) Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. *Mol Cell Endocrinol* 206, 13-22.

93. Klinge, C. M. (2001) Estrogen receptor interaction with estrogen-response elements. *Nucleic Acids Research* 29, 2905-2919

94. Safe, S. (2001) Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* 62, 231-252.

95. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. (1997) Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 277, 1508-1510.

96. Dong, L., Wang, W., Wang, F., Stoner, M., Reed, J. C., Harigai, M., Samudio, I., Kladde, M. P., Vyhlidal, C., and Safe, S. (1999) Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. *J Biol Chem* 274, 32099-32107.

97. Duan, R., Porter, W., and Safe, S. (1998) Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology* 139, 1981-1990.

98. Kim, K., Thu, N., Saville, B., and Safe, S. (2003) Domains of estrogen receptor alpha (ERalpha) required for ERalpha/Sp1-mediated activation of GC-rich promoters by estrogens and antiestrogens in breast cancer cells. *Mol Endocrinol* 17, 804-817.

99. Salvatori, L., Pallante, P., Ravenna, L., Chinzari, P., Frati, L., Russo, M. A., and Petrangeli, E. (2003) Oestrogens and selective oestrogen receptor (ER) modulators regulate EGF receptor gene expression through human ER alpha and beta subtypes via an Sp1 site. *Oncogene* 22, 4875-4881.

100. Xie, W., Duan, R., and Safe, S. (1999) Estrogen induces adenosine deaminase gene expression in MCF-7 human breast cancer cells: role of estrogen receptor-Sp1 interactions. *Endocrinology* 140, 219-227.

101. Sun, G., Porter, W., and Safe, S. (1998) Estrogen-induced retinoic acid receptor alpha 1 gene expression: role of estrogen receptor-Sp1 complex. *Mol Endocrinol* 12, 882-890.

102. Teyssier, C., Belguise, K., Galtier, F., and Chalbos, D. (2001) Characterization of the physical interaction between estrogen receptor alpha and JUN proteins. *J Biol Chem* 276, 36361-36369.

103. Jakacka, M., Ito, M., Weiss, J., Chien, P. Y., Gehm, B. D., and Jameson, J. L. (2001) Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. *J Biol Chem* 276, 13615-13621

104. McDonnell, D. P., Connor, C. E., Wijayaratne, A., Chang, C. Y., and Norris, J. D. (2002) Definition of the molecular and cellular mechanisms underlying the tissue-selective agonist/antagonist activities of selective estrogen receptor modulators. *Recent Prog Horm Res* 57, 295-316.

105. Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. (1995) Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Molecular Endocrinology* 9, 443-456

106. Webb, P., Nguyen, P., Valentine, C., Lopez, G. N., Kwok, G. R., McInerney, E., Katzenellenbogen, B. S., Enmark, E., Gustafsson, J. A., Nilsson, S., and Kushner, P. J. (1999) The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. *Mol Endocrinol* 13, 1672-1685.

107. Wilcken, N. R., Prall, O. W., Musgrove, E. A., and Sutherland, R. L. (1997) Inducible overexpression of cyclin D1 in breast cancer cells reverses the growth-inhibitory effects of antiestrogens. *Clin Cancer Res* 3, 849-854.

108. Webb, P., Valentine, C., Nguyen, P., Price, R. H., Jr., Marimuthu, A., West, B. L., Baxter, J. D., and Kushner, P. J. (2003) ERbeta Binds N-CoR in the Presence

of Estrogens via an LXXLL-like Motif in the N-CoR C-terminus. *Nucl Recept* 1, 4.

109. Stein, B., and Yang, M. X. (1995) Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. *Mol Cell Biol* 15, 4971-4979.

110. Wissink, S., van der Burg, B., Katzenellenbogen, B. S., and van der Saag, P. T. (2001) Synergistic activation of the serotonin-1A receptor by nuclear factor-kappa B and estrogen. *Mol Endocrinol* 15, 543-552.

111. Pietras, R. J., and Szego, C. M. (1977) Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* 265, 69-72.

112. Razandi, M., Pedram, A., and Levin, E. R. (2000) Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. *Mol Endocrinol* 14, 1434-1447.

113. Simoncini, T., Fornari, L., Mannella, P., Varone, G., Caruso, A., Liao, J. K., and Genazzani, A. R. (2002) Novel non-transcriptional mechanisms for estrogen receptor signaling in the cardiovascular system. Interaction of estrogen receptor alpha with phosphatidylinositol 3-OH kinase. *Steroids* 67, 935-939.

114. Aronica, S. M., Kraus, W. L., and Katzenellenbogen, B. S. (1994) Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci U S A* 91, 8517-8521.

115. Marquez, D. C., and Pietras, R. J. (2001) Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. *Oncogene* 20, 5420-5430.

116. Keen, J. C., and Davidson, N. E. (2003) The biology of breast carcinoma. *Cancer* 97, 825-833.

117. Margolese, R. G., Hortobagyi, G. N., and Buchholz, T. A. (2003) Neoplasms of the Breast. In *Cancer Medicine 6* (Kufe, D. E., Pollock, R. E., Weichselbaum, R. R., Bast, R. C. B., Gansler, T. S. G., Holland, J. F., and Frei, E., eds) pp. 1879-1949, BC Decker, Hamilton

118. Anderson, E., Clarke, R. B., and Howell, A. (2000) Estrogen Receptor in Mammary Gland Physiology. In *Endocrine Oncology* (Ethier, S. P., ed) Vol. 1 pp. 1-16, Humana Press, Ann Arbor

119. Ellis, M. J., Jenkins, S., Hanfelt, J., Redington, M. E., Taylor, M., Leek, R., Siddle, K., and Harris, A. (1998) Insulin-like growth factors in human breast cancer. *Breast Cancer Res Treat* 52, 175-184.

120. Dubik, D., and Shiu, R. P. (1992) Mechanism of estrogen activation of c-myc oncogene expression. *Oncogene* 7, 1587-1594.

121. Prall, O. W., Rogan, E. M., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. (1998) c-Myc or cyclin D1 mimics estrogen effects on cyclin E-Cdk2 activation and cell cycle reentry. *Mol Cell Biol* 18, 4499-4508.

122. Baldin, V., Lukas, J., Marcote, M. J., Pagano, M., and Draetta, G. (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev* 7, 812-821.

123. Imagawa, W., Pedchenko, V. K., Helber, J., and Zhang, H. (2002) Hormone/growth factor interactions mediating epithelial/stromal communication in mammary gland development and carcinogenesis. *J Steroid Biochem Mol Biol* 80, 213-230.

124. Nicholson, R. I., McClelland, R. A., Robertson, J. F., and Gee, J. M. (1999) Involvement of steroid hormone and growth factor cross-talk in endocrine response in breast cancer. *Endocr Relat Cancer* 6, 373-387.

125. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. (1997) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol* 17, 3629-3639.

126. Zujewski, J. (2002) Selective estrogen receptor modulators (SERMs) and retinoids in breast cancer chemoprevention. *Environ Mol Mutagen* 39, 264-270.

127. McDonnell, D. P., Chang, C. Y., and Norris, J. D. (2001) Capitalizing on the complexities of estrogen receptor pharmacology in the quest for the perfect SERM. *Ann N Y Acad Sci* 949, 16-35.

128. Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994) The retinoid receptors. In *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. B.,

Roberts, A. B., and Goodman, D. S., eds) Vol. 2 pp. 319-349, Raven Press, New York

129. Sun, S. Y., and Lotan, R. (2002) Retinoids and their receptors in cancer development and chemoprevention. *Crit Rev Oncol Hematol* 41, 41-55.

130. Altucci, L., and Gronemeyer, H. (2001) The promise of retinoids to fight against cancer. *Nat Rev Cancer* 1, 181-193.

131. Ralhan, R., and Kaur, J. (2003) Retinoids as chemopreventive agents. *J Biol Regul Homeost Agents* 17, 66-91.

132. Zusi, F. C., Lorenzi, M. V., and Vivat-Hannah, V. (2002) Selective retinoids and rexinoids in cancer therapy and chemoprevention. *Drug Discov Today* 7, 1165-1174.

133. Lee, P. P., Lee, M. T., Darcy, K. M., Shudo, K., and Ip, M. M. (1995) Modulation of normal mammary epithelial cell proliferation, morphogenesis, and functional differentiation by retinoids: a comparison of the retinobenzoic acid derivative RE80 with retinoic acid. *Endocrinology* 136, 1707-1717.

134. Marill, J., Idres, N., Capron, C. C., Nguyen, E., and Chabot, G. G. (2003) Retinoic acid metabolism and mechanism of action: a review. *Curr Drug Metab* 4, 1-10

135. Gaines, P., and Berliner, N. (2003) Retinoids in myelopoiesis. *J Biol Regul Homeost Agents* 17, 46-65.

136. Delva, L., Cornic, M., Balitrand, N., Guidez, F., Miclea, J. M., Delmer, A., Teillet, F., Fenaux, P., Castaigne, S., Degos, L., and et al. (1993) Resistance to all-trans retinoic acid (ATRA) therapy in relapsing acute promyelocytic leukemia: study of in vitro ATRA sensitivity and cellular retinoic acid binding protein levels in leukemic cells. *Blood* 82, 2175-2181.

137. Braakhuis, B. J., Klaassen, I., van der Leede, B. M., Cloos, J., Brakenhoff, R. H., Copper, M. P., Teerlink, T., Hendriks, H. F., van der Saag, P. T., and Snow, G. B. (1997) Retinoid metabolism and all-trans retinoic acid-induced growth inhibition in head and neck squamous cell carcinoma cell lines. *Br J Cancer* 76, 189-197.

138. Takase, S., Ong, D. E., and Chytil, F. (1986) Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus. 247, 328-334

139. Allenby, G., Bocquel, M. T., Saunders, M., Kazmer, S., Speck, J., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J. F., Chambon, P., and et al. (1993) Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc Natl Acad Sci U S A* 90, 30-34.

140. Mangelsdorf, D., Umesono, K., and Evans, R. (1994) The retinoid receptors. In *The Retinoids* (Sporn, M., and Goodman, D., eds) pp. 319-349, Raven Press, New York

141. Nagpal, S., and Chandraratna, R. A. (1998) Vitamin A and regulation of gene expression. *Curr Opin Clin Nutr Metab Care* 1, 341-346.

142. Keaveney, M., and Stunnenberg, H. G. (1995) Retinoic acid receptors. In *Inducible Gene Expression* (Baeuerle, P. A., ed) Vol. 2 pp. 186-229, Birkhauser, Boston

143. Rastinejad, F., Wagner, T., Zhao, Q., and Khorasanizadeh, S. (2000) Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1. *Embo J* 19, 1045-1054.

144. Mangelsdorf, D. J., Umesono, K., Kliewer, S. A., Borgemeyer, U., Ong, E. S., and Evans, R. M. (1991) A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* 66, 555-561

145. Nakshatri, H., and Bhat-Nakshatri, P. (1998) Multiple parameters determine the specificity of transcriptional response by nuclear receptors HNF-4, ARP-1, PPAR, RAR and RXR through common response elements. *Nucleic Acids Res* 26, 2491-2499.

146. Balmer, J. E., and Blomhoff, R. (2002) Gene expression regulation by retinoic acid. *J Lipid Res* 43, 1773-1808.

147. Dokmanovic, M., Chang, B. D., Fang, J., and Roninson, I. B. (2002) Retinoid-induced growth arrest of breast carcinoma cells involves co-activation of multiple growth-inhibitory genes. *Cancer Biol Ther* 1, 24-27.

148. Chen, J. Y., Penco, S., Ostrowski, J., Balaguer, P., Pons, M., Starrett, J. E., Reczek, P., Chambon, P., and Gronemeyer, H. (1995) RAR-specific

agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage- independent cell proliferation. *EMBO Journal* 14, 1187-1197

149. Takao, J., Yudate, T., Das, A., Shikano, S., Bonkobara, M., Ariizumi, K., and Cruz, P. D. (2003) Expression of NF-kappaB in epidermis and the relationship between NF-kappaB activation and inhibition of keratinocyte growth. *Br J Dermatol* 148, 680-688.

150. Witcher, M., Ross, D. T., Rousseau, C., Deluca, L., and Miller, W. H., Jr. (2003) Synergy between all-trans retinoic acid and tumor necrosis factor pathways in acute leukemia cells. *Blood* 102, 237-245.

151. Mann, K. K., Shao, W., and Miller, W. H., Jr. (2001) The biology of acute promyelocytic leukemia. *Curr Oncol Rep* 3, 209-216.

152. Picard, E., Seguin, C., Monhoven, N., Rochette-Egly, C., Siat, J., Borrelly, J., Martinet, Y., Martinet, N., and Vignaud, J. M. (1999) Expression of retinoid receptor genes and proteins in non-small-cell lung cancer. *J Natl Cancer Inst* 91, 1059-1066.

153. Wan, H., Oridate, N., Lotan, D., Hong, W. K., and Lotan, R. (1999) Overexpression of retinoic acid receptor beta in head and neck squamous cell carcinoma cells increases their sensitivity to retinoid-induced suppression of squamous differentiation by retinoids. *Cancer Res* 59, 3518-3526.

154. Xu, X. C., Sneige, N., Liu, X., Nandagiri, R., Lee, J. J., Lukmanji, F., Hortobagyi, G., Lippman, S. M., Dhingra, K., and Lotan, R. (1997) Progressive decrease in nuclear retinoic acid receptor beta messenger RNA level during breast carcinogenesis. *Cancer Res* 57, 4992-4996.

155. Widschwendter, M., Berger, J., Daxenbichler, G., Muller-Holzner, E., Widschwendter, A., Mayr, A., Marth, C., and Zeimet, A. G. (1997) Loss of retinoic acid receptor beta expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer. *Cancer Res* 57, 4158-4161.

156. Houle, B., Rochette-Egly, C., and Bradley, W. E. (1993) Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. *Proc Natl Acad Sci U S A* 90, 985-989.

157. Yang, Q., Sakurai, T., and Kakudo, K. (2002) Retinoid, retinoic acid receptor beta and breast cancer. *Breast Cancer Res Treat* 76, 167-173.

158. Xu, X. C., Ro, J. Y., Lee, J. S., Shin, D. M., Hong, W. K., and Lotan, R. (1994) Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. *Cancer Res* 54, 3580-3587.

