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Kathleen M. Eyster *Editor*

Estrogen Receptors

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Estrogen Receptors

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Preface

Estrogen is a fascinating hormone whose physiological roles extend far beyond the female reproductive system and maintenance of the species. Estrogen is known to regulate tissues such as bone, brain, the cardiovascular system, skin, liver, adipose tissue, the immune system, salivary glands, colon, male reproductive tissues, and others. Estrogen appears to have both positive and negative effects in the cardiovascular system. In the breast and uterus, the important physiological effects of estrogen on breast and endometrial growth can be diverted to stimulate the growth of tumors. Both the physiological and pathological effects of estrogen are mediated by estrogen receptors which are divided into two types: the genomic nuclear estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), and the nongenomic estrogen receptor, GPER/GPR30 and possibly other nongenomic receptors.

This volume includes chapters on a wide array of technologies that are important to advancing our understanding of the receptor-mediated actions of this important hormone. The protocols range from standard methods and important laboratory workhorses such as receptor binding assays and western blot to newer technologies such as RNAseq and proximity ligation assay, as well as many others. We have included protocols from a broad range of tissue types to demonstrate the variety of estrogen receptor effects. We hope that scientists who are intrigued by the many faces of estrogen will find the protocols detailed in this book helpful and inspirational.

About 15 years ago a visiting professor told me that there was no point in continuing to study the effects of estrogen that everything that needed to be known was known and I should move on to other things. The wide range of cutting-edge technologies described in this volume testifies to the fallacy of his statement and demonstrates that the field of estrogen receptors and estrogen actions continues to be vibrant and exciting.

I want to express my appreciation to all of the authors who wrote chapters for the book and who tolerated my requests for further explanation on the fine points of their protocols. I am also grateful to Dr. John Walker, series editor, who provided excellent guidance on how to edit a methods book as we moved through the various stages of preparation. And to the readers, I trust that you will find the protocols in this book useful and that the step-by-step instructions will give you the confidence to try new protocols and expand your research repertoire.

Vermillion, SD, USA — *Kathleen M. Eyster*

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Chapter 1

The Estrogen Receptors: An Overview from Different Perspectives

Kathleen M. Eyster

Abstract

The estrogen receptors, ERα, ERβ, and GPER, mediate the effects of estrogenic compounds on their target tissues. Estrogen receptors are located in the tissues of the female reproductive tract and breast as one would expect, but also in tissues as diverse as bone, brain, liver, colon, skin, and salivary gland. The purpose of this discussion of the estrogen receptors is to provide a brief overview of the estrogen receptors and estrogen action from perspectives such as the historical, physiological, pharmacological, pathological, structural, and ligand perspectives.

Key words Estrogenreceptors, ERα, ERβ, GPER, Review

1 Introduction

The literature contains many reviews of estrogen receptors and their ligands [1–9], as well as the effects of estrogens on specific tissue types [4, 10–31]. The reader interested in comprehensive discussion of estrogen receptors is referred to one or more of these excellent reviews. The purpose of this discussion is to provide a brief overview of the estrogen receptors and estrogen action from various perspectives.

2 The Historical Perspective

In evolutionary terms, the estrogen receptor is an ancient protein that is expressed in all vertebrates and a few invertebrates [32]. From a historical perspective, studies to discover the mechanism by which estrogen exerted its effects on target tissues took place in the 1950s and 1960s and culminated in the determination that estrogen bound to a protein in its target cells [33, 34]. Jensen and coworkers [35] describe a fascinating historical account of the early

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studies that led to the discovery of the estrogen receptor. This first estrogen binding protein is the protein now known as estrogen receptor α (ERα, also known as ER1 or Esr1). Our understanding of the binding of the estrogen-receptor complex to DNA, transcription to RNA, and subsequent protein synthesis evolved over the course of the 1960s and 1970s [33, 36, 37] to the mechanism that we recognize today of ligand-activated transcription factors. This genomic mechanism of action involves transcription and translation of genes so it is characterized by the time that it takes (hours) to develop a response as well as the persistence of the response for the lifespan of the new protein. The second estrogen receptor was identified in 1986 [38] and was named estrogen receptor β (ERβ, also known as ER2 and Esr2). Like ERα, ERβ is a ligand-activated transcription factor, so they share the slow-onset, persistent response genomic mechanism of action.

As early as the 1970s, reports appeared in the literature of actions of estrogen that occurred too rapidly to be mediated by the genomic mechanism of action [39–41]. However, these reports were pushed aside by the, at the time, seemingly more compelling genomic mechanism of action. Nevertheless, reports continued to emerge of rapid, nongenomic actions of estrogen [42] as well as nongenomic actions of other steroid hormones [43]. The ability of estrogen to mediate rapid, nongenomic actions is now widely accepted. An estrogen-responsive G protein coupled receptor called GPR30 or GPER has now been well characterized and is considered by many to be the source of the rapid actions of estrogen [44–52]. However, others have reported that the genomic receptors, ERα and ERβ, can associate with the membrane and mediate rapid actions although the mechanism of association of the genomic ER with the membrane is unclear [53]. Yet others report that mutated forms of ERα are involved in the rapid nongenomic actions of estrogen [54]. The details of rapid actions of estrogen remain controversial; it is quite possible that all of these mechanisms occur, perhaps in different cell types or differentially in physiological versus pathological situations.

3 The Physiological Perspective

We can also examine the estrogen receptor from the perspective of its physiological functions. In the same time frame as the discovery of the first estrogen receptor (late 1950s–1960s), the functional perspective of estrogen was purely as a female reproductive hormone. The reproductive functions of estrogen are the material of textbooks [55, 56]. In the historical context, an estrogenic compound was defined as a substance that could stimulate uterine growth and up-regulate synthesis of the progesterone receptor, both of which serve reproductive functions. As our understanding

of the estrogen receptors has evolved, so also has our understanding of the functions of estrogen. In addition to the requirement of estrogen in reproductive function, and therefore, its role in the survival of the species, we now know that estrogen is critical to many other physiological functions. Estrogenreceptors are expressed and estrogenic ligands produce specific effects in the cardiovascular system [10–13], brain [14, 15], bone [16, 17], liver [18, 19], adipose tissue [19–21], colon [22, 23], skin [24, 25, 57], prostate [26, 27], testes [28, 29], epididymis [30, 31], and salivary gland [4]. Thus estrogen receptors serve a truly pleiotropic array of functions.

4 The Pharmacological Perspective

From the perspective of pharmacology, estrogen receptor agonists and estrogen receptor antagonists are both clinically relevant. The clinical uses of estrogen receptor agonists are primarily in the areas of combination hormonal contraceptives and in postmenopausal hormone replacement [58]. In combination hormonal contraceptives, an estrogen is administered with a progestin in the form of a pill, patch, or vaginal ring [59, 60]. These hormonal contraceptives are highly effective in the prevention of pregnancy. Nevertheless, the pleiotropic actions of estrogens incur some adverse effects. For example, the estrogens in contraceptives increase the risk of adverse reactions in the cardiovascular system, especially in women smokers [59, 60]. The primary use of the one pure estrogen receptor antagonist that is available, fulvestrant, is in the treatment of estrogen receptor positive (ER+) breast cancer. Fulvestrant is also sometimes called a selective estrogen receptor downregulator or degrader (SERD) because its binding to receptor leads to proteasomal degradation of the receptor [61]. In addition, an intriguing family of pharmaceutical agents called selective estrogen receptor modulators (SERMs) interacts with the estrogen receptors. The SERMs act as estrogen receptor agonists in some estrogen-sensitive tissues and as antagonists in others [59, 62]. SERMs that are on the market in the USA at this time include tamoxifen, raloxifene, bazedoxifene, and ospemifene. Tamoxifen acts as an estrogen receptor antagonist in the breast and is used in the treatment of ER+ breast cancer. The drawback to tamoxifen is that it acts as an estrogen receptor agonist in the uterus [59]. Raloxifene is an estrogen receptor agonist in bone but an antagonist in breast and uterus; it is used for postmenopausal osteoporosis [59]. Ospemifene is an estrogen receptor agonist on the vaginal epithelium, endometrium, and bone and is antiestrogenic in the breast. It is used for the treatment of dyspareunia that may occur in postmenopausal women [63]. Bazedoxifene is an estrogen receptor agonist in bone, hypothalamus, vagina, and vulva and acts

as an antagonist in breast and uterus. Bazedoxifene is approved in the USA to be used in concert with conjugated estrogens for the treatment of vasomotor symptoms associated with the menopause [64]. The underlying concept is that the conjugated estrogens will alleviate the vasomotor symptoms and the bazedoxifene will prevent the adverse effects of estrogen on the breast and endometrium [64].

5 The Pathological Perspective

If we consider the estrogen receptors from the perspective of pathology, it is clear that several diseases are related to the estrogen receptor. Approximately 75 % of breast cancers express estrogen receptors [65, 66], and the presence of estrogen stimulates growth of these tumors. As mentioned above, inhibition of estrogen action in the breast is an important treatment for estrogen-sensitive breast cancer. These treatment strategies include the use of specific estrogen receptor antagonists such as fulvestrant [61], SERMs such as tamoxifen, or prevention of the synthesis of estrogen by use of aromatase inhibitors such as anastrozole, letrozole, and exemestane [59]. Similarly, endometrial cancer is also estrogen sensitive. The administration of estrogen alone for an extended period of time can cause the development of endometrial cancer in postmenopausal women [67].

The development of venous thromboembolism is another pathology associated with estrogen [58]. The risk of venous thromboembolism rises in women who take exogenous estrogens (e.g., contraceptives, postmenopausal hormone replacement therapy), as well as in situations in which endogenous estrogens are high as in women who are pregnant, or in the immediate postpartum period [68].

Autoimmune diseases occur at a higher rate in women than in men [69] and estrogen receptors play a role in the immune system [70]. Several hypotheses that been proposed to explain the discrepancy between men and women. One very intriguing idea is related to the enzyme, activation-induced deaminase (AID), which is involved in class switch recombination during antibody diversification [71]. Estrogen has been shown to activate AID [72], and may affect immune function by this mechanism. Derangement of estrogen regulation of this enzyme may be responsible for at least part of the increased risk of autoimmune disease in women.

The role of estrogen and the estrogen receptors in several other pathologies, such as cardiovascular disease and dementia, remains controversial. The rate of most cardiovascular diseases is lower in premenopausal women than in men, but rapidly rises after the menopause [73]. This disease pattern suggests that estrogen is protective to the cardiovascular system. However, studies such as

the Women's Health Initiative (WHI) showed that replacement of estrogen in postmenopausal women could trigger cardiovascular events (heart attack and stroke) [74]. Further analysis of data from the WHI study showed that the women who experienced cardiovascular events were more likely to be many years past the menopause. These data suggested that estrogen was more likely to trigger cardiovascular events in women who had been many years without the hormone and in whom cardiovascular disease may have become well-established although still silent. Thus the concept has developed that estrogen may be protective to a healthy cardiovascular system [11] but deleterious to a cardiovascular system in which disease such as atherosclerosis has developed [73, 75]. More recent data suggest that administration of estrogen soon after the menopause prevents the development of cardiovascular disease and does not precipitate CV events [12, 58, 76, 77]. A similar controversy exists regarding the effect of estrogen on dementia in postmenopausal women [78].

6 The Structural Perspective

Another perspective from which to examine the estrogen receptors is that of structure. ERα and ERβ share the same general structure, that is, a ligand-binding domain, DNA-binding domain, and two activation function (AF) domains [79]. The two receptors share a high degree of amino acid sequence homology except in the N-terminal domain (the AF-2 domain). Proteins called coactivators (e.g., NCOA1, NCOA2, NCOA3, CREBBP, PPARBP, P68, and SRA) and corepressors (e.g., NCOR1, NRIP) can interact with ligand-bound ERα or ERβ and influence the ability of the receptor to activate or inhibit expression of a gene [80–82]. Phosphorylation of the estrogen receptor can affect its activity [83]. Ligand-bound ERα and ERβ bind to the same DNA sequence, the estrogen response element (ERE), whose sequence is defined as GGTCAnnnTGACC. For many estrogen-responsive genes, the ERE may be a significant distance upstream of the start site [84–86]. The promoters of many estrogen-responsive genes may contain only a half-site sequence of the ERE rather than the complete ERE, and ligand-bound ERα or ERβ can form protein-protein complexes with other transcription factors which then bind to their own response elements in the promoters of regulated genes [87]. Transcription factors with which ERα and ERβ can interact include Sp-1 [87, 88], Ap-1 [89, 90], and NF-κB [91]. Although they utilize the same ERE and interact with the same coregulators, ERα and ERβ exhibit differential tissue distribution and different biological effects [3–5]. Thus there remain complexities to be deciphered.

In contrast, the structure of GPER is dramatically different from that of ERα and ERβ, as one would expect of the membrane protein, and of course its mechanism of action is dramatically different as well. GPER has been reported to activate several signal transduction pathways which culminate in the phosphorylation of substrate proteins, some of which are transcription factors [46]. One of the transcription factors reported to be downstream of GPER is ERα [92], suggesting an interaction between nongenomic and genomic estrogen receptors.

7 The Ligand Perspective

Another perspective from which to examine the estrogen receptors is that of their ligands. Estrogen is a generic term that typically refers to the primary endogenous estrogenic compounds 17β-estradiol, estrone, and estriol (from most to least potent). Metabolites of these estrogens have been shown to regulate ERα and ERβ [93–95], as have other endogenous substances such as 27-hydroxycholesterol [96, 97]. Exogenous substances that can activate the estrogen receptors include phytoestrogens such as genistein, daidzein, and equol [98–101] as well as environmental substances [102–104].

8 Summary

Research on the estrogen receptors has been ongoing for over five decades; this brief overview can only provide a meager glimpse of the broad range of knowledge that is available regarding these receptors. In spite of the significant quantity of information that we have about the estrogen receptors, substantial questions remain. To name just a few: if ERα and ERβ use the same ERE and interact with the same coregulators, and are expressed in many of the same tissues, what is the mechanism by which they exert different biological effects? To what extent do ERα, ERβ, and GPER interact and do those interactions differ among estrogen-sensitive tissues? Can we delineate specific functions for each of the three receptors? Knockout animals for ERα, ERβ, and GPER have answered some of the biological questions regarding individual functions of these receptors, but not all of the models agree [4, 105, 106]. In spite of the 50+ years of investigation, the field of estrogen receptor research remains strong and vibrant, with notable questions remaining to carry the field forward into the future.

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Chapter 2

Competitive Binding Assay for the G-Protein-Coupled Receptor 30 (GPR30) or G-Protein-Coupled Estrogen Receptor (GPER)

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Abstract

The role of 2-methoxyestradiol is becoming a major area of investigation because of its therapeutic utility, though its mechanism is not fully explored. Recent studies have identified the G-protein-coupled receptor 30 (GPR30, GPER) as a high-affinity membrane receptor for 2-methoxyestradiol. However, studies aimed at establishing the binding affinities of steroid compounds for specific targets are difficult, as the tracers are highly lipophilic and often result in nonspecific binding in lipid-rich membrane preparations with low-level target receptor expression. 2-Methoxyestradiol binding studies are essential to elucidate the underlying effects of this novel estrogen metabolite and to validate its targets; therefore, this competitive receptor-binding assay protocol was developed in order to assess the membrane receptor binding and affinity of 2-methyoxyestradiol.

Key words 2-Methoxyestradiol, GPR30, G-protein-coupled receptor 30, GPER, G-protein-coupled estrogen receptor, Endoplasmic reticulum membrane, Radioligand binding, Competitive binding

1 Introduction

2-Methoxyestradiol (2ME2) is a final end product of estradiol (E2) metabolism. While the affinity of E2, notably 17-β estradiol, for estrogen receptor α and β (ERα/ERβ) has been demonstrated conclusively, 2ME2 has 2000-fold less activation potential for classical genomic estrogen receptors [1]. Estrogen may also induce cellular responses through non-genomic pathways through receptors such as G-protein coupled estrogen receptor (GPER), also called G-protein-coupled receptor 30 (GPR30), a $G\alpha_i$-associated G-protein-coupled receptor [2]. In our own studies, we have found that 2ME2 retains significant binding affinity for this receptor, even though it lacks the same affinity for inducible nuclear estrogen receptors [3]. Additionally, 2ME2 is capable of inducing a number of cellular responses primarily through this receptor. In the literature, it is suggested that this receptor may be localized in

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one of three places, either the endoplasmic reticulum (ER), plasmalemma, or golgi apparatus [4–6]. In any case, for one to assess the potential binding affinity of a ligand to this novel receptor, membrane preparation is required. Studies aimed at establishing the binding affinities of steroid compounds for specific targets are difficult, as the tracers are highly lipophilic and often result in non-specific binding in lipid-rich membrane preparations with low-level target receptor expression [7]. This chapter describes a competitive receptor binding assay protocol to assess the membrane receptor binding and affinity of 2-methyoxyestradiol for the membrane estrogen receptor, GPR30. The following protocol was originally developed for use on rat liver epithelial cells, although the method should be compatible with any cell type for which GPR30 binding affinity must be determined.

2 Materials

2.1 Reagents

1. [^{3}H]-2-Methyoxyestradiol (2ME2) (American Radiolabeled Chemicals Inc., 50 Ci/mmol).
2. Unlabeled 2ME2 (16.5 mM in DMSO).
3. GPR30-specific antagonist, G15 (1.3 mM in DMSO).
4. Trypsin solution, 10× (25 g porcine trypsin peptide/L HBSS).

2.2 Total Cell Membrane Isolation

1. 50 mM Tris–HCl, pH 7.4.
2. Wheaton glass dounce tissue grinder with "B" pestle.
3. Hanks' balanced salt solution (HBSS).
4. Rubber policeman.
5. Binding buffer without bovine serum albumin: 50 mM Tris–HCl, pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 10 μg/mL bacitracin, and 2 mg/mL dextrose.
6. Low-speed and high-speed centrifuges and centrifuge tubes.

2.3 Endoplasmic Reticulum Membrane Isolation

1. Phosphate-buffered saline (PBS), pH 7.4.
2. 0.05 % porcine trypsin and 0.02 % EDTA in PBS.
3. Trypsin-neutralizing solution: soybean trypsin inhibitor.
4. MTE buffer: 270 mM mannitol, 10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, Complete Mini Protease inhibitor cocktail tablet (Roche), 1 mM phenylmethanesulfonylfluoride (PMSF) (100 mM stock in ethanol) (*see* **Note 1**).
5. Wheaton glass Dounce tissue grinder with "B" pestle.
6. Discontinuous sucrose gradient: 1.3, 1.5, and 2.0 M sucrose in 10 mM Tris–HCl, pH 7.0.

2.4 Radioligand-Binding Assay

1. Binding buffer with BSA: 50 mM Tris–HCl, pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 10 μg/mL bacitracin, 0.25 % BSA, and 2 mg/mL dextrose (*see* **Note 2**).
2. Wash buffer: 50 mM Tris–HCl, pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 10 μg/mL bacitracin, and 2 mg/mL dextrose (*see* **Note 3**).
3. Borosilicate disposable culture tubes.
4. Millipore circular nitrocellulose membrane (0.45 μm).
5. Millipore filtration unit (e.g., Model 1225 sampling manifold vacuum).
6. Scintillation fluid (Scintiverse®).
7. Scintillation counting vials.

3 Methods

Following are two specific methods, one for radioligand binding on total membrane extracts [8], and the other for assessing the binding of a given ligand (for our purposes we have used [^{3}H] 2ME2) for ER membrane localized GPR30 receptors [9]. All steps of membrane isolation must be performed at 4 °C or on ice, while radioligand binding may proceed at room temperature (22 °C).

3.1 Total Cell Membrane Preparation

1. Grow cells (rat liver epithelial cells [3]) to 90 % confluence in 100 mm cell culture plates under conditions required by the experiment.
2. Wash the cells 2× with HBSS.
3. Harvest cells in 1 mL HBSS using a rubber policeman.
4. Homogenize the cells using a Wheaton glass Dounce tissue grinder using a "B" pestle for ten strokes.
5. Collect the homogenate and centrifuge at 1000 × *g* for 7 min.
6. Transfer the supernatant to a fresh tube and centrifuge at 45,000 × *g* for 20 min.
7. Resuspend the membrane pellet in 50 mM Tris–HCl, pH 7.4 and centrifuge at 45,000 × *g* for 20 min.
8. Suspend the resulting pellet in binding buffer without BSA and use immediately for radioligand binding study or store in aliquots at −80 °C (*see* **Note 4**).
9. Determine the protein concentration before the binding assay (We use the Bio-Rad protein assay based on the Bradford method).

3.2 Preparation of Endoplasmic Reticulum Membranes

1. Grow cells (rat liver epithelial cells [3]) to 90 % confluence in a 75 cm^2 cell culture flask under conditions required by the experiment.
2. Wash the cells 1× with PBS.
3. Trypsinize the cells for 3 min in 1 mL of trypsin at 37 °C.
4. Add 1 mL of trypsin inhibitor and mix at room temperature.
5. Collect the cells in 8 mL PBS (yielding 10 mL total volume) and transfer to a 15 mL centrifuge tube.
6. Centrifugate cells at 2500 × *g* for 5 min.
7. Resuspend the cell pellet in 4 mL MTE buffer.
8. Homogenize the cell suspension using a Wheaton glass Dounce tissue grinder using a "B" pestle for ten strokes.
9. Centrifuge the resultant homogenate at 700 × *g* for 10 min at 4 °C.
10. Collect the supernatant and centrifuge at 15,000 × *g* for 10 min at 4 °C.
11. Prepare a discontinuous sucrose gradient: first layer the densest sucrose solution (2.0 M) at the bottom of the high-speed centrifuge tube, then layer the intermediate solution (1.5 M), then the least dense (1.3 M), creating three distinct layers of proportionate volumes. It is important to allow the sucrose solution to run slowly down the inside of the high-speed centrifuge tube.
12. Collect the supernatant and layer on top of the discontinuous sucrose gradient. The centrifugation requires a balanced rotor; therefore, split the supernatant sample into two parts and prepare two gradient centrifuge tubes with the same mass [9]. Centrifuge at 100,000 × *g* for 45 min at 4 °C.
13. Collect the purified ER membrane (white circular band embedded in the sucrose gradient), resuspend in binding buffer, and immediately proceed with radioligand-binding assay.
14. Alternatively, you may store aliquots of the purified ER membrane at −80 °C until needed (*see* **Note 4**).
15. Determine the protein concentration before the binding assay (we use the Bio-Rad protein assay based on Bradford method).

3.3 [^{3}H] 2-Methoxyestradiol-Binding Assay

1. Membrane samples isolated in Subheading 3.1 or 3.2 are placed in disposable borosilicate tubes in a volume of 50 μL binding buffer (*see* **Note 5**).
2. Add 400 μL of binding buffer with BSA.
3. Add 3.3 pmol [^{3}H] 2ME2 (200 nCi) in a volume of 50 μL of binding buffer (*see* **Note 6**).
4. Incubate the reaction mixture for 1 h at 22 °C. Total reaction volume should not exceed 500 μL.

5. Pass the reaction volume through a presoaked membrane filter using a Millipore filtration unit (*see* **Note 7**).
6. Wash the membrane 3× each with 2 mL wash buffer without BSA.
7. Transfer the filter to a scintillation counting vial.
8. Add 10 mL Scintiverse® scintillation fluid and mix thoroughly.
9. After 1 h, proceed to counting on a Beckman scintillation spectrometer or other suitable platform.
10. Counts (disintegrations per minute, dpm) represent total radioligand binding to the membranes.

3.4 Competitive Binding for Determining [^{3}H] 2ME2 Specificity

1. In order to determine nonspecific binding of the membranes, preincubate with 1 μM unlabeled 2ME2 in binding buffer for 10 min before the addition of the [^{3}H] 2ME2 radioligand.
2. Proceed through **steps 3–9** in Subheading 3.3.
3. Specific [^{3}H] 2ME2 binding is defined as that portion of the total binding displaced by unlabeled 2ME2.

3.5 Competitive Binding for Determining GPR30 Specificity

1. Preincubate membranes with 10 μM G15 (GPR30-specific antagonist) in binding buffer 10 min before the addition of the [^{3}H] 2ME2.
2. Proceed through **steps 3–9** in Subheading 3.3 to determine receptor specificity.
3. Specific [^{3}H] 2ME2 binding to GPR30 is defined as that portion of the total binding displaced by G15.

3.6 Binding Affinity (K_d) Calculation

1. In order to determine the affinity (K_d) of 2ME2 for GPR30, incubate a series of membrane preparations with increasing concentration of [^{3}H] 2ME2 in binding buffer (for example: 0.25, 0.5, 1.0, 2, 4, 8, 12, 25, and 50 nM).
2. Each reaction should be paired with a matching condition that was preincubated with 100–1000-fold unlabeled 2ME2. This will allow determination of nonspecific binding.
3. Obtain counts for each condition and calculate specific binding as described in Subheading 3.4.
4. Convert obtained specific DPM values to appropriate molar concentration. For example, a value of 4.4×10^5 counts should be the equivalent of 3.3 pmol of [^{3}H] 2ME2. (This assumes specific activity of the isotope at 50 μCi/nmol, and 2.2×10^6 DPM/μCi.)
5. Plot concentration of bound [^{3}H] 2ME2 (y-axis) by the concentration of free [^{3}H] 2ME2 (x-axis)—(*see* Table 1 and Fig. 1).
6. Using GraphPad Prism software, or another appropriate statistical analysis tool, analyze the data in nonlinear regression equation of one-site saturation binding: $Y = (B_{max} \times X)/(K_d + X)$.