159. Hoffman, A. D., Engelstein, D., Bogenrieder, T., Papandreou, C. N., Steckelman, E., Dave, A., Motzer, R. J., Dmitrovsky, E., Albino, A. P., and Nanus, D. M. (1996) Expression of retinoic acid receptor beta in human renal cell carcinomas correlates with sensitivity to the antiproliferative effects of 13-cis-retinoic acid. *Clin Cancer Res* 2, 1077-1082.

160. Sun, S. Y., Wan, H., Yue, P., Hong, W. K., and Lotan, R. (2000) Evidence that retinoic acid receptor beta induction by retinoids is important for tumor cell growth inhibition. *J Biol Chem* 275, 17149-17153.

161. Liu, Y., Lee, M. O., Wang, H. G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., Zhang, and X. (1996) Retinoic acid receptor beta mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Molecular & Cellular Biology* 16, 1138-1149

162. Deng, G., Lu, Y., Zlotnikov, G., Thor, A. D., and Smith, H. S. (1996) Loss of heterozygosity in normal tissue adjacent to breast carcinomas. *Science* 274, 2057-2059.

163. Roman, S. D., Clarke, C. L., Hall, R. E., Alexander, I. E., and Sutherland, R. L. (1992) Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res* 52, 2236-2242.

164. Liu, Y., Lee, M. O., Wang, H. G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X. (1996) Retinoic acid receptor beta mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol Cell Biol* 16, 1138-1149.

165. Tsou, H. C., Yao, Y. J., Xie, X. X., Ping, X. L., and Peacocke, M. (1998) Repression of transactivation of the retinoic acid receptor beta2 promoter in human breast cancer cells. *Exp Cell Res* 245, 221-227.

166. Veronesi, U., De Palo, G., Marubini, E., Costa, A., Formelli, F., Mariani, L., Decensi, A., Camerini, T., Del Turco, M. R., Di Mauro, M. G., Muraca, M. G., Del Vecchio, M., Pinto, C., D'Aiuto, G., Boni, C., Campa, T., Magni, A., Miceli, R., Perloff, M., Malone, W. F., and Sporn, M. B. (1999) Randomized trial of fenretinide to prevent second breast malignancy in women with early breast cancer. *J Natl Cancer Inst* 91, 1847-1856.

167. Veronesi, U., De Palo, G., Costa, A., Formelli, F., and Decensi, A. (1996) Chemoprevention of breast cancer with fenretinide. *IARC Sci Publ*, 87-94.

168. Li, X. S., Shao, Z. M., Sheikh, M. S., Eiseman, J. L., Sentz, D., Jetten, A. M., Chen, J. C., Dawson, M. I., Aisner, S., Rishi, A. K., and et al. (1995) Retinoic acid nuclear receptor beta inhibits breast carcinoma anchorage independent growth. *J Cell Physiol* 165, 449-458.

169. Swisshelm, K., Ryan, K., Lee, X., Tsou, H. C., Peacocke, M., and Sager, R. (1994) Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Differ* 5, 133-141.

170. van der Burg, B., van der Leede, B. M., Kwakkenbos-Isbrucker, L., Salverda, S., de Laat, S. W., and van der Saag, P. T. (1993) Retinoic acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Molecular and Cellular Endocrinology* 91, 149-157

171. Rubin, M., Fenig, E., Rosenauer, A., Menendez-Botet, C., Achkar, C., Bentel, J. M., Yahalom, J., Mendelsohn, J., and Miller, W. H., Jr. (1994) 9-cis retinoic acid inhibits growth of breast cancer cells and down-regulates estrogen receptor RNA and protein. *Cancer Research* 54, 6549-6556

172. Fontana, J. A., Nervi, C., Shao, Z.-M., and Jetten, A. M. (1992) Retinoid antagonism of estrogen-responsive transforming growth factor à and pS2 gene expression in breast carcinoma cells. 52, 3938-3945

173. Kazmi, S. M., Plante, R. K., Visconti, V., and Lau, C. Y. (1996) Comparison of N-(4-hydroxyphenyl)retinamide and all-trans-retinoic acid in the regulation of retinoid receptor-mediated gene expression in human breast cancer cell lines. *Cancer Res* 56, 1056-1062.

174. Anzano, M. A., Byers, S. W., Smith, J. M., Peer, C. W., Mullen, L. T., Brown, C. C., Roberts, A. B., and Sporn, M. B. (1994) Prevention of breast cancer in the rat with 9-cis-retinoic acid as a single agent and in combination with tamoxifen. 54, 4614-4617

175. de The, H., Vivanco-Ruiz, M. M., Tiollais, P., Stunnenberg, H., and Dejean, A. (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor beta gene. *Nature* 343, 177-180.

176. Valcarcel, R., Meyer, M., Meisterernst, M., and Stunnenberg, H. G. (1997) Requirement of cofactors for RXR/RAR-mediated transcriptional activation in vitro. *Biochim Biophys Acta* 1350, 229-234.

177. Shen, S., Kruyt, F. A., den Hertog, J., van der Saag, P. T., and Kruijer, W. (1991) Mouse and human retinoic acid receptor beta 2 promoters: sequence comparison and localization of retinoic acid responsiveness. *DNA Seq* 2, 111-119.

178. Baust, C., Redpath, L., and Schwarz, E. (1996) Different ligand responsiveness of human retinoic-acid-receptor beta-gene transcription in tumorigenic and non-tumorigenic cervical-carcinoma-derived cell lines is mediated through a large retinoic-acid-response domain. *Int J Cancer* 67, 409-416.

179. Folkers, G. E., van der Burg, B., and van der Saag, P. T. (1998) Promoter architecture, cofactors, and orphan receptors contribute to cell-specific activation of the retinoic acid receptor beta2 promoter. *J Biol Chem* 273, 32200-32212.

180. Lin, B., Chen, G. Q., Xiao, D., Kolluri, S. K., Cao, X., Su, H., and Zhang, X. K. (2000) Orphan receptor COUP-TF is required for induction of retinoic acid receptor beta, growth inhibition, and apoptosis by retinoic acid in cancer cells. *Mol Cell Biol* 20, 957-970.

181. Widschwendter, M., Berger, J., Muller, H. M., Zeimet, A. G., and Marth, C. (2001) Epigenetic downregulation of the retinoic acid receptor-beta2 gene in breast cancer. *J Mammary Gland Biol Neoplasia* 6, 193-201.

182. Lefebvre, B., Ozato, K., and Lefebvre, P. (2002) Phosphorylation of histone H3 is functionally linked to retinoic acid receptor beta promoter activation. *EMBO Rep* 3, 335-340.

183. Widschwendter, M., Berger, J., Hermann, M., Muller, H. M., Amberger, A., Zeschnigk, M., Widschwendter, A., Abendstein, B., Zeimet, A. G., Daxenbichler, G., and Marth, C. (2000) Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. *J Natl Cancer Inst* 92, 826-832.

184. Bovenzi, V., and Momparler, R. L. (2001) Antineoplastic action of 5-aza-2'-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor beta and estrogen receptor alpha genes in breast carcinoma cells. *Cancer Chemother Pharmacol* 48, 71-76.