Table 1
The numbers calculated and expressed in this table are entirely theoretical

Free [^{3}H] 2ME2 (nM)	Bound [^{3}H] 2ME2 (fmol/mg protein)
0.25	95.45
0.5	381.18
1.0	656.00
2.0	1361.54
4.0	2560.81
6.0	3363.63
12.0	5636.36
25.0	8763.00
50.0	12,210.00

Conversion of DPM to fmol was performed assuming specific activity at 2.2×10^6 DPM/μCi and 50 μCi/nmol. Protein was held constant at 0.1 mg for all reactions

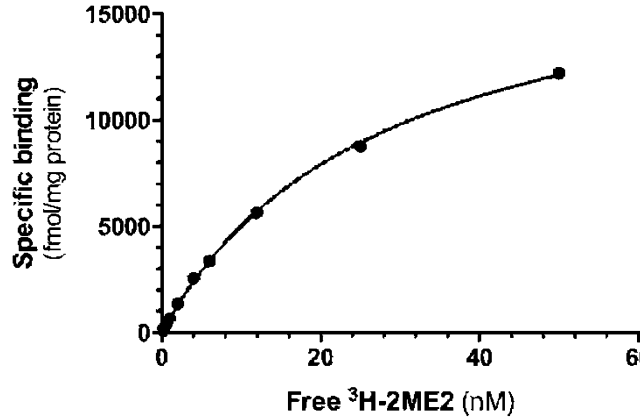

Fig. 1 Saturation binding isotherm plot of values provided in Table 1. As this is only an example using arbitrary numbers for demonstrative purposes, deviation from these calculations of B_{max} and K_d in an actual experiment can be expected

4 Notes

The methods described in this chapter were optimized in our laboratory for our particular cell type; therefore, these notes may be of interest to those who wish to adapt this protocol in other cell lines.

1. MTE buffer must be prepared fresh each day. 100 mM PMSF stock solution may be stored at room temperature.
2. Binding buffer must be prepared fresh each day before the binding assay.

3. Wash buffer and binding buffer without BSA must be prepared separately.
4. Binding affinity of membranes kept at −80 °C will probably be reduced over time.
5. Minimum protein content should be above 50 μg and up to 200 μg for a single reaction.
6. In the final reaction volume of 500 mL, 3.3 pmol is the equivalent of a 6.6 nanomolar solution of [^{3}H] 2ME2.
7. Membranes must be soaked for 1 h in binding buffer with BSA prior to use.

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Chapter 3

The Use of Real-Time Reverse Transcription-PCR for Assessing Estrogen Receptor and Estrogen-Responsive Gene Expression

Michelle L. Booze and Kathleen M. Eyster

Abstract

Real-time reverse transcription-polymerase chain reaction (RT-PCR), also known as quantitative RT-PCR (qRT-PCR), is a powerful tool for assessing gene transcription levels. The technique is especially useful for measuring estrogen receptor transcript levels as well as gene expression changes in response to estrogen stimulation as it is quick, accurate, robust, and allows the measurement of gene expression in a variety of tissues and cells. This chapter describes the protocols used for the real-time RT-PCR assay using hydrolysis (TaqMan-type) probes.

Key words Estrogenreceptors, Gene expression, Primer, Fluorogenic probe, Real-time RT-PCR, Master mix, Custom design, Normalization

1 Introduction

The purpose of real-time reverse transcription-polymerase chain reaction (RT-PCR) is the measurement of the presence of a given messenger RNA (mRNA). The procedure involves the conversion of mRNA to complementary DNA (cDNA) in an initial step, and then amplification of a specific region of the target gene, the amplicon, from the cDNA by polymerase chain reaction (PCR). Fluorescent dyes are used to visualize the amplification in "real time." This technique is more powerful than its predecessors because of its quantitative nature and because additional steps after the reaction (e.g., separation of PCR products on a gel) are not required.

A variety of choices in machines and detection methods are available; most measure the level of fluorescence as an indicator of mRNA transcript levels. The increased detection of the fluorophore directly correlates to a greater quantity of the target mRNA transcript in the starting sample [1]. The fluorophore is attached to an oligomer probe. Several oligomer probe options are available

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for this assay. These include agents that intercalate into double stranded DNA (e.g., SYBR Green) [2, 3], hydrolysis probes (e.g., Taqman probes) [4, 5], dual-hybridization probes, molecular beacons [6], and scorpions [7]. Each different probe type has its own advantages and disadvantages [8].

A real-time RT-PCR experiment using hydrolysis fluorogenic probes requires forward and reverse primers that flank the amplicon, as well as the hydrolysis probe which is an oligonucleotide that is complementary to a sequence within the amplicon. The probes are typically designed to span exon-exon junctions so as to decrease the likelihood of amplification from contaminating genomic DNA (*see* **Note 1**). The oligomer probes carry a fluorescent reporter dye at one end and a quencher dye at the other end. As long as the reporter dye and the quencher are held in close proximity by their attachment to the oligomer probe, no fluorescence is released from the reporter dye. The release of the fluorophore from the oligomer probe relies on the 5′ nuclease activity of the DNA polymerase [9]. (Taq polymerase is the most commonly used enzyme in this assay, but others are also available [10, 11].) As the DNA polymerase synthesizes the new DNA strand of the amplicon during each PCR cycle, it encounters and hydrolyzes the probe, thereby releasing the reporter dye from the quencher. This release allows the fluorophore to fluoresce which is registered by the real-time PCR machine. The quantity of increasing fluorescence that is detected with each cycle is directly proportional to the quantity of starting material (mRNA) in the initial sample [1].

The accuracy of real-time RT-PCR requires diligent care by the investigator [12, 13]. Careful experimental design from the beginning, whether working with cells or tissues, will provide the foundation needed for reproducible results. This chapter describes the use of hydrolysis probes for detecting gene expression by real-time RT-PCR (Fig. 1).

2 Materials

It is extremely important to work cleanly and mitigate the presence of RNases (*see* **Note 2**).

1. Nuclease-free water (*see* **Note 3**).
2. Primer/Probe unique to the target experimental gene (e.g., Assays on Demand, Life Technologies) or investigator-designed primers and probes (*see* **Note 4**).
3. One-step real-time RT-PCR master mix containing the appropriate polymerase (e.g., TaqMan One-Step Master Mix, Life Technologies) (*see* **Note 5**).
4. Samples of total RNA for analysis with known concentration.

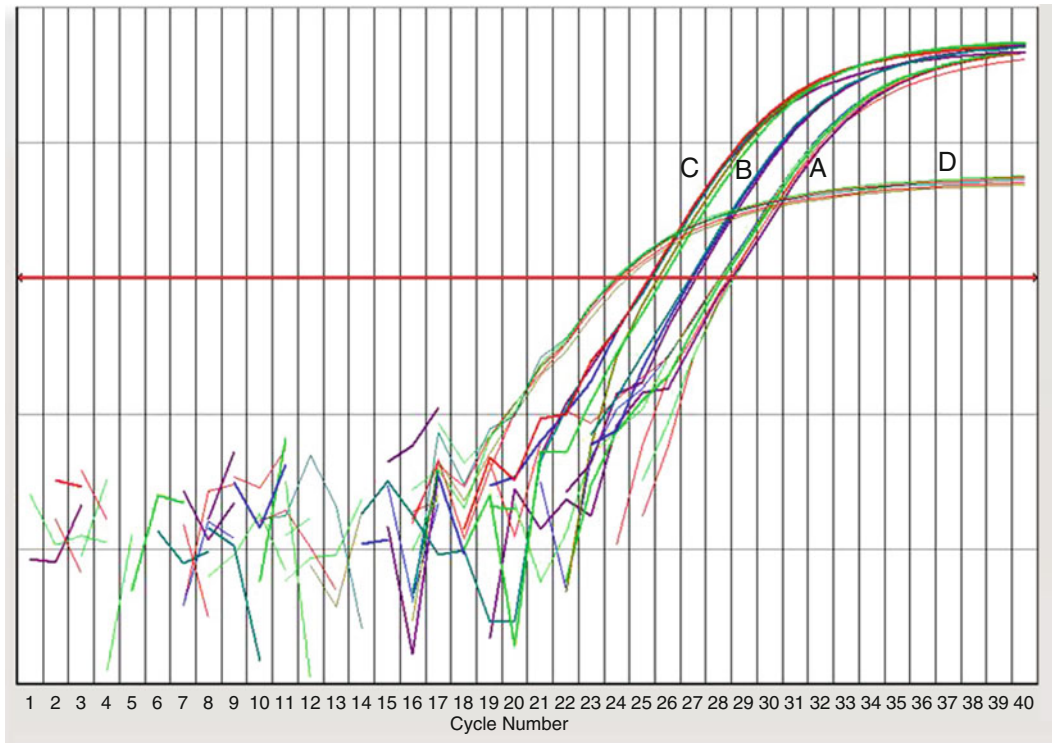

Fig. 1 Amplification curve for a one-step real-time RT-PCR reaction illustrating amplification of an experimental gene, fatty acid synthase (FAS), and a reference gene, cyclophilin. Vehicle, estradiol benzoate (EB), and ethinyl estradiol (EE) were administered by gavage for 3 weeks. Total RNA was extracted from liver and a one-step real time RT-PCR reaction was carried out on samples from control (*curve A*), EB-treated (*curve B*), and EE-treated (*curve C*) samples. EB increased FAS expression by 2.2-fold and EE increased FAS by 8.0-fold compared to control in this group of rats. *D* = housekeeping gene, cyclophilin. *N* = 5/group

5. RNase inhibitor.
6. RNA dilution pool: The same amount of RNA will be needed for each reaction and the easiest way to ensure this is to prepare an RNA dilution pool for each biological replicate and/or treatment from the concentrated mRNA samples after isolation (*see* **Note 6**). Calculate the necessary volumes of the RNA dilution pool (amount of RNA sample plus nuclease-free water) by multiplying the quantity of RNA needed per well (calculated using the validation curves described in **Note 7**) by the total volume required (6 µL/well times the number of wells. Always include the volume of one extra well for pipetting overage.). Divide this value by the concentration of RNA in the sample. For example, if you want 50 ng/well for nine wells at 6 µL/well and the concentration of RNA in the sample is 500 ng/µL, the calculation will be ((50 ng/well × 54 µL) divided by 6 µL) divided by 500 ng/µL = 0.90 µL of total RNA. Mix 0.90 µL total RNA with 53.1 µL nuclease-free

water for the first RNA dilution pool. Perform this calculation for each of the biological replicates, as the concentration of the total RNA will be different for each sample, and make an RNA pool for each (*see* **Notes 8** and **9**).

7. Master Mix pool: Make a separate Master Mix pool for each experimental and reference gene; the only difference among the Master Mix pools will be the primer-probe mix specific for a given gene. The Master Mix pool contains the following per-well volume of reagents times the number of wells: 1.25 μL primer-probe mix, 4.625 μL nuclease-free water, 0.625 μL 40× reverse transcriptase + RNase inhibitor, and 12.5 μL 2× Master Mix (*see* **Note 10**). Multiply these amounts by the number of wells needed for the assay. If less than 50 wells are needed, add one more well volume for overage. If more than 50 wells are needed, add two well volumes.
8. No-RT Master Mix pool: Make separate Master Mix pools for each experimental and reference gene for the no-RT controls. The No-RT Master Mix pools contain the following per-well volume of reagents: 1.25 μL primer-probe mix, 4.625 μL nuclease-free water, 0.625 μL RNase inhibitor, and 12.5 μL 2× Master Mix. As before, multiply these amounts by the number of wells needed for the assay. If less than 50 wells are needed, add one more well volume for overage. If more than 50 wells are needed, add two well volumes.
9. 96-Well microtiter plates and plate cover film compatible with the appropriate real time PCR machine (*see* **Note 11**).
10. Real-time PCR machine.
11. A pipette set that can dispense liquid amounts ranging from 0.1 μL to 1 mL.
12. Filter pipette tips that can dispense liquid amounts from 0.1 μL to 1 mL.

3 Methods

The instructions given here describe a one-step real-time RT-PCR reaction in which the reverse transcription reaction and the PCR cycles are carried out in the same well of the PCR plate (*see* **Note 12**). Choose the appropriate reference genes for your experiment (*see* **Note 13**). Perform a concentration-response curve (also called a validation curve) for the experimental gene and for the reference genes (*see* **Note 7**). To reduce the number of pipetting steps (and thereby reduce pipetting errors), prepare reagent pools of RNA and Master Mix (Subheading 2, **items 6–8**).

1. Design a 96-well plate map (Fig. 2) to guide experimental design. Include technical replicates of each RNA sample

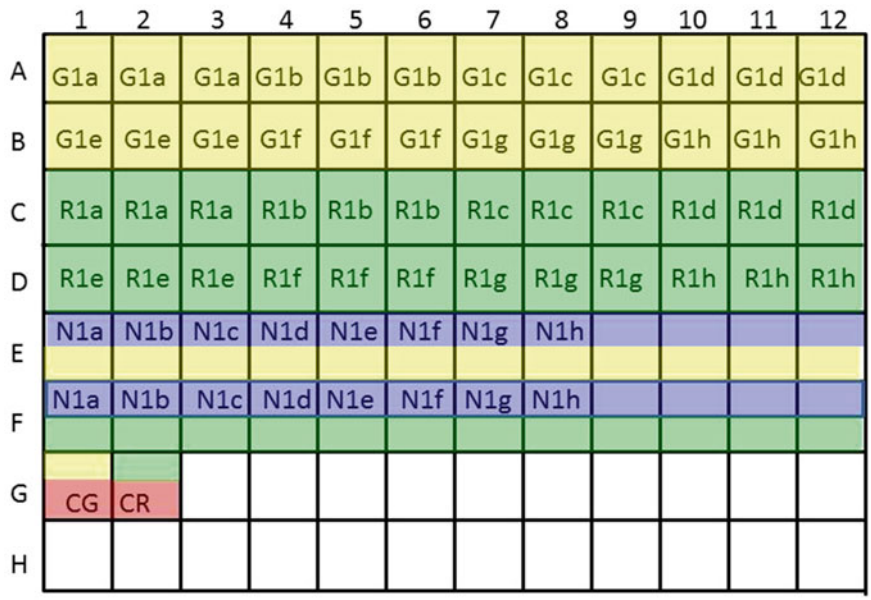

Fig. 2 96-Well plate map for a real-time RT-PCR experiment. Prepare a color-coded plate map to make it easier to find your place on the plate while pipetting. In this experimental design, *rows A* and *B*, labeled G1a–G1i, represent the experimental gene. *Rows C* and *D*, labeled R1a–R1i, represent the reference (housekeeping) gene. *Row A* illustrates four biological replicates for the vehicle control-treated sample and *row B* illustrates four biological replicates of estrogen-treated samples. Similarly, *rows C* and *D* illustrate the same four biological replicates for the vehicle control-treated and estrogen-treated samples. Triplicate wells are shown for each biological replicate. *Rows E* and *F* show a single well for each biological replicate in which the reverse transcriptase enzyme is left out of the reaction. *Row G* shows a single well for each gene (CG for the experimental gene and CR for the reference gene); no RNA is added to these wells. The reactions in *rows A–D* should yield amplification curves as in Fig. 1. No amplification should be observed in the no RT wells or in the no RNA wells. If amplification is observed in the no-RT wells, this suggests contamination of the RNA sample with genomic DNA. If amplification is observed in the no-RNA wells, it suggests that the reagents are contaminated

(preferably triplicates). Also include biological replicates from different animals or different passages of a cell line for each treatment group for the purposes of statistical analysis.

2. Control reactions: For a one-step RT-PCR reaction, each biological replicate must have a no-reverse transcription (NRT) control. Each experimental gene and each reference gene requires the inclusion of a no-RNA control (NRC) (Fig. 2).
3. Load 19 μL of the appropriate Master Mix pool or No-RT Master Mix pool into the assigned wells on the plate following the plate map.
4. Load 6 μL of the appropriate RNA pool into the assigned wells on the plate following the plate map.
5. Carefully seal the plate with the appropriate 96-well plate film (*see* **Note 14**).

6. Centrifuge the plate at 1200 × *g* for 2 min.
7. Place the plate in the real-time PCR machine and program the run as instructed by the manufacturer (*see* **Note 15**).
8. Analyze the data (*see* **Note 16**).

4 Notes

1. The precaution of designing hydrolysis probes to cross exon-exon boundaries does not provide absolute protection against contaminating genomic DNA since DNA can loop out and allow the juxtaposition of those boundaries. Therefore, it is important to eliminate genomic DNA from the RNA samples for real-time RT-PCR even when using this probe design.
2. RNases are ubiquitous and contamination with RNases will destroy your experiment. Cleanliness is key and gloves should be worn at all times. Detergents that denature RNases such as RNaseZap and RNase Away are very useful for cleaning the surfaces of the laboratory bench, pipettes, pipette tip boxes, gloved hands, and any other surfaces that may come in contact with the samples. It is recommended that a dedicated PCR hood is used; however, plates can be loaded on a bench that is dedicated to RNA work if appropriately cleaned. Use dedicated filtered pipette tips for best results.
3. Aliquot nuclease-free water into volumes of 1 mL or less to keep the stock solution clean and free of contamination. Do not use diethylpyrocarbonate-treated (DEPC) water for the real time RT-PCR reactions.
4. Several companies provide pre-designed primer-probe sets that contain forward and reverse primer as well as the oligomer probe in one solution. Often these reagents are available for human, rat, and mouse genes and some other species as well. Primer and probe oligonucleotide design programs (e.g., Primer Express, Life Technologies or Primer3, Massachusetts Institute of Technology) are available if pre-designed reagents are not available for the species or gene of interest. Primers, probes, and amplicons for real-time RT-PCR have different requirements from primers and amplicons for conventional RT-PCR, so be sure to pay attention to these specifications when designing these reagents. While the commercially designed primer/probes have a high success rate, it is important to be aware that some do fail on occasion depending on the sample being tested. Failure of amplification of any given primer/probe set could be due to an issue with the primers/probes or because a given tissue may not express the gene target. The addition of a positive control along with the mRNA samples is useful for determining the efficacy of the reaction.

5. Master mixes can be created in-house but commercially available mixes are often a better choice because of the rigorous validation of each lot. Make sure to carefully investigate which master mix is the correct fit for your experimental design and is compatible with the primer/probes sets that you plan to use.
6. The reaction volume calculations included here are for a total volume of 25 μL, a commonly used reaction volume for real-time RT-PCR. Total volume can be reduced to as low as 10 μL to conserve precious samples or to reduce reagent costs.
7. Efficiency is a term that is used to describe how well the primer and probe oligonucleotides bind with the target sequence [14]. The quality of this attachment is especially important when choosing the correct reference gene; the experimental gene and the reference genes should have comparable efficiencies. PCR efficiency is determined by performing a concentration-response curve with a range of dilutions of the starting sample [15]; for example, set up the reaction with 25, 50, 100, 150, and 200 ng of total RNA. The target gene and the reference gene should have similar concentration-response curves and efficiency should be close to 100 % [16, 17]. The best sample to use for this test is a pool that includes a representative population from the experiment, whether it is tissue or cell derived. This concentration-response curve is also known as a validation curve and it serves the additional purpose of identifying the RNA concentration that will produce a valid curve in the real time PCR reaction.
8. The RNA for real-time RT-PCR should be pure and of high quality. The sample should have been treated with DNase during the purification process to reduce the likelihood of DNA contamination. Analysis of the RNA sample on an Agilent Bioanalyzer is an excellent method of determining the quality of the RNA [18]. We have observed that RNA that has been purified on a spin column such as the RNeasy column (Qiagen) is of higher and more reproducible quality than that purified by the phenol:chloroform:isoamyl alcohol method.
9. If self-designed primer/probe sets are being used, the forward, reverse, and probe oligonucleotides will be separate. As such, the volume of water added to each reaction will have to be changed to accommodate this method.
10. Genes can be multiplexed; that is, the primer-probe sets for both the experimental gene and the reference gene can be amplified in the same well. In a multiplexed assay, adjust the volume of water in the Master Mix pool. Be aware that if one or both of the amplifications in a multiplexed reaction is especially robust, the reaction may exhaust the substrate and produce an artifactual amplification curve.

11. The 96-well microtiter plates may be machine specific. Always check to make sure that the plates and the transparent film covers are compatible with the real time PCR machine available.
12. When the sample of RNA is limited or when it is necessary to maintain long-term storage of a sample of cDNA, it may be preferable to perform a two-step reaction in which the reverse transcription reaction is carried out in a conventional PCR machine, and then the real time PCR reaction is performed using cDNA from the first step. Convenient kits are available for the reverse transcription reaction (e.g., High Capacity cDNA Archive Kit, Life Technologies); the cDNA should be purified using a spin column (e.g., QIAquick PCR Purification Kit, Qiagen) as the residual nucleotides, salts, and buffers from the RT reaction may interfere with the subsequent real-time PCR reaction. Another caveat to the two step reaction is that any evidence of potential contamination of the sample with genomic DNA will be lost.
13. Proper normalization is critical for a successful real-time RT-PCR experiment. This requires identification of a reference/housekeeping gene that does not change expression in the sample that is being tested in response to the experimental paradigm [14]. While it is common to choose a single gene such as glyceraldehyde-3-phosphate as the reference, it is important to be aware that no reference gene displays exactly the same expression across all cell types. Therefore, more than one reference gene should be validated for each new cell type or treatment [1, 13]. This is especially important in the context of estrogen treatment as many of the commonly used reference genes show a response to estrogen in a tissue-specific manner [15, 16].
14. When scheduling is an issue, it is possible to freeze a plate overnight and run the plate the next day. Pipette the plate as usual and seal with the transparent film. Wrap the plate in aluminum foil and freeze at –20 °C overnight. Allow the plate to come to room temperature and centrifuge at $1200 \times g$ for 2 min to make sure that any condensate is returned to the bottom of the well. Run the plate as usual.
15. Most machines take readings from all of the wells, whether they contain sample or not. This is useful when setting up the analysis, especially if wells are mistakenly mislabeled.
16. Several data analysis programs are available for real-time RT-PCR data (e.g., qBase, DataAssist™, Real-Time StatMiner®) which are easy to use and allow quick evaluation of the data. These programs can incorporate the use of several reference genes to normalize real time RT-PCR data. Most analysis programs do have freeware versions or free trials. Real time RT-PCR data can be manually analyzed using two methods: the

standard curve/absolute quantification method or the relative quantification method. The standard curve method is often used when assessing a small number of genes or when quantifying viral load [17, 18]. This method can also be used if an adequate reference gene cannot be found. The relative quantification method is the most common method and relies on a reference gene for normalization. The equation for this comparative threshold method is $2^{-\Delta\Delta^{Ct}} = \Delta Ct(\text{sample}) - \Delta Ct(\text{reference gene})$ [19, 20]. Perform statistical analyses on the data.

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Chapter 4

Bioinformatics Analysis of Estrogen-Responsive Genes

Adam E. Handel

Abstract

Estrogen is a steroid hormone that plays critical roles in a myriad of intracellular pathways. The expression of many genes is regulated through the steroid hormone receptors *ESR1* and *ESR2*. These bind to DNA and modulate the expression of target genes. Identification of estrogen target genes is greatly facilitated by the use of transcriptomic methods, such as RNA-seq and expression microarrays, and chromatin immunoprecipitation with massively parallel sequencing (ChIP-seq). Combining transcriptomic and ChIP-seq data enables a distinction to be drawn between direct and indirect estrogen target genes. This chapter discusses some methods of identifying estrogen target genes that do not require any expertise in programming languages or complex bioinformatics.

Key words Estrogen, ChIP-seq, Transcriptomics, Gene targets, Bioinformatics

1 Introduction

Gender disparities are associated with the risk of multiple diseases [1]. Estrogen is clearly associated with the risk of many gynecological malignancies but also has a role in modulating aspects of autoimmunity [2–4]. Therefore, understanding estrogen-regulated gene pathways is critical to understanding the pathophysiology of many diseases. This in turn requires an understanding of the dynamics of estrogen-regulated gene expression and the binding of *ESR1* and *ESR2*, the nuclear receptors through which estrogen exerts much of its effect [5].

Identifying estrogen-responsive genes is an apparently simple problem. The obvious method to use is to profile gene expression in the presence or absence of estrogen [6]. This can be performed either by expression microarray, which involves the use of tiling oligonucleotide probes and identifying the targets of RNA hybridization, and RNA-seq, which involves fragmenting RNA in cells and sequencing cDNA reverse transcribed from these RNA

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fragments [7]. However, depending on the time course used in transcriptomic experiments, this will identify both direct estradiol target genes and secondary genes modulated by those direct target genes (*see* **Note 1**).

Chromatin immunoprecipitation with massively parallel sequencing (ChIP-seq) is a technique that allows for the genomic localization of nuclear receptor binding [7, 8]. This technique uses the formation of formaldehyde cross bridges between DNA and proteins bound to nucleic acid, followed by selective sequencing of DNA fragments that have been immunoprecipitated by an antibody directed against a protein of interest. In case the of estrogen, fragments that are immunoprecipitated with antibodies against ESR1 or ESR2 can be compared with fragments immunoprecipitated by nonspecific antibodies (input control) or fragments can be compared between samples pre- and posttreatment with estrogen. Stimulation with estrogen (or estrogen receptor agonists) can be problematic as, just as in the case of transcriptomics, the duration of stimulation can be an important consideration in capturing different aspects of receptor binding (*see* **Note 2**). Remodeling of the chromatin architecture and the 3D structure of the genome are likely to be complex and time-dependent processes, which mean that the snapshot of estrogen receptor occupancy afforded by ChIP-seq may not always be representative of the underlying biology (*see* **Note 3**) [9, 10].

This chapter concentrates on basic methods of identifying direct estrogen target genes by combining transcriptomic and ChIP-seq data. The methods by which nuclear receptors are assigned to gene targets in particular cell types either in vitro or in vivo are continuously evolving both due to the availability of new techniques and the increasingly encyclopedic datasets available on genomic architecture in a multitude of cell types (*see* **Note 4**). However, here we provide a series of simple workflows that rely heavily on the Galaxy web interface and the Genomic HyperBrowser that are effective ways of identifying a set of estrogen direct gene targets with relatively high confidence [11–15]. These offer the distinct advantages that no prior knowledge of bioinformatics or programming languages is required for their use.

The first approach described explains how to identify genomic intervals for a series of genes differentially expressed in response to estrogen treatment and intersect these with ESR1 ChIP-seq-binding→ sites. The second uses a purpose-built bioinformatics tool called BETA that is able to use transcriptomic and ChIP-seq data to identify potential ESR1 target genes [16]. Both assume that the user has transcriptomic and ChIP-seq data available from their cells of interest treated with estrogen and that these data have been processed to obtain differentially expressed genes (DEGs) and significant ChIP-seq peaks. Previous chapters in this series explain how to accomplish this [17, 18].

2 Materials

1. Modern laptop or personal computer running a modern operating system with at least 60 GB of hard disc memory and 4 GB of RAM.
2. Basic software for manipulating spreadsheets (e.g., Microsoft Excel or OpenOffice Calc).
3. Internet browser software (e.g., Internet Explorer, Mozilla Firefox or Google Chrome).
4. A high-speed Internet connection.