185. Arapshian, A., Kuppumbatti, Y. S., and Mira-y-Lopez, R. (2000) Methylation of conserved CpG sites neighboring the beta retinoic acid response element may mediate retinoic acid receptor beta gene silencing in MCF-7 breast cancer cells. *Oncogene* 19, 4066-4070.

186. Sirchia, S. M., Ferguson, A. T., Sironi, E., Subramanyan, S., Orlandi, R., Sukumar, S., and Sacchi, N. (2000) Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. *Oncogene* 19, 1556-1563.

187. Lin, F., Xiao, D., Kolluri, S. K., and Zhang, X.-K. (2000) Unique anti-activator protein-1 activity of retinoic acid receptor beta. *Cancer Research* 60, 3271-3280

188. Dong, Z., Birrer, M. J., Watts, R. G., Matrisian, L. M., and Colburn, N. H. (1994) Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc Natl Acad Sci U S A* 91, 609-613.

189. Toulouse, A., Loubeau, M., Morin, J., Pappas, J. J., Wu, J., and Bradley, W. E. (2000) RARbeta involvement in enhancement of lung tumor cell immunogenicity revealed by array analysis. *Faseb J* 14, 1224-1232.

190. van der Burg, B., Slager-Davidov, R., van der Leede, B. M., de Laat, S. W., and van der Saag, P. T. (1995) Differential regulation of AP1 activity by retinoic

acid in hormone-dependent and -independent breast cancer cells. *Mol Cell Endocrinol* 112, 143-152.

191. Yang, L., Kim, H. T., Munoz-Medellin, D., Reddy, P., and Brown, P. H. (1997) Induction of retinoid resistance in breast cancer cells by overexpression of cJun. *Cancer Res* 57, 4652-4661.

192. Shang, Y., Baumrucker, C. R., and Green, M. H. (1999) Signal relay by retinoic acid receptors a and B in the retinoic acid-induced expression of insulin-like growth factor-binding protein-3 in breast cancer cells. *J Biol Chem* 274, 18005-18010

193. Ciolino, H. P., Wang, T. T., and Sathyamoorthy, N. (2000) Inhibition of aromatase activity and expression in MCF-7 cells by the chemopreventive retinoid N-(4-hydroxy-phenyl)-retinamide. *Br J Cancer* 83, 333-337.

194. Roninson, I. B., and Dokmanovic, M. (2003) Induction of senescence-associated growth inhibitors in the tumor-suppressive function of retinoids. *J Cell Biochem* 88, 83-94.

195. van der Burg, B., van der Leede, B. M., Kwakkenbos-Isbrucker, L., Salverda, S., de Laat, S. W., and van der Saag, P. T. (1993) Retinoic acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Mol Cell Endocrinol* 91, 149-157.

196. Sheikh, M. S., Shao, Z. M., Chen, J. C., Hussain, A., Jetten, A. M., and Fontana, J. A. (1993) Estrogen receptor-negative breast cancer cells transfected with the estrogen receptor exhibit increased RAR alpha gene expression and sensitivity to growth inhibition by retinoic acid. *J Cell Biochem* 53, 394-404

197. Rosenauer, A., Nervi, C., Davison, K., Lamph, W. W., Mader, S., and Miller, W. H., Jr. (1998) Estrogen receptor expression activates the transcriptional and growth-inhibitory response to retinoids without enhanced retinoic acid receptor alpha expression. *Cancer Res* 58, 5110-5116.

198. Pratt, M. A., Deonarine, D., Teixeira, C., Novosad, D., Tate, B. F., and Grippo, J. F. (1996) The AF-2 region of the retinoic acid receptor alpha mediates retinoic acid inhibition of estrogen receptor function in breast cancer cells. *J Biol Chem* 271, 20346-20352.

199. Rosenfeld, M. G., and Glass, C. K. (2001) Coregulator codes of transcriptional regulation by nuclear receptors. *J Biol Chem* 276, 36865-36868.

200. Robyr, D., Wolffe, A. P., and Wahli, W. (2000) Nuclear hormone receptor coregulators in action: diversity for shared tasks. *Mol Endocrinol* 14, 329-347.

201. Weigel, N. L., and Zhang, Y. (1998) Ligand-independent activation of steroid hormone receptors. *J Mol Med* 76, 469-479.

202. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., and et al. (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270, 1491-1494.

203. Campbell, R. A., Bhat-Nakshatri, P., Patel, N. M., Constantinidou, D., Ali, S., and Nakshatri, H. (2001) Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *J Biol Chem* 276, 9817-9824

204. Wu, X., Li, H., and Chen, J. D. (2001) The human homologue of the yeast DNA repair and TFIIH regulator MMS19 is an AF-1-specific coactivator of estrogen receptor. *J Biol Chem* 276, 23962-23968.

205. Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M. P., Chen, D., Huang, S. M., Subramanian, S., McKinerney, E., Katzenellenbogen, B. S., Stallcup, M. R., and Kushner, P. J. (1998) Estrogen receptor activation function 1 works by binding p160 coactivator proteins. *Mol Endocrinol* 12, 1605-1618.

206. Sanchez, R., Nguyen, D., Rocha, W., White, J. H., and Mader, S. (2002) Diversity in the mechanisms of gene regulation by estrogen receptors. *Bioessays, in print.*

207. Slack, J. L. (1999) Biology and treatment of acute progranulocytic leukemia. *Curr Opin Hematol* 6, 236-240.

208. Mangiarotti, R., Danova, M., Alberici, R., and Pellicciari, C. (1998) All-trans retinoic acid (ATRA)-induced apoptosis is preceded by G1 arrest in human MCF-7 breast cancer cells. *Br J Cancer* 77, 186-191.

209. Zhu, W. Y., Jones, C. S., Kiss, A., Matsukuma, K., Amin, S., and De Luca, L. M. (1997) Retinoic acid inhibition of cell cycle progression in MCF-7 human breast cancer cells. *Exp Cell Res* 234, 293-299.

210. Bischoff, E. D., Gottardis, M. M., Moon, T. E., Heyman, R. A., and Lamph, W. W. (1998) Beyond tamoxifen: The Retinoid-X-Receptor-selective ligand LGD1069 (TARGRETIN) causes complete regression of mammary carcinoma. *Cancer Research* 58, 479-484

211. Gottardis, M. M., Lamph, W. W., Shalinsky, D. R., Wellstein, A., and Heyman, R. A. (1996) The efficacy of 9-cis retinoic acid in experimental models of cancer. *Breast Cancer Res Treat* 38, 85-96.

212. Thompson, H. J., Becci, P. J., Moon, R. C., Sporn, M. B., Newton, D. L., Brown, C. C., Nurrenbach, A., and Paust, J. (1980) Inhibition of 1-methyl-1-nitrosourea-induced mammary carcinogenesis in the rat by the retinoid axerophthene. *Arzneimittelforschung* 30, 1127-1129.

213. Hunter, D. J., Manson, J. E., Colditz, G. A., Stampfer, M. J., Rosner, B., Hennekens, C. H., Speizer, F. E., and Willett, W. C. (1993) A prospective study of the intake of vitamins C, E, and A and the risk of breast cancer. *N Engl J Med* 329, 234-240.

214. Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* 240, 889-895.