3 Methods

3.1 The Genomic HyperBrowser

1. Register for the Genomic HyperBrowser (https://hyperbrowser.uio.no/hb/).
2. Prepare ChIP-seq and transciptomic datasets for upload. ChIP-seq files should be a set of tab-delimited genomic coordinates corresponding to each peak in the format:

   Chromosome Start Stop

   Transcriptomic files should be a list of differentially expressed genes (DEGs) as ENSEMBL IDs (*see* **Note 1** for methods for converting between different forms of gene ID). The datasets used for this demonstration are the ESR1-binding site data (actually ChIP-chip data) from Hurtado and colleagues and transcriptomic data from Hah and colleagues (thresholded at $q < 0.05$) [6, 19].
3. Firstly upload the ChIP-seq data as shown in Fig. 1.
4. Next use "Generate Tracks > Generate segment track from gene IDs" to obtain genomic intervals from the ENSEMBL gene IDs of DEGs. These should be uploaded into the tool as a series of comma-separated values as shown by the demo data. If necessary genomic intervals can be lifted from one genome build to another by the "Lift-Over > Convert genomic coordinates" tool.
5. Use "Operate on Genomic Intervals > Get flanks" to extend gene regions by a pre-specified number of bases in each direction (Fig. 2). A suitable distance might be 5 kb, which is analogous to the upstream region extension in the gene ontology tool GREAT [20].
6. The original intervals and flanking regions should then be concatenated into a single track using "Operate on Genomic Intervals > Concatenate" and then merged with "Operate on Genomic Intervals > Merge."

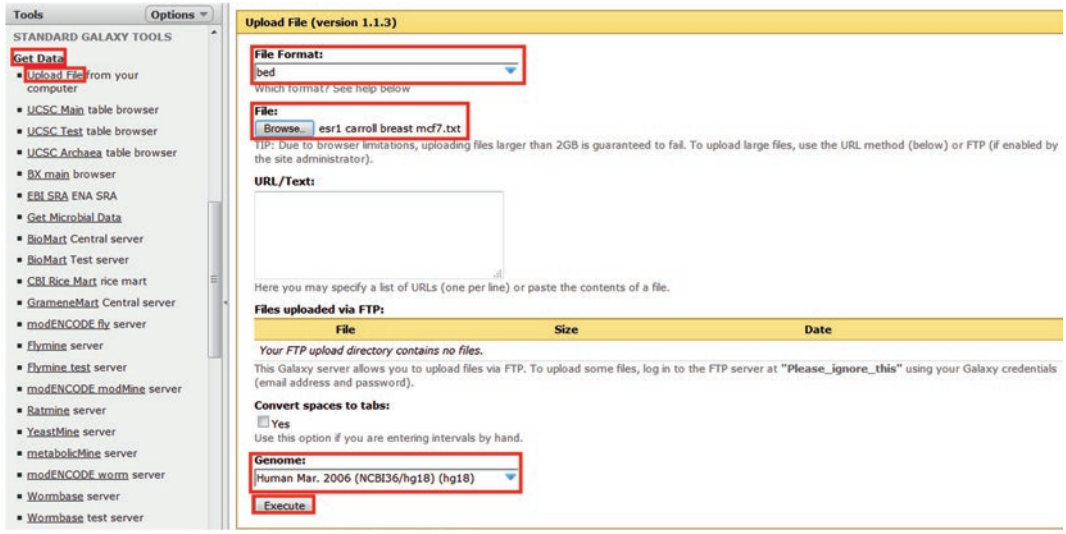

Fig. 1 Uploading files to the Galaxy/Genomic HyperBrowser server. Select "Get Data > Upload files," select the correct file type (in this case "bed" for ChIP-seq data or "txt" for transcriptomic data), select the file location using the "browse" button, select the correct genome build, and then select "Execute"

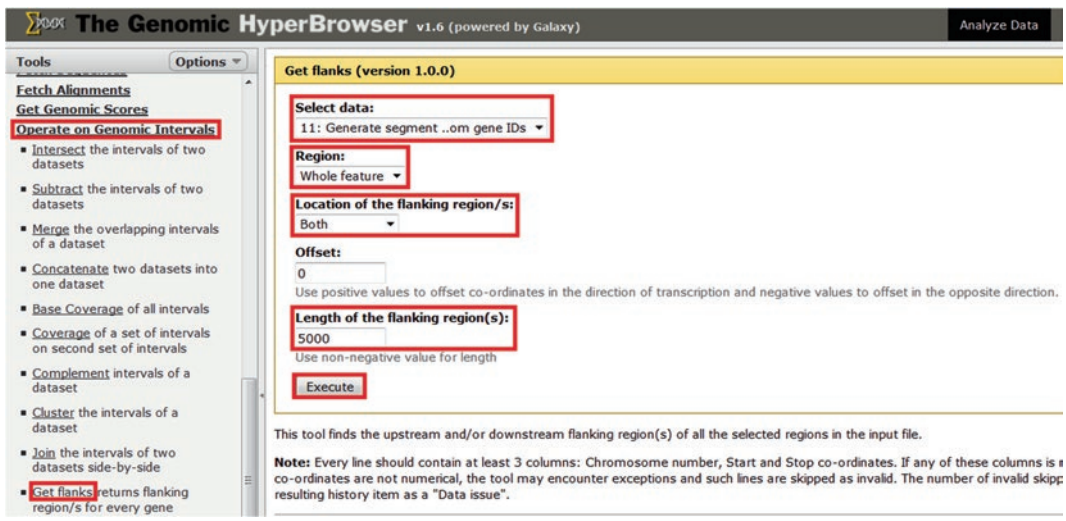

Fig. 2 Generating a track of regions flanking differentially expressed genes. Select "Operate on Genomic Intervals > Get flanks," select the desired track, select the subset of the region to flank (in this case "whole region"), select whether to extend flank from the upstream, downstream or both sides of regions, decide on the length of flanking regions, and then select "Execute"

7. An important sanity check is to ensure that estrogen receptor binding is enriched near estrogen DEGs. The Genomic HyperBrowser allows one to calculate the enrichment of estrogen receptor-binding sites with the intervals generated above (i.e., within 5 kb of estrogen DEGs). Figure 3 illustrates this

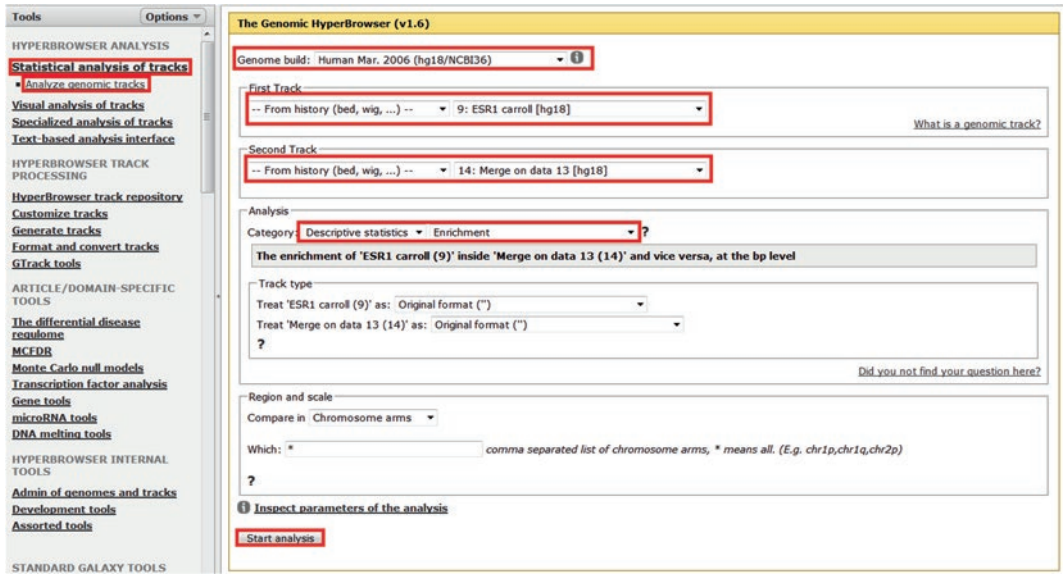

Fig. 3 Performing enrichment analysis. Select "Statistical analysis of tracks > Analyze genomic tracks," select the genome build, select that each track for analysis will be from your history and then select the appropriate track, select "Descriptive statistics," select "Enrichment," and then select "Start analysis"

process. It is possible to use the Genomic HyperBrowser to calculate an empirical *p*-value for this overlap using the same tab as for enrichment analysis but selecting "Category: Hypothesis testing," "Overlap?," a suitable null model (e.g., "Preserve segments (T2), segment lengths and inter-segment gaps (T1); randomize positions (T1) (MC)") and the number of permutations (e.g., for publication quality *p*-values ~10,000 permutations would be recommended). The region and scale tab is also important as this determines in which areas of the genome randomized tracks can fall. Leaving it at its default value (all chromosome arms) is adequate for the current sanity check. There is significant overlap between ESR1-binding sites and estrogen DEGs (2.14-fold, $p < 10^{-4}$), which suggests that there are likely to be plausible direct estrogen targets amongst the transcriptomic dataset. Note that analyses are only conducted on bed files, and so if the track of interest is not offered by the Genomic HyperBrowser as a potential track for analysis then edit that track to ensure that the track type is "bed."

8. Identifying potential direct estrogen targets is simply a matter of joining estrogen DEGs (±5 kb) to ESR1-binding sites (Fig. 4).
9. The resultant output can be pasted into a spreadsheet program and filtered to obtain unique gene IDs and their respective ESR1-binding sites.

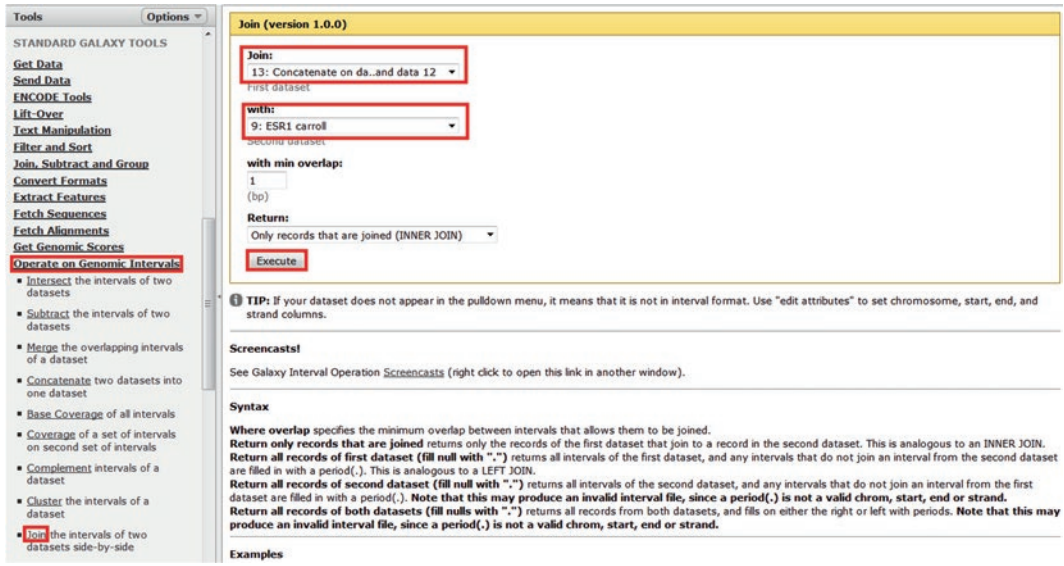

Fig. 4 Joining two tracks side by side. Select "Operate on Genomic Intervals > Join," select the required tracks, and then select "Execute"

3.2 BETA

1. Register for Galaxy/Cistrome (http://cistrome.org/ap/). This tool integrates transcription factor-binding sites with the degree of differential gene expression to predict high-confidence direct targets.
2. Prepare ChIP-seq and transcriptomic data for upload. Again, the ChIP-seq data should be a tab-delimited file in the format:

   Chromosome Start Stop

   RNA-seq data can either be directly uploaded as Cuffdiff or LIMMA output [21, 22] or formatted as a tab-delimited file with columns corresponding to gene ID, direction of change (e.g., *T*-score) and significance (e.g., FDR). Ensure that all data are present as text (gene ID) or numerical data. Some spreadsheet programs can format data in ways that will cause BETA to crash (e.g., substituting dates for numerical values).
3. Start the BETA tool running after selecting the appropriate parameters (Fig. 5). The BETA tool is available through "Integrative Analysis > BETA-plus: Binding and Expression Target prediction and motif analysis." For an initial analysis, it is recommended to leave as many settings at their default values as possible. Subsequently these can be altered to test how robust the results are to changes in, for example, the distance threshold of ESR1-binding sites to DEGs.

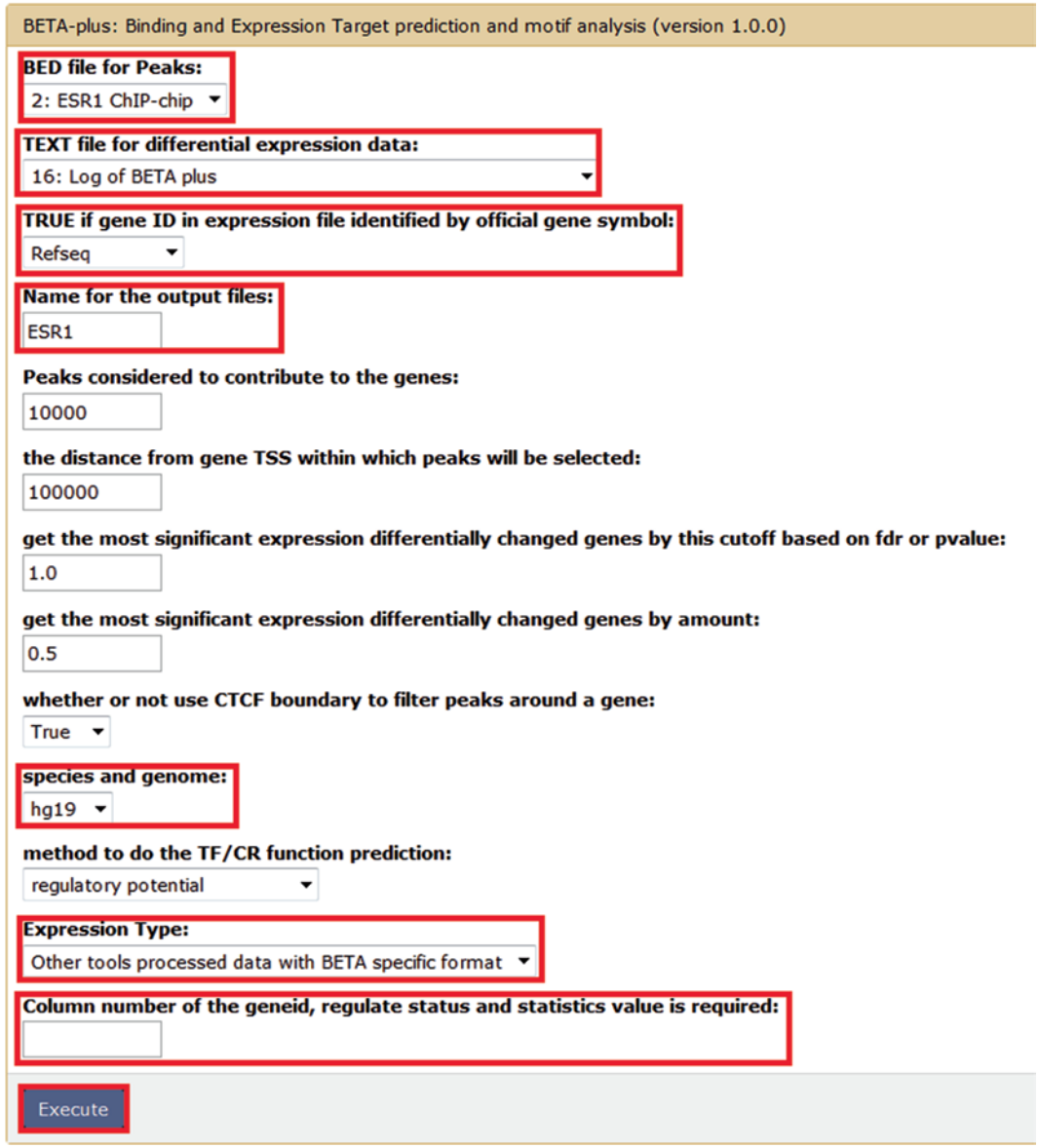

Fig. 5 Performing BETA-plus analysis. Select the track containing the ChIP-seq data, select the track containing the transcriptomic data, select the type of gene ID used (RefSeq or gene symbol), input the prefix for output files, select the genome build, select the type of transcriptomic data (i.e., the format of the track selected earlier), if the transcriptomic data is in a custom format insert a comma-separated list of numbers referencing which column is the gene ID, the direction of expression change and the significance measure (e.g., if this was a track with three columns, the first of which was the gene ID, the second of which was the $\log_2$ fold change and the third of which was the FDR, this would be 1,2,3). Finally select "Execute"

4. The output files then produce direct target predictions. These are described below:
   (a) BETA functional prediction on ESR1 ChIP-chip: A graph showing the relationship between functional rank and the number of direct targets and an associated *p*-value for up- or downregulated genes.
   (b) BETA direct targets prediction on up regulated genes: A table of up-regulated gene targets detailing the rank product score (derived from the significance score provided in the transcriptomic dataset).
   (c) BETA direct targets prediction on down regulated genes: A table of downregulated gene targets detailing the rank product score (derived from the significance score provided in the transcriptomic dataset).
   (d) Uptarget associated peaks: A list of peaks with the associated up-regulated gene target, the distance to the target gene, and a functional score.
   (e) Downtarget associated peaks: A list of peaks with the associated downregulated gene target, the distance to the target gene, and a functional score.
   (f) Motif analysis on target regions: An html output file detailing top motifs detected for multiple comparisons along with associated statistical scores.
   (g) A series of detailed motif analysis outputs: The statistical data for the above file.
   (h) Log of BETA plus: This details the input parameters and any errors encountered during the course of the analysis.

4 Notes

1. *Converting between different gene IDs*: There are multiple ways of converting between different forms of gene ID (e.g., gene symbol, RefSeq, ENSEMBL). One simple way is to use the Table Browser function in UCSC Genome Browser (http://genome.ucsc.edu/) (Fig. 6) [23]. It is possible to convert gene IDs between multiple types of gene ID using sequential conversions.
2. *Considerations for experimental design*: Replicates are essential for ChIP-seq and transcriptomic analysis when attempting to distinguish biologically meaningful variation from noise. As mentioned in the *introduction*, if using stimulation with estrogen or an estrogen receptor agonist, it is vital to decide on the time scale of stimulation to ensure that the correct cross section of binding and transcriptomic changes are sampled.

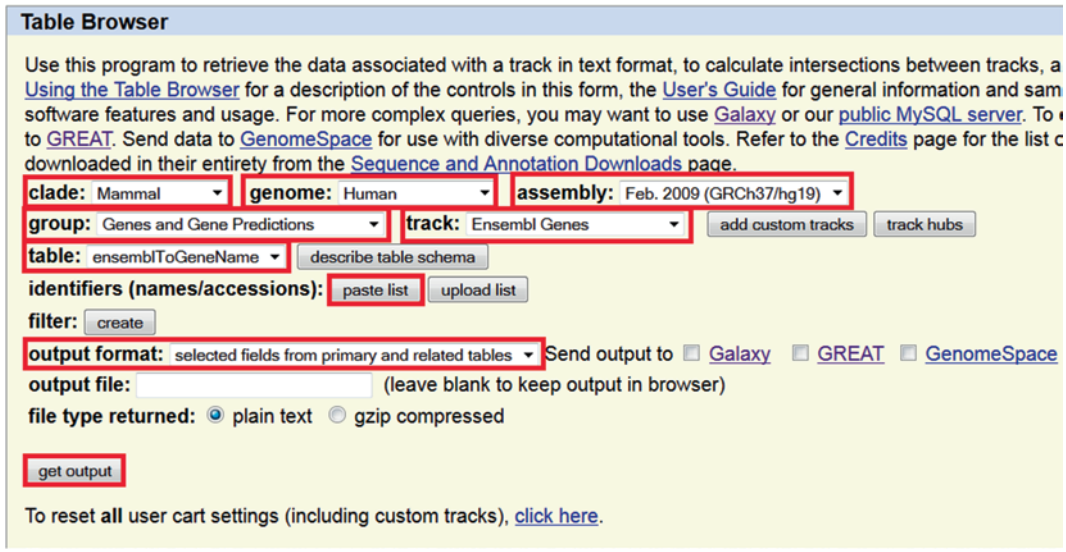

Fig. 6 Convert gene IDs from one form to another. Select the required clade, the species and the genome build. Select the "Genes and Gene Predictions" group of tables, the appropriate track (e.g., Ensembl genes), the desired table (this will be one that maps the gene ID one has to the gene ID one requires), paste the list of gene IDs (this will be checked for unknown IDs by the system), select that output should be "selected fields from primary and related tables," and then select "get output." On the resulting screen it is possible to select the desired fields to obtain the gene ID of interest

3. *Limitations of current bioinformatic methods*: There are several important limitations to consider when interpreting lists of direct gene targets. The output is only as good as the data input into the model in the first place. This can be an issue particularly for ChIP-seq datasets, which are noisy and frequently irreproducible in the main between different studies nominally using the same material and methods [24]. However, new methods for calling ChIP-seq peaks, such as irreproducibility discovery analysis, which attempt to leverage power from replicates, may help to alleviate this problem [25]. Another limitation is that distance thresholds applied in calling direct gene targets are linear, whereas it is clear that the 3D structure of chromatin is important in determining which binding sites interact with which genes [9]. Methods for considering 3D structure in enrichment analyses are available through the Genomic HyperBrowser but the interpretation of such data is not straightforward [26].
4. *Further functional annotation of direct gene targets*: As mentioned above, many of the thresholds used are rather arbitrary and so it can be informative to include other forms of functional annotation to hone down a list of potential gene targets to ones of higher confidence. ESR1 binding sites are more

likely to be consistent between different ChIP datasets if they possess a classical ESR1 recognition motif or are located in a region of open chromatin (as assessed by DNase-seq) [24]. BETA will supply a measure of motif enrichment within the peaks supplied and estrogen receptor motifs should be significantly enriched in direct gene targets. Motif scanning software such as FIMO can be used to assess whether specific ESR1-binding sites contain estrogen receptor recognition motifs and this can assist in the selection of high-confidence gene targets [27]. There is a wealth of data on chromatin state or RNA polymerase II binding in many cell types with and without estrogen stimulation available from databases like UCSC Genome Browser. These can be downloaded and intersected with candidate direct gene targets just as in Subheading 3.1 to select high-confidence direct gene targets. Gene targets containing an estrogen receptor recognition motif, a DNase hypersensitivity peak, and with a nearby RNA polymerase II ChIP-seq peak in addition to an ESR1 ChIP-seq peak are highly likely to be direct estrogen targets [28].

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Chapter 5

Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay of Cytochrome P450 2B6 in Response to Estrogen

Kwi Hye Koh and Hyunyoung Jeong

Abstract

Electrophoretic mobility shift assay (EMSA) is an invaluable tool to study interaction of proteins with DNA. Estrogens are major female hormones and modulate biological function through estrogen receptor (ER). ER regulates its target gene expression via the classical mechanism in which ER directly binds to its target gene promoter or the nonclassical mechanism involving tethering of ER to other transcription factors (such as AP-1 proteins). Here, we describe the EMSA to examine the nonclassical mechanism of ER action in regulation of a gene CYP2B6 by using competition and supershift assays.

Key words EMSA, Supershift, Estrogen, Estrogenreceptor, AP-1, CYP2B6

1 Introduction

The interaction of proteins with DNA is central to the regulation of cellular processes including gene transcription. Electrophoretic mobility shift assay (EMSA) is a tool commonly used to study protein:DNA interactions by resolving the binding complexes from the unbound DNA. By using EMSA, binding stoichiometry between the protein and DNA can be studied [1], or DNA-binding proteins (such as transcription factors) in nuclear extracts can be identified through supershift assays [2].

Estrogens are major female hormones involved in multiple biological functions including hepatic lipid synthesis or drug metabolism [2, 3]. The actions of estrogen are mediated by the estrogen receptor (ER)α and ERβ. ER, after binding to estrogen, may regulate expression of its target genes in different pathways [4]. For example, ER may bind to a *cis*-element, called the estrogen response element (ERE), of target genes and regulate gene expression. Alternatively, it may associate with other transcriptional regulators and indirectly modulate expression of target genes. In the latter case, the activation of estrogen target genes occurs in an ERE-independent manner, and transcription factors belonging to

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the activator protein-1 (AP-1) family can play an important role [5]. The transcriptional regulators in the AP-1 family include c-Jun and c-Fos, which bind to an AP-1 motif in the upstream region of target genes and regulate gene expression. Here, we describe the use of EMSA to investigate the regulatory mechanism for a hepatic drug-metabolizing enzyme, cytochrome P450 (CYP) 2B6, by using competition and supershift assays. Results from previous studies showed that estrogen induces CYP2B6 expression in human hepatocytes [2]. Through deletion and mutation assays of CYP2B6 promoter, the responsible *cis*-elements have been identified in the upstream regulatory region (e.g., −1782/−1776) of CYP2B6 [2]. The region harbors an AP-1 binding motif but not EREs [2], suggesting that the nonclassical mode of ER action plays a role in CYP2B6 regulation. EMSA was used to verify the interaction between AP-1 proteins and the regulatory region of CYP2B6.

2 Materials

All reagents should be "molecular biology grade" or better.

2.1 Preparation of End-Labeled Duplex DNA Oligonucleotide

1. 10× Annealing buffer: 10 mM Tris–HCl, 50 mM NaCl (*see* **Note 1**).
2. 10× TBE buffer: 0.89 M Tris base, 0.89 M boric acid, 20 mM ethylenediamine tetraacetic acid (EDTA).
3. T4 polynucleotide kinase (PNK) and 10× PNK buffer: 700 mM Tris–HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM dithiothreitol (DTT).
4. [γ-^{32}P] adenosine triphosphate (ATP) (3000 Ci/mmol).
5. 0.5 M EDTA.
6. TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
7. Illustra microSpin G-25 columns (GE Healthcare Life Sciences).
8. Two complementary single-stranded (ss) DNA (sense and antisense) or custom-made double-stranded (ds) DNA probes (*see* **Note 2**). The underlined are core sequences for AP-1 binding.

   CYP2B6 probe: GTGTCAGATGACACAGCACAGCA

   Consensus AP-1 binding probe: CGCTTGATGAGTCA GCCGGAA
9. Thermocycler for oligonucleotide annealing (optional).

2.2 Preparation of Nuclear Extracts from HepG2-ER Cell Lines

1. HepG2-ER cells (i.e., HepG2 cells stably expressing ERα, provided kindly by Dr. David Shapiro, University of Illinois at Urbana Champaign) (*see* **Note 3**).

2. 10× Hypotonic cell lysis buffer for protein extraction from cells: 100 mM HEPES, pH 7.9, 15 mM $MgCl_2$, 100 mM KCl (*see* **Note 4**).
3. Nuclear extraction buffer: 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25 % Glycerol (vol/vol).
4. 1 M DTT.
5. 1000× Protease inhibitor cocktail: Benzamidine (1.6 mg/ml), phenanthroline (1 mg/ml), aprotinin (1 mg/ml), pepstatin A (1 mg/ml), leupeptin (1 mg/ml), and phenylmethylsulfonyl fluoride (PMSF, 17.4 mg/ml) (*see* **Note 5**).
6. Phosphate-buffered saline (PBS), pH 7.4.
7. Microcentrifuge (Eppendorf 5415R or equivalent) and microscope.
8. Syringes (1 ml capacity) and 27-gauge needles.
9. Microscope slides and micro cover glasses.
10. Cell scrapers.
11. Microtube rotator.
12. Human recombinant ERα protein (RP-310, Thermo Scientific Pierce Products) (optional) (*see* **Note 6**).

2.3 DNA-Protein Binding and Electrophoresis

1. 5× Binding buffer: 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 250 mM NaCl, 2.5 mM DTT, 20 % glycerol (vol/vol), 0.25 mg/ml poly(dI-dC)·poly(dI-dC) (*see* **Note 7**).
2. Antibodies for supershift assay (*see* **Note 8**).
3. 30 % Acrylamide:bisacrylamide (29:1).
4. 10 % Ammonium persulfate (APS).
5. *N*,*N*,*N*,*N*′-Tetramethyl-ethylenediamine (TEMED).
6. 80 % Glycerol (vol/vol).
7. 10× gel loading buffer: 250 mM Tris–HCl, pH 7.5, 0.2 % bromophenol blue, 40 % glycerol (vol/vol).
8. Nuclease-free distilled/deionized water (ddH_2O).
9. Clear plastic wrap (Saran Wrap or equivalent) and Whatman 3MM paper.
10. Narrow gel-loading tips (e.g., Rainin Gel-Well™ tips).
11. A clear tape and a marker pen.
12. Electrophoresis power supply (250 V, 200 mA capacity is recommended). Most commercial power supplies sold for a SDS-PAGE application are suitable.
13. Vertical gel electrophoresis apparatus (*see* **Note 9**).
14. PhosphorImager cassette and imaging system (e.g., Typhoon, Molecular Dynamics).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Preparation of Nuclear Extracts from HepG2-ER Cells

1. Grow HepG2-ER cells in two 10-cm cell culture dishes to 90–100 % confluency (5–10 million cells/dish) (*see* **Note 10**).
2. Wash the cells twice with PBS.
3. Add 1 ml PBS containing protease inhibitors, scrape and collect the cells into 1.5 ml microcentrifuge tubes on ice. From this point on, keep the samples on ice or at 4 °C
4. Centrifuge for 5 min at 450 × *g* (4 °C) and discard the supernatant.
5. Measure approximate packed cell volume (PCV), the pellet volume (*see* **Note 11**).
6. Add 5× PCV volumes of lysis buffer (containing DTT and protease inhibitors).
7. Centrifuge the cells for 5 min at 450 × *g* (4 °C), discard supernatant and resuspend the pellet of packed cells in 2× PCV of lysis buffer.
8. Keep the cell suspension on ice for 15 min for cell swelling.
9. Disrupt the cells by withdrawing and pulling the cell suspension into the syringe through the 27-gauge needle five times. Avoid forming air bubbles during the process.
10. Mix an aliquot of cell suspension with an equal volume of 0.4 % Trypan blue dye (optional), and examine under microscope (*see* **Note 12**).
11. Centrifuge the disrupted cells for 20 min at 10,000–11,000 × *g* (4 °C).
12. Transfer the supernatant to a new 1.5-ml microcentrifuge tube. Save the supernatant as cytoplasmic fraction (optional).
13. Resuspend the (nuclei) pellet in 2/3× PCV volume of nuclear extraction buffer containing the DTT (0.1 M) and protease inhibitors.
14. Place the tubes on a tube rotator in a cold room or cold cabinet (4 °C) for 30 min to 1 h to allow the nuclear membrane to break.
15. Centrifuge for 20 min at 20,000–25,000 × *g* (4 °C).
16. Transfer the supernatant to a new chilled tube as the nuclear extract.
17. Snap-freeze the remaining supernatant in aliquots of 20–30 μL and store at −80 °C (*see* **Note 13**).

3.2 Probe Preparation: Annealing ssDNA and End-Labeling dsDNA

1. Mix the following components in a PCR tube (skip this step if dsDNA is custom-made): 6 μL 100 μM Sense oligonucleotide, 6 μL 100 μM Antisense oligonucleotide, 3 μL 10× Annealing buffer, ddH_2O to 30 μL.
2. Run the following program with thermocycler (skip this step if dsDNA is custom-made) (*see* **Note 14**): 2 min at 98 °C, 1 min, temperature decreased by 1.3 °C per cycle for 60 cycles, cool to 4 °C.
3. End-label the duplex DNA using T4 polynucleotide kinase (PNK): 2 μL 1.75 μM Annealed DNA duplex probe, 1 μL 10× T4 PNK buffer, 1 μL [γ-^{32}P]ATP, 1 μL T4 PNK (10,000 U/ml), ddH_2O to 10 μL.
4. Incubate at 37 °C for 15 min, then add 1 μL of 0.5 M EDTA to stop the reaction.
5. Add 89 μL of TE buffer to yield a final volume of 100 μL. Purify the isotope-labeled DNA duplex by using Illustra microSpin G-25 columns following the manufacturer's protocol. Store the purified DNA probe at −20 °C.

3.3 Preparation of Native Polyacrylamide Gel

1. Wash both panes of the glass for the electrophoresis apparatus with 70 % ethanol and wipe with laboratory wipes.
2. Assemble glass plates with spacers on sides. Using tapes, seal all sides except where a comb is placed. Clamp using binder clips.
3. Mix the following components to make 4 % native polyacrylamide gel (*see* **Note 15**): 3.3 ml 30 % Acrylamide:bisacrylamide (29:1), 1.3 ml 10× TBE buffer, 781 μL 80 % Glycerol, 188 μL 10 % APS, ddH_2O to 25 ml, gently mix, and then add 13 μL TEMED.
4. Pour the gel carefully into assembled electrophoresis unit, insert the comb, and let it polymerize for about 1 h.

3.4 Gel Electrophoresis and DNA-Protein Binding Reaction

1. Remove clips and sealing tapes from the glass plates. Before removing the comb, put clear tape on the glass plate near the bottom of the comb. Mark each well on the tape using a marker. This will be used as a visual aid later when samples are loaded.
2. Put the 4 % gel sandwich into the electrophoresis apparatus. Pre-run the gel using 0.5× TBE buffer at 250 V for at least 10 min.
3. Set up the following reactions, including reactions for positive control (e.g., consensus AP-1 probe) (Table 1): 2 μL 5× Binding buffer, *X*μL Nuclear extracts (Fig. 1, *see* **Note 16**), 1 μL 1.75 μM Unlabeled DNA probe (*see* **Note 17**) (Fig. 2), or 1 μL Antibody for supershift assay (Fig. 3, *see* **Note 18**), ddH_2O to 9 μL

Table 1
An example of experimental setup to determine AP-1 binding to CYP2B6

	Competition assay							Supershift assay										
Lane number	0	1	2	3	4	5	6	0	1	2	3	4	5	6	7	8	9	10
Gel-loading buffer (μL)	1	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–
5× Binding buffer (μL)	–	2	2	2	2	2	2	–	2	2	2	2	2	2	2	2	2	2
Nuclear extracts (μL)[a]	–	–	3	3	3	3	3	–	–	3	3	3	3	–	3	3	3	3
Cold competitor (μL) Consensus AP-1 CYP2B6	–	–	–	1	1	–	1	–	–	–	–	–	–	–	–	–	–	–
Antibody (μL) ER c-Jun c-Fos	–	–	–	–	–	–	–	–	–	–	1	1	1	–	–	1	1	1
Water (μL)	9	7	4	3	3	4	3	9	7	4	3	3	3	7	4	3	3	3
Radiolabeled probe (μL) CYP2B6 Consensus AP-1	–	1	1	1	1	1	1	–	1	1	1	1	1	1	1	1	1	1
Total volume (μL)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

[a]The amount of nuclear extract needs to be optimized

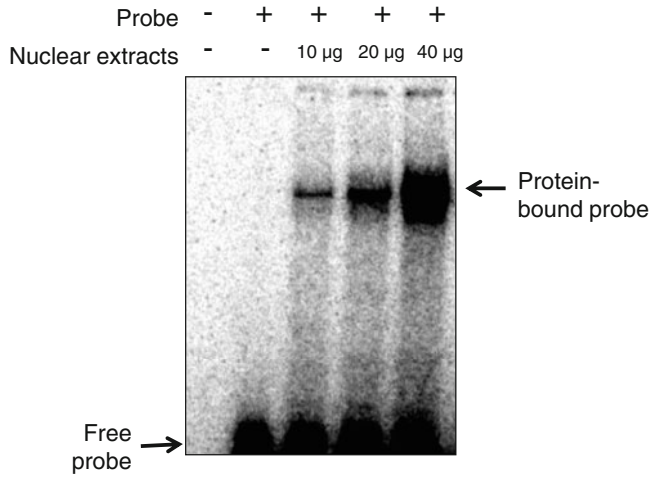

Fig. 1 Titration of nuclear extract amount for binding to CYP2B6 probe. ^{32}P-labeled CYP2B6 probe was incubated with different amounts of nuclear extracts (10, 20, or 40 μg) from HepG2-ER cells. DNA:protein mixture was resolved on a 4 % polyacrylamide gel. Electrophoresis was carried out at room temperature for 30 min. A shifted band was observed in the presence of nuclear extract (marked by the *upper arrow*) indicating the formation of protein:DNA complex. The signal intensity of the shifted band was higher with increasing quantity of binding proteins (i.e., nuclear extracts). To avoid signal saturation but to ensure robust detection of shifted bands, 30 μg of the nuclear extracts were used for competition and supershift assays

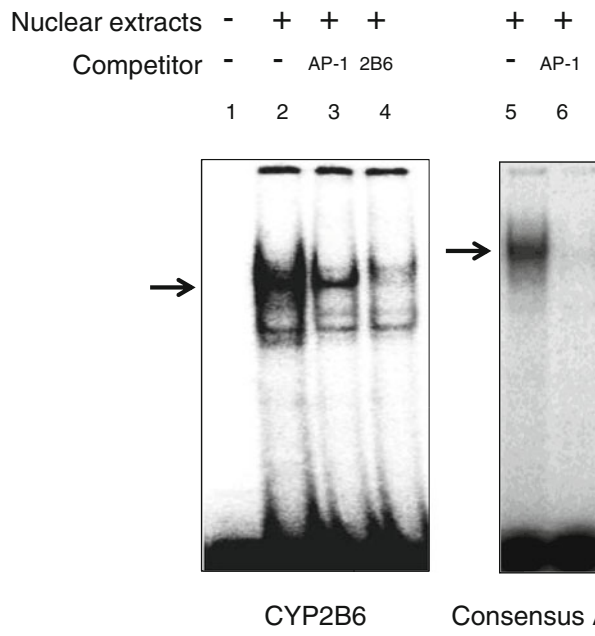

Fig. 2 Competition assays for AP-1 binding to CYP2B6. The specificity of protein:DNA binding was examined by competition assays. ^{32}P-labeled CYP2B6 or consensus AP-1 probe was incubated with nuclear extracts (30 μg) of HepG2-ER in the presence or absence of 50-fold molar excess of unlabeled consensus AP-1 probe (AP-1) or CYP2B6 probe (2B6). The DNA:protein mixture was resolved on 4 % polyacrylamide gels. The signal intensity of the shifted bands for CYP2B6 probe was significantly reduced upon the addition of unlabeled CYP2B6 (lane 2 vs. 3) or AP-1 probe (lane 2 vs. 4), indicating that (1) the interaction between the probe and proteins is specific, and (2) the proteins bound to CYP2B6 probe can bind to AP-1 probe as well. The signal intensity of shifted bands for consensus AP-1 probe was also reduced upon addition of unlabeled AP-1 (lane 5 vs. 6) as expected. These results suggest that AP-1 proteins bind to the CYP2B6 probe

4. Incubate the reactions at room temperature for 10 min (*see* **Note 19**).
5. Add 1 μL of the ^{32}P-labeled DNA probe (Table 1) to each reaction and incubate the reactions for 20 min at room temperature.
6. Load the samples to the gel. The marked line for each well serves as a guide for loading. Load the gel-loading buffer in the first lane (i.e., lane 0 in Table 1) as an indicator of DNA running (*see* **Note 20**).
7. Run the gel at room temperature for 30–60 min until bromophenol blue runs at least halfway down the length of the gel.
8. Open the gel plates by using a spatula and place the gel on two sheets of Whatman 3MM paper.

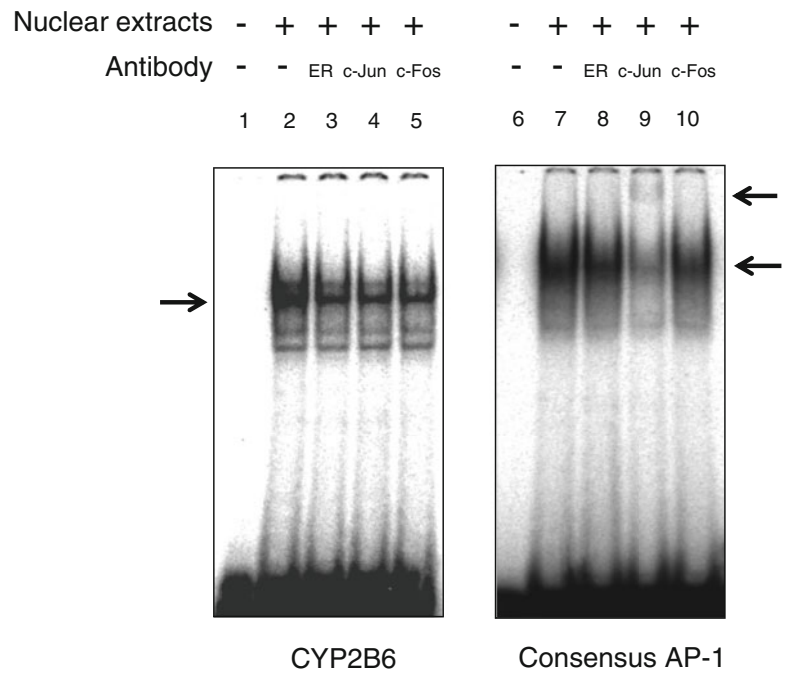

Fig. 3 Supershift assays for AP-1 binding to CYP2B6. Binding of AP-1 proteins to CYP2B6 was further examined by using supershift assays, and the identity of proteins interacting with the CYP2B6 probe was determined by using antibodies. ^{32}P-labeled CYP2B6 or consensus AP-1 probe was incubated with nuclear extracts (30 μg) of HepG2-ER in the presence or absence of antibodies against ERα or AP-1 proteins (c-Jun and c-Fos). The DNA:protein mixture was resolved on 4 % polyacrylamide gels. *Upper and lower arrows* indicate the locations of supershifted and shifted bands, respectively. The signal intensity of shifted bands for CYP2B6 probe was reduced upon addition of antibody against ERα, c-Jun, or c-Fos (lane 2 vs. 3, 4, or 5), likely resulting from the formation of a ternary complex among ERα/AP-1 proteins, DNA, and antibody. However, a supershifted band is not observed. Due to the large size, the ternary complex is often trapped in the loading well such that supershifted bands do not appear. When consensus AP-1 probe was used for protein binding, the signal intensity of shifted bands was reduced in the presence of c-Jun antibody (lane 7 vs. 9), accompanied by the appearance of supershifted band (lane 9, *upper arrow*). This finding was not observed when antibodies against ERα or c-Fos were added to the reaction (lane 7 vs. 8 and 10). These results indicate that the consensus AP-1 probe used in this study binds to c-Jun (but not to c-Fos) in nuclear extracts from HepG2-ER cells

9. Cover the other side of the gel with Saran Wrap and dry on a gel dryer at 80 °C for 1 h.
10. Expose the gel to a PhosphorImager screen overnight (*see* **Note 21**).
11. Visualize the screen by PhosphorImager.

4 Notes

1. Alternatively, the commercially available NEB buffer 2 (final concentrations: 10 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) may be used.
2. The length of DNA oligonucleotide for DNA-protein binding reaction used in our studies is 25–35 base pairs (bp) because protein-binding DNA sequences are typically 4–30 bp. Longer probes can be used if the binding sequence is unknown or if multiple regulatory regions are being studied. Double-stranded DNA probes can be generated by annealing two complementary single-stranded oligonucleotides or custom-made. In the latter case, oligonucleotides of high quality (e.g., cartridge, HPLC, or PAGE-purification) are recommended because the DNA probes of low purity can cause smearing of bands during electrophoresis (which can be interpreted as nonspecific interactions between the protein and DNA).
3. HepG2-ER cells were used in our study as liver was the target organ of interest. Basal ERα expression in parental HepG2 cells is known to be minimal. The cell lines should be chosen based on target tissues of interest.
4. Cell lysis buffer should be prepared freshly by adding DTT (final concentration: 1 mM) and protease inhibitor cocktail. On the day of nuclear protein extraction, all buffers and stock solutions should be kept on ice or at 4 °C before use.
5. These compounds exhibit poor chemical stability and water solubility. Prepare the stock solution containing benzamidine, phenanthroline, aprotinin, pepstatin A, and leupeptin using ethanol as solvent. PMSF stock solution is prepared separately in isopropanol. These stock solutions should be stored at –80°C. Alternatively, commercially available protease inhibitor cocktails may be used.
6. As the source of DNA-binding protein, either crude nuclear extract (e.g., from HepG2-ER cells) or recombinant protein can be used. Recombinant ERα protein (1–5 ng per reaction) can be used if a direct ERα binding to the DNA is suspected (e.g., the presence of putative ERE). In this case, the following dsDNA that harbors a consensus ERE can be used as a positive control probe: GGATCTAGGTCACTGTGACCCCGGATC (underlined is core sequence for ERα binding). When recombinant ERα protein is used as a protein source, a nonclassical mode of ERα action (e.g., ERα regulating target gene expression via tethering to AP-1 proteins) is apparently not captured. One can determine the involvement of nonclassical ERα action by performing transient transfection assays of ERα mutants or mutation/deletion assays of target gene promoter (please refer

to [2] for examples of such experiments). When nonclassical ERα action is suspected, nuclear extract should be used as a protein source as it contains other transcription factors (e.g., AP-1 proteins) that ERα binds to.

7. The 5× binding buffer is typically aliquoted in 100 μL, stored at −20 °C, and used within a month. Poly(dI-dC)·poly(dI-dC) reduces nonspecific binding of proteins to DNA probe. The amount of poly(dI-dC)·poly(dI-dC) in a binding reaction needs to be optimized. Typical amounts of poly(dI-dC)·poly(dI-dC) range from 25 to 2000 ng per each reaction [6].
8. For supershift assays, it is recommended to use concentrated antibody, so that small volumes (1–1.5 μL) can be used for each reaction. When possible, choose antibodies known to function in EMSA. Antibodies that are effective for western blotting (e.g., detecting denatured proteins) may not work for EMSA. We have successfully used the following antibodies for supershift assay: ERα (sc-543x), c-Jun (sc-44x), and c-Fos (sc-52x) (Santa Cruz Biotechnology).
9. Most vertical gel electrophoresis systems are suitable, e.g., dual slab vertical electrophoresis system (Model JVD-80, Shelton Scientific). We use 1.5-mm spacers, 18-well combs, and 18 cm × 16 cm gels for EMSA.
10. One dish of confluent HepG2-ER cells provides >1 mg of concentrated nuclear proteins. HepG2-ER cells, cultured in estrogen-free (e.g., phenol-red free) media, were treated with 17β-estradiol (0.1–1 μM) for 45 min before harvesting the cells.
11. The packed volume of cells is approximately 100 μL for 10^7 cells.
12. Trypan blue will provide a better contrasting background. Cell lysis is manifested by the presence of numerous cellular debris and small spherical structures (i.e., nuclei) that lack cytoplasm and cell membrane.
13. When needed for binding reactions, thaw the frozen nuclear extracts on ice or at 4 °C.
14. Alternatively, a dry heat block can be used. Place the tubes in a heat block at 90–95 °C for 3–5 min. Turn off the heat block and let it cool down to room temperature. It typically takes about 1 h. If custom-made dsDNA is used, the annealing step is not necessary.
15. The percentage of polyacrylamide gels used in EMSA varies depending on the size of the DNA probe and the required resolution of the DNA:protein complexes. It typically ranges from 4 % to 7 %. Higher percentage gels exhibit better resolution. For example, two closely migrating DNA:protein complexes that appear as a single diffuse complex on a 4 % gel can

be resolved on a 7 % gel. However, higher percentage gels require longer electrophoresis times. We typically start testing with a low percentage gel and increase the percentage if a higher electrophoretic resolution is needed.

16. The amounts of nuclear extracts need to be titrated, typically ranging from 1 to 30 μg/reaction. As components in the nuclear extraction buffer may affect the binding between DNA and proteins, the volume of nuclear extracts should be kept at minimum (i.e., no more than 60 % of total reaction volume). If the protein concentration of nuclear extracts is low, increase the total reaction volume up to 19 μL.
17. This amount of unlabeled DNA represents 50-fold molar excess of the labeled DNA probe. For competition assays, 25- to 100-fold molar excess amounts of unlabeled DNA are typically used.
18. Binding of antibody to the protein may lead to the appearance of supershifted band (Fig. 3, lane 9) or decreased signal intensity of shifted band (Fig. 3, lane 3–5). The latter could be due to the large size of binding complexes (e.g., ERα/AP-1 complex) [7] that remain trapped at the bottom of the loading well.
19. The temperature for binding reaction needs to be optimized for the reproducibility of experimental results.
20. It is not recommended to add gel-loading buffer to binding reactions because the dyes may interfere with the binding of certain proteins to DNA. Free probes run at the same mobility as bromophenol blue in gel-loading buffer.
21. As an alternative, the dried gel can be exposed to film and then bands visualized on the developed film.

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Chapter 6

Chromatin Immunoprecipitation Assay to Identify Genomic Binding Sites of Regulatory Factors

Meike Wagner, Johannes Jung, Michael Koslowski, Özlem Türeci, Vijay K. Tiwari, and Ugur Sahin

Abstract

DNA–protein interactions are vital to fundamental cellular events including transcription, replication, DNA repair, and recombination. Thus, their study holds the key to our understanding of mechanisms underlying normal development and homeostasis as well as disease. Transcriptional regulation is a highly complex process that involves recruitment of numerous factors resulting in formation of multi-protein complexes at gene promoters to regulate gene expression. The studied proteins can be, for example, transcription factors, epigenetic regulators, co-activators, co-repressors, or ligand-activated nuclear receptors as estrogen receptor-α (ERα) bound either directly to the DNA or indirectly by interaction with other DNA-bound factors. Chromatin immunoprecipitation (ChIP) assay is a powerful method to study interactions of proteins and a specific genomic DNA region. Recruitment of ERα to promoters of estrogen-dependent genes is a common mechanism to activate or enhance gene transcription in breast cancer thus promoting tumor progression. In this chapter, we demonstrate a stepwise protocol for ChIP assay using binding of ERα to its genomic targets after stimulation with 17β-estradiol (E_2) in breast cancer cells as an example.