215. Lacroix, A., and Lippman, M. E. (1980) Binding of retinoids to human breast cancer cell lines and their effects on cell growth. *Journal of Clinical Investigation* 65, 586-591

216. Zhu, W. Y., Jones, C. S., Amin, S., Matsukuma, K., Haque, M., Vuligonda, V., Chandraratna, R. A., and De Luca, L. M. (1999) Retinoic acid increases tyrosine phosphorylation of focal adhesion kinase and paxillin in MCF-7 human breast cancer cells. *Cancer Res* 59, 85-90

217. Niu, M. Y., Menard, M., Reed, J. C., Krajewski, S., and Pratt, M. A. (2001) Ectopic expression of cyclin D1 amplifies a retinoic acid-induced mitochondrial death pathway in breast cancer cells. *Oncogene* 20, 3506-3518

218. Budd, G. T., Adamson, P. C., Gupta, M., Homayoun, P., Sandstrom, S. K., Murphy, R. F., McLain, D., Tuason, L., Peereboom, D., Bukowski, R. M., and Ganapathi, R. (1998) Phase I/II trial of all-trans retinoic acid and tamoxifen in patients with advanced breast cancer. *Clin Cancer Res* 4, 635-642

219. Jing, Y., Zhang, J., Waxman, S., and Mira-y-Lopez, R. (1996) Upregulation of cytokeratins 8 and 18 in human breast cancer T47D cells is retinoid-specific and retinoic acid receptor-dependent. *Differentiation* 60, 109-117

220. Zhou, Q., Stetler-Stevenson, M., and Steeg, P. S. (1997) Inhibition of cyclin D expression in human breast carcinoma cells by retinoids in vitro. *Oncogene* 15, 107-115

221. de The, H., del Mar Vivanco-Ruiz, M., Tiollais, P., Stunnenberg, H., and Dejean, A. (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor á gene. *Nature* 343, 177-180

222. Lee, M. O., Han, S. Y., Jiang, S., Park, J. H., and Kim, S. J. (2000) Differential effects of retinoic acid on growth and apoptosis in human colon cancer cell lines associated with the induction of retinoic acid receptor beta. *Biochem Pharmacol* 59, 485-496.

223. Sun, S. Y., Wan, H., Yue, P., Hong, W. K., and Lotan, R. (2000) Evidence that retinoic acid receptor beta induction by retinoids is important for tumor cell growth inhibition. *J Biol Chem* 275, 17149-17153

224. Galipeau, J., Li, H., Paquin, A., Sicilia, F., Karpati, G., and Nalbantoglu, J. (1999) Vesicular stomatitis virus G pseudotyped retrovector mediates effective in vivo suicide gene delivery in experimental brain cancer. *Cancer Res* 59, 2384-2394.

225. Ory, D. S., Neugeboren, B. A., and Mulligan, R. C. (1996) A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc Natl Acad Sci U S A* 93, 11400-11406.

226. Paquin, A., Jaalouk, D. E., and Galipeau, J. (2001) Retrovector encoding a green fluorescent protein-herpes simplex virus thymidine kinase fusion protein serves as a versatile suicide/reporter for cell and gene therapy applications. *Hum Gene Ther* 12, 13-23.

227. Andrews, N. C., and Faller, D. V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* 19, 2499

228. Zinn, K., DiMaio, D., and Maniatis, T. (1984) Identification of two distinct regulatory regions adjacent to the human á-interferon gene. *Cell* 34, 865-879

229. Jiang, S. Y., and Jordan, V. C. (1992) Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. *J Natl Cancer Inst* 84, 580-591.

230. Mangelsdorf, D. J., and Evans, R. M. (1995) The RXR heterodimers and orphan receptors [Review]. *Cell* 83, 841-850

231. Berry, M., Metzger, D., and Chambon, P. (1990) Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *Embo J* 9, 2811-2818

232. Tzukerman, M. T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M. G., Stein, R. B., Pike, J. W., and McDonnell, D. P. (1994) Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol* 8, 21-30.

233. Liu, H., Lee, E. S., Deb Los Reyes, A., Zapf, J. W., and Jordan, V. C. (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. *Cancer Res* 61, 3632-3639

234. Dauvois, S., Danielian, P. S., White, R., and Parker, M. G. (1992) Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. *Proc Natl Acad Sci U S A* 89, 4037-4041.

235. Howell, A., Osborne, C. K., Morris, C., and Wakeling, A. E. (2000) ICI 182,780 (Faslodex): development of a novel, "pure" antiestrogen. *Cancer* 89, 817-825

236. Hall, J. M., Couse, J. F., and Korach, K. S. (2001) The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J Biol Chem* 276, 36869-36872

237. Wijayaratne, A. L., and McDonnell, D. P. (2001) The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by

agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 276, 35684-35692.

238. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 277, 965-968.

239. Onate, S. A., Boonyaratanakornkit, V., Spencer, T. E., Tsai, S. Y., Tsai, M. J., Edwards, D. P., and O'Malley, B. W. (1998) The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J Biol Chem* 273, 12101-12108.

240. Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J. C., and Auwerx, J. (1999) p300 interacts with the N- and C-terminal part of PPARgamma2 in a ligand-independent and -dependent manner, respectively. *J Biol Chem* 274, 7681-7688.

241. Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. H., Jr., and Evans, R. M. (1998) Role of the Histone deacetylase complex in acute promyelocytic leukaemia. *Nature* 391, 811-814

242. Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) Steroid receptor induction of gene transcription: a two-step model. *Proc Natl Acad Sci U S A* 94, 7879-7884

243. Sirchia, S. M., Ren, M., Pili, R., Sironi, E., Somenzi, G., Ghidoni, R., Toma, S., Nicolo, G., and Sacchi, N. (2002) Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. *Cancer Res* 62, 2455-2461.

244. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R., and Chambon, P. (1987) Functional domains of the human estrogen receptor. *Cell* 51, 941-951.

245. Kushner, P. J., Hort, E., Shine, J., Baxter, J. D., and Greene, G. L. (1990) Construction of cell lines that express high levels of the human estrogen receptor and are killed by estrogens. 4, 1465-1473

246. Lundholt, B. K., Briand, P., and Lykkesfeldt, A. E. (2001) Growth inhibition and growth stimulation by estradiol of estrogen receptor transfected human breast epithelial cell lines involve different pathways. *Breast Cancer Res Treat* 67, 199-214

247. Liu, G., Wu, M., Levi, G., and Ferrari, N. (1998) Inhibition of cancer cell growth by all-trans retinoic acid and its analog N-(4-hydroxyphenyl) retinamide: a possible mechanism of action via regulation of retinoid receptors expression. *Int J Cancer* 78, 248-254

248. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 59, 477-487.

249. Lee, H., Jiang, F., Wang, Q., Nicosia, S. V., Yang, J., Su, B., and Bai, W. (2000) MEKK1 activation of human estrogen receptor alpha and stimulation of the agonistic activity of 4-hydroxytamoxifen in endometrial and ovarian cancer cells. *Mol Endocrinol* 14, 1882-1896.

250. Tahayato, A., Lefebvre, P., Formstecher, P., and Dautrevaux, M. (1993) A protein kinase C-dependent activity modulates retinoic acid-induced transcription. *Mol Endocrinol* 7, 1642-1653.