Key words Chromatin, ChIP assay, DNA–protein interactions, Gene regulation, Estrogensignaling, Cancer, Estrogenreceptorα

1 Introduction

DNA–protein interactions are critical to various fundamental cellular events including transcription, replication, DNA repair, and recombination. Therefore, identifying genomic regions bound by regulatory factors provides capacity to directly link the function of these proteins to particular processes at the associated loci. The DNA-binding potential of proteins is generally reflected in discrete conserved domains within their tertiary structure. Several common DNA-binding domains include zinc fingers, basic helix-loop-helix motifs, and leucine zippers. Furthermore, a number of proteins contain multiple DNA-binding domains or employ adapter proteins to facilitate a high efficiency binding to DNA. Chromatin

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immunoprecipitation (ChIP) assay is a very powerful method to measure these interactions. Following enrichment of protein-bound DNA sites, it is possible to either perform quantitative PCRs for specific loci of interest (ChIP-qPCRs) or next-generation sequencing to identify genome-wide binding sites (ChIP-Seq) [1, 2]. Thus, ChIP assay continues to revolutionize our understanding of mechanisms that are critical to normal development and homeostasis and its misregulation in disease. Gene regulation is a highly complex process that involves recruitment of various factors resulting in formation of multi-protein complexes at gene promoters to regulate transcription. These proteins include transcription factors, epigenetic regulators, co-activators, co-repressors, or ligand-activated nuclear receptors such as estrogen receptor-α (ERα). The proteins may be bound either directly to the DNA or indirectly by interacting with other DNA-bound factors. Signaling via estrogens plays a pivotal role in reproductive physiology and cancer. In hormone-responsive breast cancer, which accounts for ~70 % of all diagnosed breast cancers, estrogens stimulate growth of breast cancer cells by activating transcription of estrogen-regulated genes [3, 4]. Thus, it is crucial to identify estrogen-regulated genes and signaling pathways in order to understand tumor progression in mammary tissues and to design effective strategies for the treatment of hormone-responsive breast cancer. The genomic effects of estrogens are mediated by binding to ERα or ERβ [4, 5]. Upon ligand activation, ERα and ERβ undergo conformational changes that promote dimerization and binding to specific inverted palindromic DNA sequences, the estrogen response elements (ERE), in the promoter region of target genes. ERα and ERβ can homo- or heterodimerize and bind to the same DNA sequences with different consequences. In the following, we will concentrate on ERα. Many of the EREs found in the genome are imperfect EREs or non-palindromic ERE half sites that exist in combination with GC-rich motifs or activating protein 1 (AP-1) sites and are bound by ERα-specificity protein 1 (SP1) or ERα-AP-1 complexes [6–8]. The DNA-bound ERα interacts with other transcription factors, co-activators or co-repressors, and chromatin remodeling complexes thus stabilizing the formation of a transcription preinitiation complex at the ERE. In addition, ERα has been found to activate transcription of genes that do not feature ERE sites in their promoter. Such non-classical signaling has been reported for ~35 % of human estrogen-responsive genes. In this case, ERα is indirectly associated with the promoters by interaction with other DNA-binding transcription factors like SP1, AP-1 or NF-κB, which was shown for several genes including *c-fos*, *cyclin D1* or *human prolactin receptor* [7, 9]. Recently, we identified *placenta-specific 1* (*PLAC1*) to be regulated in a non-classical pathway by tethering ERα to DNA-bound SP1 and CCAAT/enhancer binding protein β-2 (C/EBPβ-2) resulting in recruitment of the nuclear receptor co-activator 3 (NCOA3) and further

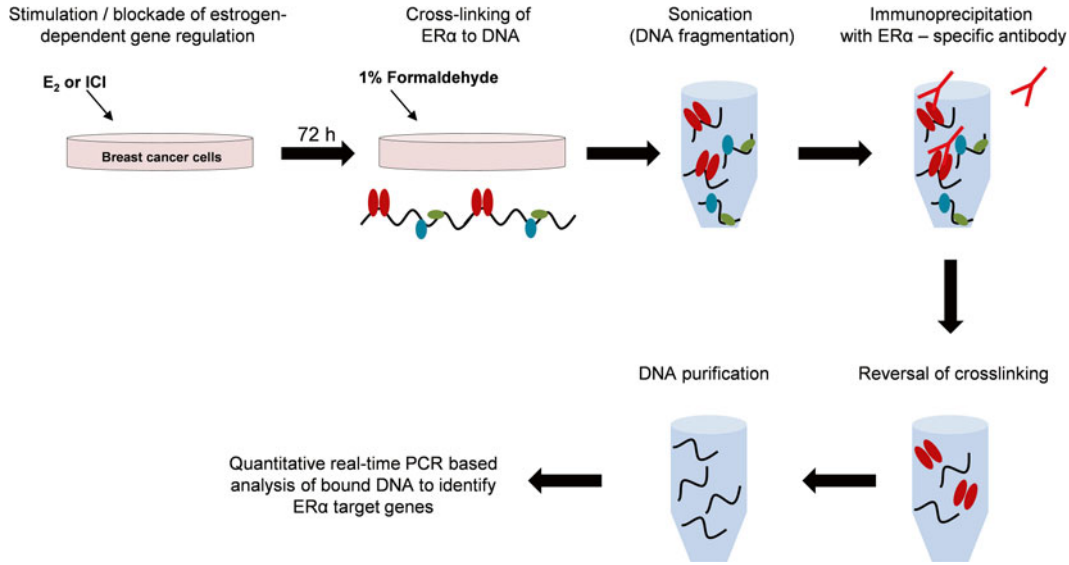

Fig. 1 Schematic representation of ChIP assay with ERα in breast cancer cells

binding of transcription factor II B (TFIIB) and RNA-Polymerase II in MCF-7 breast cancer cells [10, 11]. Thus, estrogen signaling offers a very good example to study how external signaling modulates gene expression via directly affecting the binding of gene regulatory proteins onto DNA.

ChIP assay is a convenient method to identify the enrichment of transcription factors or histone modifications at specific genomic regions or at the genome-wide level [12, 13]. Here, we demonstrate a stepwise protocol for ChIP assay using binding of ERα to its genomic targets in estrogen-responsive breast cancer cells as an example. A schematic representation of ChIP assay with ERα is shown in Fig. 1. The direct as well as indirect binding of ligand-activated ERα to the promoter of a target gene can be efficiently studied in vivo using conventional cross-linking ChIP. The ChIP assay procedure involves the reversible covalent cross-linking of proteins to DNA in living cells by formaldehyde. The chromatin is sheared by sonication to generate DNA fragments of ~300 bp in average size. To ensure efficient ChIP, the size of the DNA fragments should be analyzed by agarose gel electrophoresis prior immunoprecipitation. If sheared DNA shows the appropriate fragment size, the protein–DNA complex of choice is immunoprecipitated by a specific antibody. To recover the DNA, cross-linking of DNA and proteins has to be reversed and bound DNA fragments are purified. DNA purified from ChIP with ERα is analyzed by quantitative real-time PCR to determine the relative occupancy of ERα at the target promoter. To quantify the increase of ERα enriched at the promoter of the target gene in response to E_2, we normalize to unstimulated control cells. It is highly recommended

to include cells treated with the complete ER blocker ICI 182,780 (ICI) to detect specific recruitment of ERα to the analyzed target gene. In case the genomic targets are unknown, immunoprecipitated DNA can be analyzed by next-generation sequencing (ChIP-Seq) [14–17] which has been done for ERα and ERβ [18–20]. For ChIP-Seq, it is recommended to use smaller DNA fragments of ~200 bp to achieve a high coverage of sequencing. In our hands, ChIP with ERα worked efficiently when cross-linked with formaldehyde. However, when the immunoprecipitated proteins are present in large multi-protein complexes, it may be more efficient to use a double cross-linking with formaldehyde and a second cross-linker, for example ethylene glycolbis[succinimidyl succinate] (EGS) [21] that has a longer spacer arm than formaldehyde and can better crosslink proteins that are farther from the DNA. The analysis of histones and some transcription factors that are strongly bound to DNA and do not need cross-linking can be performed by native ChIP. Using native ChIP, cross-linking is not performed and native chromatin is fragmented by nuclease digestion using, for example, micrococcal nuclease instead of sonication [22, 23]. However, most of the non-histone proteins are not retained on the DNA using this procedure. For ChIP with ERα, cross-linking ChIP is the preferred method.

For ChIP assay with ERα, any ER-positive cancer cell line can be used. The most prominent ER-positive breast cancer cell line is MCF-7 [24, 25]. Moreover, it is useful to include an ER-negative tumor cell line, for example SK-BR-3 breast cancer cells, to exclude ERα-non-specific effects of E_2-stimulation. In this chapter, we describe in detail the ChIP assay with ERα in MCF-7 cells and the use of SK-BR-3 cells as ER-negative control cell line.

2 Materials

Prepare all solutions using ultrapure water (resistivity 18.2 MΩ) and analytical grade reagents. All solutions can be prepared and stored at room temperature (RT) unless otherwise indicated.

1. For cell culture: 10 cm cell culture dishes, MCF-7 and SK-BR-3 breast cancer cells (ATCC), phenol red-free Dulbecco's modified eagle medium (DMEM), charcoal stripped fetal bovine serum (FBS), 17β-estradiol (E_2), ICI 182,780 (ICI).
2. TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
3. Protein A or G agarose beads provided as 50 % gel slurry (Merck Millipore), store at 4 °C.
4. tRNA, 10 mg/mL stock solution, store at −20 °C.
5. Bovine serum albumin (BSA), 10 mg/mL stock solution, store at −20 °C.

6. Protease inhibitor cocktail (PIC) 50×, Complete EDTA-free tablets (Roche), dissolve one tablet in 1 mL ultrapure water and store at −20 °C.
7. 2.5 M glycine, pH 2.5.
8. Formaldehyde solution, minimum 36.5 %.
9. 1× Dulbecco's PBS without calcium and magnesium.
10. Fixation buffer: 50 mM HEPES/NaOH, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, add freshly before use: 1 % formaldehyde (final concentration). Store at 4 °C.
11. Lysis buffer: 50 mM HEPES/KOH, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), add freshly before use: 1× PIC (final concentration). Store at 4 °C.
12. Hoechst 33342 working solution: 200 ng/mL in PBS.
13. Proteinase K, 10 mg/mL stock solution, store at −20 °C.
14. For DNA precipitation: absolute ethanol, 3 M NaOAc, pH 5.2, glycogen (20 mg/mL).
15. TBE 5×: 54 g Tris base, 27.5 g boric acid, 20 mL of 0.5 M EDTA, pH 8.0, adjust to a final volume of 1 L.
16. 1.2 % agarose gel: 1.2 g agarose/100 mL 1× TBE containing 4 μL SYBR safe DNA gel staining.
17. Anti-ERα antibodies: monoclonal mouse anti-ERα; clone 33 (Abcam) or rabbit polyclonal anti-ERα (H-20) (Santa Cruz) have been shown to work in ChIP assay.
18. Elution buffer: 1 % SDS, 0.1 M $NaHCO_3$.
19. LiCl wash buffer: 0.25 M LiCl, 0.5 % NP-40, 0.5 % sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8. Store at 4 °C.
20. Wash buffer 1: 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5 mM EGTA, 0.25 % Triton X-100. Store at 4 °C.
21. Wash buffer 2: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl. Store at 4 °C.
22. Phenol solution (equilibrated with 10 mM Tris–HCl, pH 8.0, 1 mM EDTA).
23. Chloroform/isoamyl alcohol (IAA) 24:1.
24. Rotating wheel (such as Kisker Biotech).
25. QuantiTect SYBR green PCR kit (Qiagen).
26. Quantitative real-time PCR instrument (e.g., ABI 7300 Real-Time PCR Detection System, Applied Biosystems or equivalent).
27. Wide bore tips (300 μL).
28. 1.5 and 2 mL Eppendorf tubes, 15 mL conical tubes.

29. Semi-micro UV-cuvettes (1.5–3 mL).
30. Syringe (5 mL) and Sterican® needle (size 18, G 26×1″, Ø 0.45×25 mm) (B. Braun).
31. Bath sonicator (such as Bioruptor Plus, Diagenode).
32. Fluorometer (e.g., Hoefer™ DQ 300) and spectrophotometer (e.g., NanoDrop 2000).

3 Methods

3.1 Pre-blocking of Protein A or G Agarose Beads (See Note 1)

1. Pre-cool TE buffer to 4 °C. Denature 100 µL tRNA for 5 min at 95 °C and transfer immediately to 4 °C until use. Thaw BSA on ice.
2. Flip the tube with protein A/G agarose beads several times before use to obtain a homogenous bead suspension. Transfer 1 mL of protein A/G agarose beads using wide-bore tips in a 2 mL Eppendorf tube and add 1 mL TE buffer. Invert the tube 10 times and centrifuge for 2 min at 11,000×*g* at 4 °C, then leave 2 min on ice until beads have settled. Aspirate supernatant. Be careful to not remove beads! Repeat washing step once.
3. Resuspend the protein A or G agarose beads in 1 mL TE buffer, add 100 µL BSA (10 mg/mL stock) and 100 µL tRNA (10 mg/mL stock).
4. Incubate for 2 h or overnight at 4 °C on a rotating wheel.
5. Aspirate supernatant and wash 3 times with 1 mL TE. Centrifuge for 2 min at 11,000×*g* at 4 °C, then leave 2 min on ice until beads have settled.
6. Add 500 µL TE final and keep as 50 % slurry at 4 °C.

3.2 Preparation of Cells

1. Seed MCF-7 and SK-BR-3 cells in an appropriate density in 10 cm cell culture dishes (we use 3×10^6 MCF-7 cells and 4×10^6 SK-BR-3 cells per 10 cm dish) and grow the cells in phenol red-free DMEM supplemented with 10 % charcoal stripped FBS for 72 h at 37 °C, 5 % CO_2 to ~80 % confluency (*see* **Notes 2** and **3**).
2. 12 h prior starting the ChIP assay, add 100 nM E_2 and/or 5 µM of the complete estrogen receptor blocker ICI to the cells to stimulate or block ERα-dependent gene expression (*see* **Note 4**).

3.3 Cross-Linking

1. Collect medium from cell culture dish per condition and add 1/10th fixation buffer supplemented with 1 % formaldehyde (*see* **Note 5**). Redistribute medium carefully to the dish and incubate for 10 min at RT.
2. Stop the cross-linking reaction by addition of glycine (2.5 M) to a final concentration of 0.125 M in the medium and incubate for 10 min at 4 °C.

3.4 Preparation of Chromatin

1. Perform the following steps at 4 °C or on ice. All volumes are given per 10 cm plate. Pre-cool PBS to 4 °C.
2. Wash the cells twice with 4 mL cold PBS. Finally, add 3 mL PBS, scrape cells off and collect in conical tubes on ice.
3. Pellet cells by centrifugation at 600 × *g* for 7 min at 4 °C.
4. Resuspend the cell pellet in 10 mL wash buffer 1 to remove cytoplasm and incubate for 5 min on ice. Centrifuge again at 600 × *g* for 7 min at 4 °C.
5. Resuspend the cell pellet in 10 mL wash buffer 2 and incubate for 15 min on ice (*see* **Note 6**). Centrifuge again at 600 × *g* for 7 min at 4 °C.
6. Resuspend the cell pellet in 900 µL cold lysis buffer containing freshly added PIC per plate and incubate on ice for 3 h to ensure cell lysis (*see* **Note 7**).
7. Sonicate 300 µL aliquots of chromatin per 1.5 mL tube using a bath sonicator (e.g., Bioruptor Plus) with 30 s on/45 s of off pulses at high power for 15–25 cycles (*see* **Note 8**).
8. Centrifuge sheared chromatin at 14,000 × *g* for 10 min at 4 °C and transfer supernatant into a new tube.
9. To quantify the chromatin concentration, mix 10 µL of sheared chromatin with 490 µL TE and 500 µL Hoechst 33342 working solution (200 ng/mL) and measure the UV absorbance using a fluorometer (*see* **Note 9**). Chromatin can be stored at 4 °C overnight or for long term at −80 °C.

3.5 Analysis of DNA Fragment Size

1. In order to analyze the DNA fragment size, cross-linking of sheared DNA to bound proteins has to be reversed. Therefore, first add 2 µL RNase A to 100 µL of 200 ng/µL chromatin and incubate for 30 min at 37 °C.
2. Add 10 µL 10 % SDS, 2 µL 5 M NaCl and 2 µL Proteinase K and incubate at 55 °C for 2 h and 65 °C overnight with shaking.
3. (Optional: one can perform phenol/chloroform extraction for attaining higher purity at this point).
4. To precipitate DNA, add 200 µL of absolute ethanol, 10 µL of 3 M NaOAc, pH 5.2, and 1 µL of glycogen and centrifuge for 2 h at 14,000 × *g* and 4 °C.
5. Discard supernatant and wash with 350 µL of 70 % ethanol and centrifuge for 15 min, 14,000 × *g* at 4 °C. Repeat this step and make sure to remove all of the residual ethanol and air dry the pellet for 30 min.
6. Resuspend the pellet in 40 µL TE and measure DNA concentration using a spectrophotometer. To visualize shearing efficiency and fragment size, load 1 µg and/or 2 µg DNA on a 1.2 % agarose gel (Fig. 2). The prominent DNA band should be located

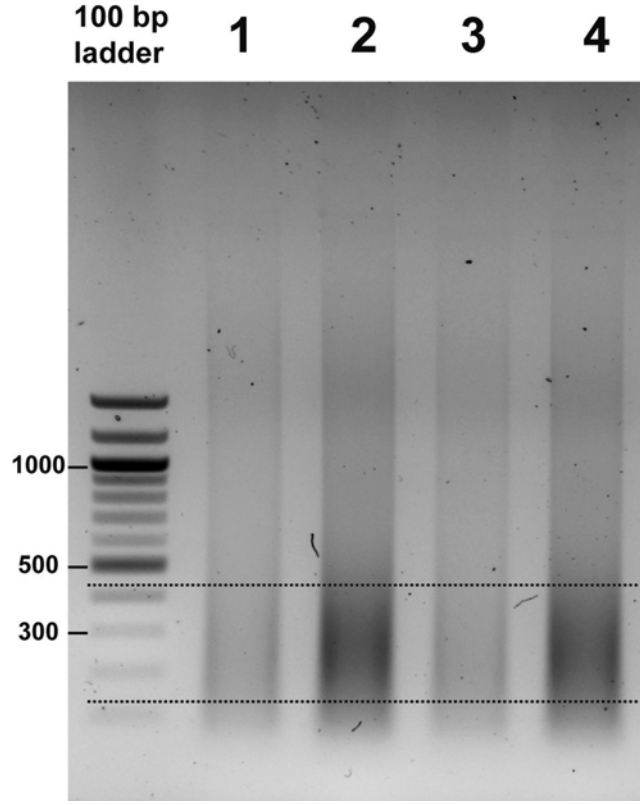

Fig. 2 Agarose gel (1.2 %) showing sonicated genomic DNA with fragments of ~300 bp average size. *Left*: 100 bp DNA ladder. *Lane 1–4*: Sonicated genomic DNA from SK-BR-3 cells is shown, 1 μg DNA was loaded onto *lane 1* and *3* and 2 μg DNA was loaded onto *lane 2* and *4*

at approximately 300 bp, confirming a good sonication (*see* **Note 10**). If bulk DNA fragments are not in this range, repeat **steps 7–9** in Subheading 3.4 and steps in Subheading 3.5.

3.6 Chromatin Immunoprecipitation

1. Use 70 μg of chromatin (from **step 9**, Subheading 3.4) in a total volume of 600 μL filled up with lysis buffer supplemented with fresh PIC per ChIP sample. Thus, chromatin concentration has to be at least 120 ng/μL.
2. In order to reduce non-specific binding, perform pre-clearing of the chromatin with pre-blocked protein A/G agarose beads. Add 40 μL pre-blocked protein A/G agarose beads per sample and incubate for 1 h at 4 °C on a rotating wheel.
3. Pellet the beads by centrifugation at 11,000 × *g* for 2 min at 4 °C. Place tubes for 2 min on ice and transfer supernatant into a new tube. Save 1 % of pre-cleared chromatin from each sample as "input" and store at −20 °C until use (*see* **Note 11**).

4. Add 5 μg of the antibody to the pre-cleared chromatin and incubate overnight (16 h) at 4 °C on a rotating wheel.
5. On the next day, add 40 μL of pre-blocked protein A/G agarose beads to bind the immunoprecipitated antibody–protein–DNA complexes and incubate for 3 h at 4 °C on a rotating wheel.
6. Centrifuge at 11,000 × *g* for 2 min at 4 °C, place on ice for 2 min and discard supernatant containing the unbound, non-specific DNA.
7. Resuspend the beads in 1 mL cold lysis buffer and incubate for 5 min on a rotating wheel at RT. Centrifuge at 11,000 × *g* for 2 min at 4 °C, place on ice for 2 min and discard supernatant. Repeat this washing step.
8. Wash the beads with 1 mL of cold LiCl buffer and incubate for 5 min on a rotating wheel at RT. Centrifuge at 11,000 × *g* for 2 min at 4 °C, place on ice for 2 min and discard supernatant.
9. Transfer the beads into a new tube in 1 mL cold TE buffer. Centrifuge at 11,000 × *g* for 2 min at 4 °C, place on ice for 2 min and remove all supernatant with a gel loading tip.
10. Add 160 μL of fresh elution buffer to the beads and incubate for 20 min at RT on a rotating wheel.
11. Centrifuge at 11,000 × *g* for 2 min at RT and transfer supernatant to a new 1.5 mL tube.
12. Repeat the elution step by adding 150 μL of fresh elution buffer to the beads and incubate again for 20 min at RT on a rotating wheel. Centrifuge at 11,000 × *g* for 2 min at RT and transfer supernatant to the eluate from **step 11**. The final volume of the eluate should be 300 μL.

3.7 Reversal of Cross-Linking and DNA Recovery

1. Thaw input from **step 3** in Subheading 3.6 and raise volume to 100 μL with TE buffer.
2. Add 0.2 mg/mL RNase A (final concentration) to input and ChIP samples. Incubate for 30 min at 37 °C.
3. For ChIP fraction, add 6 μL of 0.5 M EDTA, 12 μL of 1 M Tris–HCl, pH 6.5 and 1.5 μL of 10 mg/mL Proteinase K and incubate at 55 °C for at least 2.5 h, then overnight at 65 °C.
4. For input samples, add 5 μL of 20 % SDS, 2 μL of 5 M NaCl and 2 μL of 10 mg/mL Proteinase K and incubate at 55 °C for at least 2.5 h, then overnight at 65 °C.
5. On the next day, add 200 μL TE to input samples to raise the volume to 300 μL.
6. Recover DNA by phenol/chloroform extraction (*see* **Note 12**). Add 300 μL of phenol to each sample and vortex vigorously. Centrifuge at 16,000 × *g* for 10 min at RT. Transfer the upper aqueous phase into a new tube.

7. Add 300 µL of 24:1 chloroform/IAA to each sample and vortex rigorously. Centrifuge at 16,000 × *g* for 10 min at RT. Transfer the upper aqueous phase into a new tube.
8. Precipitate DNA by adding 600 µL of cold 100 % ethanol, 30 µL of 3 M NaOAc, pH 5.2, and 1 µL of glycogen and centrifuge at 14,000 × *g* for 2 h at 4 °C. Discard supernatant.
9. Wash pellet by adding 350 µL of 70 % ethanol and centrifuge at 14,000 × *g* for 15 min at 4 °C. Discard supernatant and centrifuge again for 5 min at 4 °C.
10. Remove the residual ethanol with a pipette and air-dry the pellet for 30 min.
11. Dissolve DNA in 40 µL TE and store at −20 °C until use.
12. Analyze presence of ERα at the desired gene promoter using gene-specific primers by PCR. We recommend performing quantitative real-time PCR using SYBR green to detect the occupancy of ERα at the gene promoters of interest (*see* **Note 13**). Dilute the DNA from ChIP and input samples 1:5 for use in PCR. The signal from the ChIP samples is normalized to the signal from the respective input samples. To calculate the relative occupancy of ERα at the desired promoter upon E_2 treatment, normalize the signals from E_2-, E_2/ICI- and ICI-treated cells to untreated control cells.

4 Notes

1. Pre-blocking of protein A or G agarose beads is necessary to reduce background of non-specific binding to the beads during ChIP assay. Beads can be stored at 4 °C for up to 3 weeks but it is recommended to prepare beads freshly before starting a new ChIP assay. Alternatively, pre-blocked beads can be purchased. Depending on the species and IgG subclass of the used antibodies, protein A or G agarose beads are appropriate [26].
2. It is crucial to use phenol red-free medium and charcoal-stripped FBS when analyzing estradiol-dependent gene regulation as phenol red is known to be a weak estrogen and conventional (non-charcoal-stripped) FBS contains estrogens that could lead to false-positive results. At least, cells should be cultivated 72 h prior starting of treatment with E_2 in phenol red-free medium supplemented with charcoal-stripped FBS to avoid any side effects caused by estrogens in the conventional media.
3. Cell confluency should be about 80 % at the time of cross-linking. For optimal results, $5–10 \times 10^6$ cells should be used per ChIP sample. It is essential to determine the optimal cell

density seeded at the beginning of the experiment for every cell line individually to obtain reproducible sonication results.

4. Appropriate controls as the complete estrogen receptor blocker ICI should be carried along to detect specific recruitment of ERα to gene promoters by E_2. Additionally, a control using both E_2 and ICI can be used to ensure specific activation of ERα by E_2 that is blocked by ICI.
5. Formaldehyde should be added freshly directly prior use to fixation buffer: for 10 mL, add 2.9 mL formaldehyde (37 %) to 7.1 mL of fixation buffer at room temperature. It is crucial to use molecular biology grade formaldehyde with a high purity.
6. To avoid clotting of the cell pellet, first add 2 mL wash buffer 2 and dissociate pellet by a syringe and needle. For resuspension of the pellet, add 8 mL of wash buffer 2.
7. Incubation time can be extended to up to 48 h.
8. The optimal cycle number for sonication of the chromatin has to be determined for every cell line. For this, grow cells to 80 % confluency in 10 cm cell culture dishes and prepare chromatin, test different cycle numbers (e.g., 15/20/25 cycles) and analyze DNA fragment size on a 1.2 % agarose gel. The cycle number that gives rise to fragments of ~300 bp in size should be used.
9. The quantification of chromatin is based on fluorometric measurement of UV absorbance of the DNA contained in the sonicated chromatin (e.g., UV-channel of fluorometer). Multiply the value that the fluorometer reads with the value derived from a chromatin calibration standard according to the manufacturer's instruction to obtain the chromatin concentration (e.g., in ng/μL). As an alternative to chromatin quantification, a fixed cell number can be used per ChIP. We recommend using a defined amount of chromatin as this might compensate for cell loss during preparation steps of replicates and leads to a higher reproducibility between different experiments.
10. The analysis of the fragment size is recommended but can be skipped if sonication of chromatin is established for a certain cell type. The best results are obtained, when ChIP experiments are performed with samples of ~300 bp DNA length. To analyze the fragment size by agarose gel electrophoresis, use sheared DNA after reversal of cross-linking since proteins bound to DNA are known to affect the separation of the DNA in agarose gels (Fig. 2).
11. The input contains the total genomic DNA fraction that will be used for ChIP and serves as a control in subsequent quantitative PCR.

12. As an alternative to phenol/chloroform extraction, DNA can be recovered by column purification using commercially available kits (e.g., PCR purification kit from Qiagen). Notably, columns have a selection bias based on size of fragment resulting in retention of fragments of particular sizes. This may cause faults in subsequent quantitative real-time PCR. Moreover, phenol/chloroform extraction results in a higher amount of DNA that is recovered. Thus, phenol/chloroform extraction should be the preferred method, especially when very low amounts of DNA are expected.
13. Optimal PCR primers should be designed to amplify a DNA fragment of the desired promoter region of 75–150 bp in size, have a melting temperature (T_m) of ~60 °C and GC content of ~50 %. A negative control region 1–2 kb upstream from the target region in which no binding sites for ERα are predicted should also be analyzed. Moreover, intergenic control regions can be used as a background control. Normalization can be performed either against input only (fold enrichment of ERα vs. input) or against input and an intergenic control (fold enrichment of ERα vs. background). Furthermore, known positive control genes can be included to ensure that the antibody works in ChIP. For ChIP with ERα, a good positive control gene is pS2 (for primer sequences, *see* ref. 27).