251. Lee, C. S., deFazio, A., Ormandy, C. J., and Sutherland, R. L. (1996) Inverse regulation of oestrogen receptor and epidermal growth factor receptor gene expression in MCF-7 breast cancer cells treated with phorbol ester. *J Steroid Biochem Mol Biol* 58, 267-275

252. Oh, A. S., Lorant, L. A., Holloway, J. N., Miller, D. L., Kern, F. G., and El-Ashry, D. (2001) Hyperactivation of MAPK induces loss of ERalpha expression in breast cancer cells. *Mol Endocrinol* 15, 1344-1359

253. Delmotte, M. H., Tahayato, A., Formstecher, P., and Lefebvre, P. (1999) Serine 157, a retinoic acid receptor alpha residue phosphorylated by protein kinase C in vitro, is involved in RXR.RARalpha heterodimerization and transcriptional activity. *J Biol Chem* 274, 38225-38231.

254. Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J. M., and Chambon, P. (1997) Stimulation of RAR alpha activation function AF-1 through binding to

the general transcription factor TFIIH and phosphorylation by CDK7. *Cell* 90, 97-107.

255. Feng, Q., and Zhang, Y. (2001) The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes. *Genes Dev* 15, 827-832.

256. Speirs, V., Parkes, A. T., Kerin, M. J., Walton, D. S., Carleton, P. J., Fox, J. N., and Atkin, S. L. (1999) Coexpression of estrogen receptor alpha and beta: poor prognostic factors in human breast cancer? *Cancer Res* 59, 525-528.

257. Sheikh, M. S., Shao, Z. M., Li, X. S., Ordonez, J. V., Conley, B. A., Wu, S., Dawson, M. I., Han, Q. X., Chao, W. R., and Quick, T. (1995) N-(4-hydroxyphenyl)retinamide (4-HPR)-mediated biological actions involve retinoid receptor-independent pathways in human breast carcinoma. *Carcinogenesis* 16, 2477-2486

258. Rousseau, C., Pettersson, F., Couture, M. C., Paquin, A., Galipeau, J., Mader, S., and Miller, W. H., Jr. (2003) The N-terminal of the estrogen receptor (ERalpha) mediates transcriptional cross-talk with the retinoic acid receptor in human breast cancer cells. *J Steroid Biochem Mol Biol* 86, 1-14.

259. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 82, 1107-1112.

260. Jiang, S.-Y., and Jordan, V. C. (1992) Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. *Journal of the National Cancer Institute* 84, 580-591

261. Fontana, J. A., Nervi, C., Shao, Z. M., and Jetten, A. M. (1992) Retinoid antagonism of estrogen-responsive transforming growth factor alpha and pS2 gene expression in breast carcinoma cells. *Cancer Res* 52, 3938-3945.

262. van Agthoven, T., Timmermans, M., Foekens, J. A., Dorssers, L. C., and Henzen-Logmans, S. C. (1994) Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and

malignant human breast tissues using dual staining immunohistochemistry. *Am J Pathol* 144, 1238-1246.

263. Speirs, V. (2002) Oestrogen receptor beta in breast cancer: good, bad or still too early to tell? *J Pathol* 197, 143-147.

264. Tonetti, D. A., Rubenstein, R., DeLeon, M., Zhao, H., Pappas, S. G., Bentrem, D. J., Chen, B., Constantinou, A., and Craig Jordan, V. (2003) Stable transfection of an estrogen receptor beta cDNA isoform into MDA-MB-231 breast cancer cells. *J Steroid Biochem Mol Biol* 87, 47-55.

265. Yang, L. M., Tin, U. C., Wu, K., and Brown, P. (1999) Role of retinoid receptors in the prevention and treatment of breast cancer. *J Mammary Gland Biol Neoplasia* 4, 377-388.

266. Agadir, A., Chen, G., Bost, F., Li, Y., Mercola, D., and Zhang, X. (1999) Differential effect of retinoic acid on growth regulation by phorbol ester in human cancer cell lines. *J Biol Chem* 274, 29779-29785.

267. Young, M. R., Yang, H. S., and Colburn, N. H. (2003) Promising molecular targets for cancer prevention: AP-1, NF-kappa B and Pdcd4. *Trends Mol Med* 9, 36-41.

268. Shaulian, E., and Karin, M. (2002) AP-1 as a regulator of cell life and death. *Nat Cell Biol* 4, E131-136.

269. Ogawa, S., Inoue, S., Watanabe, T., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. (1998) Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor ofestrogen action in human. *Nucleic Acids Res* 26, 3505-3512.

270. Afonja, O., Raaka, B. M., Huang, A., Das, S., Zhao, X., Helmer, E., Juste, D., and Samuels, H. H. (2002) RAR agonists stimulate SOX9 gene expression in breast cancer cell lines: evidence for a role in retinoid-mediated growth inhibition. *Oncogene* 21, 7850-7860.

271. Adamo, M. L., Shao, Z.-M., Lanau, F., Chen, J.-C., Clemmons, D. R., Roberts, C. T., Jr., LeRoith, D., and Fontana, J. A. (1992) Insulin-like growth factor-I (IGF-I) and retinoic acid modulation of IGF-binding proteins (IGFBPs): IGFBP-

2, -3, and -4 gene expression and protein secretion in a breast cancer cell line. *Endocrinology* 131, 1858-1866

272. Sheikh, M. S., Shao, Z.-M., Hussain, A., Clemmons, D. R., Chen, J.-C., Roberts, C. T., Jr., LeRoith, D., and Fontana, J. A. (1993) Regulation of insulin-like growth factor-binding-protein-1, 2, 3, 4, 5, and 6: synthesis, secretion, and gene expression in estrogen receptor-negative human breast carcinoma cells. *Journal of Cellular Physiology* 155, 556-567

273. Christine Pratt, M. A., Niu, M., and White, D. (2003) Differential regulation of protein expression, growth and apoptosis by natural and synthetic retinoids. *J Cell Biochem* 90, 692-708.

274. Seewaldt, V. L., Johnson, B. S., Parker, M. B., Collins, S. J., and Swisshelm, K. (1995) Expression of retinoic acid receptor beta mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. *Cell Growth Differ* 6, 1077-1088.

275. Agadir, A., Shealy, Y. F., Hill, D. L., and Zhang, X. (1997) Retinyl methyl ether down-regulates activator protein 1 transcriptional activation in breast cancer cells. *Cancer Res* 57, 3444-3450.

276. Zhou, G., Hashimoto, Y., Kwak, I., Tsai, S. Y., and Tsai, M. J. (2003) Role of the steroid receptor coactivator SRC-3 in cell growth. *Mol Cell Biol* 23, 7742-7755.

277. Min, G., Kim, H., Bae, Y., Petz, L., and Kemper, J. K. (2002) Inhibitory cross-talk between estrogen receptor (ER) and constitutively activated androstane receptor (CAR). CAR inhibits ER-mediated signaling pathway by squelching p160 coactivators. *J Biol Chem* 277, 34626-34633.

278. Lehmann, J. M., Zhang, X. K., Graupner, G., Lee, M. O., Hermann, T., Hoffmann, B., and Pfahl, M. (1993) Formation of retinoid X receptor homodimers leads to repression of T3 response: hormonal cross talk by ligand-induced squelching. *Molecular & Cellular Biology* 13, 7698-7707

279. Zhang, Z., and Teng, C. T. (2001) Estrogen receptor alpha and estrogen receptor-related receptor alpha1 compete for binding and coactivator. *Mol Cell Endocrinol* 172, 223-233.

280. Lawrence, J. A., Adamson, P. C., Caruso, R., Chow, C., Kleiner, D., Murphy, R. F., Venzon, D. J., Shovlin, M., Noone, M., Merino, M., Cowan, K. H., Kaiser, M., O'Shaughnessy, J., and Zujewski, J. (2001) Phase I clinical trial of alitretinoin and tamoxifen in breast cancer patients: toxicity, pharmacokinetic, and biomarker evaluations. *J Clin Oncol* 19, 2754-2763.

281. Saez, C. G., Velasquez, L., Montoya, M., Eugenin, E., and Alvarez, M. G. (2003) Increased gap junctional intercellular communication is directly related to the anti-tumor effect of all-trans-retinoic acid plus tamoxifen in a human mammary cancer cell line. *J Cell Biochem* 89, 450-461.

282. Freemantle, S. J., Spinella, M. J., and Dmitrovsky, E. (2003) Retinoids in cancer therapy and chemoprevention: promise meets resistance. *Oncogene* 22, 7305-7315.

283. Yang, Q., Shan, L., Yoshimura, G., Nakamura, M., Nakamura, Y., Suzuma, T., Umemura, T., Mori, I., Sakurai, T., and Kakudo, K. (2002) 5-aza-2'-deoxycytidine induces retinoic acid receptor beta 2 demethylation, cell cycle arrest and growth inhibition in breast carcinoma cells. *Anticancer Res* 22, 2753-2756.

284. Coffey, D. C., Kutko, M. C., Glick, R. D., Butler, L. M., Heller, G., Rifkind, R. A., Marks, P. A., Richon, V. M., and La Quaglia, M. P. (2001) The histone deacetylase inhibitor, CBHA, inhibits growth of human neuroblastoma xenografts in vivo, alone and synergistically with all-trans retinoic acid. *Cancer Res* 61, 3591-3594.

285. Belinsky, S. A., Klinge, D. M., Stidley, C. A., Issa, J. P., Herman, J. G., March, T. H., and Baylin, S. B. (2003) Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. *Cancer Res* 63, 7089-7093.

286. Kennedy, C., Byth, K., Clarke, C. L., and deFazio, A. (2002) Cell proliferation in the normal mouse mammary gland and inhibition by phenylbutyrate. *Mol Cancer Ther* 1, 1025-1033.

287. Yu, K. H., Weng, L. J., Fu, S., Piantadosi, S., and Gore, S. D. (1999) Augmentation of phenylbutyrate-induced differentiation of myeloid leukemia cells using all-trans retinoic acid. *Leukemia* 13, 1258-1265.

288. Wang, Q., Yang, W., Uytingco, M. S., Christakos, S., and Wieder, R. (2000) 1,25-Dihydroxyvitamin D3 and all-trans-retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. *Cancer Res* 60, 2040-2048.

289. Vivat-Hannah, V., You, D., Rizzo, C., Daris, J. P., Lapointe, P., Zusi, F. C., Marinier, A., Lorenzi, M. V., and Gottardis, M. M. (2001) Synergistic cytotoxicity exhibited by combination treatment of selective retinoid ligands with taxol (Paclitaxel). *Cancer Res* 61, 8703-8711.

290. Offterdinger, M., Schneider, S. M., and Grunt, T. W. (2003) Heregulin and retinoids synergistically induce branching morphogenesis of breast cancer cells cultivated in 3D collagen gels. *J Cell Physiol* 195, 260-275.

APPENDIX A

Table A.1. Genes differentially regulated by treatment with 10^{-5} M RA for 24 h in S30 (ERα-positive cells). Differential gene expression was identified using the Atlas™ Human Cancer cDNA Expresssion Array.

		Name of Gene	Array Position
Upregulated genes	**1**	CDK4	A1d
_____	**2**	GRB2 isoform	A6c
	3	GRB-1R/GRB10	A6d
	4	cytokeratin 18 (K18)	A7b
	5	c-myc binding protein	A6i
	6	TNF receptor 1-associated protein (TRADD)	B1n
	7	TIMP-3 (mitogen-inducible gene 5)	E2d
Downregulated genes	**8**	AXL tyrosine kinase receptor	C5b
_____	**9**	CD59	D2d
	10	macrophage migration inhibitory factor (MIF)	F5n