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Chapter 7

Chromatin Immunoprecipitation with Estrogen Receptor 1 and the Promoter of *Greb1* in TM4 Sertoli Cells

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Abstract

Chromatin immunoprecipitation (ChIP) is a technique to investigate the interaction between proteins and DNA within the natural chromatin context of the cell. There are two previously identified two canonical estrogen response elements (ERE1 and ERE2) present in the 5′-flanking region of the *Greb1* gene which is a known estrogen-responsive gene. ChIP results showed the physical interaction between estrogen receptor I (ESR1) and EREs in the *Greb1* promoter in TM4 mouse Sertoli cells. This chapter describes the protocol for chromatin immunoprecipitation applied to the estrogen response elements in the *Greb1* promoter.

Key words Chromatin immunoprecipitation, Estrogen receptor, Estrogen response element, *Greb1*, Sertoli cell

1 Introduction

Nuclear hormone receptors, such as estrogen receptors, are hormone-responsive transcription factors that directly interact with DNA and regulate transcription. Previous methods such as radioactively labeled ligands, nitrocellulose filter binding assay, electrophoretic mobility shift assay, and DNA footprinting assay are difficult to adapt for genome-wide analysis. Chromatin immunoprecipitation (ChIP) is a type of immunoprecipitation technique to investigate the interaction between proteins and DNA to a specific region of the genome within the natural chromatin context of the cell. It aims to determine whether specific proteins are associated with specific genomic regions, such as transcription factors on promoters. The development of ChIP-based assays has opened up the field for gene-specific and genome-wide in vivo analyses in an unprecedented manner via ChIP-chip, Chip-seq technique [1]. Thus ChIP is a powerful and versatile tool in modern molecule endocrinology research. The estrogen receptors (ESR1 and ESR2, also known as ERα and ERβ) are members of the superfamily

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of nuclear receptors. After binding of estrogen to the estrogen-binding site on the receptors, the receptors dimerize and translocate to the nuclei of the target cells, and bind to specific regions on chromatin, the so-called estrogen response element (ERE). The interactions of ESRs and EREs to modulate the expression of specific genes have been extensively studied over the years [2]. *Greb1* (*g*ene *r*egulated by *e*strogen in *b*reast cancer protein 1), a known estrogen-responsive gene, was initially identified as an estrogen target gene in human breast tumor cells through a genome-wide screen [3, 4]. Two previously identified canonical EREs (ERE1 and ERE2) were present in the *Greb1* gene at −3289 and −7666 bp upstream of its transcription start site, respectively [3, 5]. TM4 Sertoli cells express *Greb1* and its expression is estrogen-inducible. Therefore, *Greb1* was chosen as an endogenous reporter gene for functional analysis of ESR1 in mediating estrogen action to regulate target gene expression in the mouse TM4 Sertoli cell line [6]. The physical interaction between ESR1 and EREs in the *Greb1* promoter suggests that ESR1 contributes to the upregulation of *Greb1* expression in TM4 cells through a genomic action. It provides further evidence for the presence of functional ESR1 in mouse Sertoli cells. This chapter describes the protocol for chromatin immunoprecipitation applied to the estrogen response elements in the *Greb1* promoter.

2 Materials

2.1 TM4 Cell Culture

1. TM4 Sertoli cells: American Type Culture Collection.
2. Complete cell culture medium: 1:1 mixture of Dulbecco's Modified Eagle medium (DMEM)/F-12 HAM supplemented with 10 % fetal bovine serum (FBS) and antibiotic-antimycotic (10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of Fungizone® Antimycotic).
3. 0.25 % Trypsin-EDTA.
4. Hanks balanced salt solution (HBSS).
5. Cell culture wares and equipment.
6. Hemocytometer and microscope for cell counting.
7. 17β-estradiol (E2, 10^{-10} mol/L).

2.2 Chromatin Immunoprecipitation

1. Imprint Ultra ChIP kit (Sigma). The kit contains Seq Sepharose, dialysis buffer, cell lysis buffer, nuclear lysis buffer, IP (immunoprecipitation) dilution buffer, IP wash buffer, IGEPAL (Octylphenoxypolyethoxyethanol), AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), PIC (protease inhibitor cocktail), DNA purification columns, 10 mg/mL RNase A,

DNA binding buffer, DNA wash buffer, DNA elution buffer, 5 M NaCl, 1 M sodium bicarbonate, 10 % sodium dodecyl sulfate, bovine serum albumin, 1.25 M glycine, salmon testes DNA, rabbit and mouse immunoglobulin G.

2. 10 % IGEPAL: prepare in 1 mL purified water (resistivity 18.2 MΩ) and store at room temperature.
3. 0.1 M AEBSF: reconstitute a 25 mg/bottle with 1.043 mL purified water. Keep on ice during use. Store at –20 °C.
4. PIC (protease inhibitor cocktail). Thaw and bring to room temperature for use as it will precipitate on ice. Store at –20 °C.
5. Cell lysis buffer: Add 100 μL of 10 % IGEPAL per mL of cell lysis buffer for a final concentration of 1 % IGEPAL. Just before use, add 10 μL of 0.1 M AEBSF and PIC/mL of cell lysis buffer. Mix well and keep on ice.
6. Nuclei lysis buffer: Just before use, add 1/100 final volume of 0.1 M AEBSF and PIC to nuclear lysis buffer. Mix well and keep at room temperature, as it will precipitate on ice.
7. 37 % formaldehyde.
8. Glass Dounce homogenizer.
9. Sonicator (such as the dismembrator model 150).
10. ESR1 antibody (H-184, Santa Cruz Biotechnology).
11. Heat block or waterbath capable of 67 °C, 100 °C and 37 °C.
12. Rotating platform that can hold 0.5 and 1.5 mL tubes.
13. 1.5, 15, and 50 mL screw-cap conical tubes.
14. 200 proof ethanol.
15. Phosphate buffered saline (PBS), pH 7.2.
16. DNA wash buffer: add 24 mL ethanol to the concentrated DNA wash buffer before use.
17. Preparation of blocked Staph-Seq solution (*see* **Note 1**).
    (a) Pre-cool microcentrifuge to 4 °C.
    (b) Thaw 0.1 M AEBSF and keep it on ice.
    (c) Reconstitute 20 mg bovine serum albumin (BSA) with 1 mL of purified water and keep on ice. Store at –20 °C after use.
    (d) Thaw sonicated salmon sperm DNA from –20 °C and keep on ice.
    (e) Rehydrate one vial of lyophilized Staph-Seq with 200 μL of purified water for 15 min at room temperature, vortex briefly and resuspend with a pipette until no clumps are visible.

(f) Transfer 100 μL of the suspension to a 0.5 mL tube then add 5 μL of BSA, 10 μL of salmon sperm DNA, and 1 μL of 0.1 M AEBSF, and mix by a pipette.

(g) Incubate on a rotating platform at room temperature for 2 h or at 4 °C for at least 3 h to overnight.

(h) Transfer to a 1.5 mL tube and pellet Staph-Seq at 18,407 × *g* at 4 °C for 3 min and remove supernatant.

(i) Wash the pellet twice by resuspending in 1 mL dialysis buffer and re-pelleting (18,407 × *g* at 4 °C for 3 min).

(j) After the last wash, resuspend the pellet in 100 μL dialysis buffer and 1 μL of 0.1 M AEBSF and mix by pipetting. Washed and blocked Staph-Seq may be stored at 4 °C. It can be used for up to 2 weeks.

18. IP dilution buffer: Each ChIP sample will require 400 μL of IP dilution buffer. For each 400 μL of buffer, add 4 μL 0.1 M AEBSF and 4 μL PIC. Warm to room temperature and mix well before using.
19. Dialysis wash buffer: Each ChIP sample will require 1000 μL of dialysis wash buffer. For each 1000 μL of dialysis wash buffer add 10 μL 0.1 M AEBSF. Keep on ice.
20. IP wash buffer: Each ChIP sample will require 4000 μL of IP wash buffer. For each 4000 μL of IP wash buffer, add 40 μL 0.1 M AEBSF. Keep at room temperature as it will precipitate on ice.
21. IP elution buffer: 85 μL of purified water, 10 μL of 10 % SDS, 5 μL of 1 M $NaHCO_3$ per each 100 μL needed. Prepare fresh before use. Calculate 100 μL of IP elution buffer needed for each ChIP sample plus 100 μL for the "10 % Input" sample.

2.3 Polymerase Chain Reaction (PCR)

1. PCR reagents: 5× Green GoTaq Flexi buffer, Taq DNA Polymerase, 10 mM dNTPs (dATP, dCTP, dGTP, and dTTP), 25 mM $MgCl_2$, highly purified water (resistivity 18.2 MΩ).
2. PCR primers: 50 mM; custom synthesized (*see* **Note 2**):

   ERE1 Forward: 5′-TTGGAAGATCCACCGCAAACT-3′

   Reverse: 5′-AGACAGGCTCGGGCATGTATC-3′

   ERE2 Forward: 5′-TCACCCACAGTGCTGCGAGA-3′

   Reverse: 5′-GCCCTTGACCGAGGAGATGA-3′
3. Spectrophotometer.
4. 2 % agarose gel in Tris/Borate/EDTA (1.3 M Tris, 450 mM boric acid and 25 mM EDTA, TBE) buffer.
5. Gel box to run agarose gels and UV trans-illuminator.
6. Thermal cycler PCR machine.
7. DNA purification columns.

3 Methods

3.1 Cell Culture and Cross-Linking

1. Culture the TM4 cell line in complete medium in 500 mm^2 flasks. Maintain the cells in a humidified incubator at 37 °C with 5 % CO_2.
2. To stimulate the ligand-bound receptors to bind to the ERE, change the cell culture medium to phenol red- and serum-free DMEM, then incubate the cells with 17β-estradiol (E2, 10^{-10} mol/L) for 16 h.
3. Harvest the cells at 80 % confluence (*see* **Note 3**). Remove medium from the flasks and wash the cells with 70 mL HBSS.
4. Trypsinize the cells with 9 mL of trypsin-EDTA solution. When cells become round (approximately 5 min), add 31 mL of pre-warmed complete medium to immediately stop the trypsinization.
5. Disperse the cells by repeatedly pipetting up and down; transfer the cells to a 50 mL conical tube. Count the cells to determine the cell number using a hemocytometer under a microscope.
6. Move the cells to the fume hood and add 1.2 mL of formaldehyde to a final concentration of 1 %. Close the lid tightly and invert rocking for 10 min at room temperature (*see* **Note 4**).
7. In the fume hood, add 4.1 mL of 10× 1.25 M glycine to stop the cross-linking reaction, and continue to invert rocking for 5 min at room temperature. The color of the solution will become lighter.
8. Centrifuge the cells at 200 × *g* for 10 min at 4 °C. Discard the formaldehyde in a marked closed chemical waste container in the fume hood, and carefully aspirate supernatant so as to not lose the cell pellet.
9. Wash the cells by resuspending the pellet in 50 mL of ice-cold 1× PBS.
10. Centrifuge the cells at 200 × *g* for 10 min at 4 °C and aspirate the supernatant.
11. Snap -freeze the cross-linked cells in liquid nitrogen and store at –80 °C.

3.2 Preparation of Chromatin from Cross-Linked Cells

1. Pre-cool the microfuge to 4 °C, cool the glass Dounce homogenizer on ice, and chill the appropriate size polystyrene tube for sonication (1.5 mL tube for 100–300 μL cell suspension, 15 mL tube for 500–1000 μL cell suspension).
2. Thaw the frozen cross-linked and washed cell pellet from –80 °C rapidly by rubbing between hands and place on ice.
3. Resuspend cells with freshly prepared cell lysis buffer, use 1 mL per 50 million cells, incubate on ice for 20 min and flick the tube occasionally to resuspend the cells.

4. Load up to 1 mL of the cell suspension to a 2 mL glass Dounce homogenizer on ice and stroke with the B pestle for approximate 15 times to release nuclei and disperse cell clumps.
5. Transfer sample to 1.5 mL tubes.
6. Centrifuge the sample at 2500 × *g* for 5 min at 4 °C to pellet the nuclei. Aspirate the supernatant carefully.
7. Resuspend the nuclei in 0.5 mL of freshly prepared nuclei lysis buffer. Use 1 mL buffer per 100×10^6 cells.
8. Transfer the nuclei immediately after complete resuspension into a 15 mL pre-chilled polystyrene tube in preparation for sonication. Incubate on ice for 10 min.
9. Chromatin sonication: Fragment the cross-linked chromatin by sonication using a sonic dismembrator for 30 min with 30-s sonication burst and 30-s rest on ice during entire sonication procedure (*see* **Note 5**).

3.3 Cross-Link Reversal and DNA Purification

1. Add 40 μL purified water to each 10 μL sonicated chromatin sample.
2. Reverse the cross-linking by adding 4.8 μL of 5 M NaCl and boil for 15 min.
3. Cool the DNA sample to room temperature, then add 1 μL of 10 mg/mL RNase A and incubate at 37 °C for 10 min.
4. Add 250 μL of DNA binding buffer.
5. Assemble a DNA purification column in a collection tube and transfer the DNA and binding solution to the column.
6. Centrifuge at 12,000 × *g* for 1 min at room temperature.
7. Discard the solution from the collection tube and wash the DNA by adding 200 μL of wash buffer to the column.
8. Centrifuge at 12,000 × *g* for 1 min at room temperature.
9. Transfer the column to a clean collection tube and add 10 μL of DNA elution buffer to the column.
10. Elute the DNA by centrifugation at 12,000 × *g* for 1 min at room temperature.
11. Calculate the DNA concentration by spectrophotometry.
12. Load 5 μL of sonicated and purified DNA and a DNA size marker on a 2 % agarose gel and electrophorese to determine the size range of the DNA smear. The sonication condition that gives a smear of DNA sizes from 200 bp to 1 kb with a peak around 500 bp will be used for ChIP-PCR reactions.
13. Transfer sonicated and purified chromatin to 1.5 mL centrifuge tubes.
14. Centrifuge the samples at 18,407 × *g* for 10 min at 4 °C.
15. Transfer supernatant to several new 1.5 mL tubes and store at −80 °C.

3.4 Pre-clearing of Cross-Linked Chromatin

1. Use blocked Staph-Seq to pre-clear the chromatin. Prepare the required amount of blocked Staph-Seq solution the evening before starting the ChIP (Subheading 2.2, **item 17**).
2. Place 3 × 100 μL fresh chromatin on ice. For chromatin that has been frozen, thaw rapidly by floating it briefly in room temperature water. Transfer 10 μL of the chromatin to a separate tube and store on ice for purifying "input" DNA control in cross-link reversal later.
3. Vortex blocked Staph-Seq briefly and resuspend uniformly with a pipette. Add 10 μL of blocked Staph-Seq to each 100 μL chromatin. For example, incubate 300 μL chromatin with 30 μL blocked Staph-Seq.
4. Incubate on a rotating platform at 4 °C for not longer than 15 min.
5. Centrifuge at 18,407 × *g* for 5 min at 4 °C in a microfuge.
6. Transfer the supernatant to a new tube.

3.5 Antibody Binding (See Note 6)

1. Prepare IP dilution buffer (Subheading 2.2, **item 18**).
2. Divide pre-cleared, cross-linked chromatin equally into 3 × 2 mL screw-cap tubes, and dilute chromatin 1:5 with IP dilution buffer before adding antibody to reduce non-specific binding. For each 100 μL chromatin add 400 μL IP dilution buffer and 1 μg (1–10 μL) ESR1 antibody or nonspecific rabbit IgG (as a control).
3. Incubate overnight on a rotating platform at 4 °C.

3.6 Immunoprecipitation

1. Preheat the water bath to 67 °C. Precool the microfuge to 4 °C.
2. Prepare dialysis wash buffer (Subheading 2.2, **item 19**) and IP wash buffer (Subheading 2.2, **item 20**).
3. Add 10 μL of blocked/washed Staph-Seq to each incubated chromatin-antibody sample to pull-down the antibody-bound chromatin complexes. Incubate on a rotating platform for no longer than 15 min at room temperature.
4. Transfer samples to new 1.5 mL tubes.
5. Centrifuge samples at 18,407 × *g* for 4 min at 4 °C in a microfuge. Discard the supernatants.
6. Wash the pellets twice with 1 mL of freshly prepared dialysis buffer. For each wash, resuspend the pellet thoroughly by pipetting at least 7 times with a pipette and then microfuge at 18,407 × *g* for 3 min at 4 °C (*see* **Note 7**).
7. Wash 4 times with 1 mL each of IP wash buffer.
8. After the last wash, gently aspirate the buffer and leave 10–20 μL of buffer above the pellet so as not to lose any Staph-Seq-bound sample. Recap the tubes, centrifuge the tubes again, and carefully aspirate any trace of buffer.

3.7 Elution

1. Prepare fresh IP elution buffer (Subheading 2.2, **item 21**).
2. Elute antibody/protein/DNA complexes by adding 50 μL of IP elution buffer to each sample. Vortex moderately for 15 min and centrifuge at 18,407 × *g* for 3 min at room temperature. Save the supernatants! Transfer supernatants to clean 1.5 mL tubes.
3. Repeat the elution step and pool the eluates in the same tube (100 μL total).

3.8 Cross-Link Reversal

1. Centrifuge the samples again at 18,407 × *g* for 5 min to remove any trace of Staph-Seq. Transfer the supernatants (approximately 100 μL) to fresh 1.5 mL tubes.
2. Add 12 μL of 5 M NaCl to each tube for a final concentration of 0.54 M NaCl.
3. To prepare the "input" sample, take the 10 % input that was saved in **step 2** Subheading 3.4, bring the volume to 100 μL with elution buffer. Add 12 μL of 5 M NaCl.
4. Incubate all samples in a 67 °C water bath overnight (*see* **Note 8**).

3.9 DNA Purification

1. Cool samples if proceeding directly from crosslink reversal. Add 1 μL of RNase A, and incubate at 37 °C for 15 min.
2. Purify DNA from each ChIP and input sample using DNA purification columns and reagents. Assemble one column in a collection tube per sample.
3. Add 500 μL of DNA binding buffer to each sample and vortex.
4. Transfer each sample to the DNA purification column/collection tube assembly and centrifuge at 12,000 × *g* at room temperature for 1 min. Discard the flow through liquid and return column to the collection tube.
5. Wash the column by adding 500 μL of DNA wash buffer (Subheading 2.2, **item 16**) and centrifuge at 12,000 × *g* at room temperature for 1 min. Discard the flow through liquid and return column to the collection tube.
6. Repeat the wash step one more time.
7. Centrifuge the columns for an additional 1 min at 12,000 × *g* to remove any trace of DNA wash buffer.
8. Transfer columns to fresh labeled 1.5 mL clear-view tubes.
9. Elute each sample by adding 30 μL DNA elution buffer to each column.
10. Centrifuge samples at 12,000 × *g* at room temperature for 1 min. The purified DNA can be used directly for PCR or stored at −20 °C for later use.

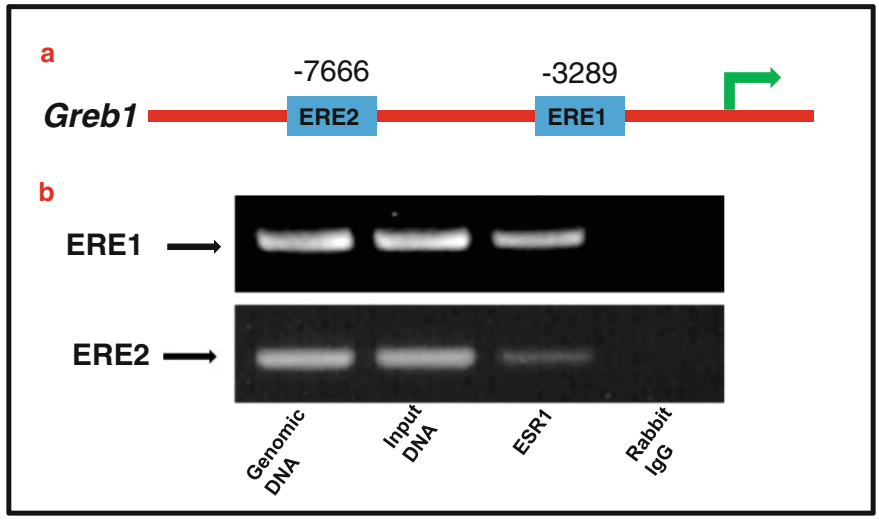

Fig. 1 (**a**) Schematic diagram illustrates the positions of two consensus estrogen-response elements (ERE) in the 5′ flanking regions of mouse *Greb1* gene in mouse chromosome 2. Two sets of PCR primers were designed to amplify the DNA sequences in the *Greb1* promoter of these two EREs, respectively. (**b**) ChIP of ESR1 on the two EREs in the *Greb1* promoter in mouse TM4 Sertoli cell line. ChIP was performed using a polyclonal antibody against ESR1. Mouse genomic DNA and input DNA serve as positive technical controls and rabbit IgG is a negative procedural control. The ChIP results show that ESR1 specifically binds to both ERE1 and ERE2 of the *Greb1* promoter in mouse Sertoli cell line TM4

3.10 PCR Analysis (See Note 9*)*

1. A total of 20 μL PCR mix is required for each sample. The mix is composed of 1 μL of sample or control DNA, 12 μL of purified water, 4 μL green GoTaq Flexi buffer, 1.5 μL $MgCl_2$, 0.5 μL dNTPs, 0.4 μL each of forward and reverse ERE1 or ERE2 primers, and 0.2 μL of Taq DNA polymerase.
2. PCR program: Step 1, 95 °C 3 min, step 2, (95 °C 30 s, 60 °C 30 s, 72 °C 1 min) × 40, step 3, 72 °C 7 min.
3. Electrophorese the amplified products in 2 % agarose gels. Stain the gels with ethidium bromide and photograph using a UV digital imager (Fig. 1).

4 Notes

1. Do not let pellets dry out during this procedure. Add fresh wash buffer to each tube immediately after the previous wash buffer has been removed. Please aspirate gently with a pipette when removing supernatant from Staph-Seq pellets and do not use vacuum for aspiration as you may lose the Staph-Seq. It is advised to use wide-mouth pipette tips for dispensing to prevent clogging of the tips. To reduce the background in ChIP, overnight blocking incubation at 4 °C is recommended. The resuspended Staph-Seq may be stored at 4 °C for up to a month.

2. The PCR primer design is an important part in ChIP experiment. The promoter region of the target gene which interacts with ESR1 must be determined first. The PCR primers described here were designed according to sequences obtained from GenBank using the Vector NTI 12.0 program (Life Technologies). A BLAST search of the genomic sequences in the RefSeqGene Nucleotide was performed to make sure the sequences amplified a unique genomic region. ChIP PCR primers have special requirements: (1) The focus is usually on the promoter region of a gene, which is mostly GC-rich, (2) The PCR product size should not be too big and amplified fragments should be between 150 and 250 bp, (3) Primers should have a size of 20- to 24-mer oligonucleotides, (4) Primers should have a melting temperature (Tm) of 58–60 °C, (5) GC content should be 40–70 %, (6) Primers should be tested with genomic DNA before using them for ChIP PCR.
3. Cell growth conditions are very important because they will affect the binding of transcription factors and promoters. The cells are generally in the best condition around 70–80 % confluence.
4. Too little or too much formaldehyde cross-linking will significantly affect the outcomes. The duration of formaldehyde treatment varies depending on cell types. Longer treatment will yield more protein/DNA cross-links but could hinder sonication. However, not enough treatment could produce a negative result. As an alternative method of cross-linking, the cells can be fixed directly in the flask and rocked gently at RT for 10 min. Use a cell scraper to collect the cells on ice.
5. Sonication must be optimized for different cell lines and different sonicators used. We use the sonic dismembrator model 150 (Fisher Scientific) which is a probe-type sonicator. Avoid foaming and keep the sample on ice at all times during sonication.
6. Adding tenfold excess PIC to IP dilution buffer will precipitate chromatin and result in reduced yields. High-quality, high-affinity, and high-titer antibody should be used. Polyclonal antibodies are preferred over monoclonal antibodies because epitope masking can occur during cross-linking. Immunoprecipitation-qualified antibodies are suitable and have the highest chance for success. Successful ChIPs with lower yields may be obtained even with a short 2-h incubation at 4 °C with a good ChIP-validated antibody, but overnight incubation is recommended for maximum yields.
7. Efficient washing is critical to reduce background. For each wash, resuspend the Staph-Seq pellet thoroughly by pipetting at least 5–7 times and aspirate the supernatant carefully. Do not allow pellets to dry out, and add fresh wash buffer to each tube immediately after the previous wash buffer has been removed.

8. Incubating the samples in a 67 °C waterbath overnight is recommended because incomplete reversal of cross-links could result in a negative result.
9. PCR analysis may produce weak target signal and high mock signal. It is not uncommon to capture non-specific DNA or long chromatin fragments. Therefore, PCR primer designing is very important. The number of PCR cycles needs to be titrated to achieve optimal results.

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Chapter 8

Chromatin Immunoprecipitation-Sequencing (ChIP-seq) for Mapping of Estrogen Receptor-Chromatin Interactions in Breast Cancer

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Abstract

Chromatin immunoprecipitation-sequencing (ChIP-Seq) is a powerful tool which combines the established method of ChIP with next-generation sequencing (NGS) to determine DNA-binding sites of a protein of interest on a genome-wide level, importantly, allowing for de novo discovery of binding events. Here we describe ChIP-seq using the well-established example of estrogen receptor-α mapping in the MCF7 breast cancer cell line.

Key words Chromatin, Estrogen receptor, Next-generation sequencing, Promoters, Enhancers, Chromatin immunoprecipitation

1 Introduction

DNA-interacting proteins are essential for gene regulation, chromatin stability and DNA replication. Determining where these proteins bind to the genome in normal and disease states can help us to better understand the factors that contribute to disease initiation and progression and ultimately can identify therapeutic targets and aid treatment decisions. In order to do this, cells or tissues are cross-linked with formaldehyde, the nuclei are isolated and the chromatin is sheared by sonication to fragments of approximately 500 bp in size. The chromatin is then incubated with an antibody raised against the protein of interest. After incubation, the antibody-chromatin complex is captured on Protein A or G beads and unbound chromatin is washed away. The antibody-chromatin complex is eluted; then the DNA is purified and amplified. The first step in amplification is the ligation of DNA adaptors to the ChIP enriched chromatin. The adaptors are then used for priming a PCR reaction; they also possess the correct sequence to physically associate with the flow cell of the sequencer.