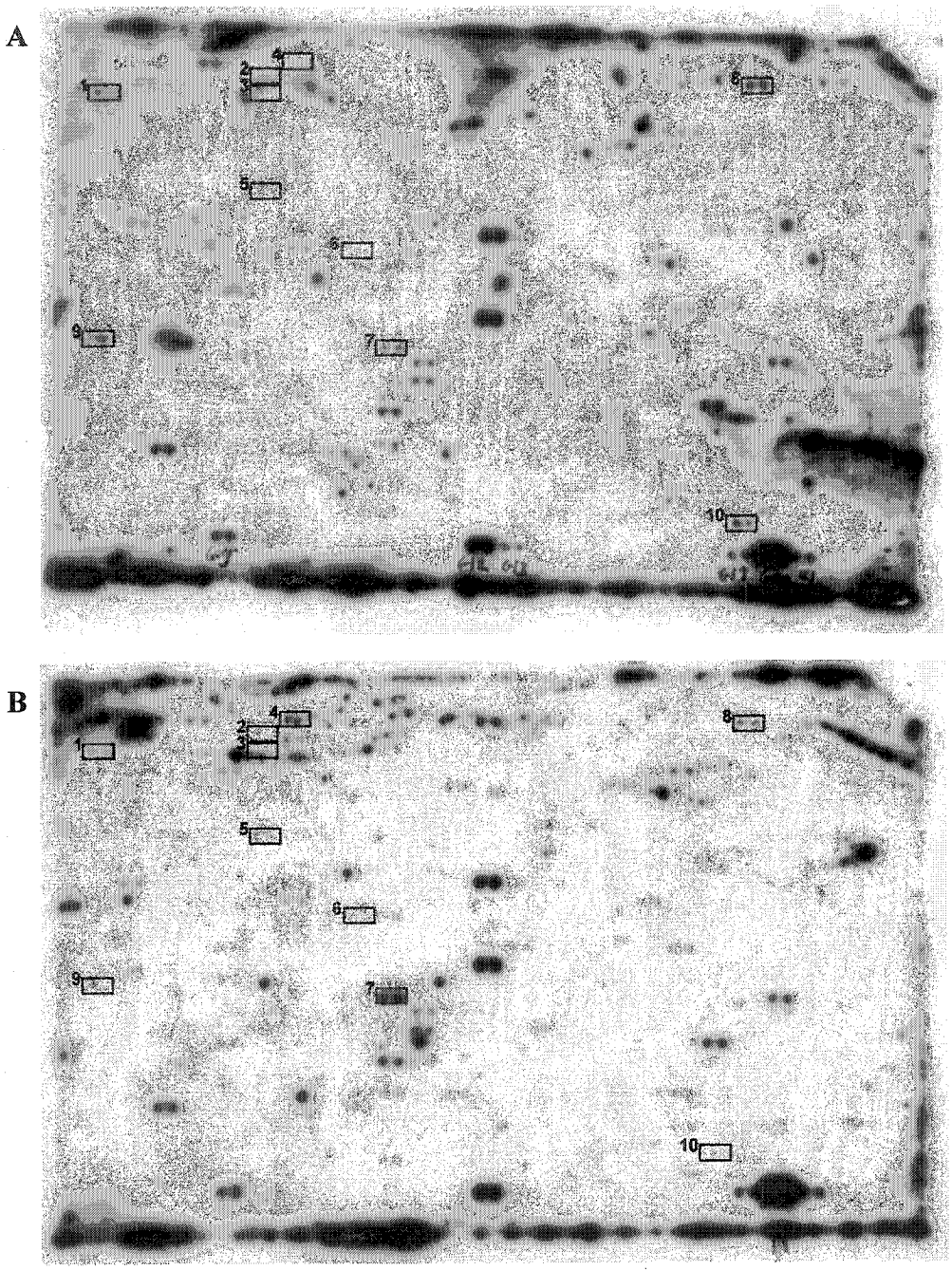

Figure A.1. Differential gene expression patterns in an ERα-positive (S30) human breast cancer cell line treated with (A) vehicle or (B) 10^{-5} M RA for 24 h. Poly A^+ RNA was isolated from the indicated cell lines. ^{32}P-labeled DNA probes were generated from each poly A^+ RNA and hybridized to the Atlas™ Human Cancer Array according to the User Manual. Blots were exposed for 7 days.

HÔPITAL GÉNÉRAL JUIF
SIR MORTIMER B. DAVIS
JEWISH GENERAL HOSPITAL

UNIVERSITÉ
McGILL
UNIVERSITY

INSTITUT LADY DAVIS DE RECHERCHES MÉDICALES
LADY DAVIS INSTITUTE FOR MEDICAL RESEARCH

MARK A. WAINBERG, O.C., Ph.D., F.R.S.C.
DIRECTOR OF RESEARCH

PROFESSOR OF MEDICINE
DIRECTOR,
McGILL UNIVERSITY AIDS CENTRE

February 1, 2004

RE: Caroline Rousseau
Thesis submission to McGill University

To Whom It May Concern:

The following will confirm the safety and ethical conditions with regard to all laboratories at the Lady Davis Institute for Medical Research of the Sir Mortimer B. Davis Jewish General Hospital.

The Lady Davis Institute for Medical Research is a free-standing biomedical research institute attached to the Sir Mortimer B. Davis Jewish General Hospital, a major adult teaching hospital affiliated with McGill University. There is approximately 110,000 sq. ft. of laboratory space occupied by over 65 independent investigators all of whom hold McGill University appointments in appropriate departments. At the present time we have approximately 100 graduate students and post-doctoral fellows registered with McGill University. Total external funding is in the range of $18 million (exclusive of overhead), which is large by Canadian standards.

Professor Andrew Mouland functions as Chair of our Health and Safety Committee, while Professor John Hiscott, a virologist, heads the subcommittee of the Health and Safety Committee on Biohazards.

Dr. Mouland affirms the safety program which addresses the question of radioactive material licence and Dr. Hiscott affirms the question of the use of recombinant DNA in the experiments. Dr. Lorraine Chalifour affirms the ethical use of animals.

As a constituent part of McGill University the Institute follows and meets all of the requirements of the *McGill Laboratory Biosafety Manual*. For those rare circumstances not covered by the *McGill Laboratory Biosafety Manual* we use the following as reference guidelines:

- *Biosafety in Microbiological and Biomedical Laboratories*, 3rd edition (1993). U.S. Department of Health and Human Services (Public Health Service), Centers for Disease Control and Prevention, and the National Institutes of Health. U.S. Government Printing Office, Washington, D.C.

- *Laboratory Biosafety Manual*, 2nd edition (1993). World Health Organization, Geneva, Switzerland.

3755, CHEMIN DE LA CÔTE-STE-CATHERINE, MONTRÉAL, QUÉBEC H3T 1E2
TÉL.: (514) 340-8260 FAX: (514) 340-7502

- *Laboratory Biosafety Guidelines* (1990). Medical Research Council of Canada and Laboratory Centre for Disease Control, Health Protection Branch, Health Canada, Ottawa, Canada.

- *Laboratory Safety*, 2nd edition (1995). D.O. Fleming, J.H. Richardson, J.J. Tullis and D. Vesley, editors. ASM Press, Washington, D.C.

- *Guidelines for Research Involving Recombinant DNA Molecules* (1996). U.S. Department of Health and Human Services, National Institutes of Health, Bethesda, M.D.

Regular inspection of safety procedures are conducted by the Canadian Nuclear Safety Commission (CNSC) (annual), Contex Environment (laboratory health and safety consultants – several times per year), the Sir Mortimer B. Davis Jewish General Hospital Chairman of Infection Control – Dr. Mark Miller (monthly), the Canadian Council on Animal Care (bi-annually), the McGill animal Care Committee (monthly), as well as by the Hospital Fire Marshal – Mr. Thomas Prokos, and the City of Montreal Fire Department (semi-annually).

The Lady Davis Institute (LDI) laboratories in which the research will be conducted are specially designed and equipped for scientific research and are used solely for that purpose. Specialized facilities are provided for the safe conduct of scientific research, including but not limited to, waste disposal facilities for biohazardous, radioactive and chemical wastes, a level 3 containment facility and state-of-the-art animal facilities. An automatic sprinkler system (water) is installed throughout the building. Fire extinguishers are located in several locations on each of six floors, as are fire alarms. Fire doors and alarm pulls are marked. In general, the laboratories and animal rooms are designed to have pressure differentials negative to adjacent areas. Fume hoods are monitored on a semi-annual basis. Fume hoods exhaust to the outside. Biological safety cabinets are certified semi-annually.

The use of chemicals is governed by McGill University regulations which are distributed to each laboratory. Standing operative procedures have been formulated to address actions to be taken after spills or accidents involving potentially hazardous materials under the supervision of the LDI Health and Safety Committee. Training which meets the requirements of various inspection teams is undertaken twice annually and the Health and Safety Committee meets monthly. Radiation worker training is offered on a regularly scheduled basis consistent with the requirements of the CNSC. Dr. Richard Latt, Director of the McGill Animal Resources Centre, is employed as a part-time veterinary consultant. All personnel working with animals hold a certificate as an Animal Health Technician as provided under the laws of the Province of Quebec. The Institute provides hepatitis immunization to all employees potentially exposed to human blood or blood products.

Access to the laboratory is restricted at the discretion of the PI when experiments are in progress. No food or cosmetics are allowed within the laboratory. The laboratory is equipped with a sink for hand washing and for disposal of properly decontaminated experimental materials. Laboratory personnel receive appropriate training and instruction from the PI concerning potential hazards associated with the work, prior to initiating research. Personnel wear suitable protective clothing and gloves when handling biohazardous materials. Cell cultures and tissue specimens are transported and stored in leak-proof containers. Work surfaces are decontaminated (bleach or other disinfectants) daily, and after any spills of biohazardous materials. Manipulations with the human cancer cell lines are carried out in a biological safety cabinet (Class II). All cell culture material wastes are decontaminated by autoclaving