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After amplification, DNA of 300–400 bp is selected and quantified so that it can be analysed by an Illumina sequencer. By using this technique to map ER-binding events in breast cancer cells lines and primary tumor samples, we are able to identify between 20,000 and 50,000 estrogen receptor-α (ER)-binding sites [1].

Since its discovery in 1984 [2], chromatin immunoprecipitation has been widely used to identify interactions between numerous proteins and DNA from many cell lines and tissues. The first experiments mapped RNA polymerase II binding at promoters in bacteria by coupling ChIP with microarrays that covered regions of promoter DNA sequence. This was a technical leap for the transcription factor field, but it was limited by the fact that some prior knowledge of where the protein might bind was required in order to design the probes for hybridization. ChIP was later coupled with PCR, with primers designed against regions that the proteins were thought to bind, again requiring some a priori knowledge as to where the proteins of interest might be binding [3, 4] permitting validation of putative binding regions. In 2006, the first ChIP-chip (ChIP coupled with microarray) experiments were published, resulting in the discovery of protein binding regions, initially on a chromosomal level and subsequently on a whole-genome level [5–8]. The power of this technique is that it provided insight into ER occupancy sites without any prior knowledge of where DNA contact points might occur, resulting in a redefinition of ER transcriptional activity. Specifically, the majority of ER-binding sites were found to occur at distal enhancer regions; only 5 % of binding sites were at promoters, where most of the previous work assessing ER transcriptional activity had been focused [3, 4]. The large number of binding sites for ER permitted the discovery of overrepresented DNA motifs, which in turn led to the discovery of pioneer factors and other binding partners for ER [5]. Recent increases in the quantity of data and reduction in cost associated with high-throughput sequencing technology has allowed it to be coupled with ChIP, providing a robust and cost-effective way of mapping transcription factor binding sites. In this chapter we describe ChIP-seq using the example of estrogen receptor-α mapping in the MCF7 breast cancer cell line.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistivity of 18 MΩ cm at 25 °C). Prepare and store all reagents at 4 °C (unless stated otherwise). Follow all waste disposal regulations when disposing of waste materials.

2.1 Cross-Linking

1. 1 % formaldehyde in DMEM/growth media (*see* **Note 1**).
2. 1 M glycine, pH 7.5.

3. Ice cold PBS with protease inhibitor (Complete, EDTA-free, Roche).
4. Dounce homogenizer, if preparing primary tissue.

2.2 Cell Lysis and Chromatin Fractionation

1. Lysis buffer 1 (LB1): 50 mM HEPES–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.5 % Igepal CA-630, 0.25 % Triton X-100 (*see* **Note 2**). To prepare 100 mL, take 64.5 mL deionized water and add 5 mL 1 M HEPES–KOH, pH 7.5, 2.8 mL 5 M NaCl, 0.2 mL 0.5 M EDTA, 20 mL 50 % glycerol, 5 mL 10 % Igepal CA-630, 2.5 mL 10 % Triton X-100 (*see* **Note 3**).
2. Lysis buffer 2 (LB2): 10 mM Tris–HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA. To 94.7 mL deionized water add 1 mL 1 M Tris–HCl, pH 8.0, add 4 mL 5 M NaCl, add 0.2 mL 0.5 M EDTA, add 0.1 mL 0.5 M EGTA (*see* **Note 3**).
3. Lysis buffer 3 (LB3): 10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1 % Na-deoxycholate, 0.5 % N-lauroylsarcosine. To 93.2 mL deionized water add 1 mL 1 M Tris–HCl, pH 8.0, 2 mL 5 M NaCl, 0.2 mL 0.5 M EDTA, 0.1 mL 0.5 M EGTA, 1 mL 10 % Na-deoxycholate, 2.5 mL 20 % N-lauroylsarcosine (*see* **Note 3**).
4. Water bath or tip sonicator (such as Diagenode Bioruptor).
5. 10 % Triton X-100 in LB3 (*see* **Note 4**).
6. Protein A or Protein G beads (*see* **Note 5**).
7. Blocking buffer: PBS, 0.5 % bovine serum albumin (BSA) (w/v). Filter the solution through a 0.22 μm filter (*see* **Note 6**).
8. 10 μg antibody per sample (*see* **Note 7**). For ERα antibody sc-543 (Santa Cruz) has been shown to work for ChIP.
9. Nanodrop spectrophotometer or similar spectrophotometer.
10. 2 mL phase lock tube (Scientific Laboratory Services).

2.3 Washes, Reverse Cross-Linking, and Elution

1. RIPA: 50 mM HEPES–KOH, pH 7.5, 500 mM LiCl, 1 mM EDTA, 1 % NP-40 or Igepal CA-630 (*see* **Note 8**), 0.7 % Na-deoxycholate (*see* **Note 9**). Store at 4 °C.
2. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
3. Elution buffer: 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1 % SDS. Store at room temperature.
4. Water bath at 65 °C.
5. RNase A, 1 mg/mL.
6. Proteinase K, 20 mg/mL.
7. Phenol-chloroform-isoamyl alcohol.
8. 100 % ethanol.
9. 70 % ethanol in nuclease-free water.
10. Glycogen, 20 μg/μL.

2.4 Library Preparation and Sequencing

1. Illumina TruSeq kit (IP-202-1012) or equivalent.
2. Ampure beads (Agencourt AMPure XP, A63880) or equivalent.
3. 80 % ethanol in nuclease free water.
4. PCR machine.
5. 2 % agarose gels: Add 1 g agarose to 50 mL TAE buffer. Add 5 μL SybrSafe.
6. Loading buffer for agarose gels: 50 % glycerol, 0.25 % xylene cyanol.
7. DNA molecular weight ladder.
8. Dark reader (Claire Chemical Research) or UV light.
9. MinElute Gel Extraction kit (Qiagen 28604) or equivalent.
10. Reagents and standards for qPCR such as the Kapa kit.
11. Agilent Bioanalyzer and DNA 1000 kit.
12. Real-time PCR machine.

3 Methods

Carry out all procedures on ice or at 4 °C unless stated otherwise.

3.1 Prepare Magnetic Beads

1. Vortex beads, and then add 100 μL of beads per immunoprecipitation (IP) sample to a 2 mL, round-bottomed centrifuge tube.
2. Place in a magnetic stand, on ice. Wait 30 s for all of the beads to migrate to the side of the tube (*see* **Note 10**).
3. When the supernatant is clear, remove the liquid.
4. Wash the beads in 1 mL blocking buffer. Remove the magnet to wash and ensure that the beads are resuspended.
5. Repeat washing step two more times.
6. Resuspend the beads in 500 μL blocking buffer and add 10 μg antibody (for ERα use sc-543, Santa Cruz).
7. Invert the vial to mix.
8. Rotate at 4 °C at 10 rpm overnight (*see* **Note 11**).

3.2 Cross-Linking Cells (for Tumor Tissues See Note 12)

1. Preheat media (*see* **Note 1**) (without formaldehyde, phenol red, or any additives) to 37 °C for cross-linking.
2. Just before use, add formaldehyde (final concentration 1 %) to media in fume hood.
3. Remove growth media from cells. In fume hood, add cross-linking media: 8 mL to a 10 cm dish, or 15 mL to a 15 cm dish so as to fully cover the cells on the plates and not let patches dry out.
4. Incubate at room temperature for 10 min.

5. Quench with 1 M glycine, pH 7.5, in a ratio of glycine to formaldehyde of 1:10 (1.5–15 mL of formaldehyde in the dish).
6. Pour off the formaldehyde into a beaker for disposal and wash cells twice with ice-cold PBS.
7. Keep cells on a tray of ice and pipette off excess PBS.
8. Add 500 µL ice-cold PBS with protease inhibitors and scrape the cells off of the dish. Transfer to a 1.5 mL centrifuge tube (*see* **Note 13**).
9. Centrifuge at 2000 × *g* for 3 min at 4 °C.
10. Remove the supernatant and resuspend the pellet in 500 µL PBS with protease inhibitors (per 15 cm dish).
11. Repeat centrifugation and remove supernatant.
12. At this point, cells can be stored by flash freezing in dry ice and storing at –80 °C (for up to 6 months). Alternatively, proceed to sonication without freezing.

3.3 Cell Lysis, Chromatin Fractionation, and Immunoprecipitation

1. Thaw pellets on ice if needed.
2. Add 1 mL lysis buffer 1 (LB1) with protease inhibitors to cell pellet (per 15 cm dish) and resuspend.
3. Rotate at 4 °C for 10 min.
4. Centrifuge at 2000 × *g* for 5 min at 4 °C.
5. Resuspend pellet in 1 mL LB2 with protease inhibitors.
6. Rotate at 4 °C for 5 min.
7. Centrifuge at 2000 × *g* for 5 min at 4 °C.
8. Resuspend pellet in 300 µL LB3 with protease inhibitors (per 15 cm dish).
9. Sonicate, 30 s on and 30 s off, on high power for 15 min. Ensure that you add ice to water in the sonicator (*see* **Note 14**).
10. Take a 10 µL aliquot of sonicated chromatin, reverse the crosslinking (incubate at 95 °C, 10 min), and run 5 µL on an agarose gel to confirm sufficient sonication. Fragment sizes should show a smear with the majority of DNA below 500 bp (Fig. 1).
11. Quantify reverse crosslinked material using a spectrophotometer such as the Nanodrop (*see* **Note 15**).
12. Add 30 µL 10 % Triton X-100 to sonicated lysate.
13. Centrifuge, in a benchtop centrifuge at 20,000 × *g* for 10 min at 4 °C.
14. Wash the preincubated antibody-bead mixture (from **step 8**, Subheading 3.1) three times with 1 mL blocking buffer to remove unbound antibody.
15. Resuspend the antibody-bead mixture in 100 µL blocking buffer.

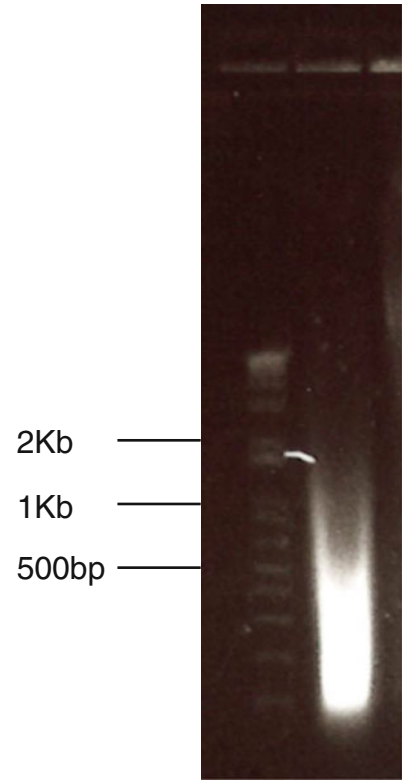

Fig. 1 Gel electrophoresis of reverse-cross-linked DNA after sonication

16. Carefully remove the supernatant from the sonicated lysate and add to a fresh 2 mL tube. Discard the pellet.
17. Dilute the sonicated lysate supernatant with LB3 to 1 mL to a final concentration of 1 % Triton X-100. Add 1× protease inhibitors.
18. Remove 50 μL of the sample as the input sample; store the input sample at 4 °C.
19. Add antibody bound beads (from **step 15**, Subheading 3.3) to the cell lysate. Rotate at 4 °C overnight.

3.4 Reverse Cross-Link and Harvest

1. Place tube in a magnetic stand, on ice, and wait 30 s for all of the beads to migrate to the side of the tube. Remove the supernatant; this is the unbound fraction.
2. Wash the beads six times in 1 mL RIPA buffer. After each wash ensure that the beads are resuspended (*see* **Note 16**).
3. Wash once with 1 mL TE. Remove TE and pulse spin in picofuge and then remove any remaining liquid (*see* **Note 17**).
4. Add 200 μL elution buffer and vortex.
5. Remove input sample from refrigerator. Add 150 μL of elution buffer.

6. Incubate both ChIP and input samples at 65 °C in a water bath for 6–18 h to reverse the cross-linking. Vortex briefly every 5 min for the first 15 min (*see* **Note 18**).

3.5 Removal of Proteins and RNA

1. After incubation, vortex the samples and centrifuge briefly.
2. Place the tube on a magnetic rack and transfer 200 µL of the supernatant from each sample to a clean 1.5 mL tube. Follow the same procedure for all samples including the input sample.
3. Add 200 µL TE to the supernatant samples.
4. Add 8 µL of RNase A, mix, and incubate at 37 °C for 30 min.
5. Add 4 µL of Proteinase K, mix, and incubate 55 °C for 1–2 h.
6. Centrifuge briefly on a desktop centrifuge.
7. In a fume hood, add 400 µL phenol-chloroform-isoamyl alcohol. Mix well. The supernatant will turn cloudy.
8. Separate the phases with 2 mL phase lock tube (Scientific Laboratory Services) as per the manufacturer's instructions.
   (a) Spin phase lock column before use at 10,000 × *g* for 1 min, in benchtop centrifuge.
   (b) Add 800 µL of sample from **step 7**, Subheading 3.4.
   (c) Spin at 10,000 × *g* for 5 min, in a benchtop centrifuge.
9. In a fume hood, remove the upper layer (~400 µL) into a 1.5 mL centrifuge tube containing 16 µL of 5 M NaCl (final concentration 200 mM) and 2 µL glycogen (20 µg/µL) and mix well. Glycogen yields a visible pellet.
10. Add 800 µL 100 % ethanol and leave at −80 °C for a minimum of 30 min. This improves precipitation. Samples can be left at this step overnight.
11. Centrifuge, in a benchtop centrifuge, at 4 °C maximum speed for 15 min (*see* **Note 19**).
12. Remove the supernatant and rinse the pellet with 500 µL cold 70 % ethanol. Do not resuspend the pellet at this point; it can be very small.
13. Centrifuge, in a benchtop centrifuge, for 5 min, full speed at 4 °C.
14. Remove the ethanol, then air dry pellet at room temperature. This should not take more than 10 min. Be careful not to over dry the pellet.
15. Resuspend the pellet in 50 µL* 10 mM Tris–HCl, pH 8.0, heated to 65 °C. Mix well and leave at 65 °C for 1 min. Vortex gently. Samples can be stored at −20 °C. *If the library preparation is to be carried out by another facility, this volume may be too high. Resuspend in appropriate volume.
16. Quantify the input sample by spectrophotometry. Use 50 ng of the input for amplification in the following steps (*see* **Note 20**).

3.6 Amplification of the Sample

1. Perform end repair (*see* **Note 21**): Using the Illumina TruSeq ChIP kit, add 10 μL resuspension buffer to 50 μL ChIP sample or 50 ng input sample (from **steps 15** and **16**, Subheading 3.5).
2. Add 40 μL end repair mix. Mix well, and then transfer sample to 0.2 mL PCR tube (and for the remainder of the library preparation use 0.2 mL tubes).
3. Incubate in thermal cycler for 30 min at 30 °C.
4. Purify with beads: Vortex AMPure XP beads.
5. Add 160 μL AMPure beads to 100 μL sample.
6. Pipette ten times to mix sample.
7. Incubate at room temperature for 15 min.
8. Place on a magnetic stand at room temperature until liquid appears clear.
9. Set pipette to 127.5 μL and remove liquid from the beads. Discard the liquid. Repeat. Continue to the next step even if some liquid remains in the tube.
10. With tubes on magnetic stand, add 200 μL freshly prepared 80 % EtOH to sample. Do not disturb the beads; just wash back and forth.
11. Incubate for 30 s at room temperature and remove all supernatant.
12. Repeat the ethanol wash.
13. Remove the tube from the magnetic stand and leave it at room temperature until dry (about 15 min).
14. Resuspend the dried pellet in 18 μL resuspension buffer and pipette ten times to mix.
15. Incubate at room temperature for 2 min.
16. Place the tube on the magnetic stand until the liquid appears clear and remove 15 μL to a fresh tube.
17. Discard the beads. The protocol can be stopped here and samples stored at −20 °C if required.
18. Adenylate the 3′ ends: Add 2.5 μL resuspension buffer to 15 μL of sample.
19. Add 12.5 μL A-tailing mix and mix well.
20. Incubate in a thermal cycler 37 °C for 30 min, 70 °C for 5 min, hold at 4 °C.
21. Ligate adapters: Add 2.5 μL resuspension buffer to each tube.
22. Add 2.5 μL DNA ligase mix. Return DNA ligase to the freezer immediately after use.
23. Add 2.5 μL adapter index to each tube. When multiplexing, use different, compatible adapters. Consult the Illumina multiplexing guidelines before choosing which adapters to use (*see* **Note 22**).

24. Change gloves and clean pipette after adding each adapter. Mix well.
25. Incubate for 10 min at 30 °C.
26. Add 5 μL stop ligase mix to each tube to inactivate ligation. Mix well.
27. Purify with beads: Vortex AMPure XP beads.
28. Add 42.5 μL AMPure beads to 42.5 μL sample.
29. Pipette ten times to mix sample.
30. Incubate at room temperature for 15 min.
31. Place on a magnetic stand at room temperature until the liquid appears clear.
32. Set pipette to 80 μL and remove some liquid from beads. Discard the liquid. Continue to next step even if some liquid remains in the tube.
33. With tubes on magnetic stand, add 200 μL freshly prepared 80 % EtOH to sample. Do not disturb the beads. Just wash back and forth.
34. Incubate for 30 s at room temperature and remove all of the supernatant.
35. Repeat the ethanol wash.
36. Remove the tube from the magnetic stand and leave the sample at room temperature until dry (about 15 min).
37. Resuspend the dried pellet in 53 μL resuspension buffer. Pipette ten times to mix.
38. Incubate at room temperature for 2 min.
39. Place the tube on the magnetic stand until the liquid appears clear. Remove 50 μL of the sample to a fresh tube.
40. Discard the beads.
41. Repeat the purification with beads (*see* **Note 23**): Vortex AMPure XP beads.
42. Add 50 μL AMPure beads to 50 μL sample.
43. Pipette ten times to mix sample.
44. Incubate at room temperature for 15 min.
45. Place on a magnetic stand at room temperature until the liquid appears clear.
46. Set pipette to 95 μL and remove some liquid from beads. Discard the liquid. Continue to the next step even if some liquid remains in tube.
47. With tubes on magnetic stand, add 200 μL freshly prepared 80 % EtOH to the sample. Do not disturb the beads; just wash back and forth.

48. Incubate for 30 s at room temperature and remove all of the supernatant.
49. Repeat the ethanol wash.
50. Remove the tube from the magnetic stand and leave it at room temperature until dry (about 15 min).
51. Resuspend the dried pellet in 23 μL resuspension buffer. Pipette ten times to mix.
52. Incubate at room temperature for 2 min.
53. Place the tube on the magnetic stand until the liquid appears clear. Remove 20 μL to a fresh tube.
54. Discard the beads. Protocol can be stopped here and samples stored at −20 °C if required.
55. Enrich DNA fragments: Add 5 μL PCR primer cocktail to each sample tube.
56. Add 25 μL PCR master mix and mix well.
57. Run PCR amplification as follows:
    (a) Step 1: 98 °C for 30 s
    (b) Step 2: 98 °C for 10 s
    (c) Step 3: 60 °C for 30 s
    (d) Step 4: 72 °C for 30 s
    (e) Step 5: go to **step 2**, 17 times (*see* **Note 24**)
    (f) Step 6: 72 °C for 5 min
    (g) Step 7: Hold at 4 °C
58. Purify with beads: Vortex AMPure XP beads.
59. Add 50 μL AMPure beads to 50 μL sample.
60. Pipette ten times to mix sample.
61. Incubate at room temperature for 15 min.
62. Place on a magnetic stand at room temperature until liquid appears clear.
63. Set pipette to 95 μL and remove some liquid from beads. Discard the liquid. Continue even if some liquid remains in tube.
64. With tubes on magnetic stand, add 200 μL freshly prepared 80 % EtOH to sample. Do not disturb the beads; just wash back and forth.
65. Incubate for at 30 s at room temperature and remove all supernatant.
66. Repeat the ethanol wash.
67. Remove the tube from the magnetic stand and leave at room temperature until dry (for about 15 min).

68. Resuspend the dried pellet in 33 μL resuspension buffer. Pipette ten times to mix.
69. Incubate at room temperature for 2 min.
70. Place the tube on a magnetic stand until the liquid appears clear. Remove 30 μL to a fresh tube.
71. Discard the beads. The protocol can be stopped here and samples stored at −20 °C if required.
72. Pour gels and prepare samples for electrophoresis: Cast 50 mL 2 % gel. Pour the gels and allow gel to set.
73. Add 3 μL loading buffer to 8 μL DNA ladder (*see* **Note 25**). Load 11 μL to the first well. Leave a lane between the ladder and first sample.
74. To prepare samples, add 10 μL loading buffer to 30 μL of each sample and load entire volume into the well. Leave at least one lane between samples.
75. Run the gel at 120 V for 45 min.
76. Visualize the gels on a UV light or dark reader.
77. Excise the fragments between 300 and 400 bp.
78. Purify with QIAgen MinElute gel extraction kit: Weigh the gel slices. Add 3 volumes of buffer QG (e.g., 300 μL buffer to 100 mg gel).
79. Vortex the sample and incubate at room temperature (not 50 °C) for 10 min, vortexing every 2 min.
80. Add 1 volume isopropanol, mix, and add to 2 mL column (kept at 4 °C; the column takes a maximum volume of 700 μL).
81. Centrifuge for 1 min at full speed, discard flow through, and add another 500 μL QG buffer.
82. Centrifuge for 1 min at full speed, discard flow through, and add 750 μL buffer PE.
83. Allow to stand for 3 min, and then centrifuge for 1 min at full speed.
84. Discard flow through, and then centrifuge again to dry the column.
85. Put the column in fresh 1.5 mL centrifuge tube and add 20 μL EB buffer prewarmed to 50 °C.
86. Allow to stand for 1 min, and then centrifuge for 1 min at full speed.
87. Sample can be stored at −20 °C (*see* **Note 26**).

3.7 Quantification by qPCR and Submission

Use qPCR as follows to quantify the sample DNA and the Bioanalyzer as per manufacturers instructions to determine the average size of the library.

1. For qPCR, dilute the sample library 1:1000 in 10 mM Tris–HCl, pH 8.0, 0.05 % Tween 20.
2. Make further dilutions to 1:2000, 1:10,000 and 1:20,000. Use these dilutions for quantification.
3. Add 1 mL of Illumina primer premix (10×) to 5 mL bottle of KAPA SYBRFAST qPCR master mix and mix well. (This is only done the first time the kit is used.)
4. Set up the reaction mixture in duplicate in 96-well qPCR plate. For each 20 μL reaction, include 12 μL KAPA SYBRFAST qPCR mix containing primer premix, 4 μL PCR-grade water, and 4 μL diluted library or DNA standard.
5. Depending on your qPCR machine you may be able to specify your standard wells and the concentration:
   (a) Std 1: 20 pM
   (b) Std 2: 2 pM
   (c) Std 3: 0.2 pM
   (d) Std 4: 0.02 pM
   (e) Std 5: 0.002 pM
   (f) Std 6: 0.0002 pM
6. Run qPCR program:
   (a) Step 1: 95 °C for 5 min
   (b) Step 2: 95 °C for 30 s
   (c) Step 3: 60 °C for 45 s
   (d) Step 4: Go to **step 2**, 34 times
7. Reaction efficiency for standards needs to be in the range of 90–110 %.
8. Estimating fragment size: Analyze 1 μL of sample on a Bioanalyzer.
9. Follow the Agilent DNA 1000 kit protocol (*see* **Note 27**).
10. Calculations: Take average concentration in pM = A.
11. Size adjust concentration: $A \times 452$/average fragment length (from Bioanalyzer) = W.
12. $W \times$ dilution factor = concentration of undiluted library stock (pM).
13. Mix all libraries to an equal concentration and submit for sequencing as per guidelines of individual sequencing facilities (*see* **Note 28**).

3.8 Sequencing

Advances in next-generation sequencing mean that as of 2015, it is possible to obtain 200 million (50 bp) reads of sequence from a single lane on a flow cell. Therefore when performing ChIP it is possible to multiplex up to ten samples and still obtain 20 million

reads per sample from one flow cell, which is usually sufficient to identify peaks even at weakly bound sites. The Illumina MiSeq is also now an option for running ChIP-seq samples, as this can provide up to 25 M (50 bp) reads from one flow cell. Details of the chemistry and sequencing process [9] are beyond the scope of this chapter.

3.9 Data Analysis

1. The usual starting point for data analysis is raw sequencing reads in FASTQ format [10] (*see* **Note 29**).
2. Given the raw sequencing reads, it is sensible to run quality control analyses using packages such as FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) or ChIPQC (specialized for ChIP experiments) http://www.bioconductor.org/packages/release/bioc/html/ChIPQC.html [11].
3. Alignment to a reference genome is the usual next step. It is essential to ensure that all analyses in a given comparison are made with respect to the same reference genome. Visit the Genome Reference Consortium website for standardized reference genomes for common model organisms, and standardized naming (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/). Many software packages are available to align sequences to the reference genome. BWA is a common choice for ChIP-seq [12] (http://bio-bwa.sourceforge.net/). For this analysis of ER binding, MAQ was used (http://maq.sourceforge.net/maq-man.shtml) (*see* **Note 30**).
4. For an initial look at the data, view the data in a wiggle or bedgraph format in a genome browser such as UCSCs (Fig. 2) (*see* **Note 31**). Alternatively a desktop software package such as IGV may be useful (http://www.broadinstitute.org/igv/). Software such as bedtools can be used to generate "wiggle" or "bedgraph" format (https://github.com/arq5x/bedtools2); the UCSC Genome Browser site also provides facilities to convert to the newer and more convenient BigWig format.

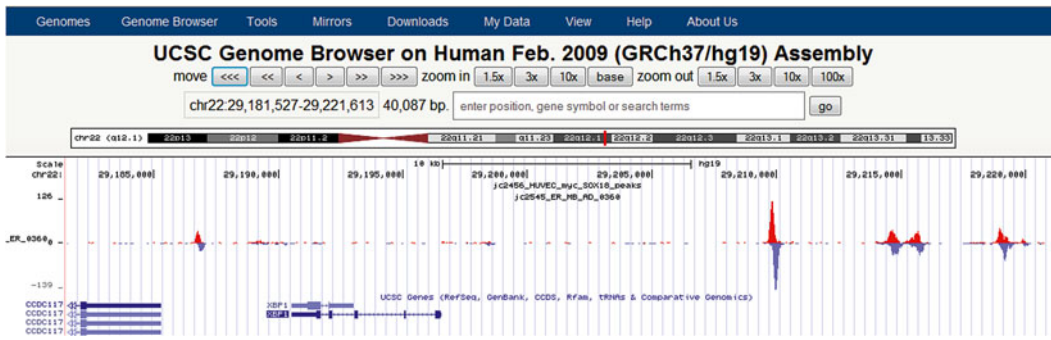

Fig. 2 Bedgraph (bgr) file displayed in the UCSC genome browser showing a successful ER ChIP, with peaks at the XBP enhancer region

5. Subsequently, to determine enrichment of the estrogen receptor over the input across the whole-genome analysis is performed using MACS or HOMER (*see* **Note 32**). So-called peak calling (determining regions of enrichment, as seen in Fig. 2) is not yet a very-well-solved problem: different choices of software, or of parameters for a given piece of software, frequently provide widely differing answers, so caution is needed in interpretation, and above all a sound experimental design, including replicates.
6. Heatmaps (Fig. 3) are a way of visualizing many hundreds of binding sites simultaneously from ChIP-seq data in a window of 5 or 10 kb around a peak (*see* **Note 33**).
7. Further downstream analysis might include examining motifs present in different classes of peaks, differential binding under different conditions, or enrichment for peaks close to interesting classes of genes (*see* **Note 34** for suggested tools).

4 Notes

1. Use the same growth media that your cells are in, without any FBS or additives. Warm at 37 °C before cross-linking and add formaldehyde just before use.
2. As Triton X-100 is very viscous, cut the end of the pipette with a clean scalpel to aid in dispensing. Be patient and wait for the total volume to be withdrawn before removing pipette from the bottle.
3. Aliquot what you need from your stock solution and add fresh protease inhibitor on the day of use.
4. Make up in advance, as the Triton X-100 takes some time to dissolve. Pipette as in **Note 2** into the LB3 and leave for 10 min at 37 °C. Briefly vortex and leave on ice or store at 4 °C until use. Ensure that the solution has cooled down before use.
5. Choose Protein A or G beads, following the antibody compatibility table (Life Technologies http://www.lifetechnologies.com/uk/en/home/life-science/protein-expression-and-analysis/protein-sample-preparation-and-protein-purification/proteinspproteiniso-misc/protein-isolation/immunoprecipitation-using-dynabeads-protein-a-or-protein-g.html). Ensure that the beads do not dry out.
6. It is important to filter your solution using a 0.22 μm filter. If the PBS 0.25 % BSA becomes cloudy or shows signs of contamination, replace with fresh stock.
7. The antibody you use is critical to your ChIP. Where possible use antibodies that have already been published for ChIP. When performing ChIP for the first time run a positive control

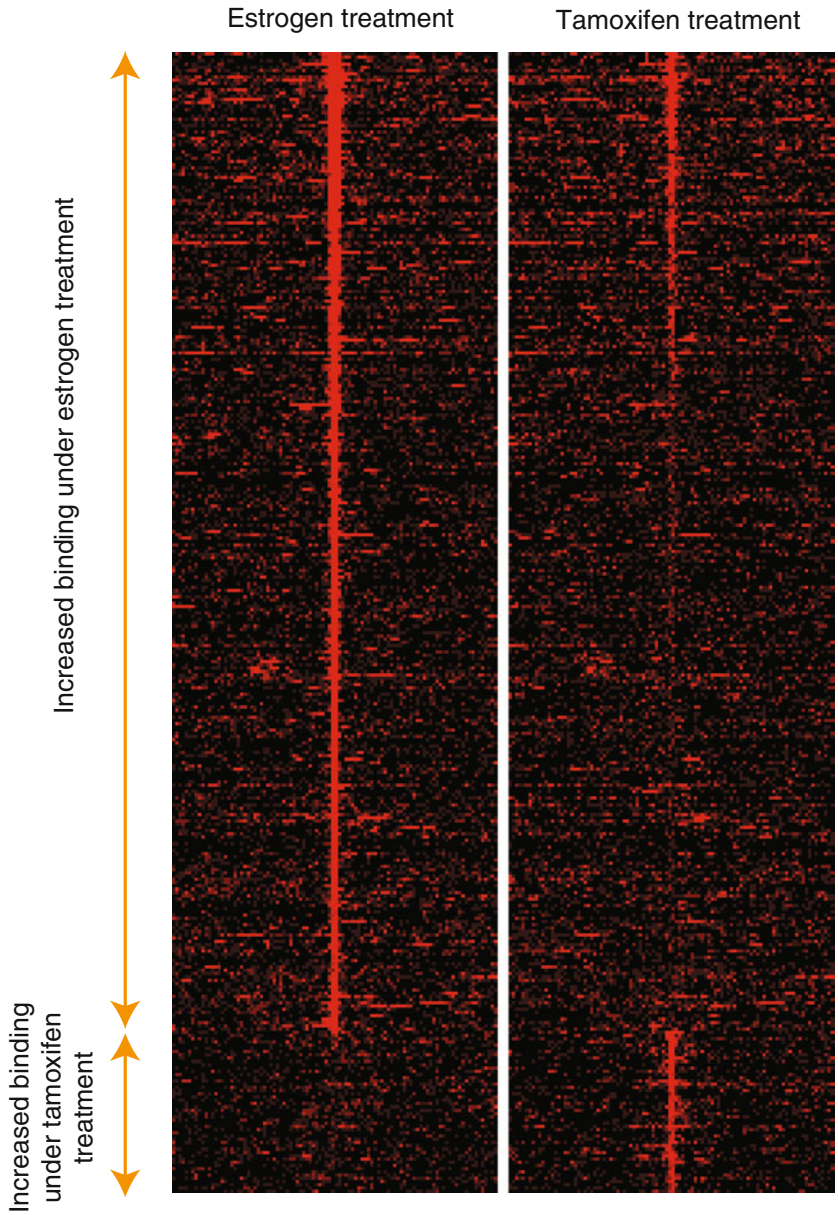

Fig. 3 Heatmap showing binding peak intensity of ER-binding events in MCF7 breast cancer cells treated with estrogen or tamoxifen. *Each panel* in the figure represents ±5 kb regions from the center binding event

along with your samples. Anti-H3K4me3 (ab8580, Abcam) recognizes histone methylation, which is present in many cell types and shows good enrichment [13]. 2 μg is the minimum we would recommend per ChIP. Our experience suggests that additional antibody (up to 10 μg) improves ChIP-seq signal.

8. As Igepal is very viscous cut the end of the pipette with a clean scalpel or scissors to aid in dispensing. Be patient and wait for

the total volume to be withdrawn before removing the pipette from the bottle.

9. There are different forms of sodium deoxycholate, we have had good experience with Sigma-Aldrich D6750.
10. Briefly vortex to resuspend beads, but after addition of the antibody do not vortex as this may disrupt the antibody. Gently invert tube to resuspend beads. It is important not to let beads dry out.
11. Some antibodies can be incubated for shorter times (4 h at 4 °C or 1 h at room temperature). This needs to be optimized for individual antibodies.
12. We have tried to select tumor material that has a high cellularity, but our experience suggests that the larger tumors produce better quality data. Sometimes the tumor will have a lot of fatty material and will be difficult to homogenize with the Dounce.
    (a) In a category 2 hood, mince fresh or flash frozen tissue quickly (if size allows) with a razor blade.
    (b) Add tumor tissue to 5 mL freshly prepared 1 % formaldehyde in DMEM/growth media.
    (c) Incubate at room temperature for 10 min.
    (d) Add 0.5 mL of 1 M glycine to quench the formaldehyde.
    (e) Rinse the tumor tissue with ice-cold PBS.
    (f) Dounce tumor tissue with first the loose and then the tight pestle.
    (g) Centrifuge in a 15 mL tube at 2500 × *g* for 5 min.
    (h) Repeat PBS wash.
    (i) Proceed with lysis in LB1 (**step 2**, Subheading 3.2).
13. We use Corning, Costar cell lifters to scrape. If working with multiple plates for a ChIP, you can combine cells into a 15 mL centrifuge tube to reduce the number of tubes being handled. Be sure not to mix samples. Scale up the amounts of PBS for the next wash and LB1, LB2 and LB3 you use, respectively. For example, if you have four plates of cells, and each has been scraped in 500 μL of PBS, place all into a 15 mL centrifuge tube, spin, resuspend in 4 mL of LB1 and subsequently LB2, and then resuspend in 1.2 mL LB3. At the sonication step, if using a water bath sonicator, be sure to split the sample again into four tubes of 300 μL to ensure sufficient sonication.
14. Sonication time is variable, depending on the cell line and the volume in the tube. This requires optimization for your own equipment and cell line. We would recommend optimizing sonication time prior to performing the ChIP. It is essential to achieve good sonication as the more small fragments you have, the more of your IP will be in your final sample (after amplification only

the 300–400 bp fragments are submitted; this size includes the adaptor sequences). It is however a balance between enough sonication to get small fragments and not over-sonicating to the point which can compromise the integrity of the protein-DNA interactions. If using a tip sonicator, the presence of bubbles can mean that the sonication is incomplete. If you see bubbles appearing in your sample, pause the sonication and adjust the tip, as it may be making contact with the tube and continue.

15. Quantifying how much chromatin you have at this point is important if you are looking at quantitative differences between ChIP seq samples. Once you have quantified, add the same amount of chromatin to each IP (use as much as the lowest sample allows).
16. Try to avoid resuspending with the pipette as a lot of beads can get lost at this stage. Gently invert tube to resuspend beads. It is important not to let beads dry out. A vacuum pump can be used at this stage to reduce the time taken to perform the washes. As the beads stick to the side of the tube where the magnet is located, if you are careful they will not get sucked away.
17. It is critical to remove residual TE. Make sure the lid of the tube does not contain any TE.
18. Six hours is the minimum time for reverse cross-linking at 65 °C. Incubating for less than this will compromise your experiment. We recommend overnight.
19. Always place the tube in the rotor with the hinge of the cap to the outside so the pellet can be easily visualised below the hinge.
20. It is difficult to quantitate how much DNA is in the ChIP sample. We recommend using all of the sample for library amplification.
21. Clean pipettes in fume hood with DNAzap before starting. Clean work bench and racks with 70 % ethanol to prevent any contamination during library preparation. If you have frozen your samples previously, ensure that you centrifuge all tubes after defrosting.
22. Adaptor pooling guidelines can be found in the TruSeq® sample preparation pooling guide from Illumina http://supportres.illumina.com/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqsampleprep/truseq_sample_prep_pooling_guide_15042173_a.pdf
23. Do not skip this step. Two bead washes are required.
24. The number of cycles here can be reduced to 11 if previously experiments show a high duplication rate in the library.
25. Use glycerol rather than dye, as dye runs at 100–200 and interferes with visualization of chromatin.

26. It is advisable at this point to take 1 μL of the sample, dilute 1:100, and perform qPCR to check enrichment at known binding sites. For ER the TFF1 promoter and XBP enhancer are good binding sites.

    TFF1 Forward: CACCCCGTGAGCCACTGT

    TFF1 Reverse: CTGCAGAAGTGATTCATAGTGAGAGAT

    XBP1 Forward: ATACTTGGCAGCCTGTGACC

    XBP1 Reverse: GGTCCACAAAGCAGGAAAAA

    Control Forward: ACCCTCCAAAATTCTTCTGC

    Control Reverse: ATGAGCATCTGAGAGCAAGC

    Use the promoter and a control region (with no binding) to determine your enrichment. If you have tenfold or less enrichment over input at these sites it is unadvisable to continue with the sequencing, as in our experience this does not result in identification of peaks following sequencing.

27. The bioanalyzer is advantageous over a standard spectrophotometer as it gives the size distribution of the library.

28. Once you have combined all of your libraries, it is prudent to use the qPCR again to check the concentration of your final library before sequencing. Some sequencing facilities may provide this service before they run the sequencing.

29. Other pipelines are available to obtain sequences from the raw data files [14, 15].

30. A number of algorithms are available to align sequences to the genome [16]. When using MAQ, remove regions with a mapping quality of 0. Use uniquely mapped reads for subsequent analysis (unless studying repeat regions). It is also worth noting for quality control purposes how much of each sample does not align to the genome of interest and the duplication rate. If the duplication rate is high, if you need to repeat the experiment, you can decrease the number of cycles in subsequent amplification steps.

31. Wiggle files, bedgraph or GFF files can be viewed in the UCSC genome browser (http://genome.ucsc.edu/).

32. Many peak calling programs are available. It is important to understand the limitations of each [16, 17]. We usually use MACS (http://liulab.dfci.harvard.edu/MACS/) and HOMER (http://homer.salk.edu/homer/index.html). It is prudent to perform peak calling using two peak callers and overlap the two data sets to reduce peak caller bias. When studying histone marks it is advisable to use Control based ChIP-seq analysis tool (CCAT2.0 [18]) or SICER (http://home.gwu.edu/~wpeng/Software.htm). Programs such as MACS and HOMER support searching for broad peaks, however, they are not specifically designed for it.

33. Heatmaps are useful tool to visualise the entire ChIP-seq data set in one figure. They can be generated using clustering software once the data matrix has been generated using HOMER (http://homer.salk.edu/homer/chipseq/heatmap.html).
34. Three commonly used motif analysis programs are the MEME suite (http://meme.nbcr.net/meme/) [19], HOMER (mentioned above in the context of peak calling), and RSAT (http://rsat.ulb.ac.be/rsat//RSAT_home.cgi) [20]. For differential binding analysis, the DiffBind package might be useful (http://www.bioconductor.org/packages/release/bioc/html/DiffBind.html, Stark R and Brown G (2011). *DiffBind: differential binding analysis of ChIP-Seq peak data*). Regulatory relationships may be examined with a variety of tools, such as CEAS (http://liulab.dfci.harvard.edu/CEAS/) or GREAT (http://bejerano.stanford.edu/great/public/html/) [21].

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Chapter 9

RNA-Seq Experiment and Data Analysis

Hanquan Liang and Erliang Zeng

Abstract

With the ability to obtain tens of millions of reads, high-throughput messenger RNA sequencing (RNA-Seq) data offers the possibility of estimating abundance of isoforms and finding novel transcripts. In this chapter, we describe a protocol to construct an RNA-Seq library for sequencing on Illumina NGS platforms, and a computational pipeline to perform RNA-Seq data analysis. The protocols described in this chapter can be applied to the analysis of differential gene expression in control versus 17β-estradiol treatment of in vivo or in vitro systems.

Key words RNA-Seq, Next-generation sequencing, Data analysis, Bioconductor, Statistical analysis, Differentially expressed genes

1 Introduction

Recent advancements in next-generation sequencing (NGS) technologies enable sequencing of tens of millions to billions of cDNA fragments generated from RNA, offering great opportunity to directly quantify entire messenger RNA (mRNA) from a sample [1, 2]. In high-throughput transcript data a large number of transcripts are concurrently sensed using fragments of cDNAs (called reads), with the idea that abundance of a transcript is estimated by integrating the counts of reads likely to be produced from the transcript [3–6]. If the reads are uniquely associated with a transcript, estimating its expression value is relatively simple—roughly speaking, the total read counts associated with a transcript divided by the base length of the transcript with a fixed scaling gives an estimate [4]. There are many platforms that can be used for NGS and many pathways for data analysis. This chapter describes how to prepare an RNA-Seq library for sequencing on Illumina platforms [7], and how to use a computational pipeline for estimating expression of transcripts and for statistical analysis to discover differentially expressed genes (DEGs). Starting with total RNA, the library construction steps include mRNA purification and fragmentation,

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first-strand cDNA synthesis, and second-strand cDNA synthesis. The double-strand cDNA is then ligated to adapters, and enriched with PCR amplification. The computational pipeline for RNA-Seq data analysis consists of steps for data quality assessment, read aligning and gene expression estimating, and statistical analysis. The protocols described in this chapter can be applied to the analysis of differential gene expression in control versus 17β-estradiol treatment of in vivo or in vitro systems.

2 Materials

1. Samples of purified total RNA from control and 17β-estradiol treated cells or tissues.
2. Oligo (dT)25 magnetic beads and magnetic stand (Invitrogen).
3. Washing Buffer B, comes with Oligo (dT)25 magnetic beads reagent (Invitrogen).
4. Binding Buffer, comes with Oligo (dT)25 magnetic beads reagent (Invitrogen).
5. 10 mM Tris–HCl, pH 7.5 (Invitrogen).
6. 10× RNA Fragmentation Buffer (New England Biolabs).
7. 10× RNA Fragmentation Stop Solution (New England Biolabs).
8. Random hexamers (100 pmol/μL).
9. First-Strand Reaction Buffer (Invitrogen): 4 μL 5× first-strand buffer, 2 μL 100 mM dithiothreitol, 1 μL dNTP mix, 1 μL SuperScript II per reaction tube.
10. dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP).
11. Second-strand reaction buffer: 51 μL nuclease-free water, 20 μL 5× second-strand reaction buffer, 2 μL dNTP mix, 5 μL *E. coli* DNA Polymerase I (10 U/μL) (New England Biolabs, NEB), 1 μL *E. coli* DNA Ligase (10 U/μL) (NEB), 1 μL *E. coli* RNase H (5 U/μL) (NEB) per reaction tube.
12. dA tailing mix: 5 μL 10× A-tailing buffer, 10 μL 1 mM dATP, 3 μL Klenow fragment (3′→5′ exo-) per reaction (NEB).
13. End repair reaction mix: 25 μL nuclease-free water, 10 μL 10× phosphorylation reaction buffer, 4 μL dNTP mix, 5 μL T4 DNA polymerase, 1 μL *E. coli* DNA polymerase I, Large (Klenow) Fragment, 5 μL T4 Polynucleotide Kinase (New England Biolabs).
14. T4 DNA ligation mix: 5 μL nuclease-free water, 3 μL 10× T4 DNA ligation buffer (NEB), 1 μL Illumina Adapters (Illumina), 1 μL T4 DNA ligase (NEB).
15. Universal Primer Mix (10 μM each forward & reverse).

16. 2× Phusion High-Fidelity PCR Master Mix (Thermo Scientific).
17. QIAquick PCR Purification Kit (Qiagen).
18. RNeasy MinElute Cleanup Kit (Qiagen).
19. AMPure XP beads (Beckman Coulter).
20. Nuclease-free water.
21. 80 % ethanol.
22. Qubit Fluorometer (Invitrogen).
23. Thermal cycler.
24. Agilent 2100 Bioanalyzer (Agilent).

3 Methods

3.1 RNA-Seq Experiment

Before starting, it is highly recommended to assess the total RNA quality of the samples using an Agilent Bioanalyzer 2100. To achieve the best results, the RNA Integrity Number (RIN) estimated by the Bioanalyzer should be 8 or higher. Low quality samples may yield seemingly good libraries and good sequencing reads, but analysis of such data is challenging and the results may be misleading. In the second-strand cDNA synthesis reaction, the second strand of cDNA is synthesized and the RNA templates are removed.

In the mRNA purification steps, messenger RNA with poly-A tails will be captured, while other RNA components (rRNA, tRNA) will be removed from the samples. For mRNA fragmentation, the mRNA is cleaved into small pieces by heating in divalent metal cation buffer. The first-strand cDNA synthesis steps will generate the first strand of cDNA using reverse transcriptase and random primers. The second-strand cDNA synthesis steps generate the second strand of cDNA and removes the RNA templates from the reaction. In the end repair steps, the ends of double-strand cDNA fragments are converted into blunt ends. An adenine (A) base is then added to the 3′-end of blunt double strand cDNA to facilitate adapter ligation. The adaptor ligated cDNA is then enriched and amplified by PCR to achieve a sufficient amount of library. Finally the library is quantified and its quality is checked.

1. For mRNA purification, dilute 100–1000 ng of total RNA with nuclease-free water to a final volume of 50 μL (*see* **Note 1**).
2. Add 50 μL resuspended oligo-dT beads (prepared according to the manufacturer's recommendation if necessary) to each RNA sample.
3. Place the samples in a thermal cycler and heat at 65 °C for 5 min, and then 4 °C on hold. Remove the tubes from the thermal cycler after the temperature reaches 4 °C.
4. Incubate samples at room temperature for 5 min (*see* **Note 2**).

5. Place the samples on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully remove and discard supernatants without disturbing the beads (*see* **Note 3**).
6. Remove the tubes from the magnetic stand. Resuspend the beads with 200 μL washing buffer B.
7. Place the tubes on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully remove and discard the supernatants without disturbing the beads.
8. Remove the tubes from the magnetic stand. Resuspend the beads with 50 μL 10 mM Tris–HCl buffer.
9. Place the tubes in a thermal cycler. Heat the samples at 80 °C for 2 min, and then 25 °C on hold. In this step, binding is disrupted and RNA is released into the supernatant.
10. Remove the tubes from the thermal cycler. Add 50 μL binding buffer and resuspend beads.
11. Incubate the samples at room temperature for 5 min. In this step, the mRNA rebinds to the poly-dT beads.
12. Place the tubes on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully remove and discard the supernatants without disturbing the beads.
13. Remove the tubes from the magnetic stand. Resuspend the beads with 200 μL washing buffer B.
14. Place the tubes on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully remove and discard the supernatants without disturbing the beads (*see* **Note 4**)
15. Remove the tubes from the magnetic stand. Resuspend the beads with 20 μL of nuclease-free water.
16. Place the tubes on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully transfer 18 μL of the supernatant to new tubes.
17. For mRNA fragmentation, add 2 μL 10× RNA fragmentation buffer to the samples.
18. Place the tubes in a thermal cycler and incubate at 94 °C for 5 min. Immediately transfer the tubes to ice (*see* **Note 5**).
19. Add 2 μL 10× RNA fragmentation stop solution.
20. Clean up fragmented RNA using RNeasy MinElute columns following the manufacturer's instructions. Elute fragmented mRNA with 12 μL nuclease-free water.

21. For first-strand cDNA synthesis, add 1 μL random hexamers. Incubate at 70 °C for 10 min, and quick-chill on ice.
22. Add 8 μL first-strand cDNA reaction mixture to each reaction.
23. Incubate at 25 °C for 10 min, 42 °C for 50 min, 70 °C for 15 min, hold at 4 °C (*see* **Note 6**).
24. For second-strand cDNA synthesis, add 80 μL of second-strand reaction mixture to each reaction.
25. Place the tubes in a thermal cycler at 16 °C for 2 h.
26. Purify the double-stranded cDNA using the QIAquick PCR Purification Kit following the manufacturer's directions and elute in 50 μL nuclease-free water.
27. For the end repair steps, add 50 μL of end repair reaction mix to each double-stranded cDNA sample.
28. Incubate at 20 °C for 30 min.
29. Purify end-repaired double-stranded cDNA using the QIAquick PCR Purification Kit and elute in 32 μL nuclease-free water.
30. To carry out the dA tailing step, add 18 μL of dA tailing mix to each 32 μL of end-repaired cDNA.
31. Incubate at 37 °C for 30 min.
32. Purify dA-tailed double-stranded cDNA using QIAquick PCR Purification Kit and elute in 20 μL nuclease-free water.
33. To ligate the adapters, add 10 μL of T4 DNA ligation mix to each dA-tailed sample (20 μL) (*see* **Note 7**).
34. Incubate the samples in a thermal cycler at 30 °C for 10 min.
35. Add 1.2× (42 μL) resuspended AMPure XP beads to each reaction and mix thoroughly by pipetting. Incubate at room temperature for 10 min.
36. Place the tubes on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully remove and discard the supernatants without disturbing the beads.
37. With the tubes on the magnetic stand, add 200 μL of 80 % freshly prepared ethanol.
38. Incubate at room temperature for 30 s, and then discard all supernatant without disturbing the beads.
39. Repeat ethanol wash one more time.
40. Keep the tubes on the magnetic stand, leave lids open and air-dry the beads for 10 min (but do not over dry).
41. Remove tubes from magnetic stand. Suspend beads with 52 μL nuclease-free water, and then incubate at room temperature for 2 min.

42. Place the tubes on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully transfer 50 μL of the supernatant to new tubes.
43. Perform size selection by purifying the adapter-ligated DNA with 1× (50 μL) AMPure XP beads (**steps 35–40**) (*see* **Note 8**).
44. Remove tubes from magnetic stand. Suspend beads with 25 μL nuclease-free water, and then incubate at room temperature for 2 min.
45. Place the tubes on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully transfer 23 μL of the supernatant to new tubes.
46. For PCR amplification of the cDNA samples, add 2 μL Universal Primer Mix and 25 μL of 2× Phusion High-Fidelity PCR Master Mix to each adapter-ligated cDNA (23 μL).
47. Place the samples in a thermal cycler and program the cycles as follows (*see* **Note 9**):
    (a) 98 °C for 30 s
    (b) 98 °C for 10 s
    (c) 60 °C for 30 s
    (d) 72 °C for 30 s
    (e) Go to **step** (**b**) for 14×
    (f) 72 °C for 5 min
    (g) Hold at 10 °C
48. Purify the PCR-enriched library with 1× (50 μl) AMPure XP beads (**steps 35–40**).
49. Remove tubes from magnetic stand. Suspend beads with 22 μL nuclease-free water, and then incubate at room temperature for 2 min.
50. Place the tubes on a magnetic stand for 5 min or until the solution is clear. Keeping the tubes on the magnetic stand, carefully transfer 20 μL of the supernatant to new tubes.
51. Use an Agilent Bioanalyzer to check the quality of the library and to estimate library size (*see* **Note 10**).
52. Quantify library using Qubit Assay (Invitrogen) by following the manufacturer's documents.
53. Based on library size and concentration, dilute library to 20 nM. Pool libraries if necessary (*see* **Note 11**).
54. Store the library at −20 °C.
55. Perform sequencing by following the manufacturer's protocol. This library is designed for sequencing on the Illumina platform. Alternatively, send the samples out for sequencing.

3.2 RNA-Seq Data Analysis

A functional laptop/desktop with 4GB or larger of RAM is required for running the computational pipeline. The computational pipeline for analysis of RNAseq data described here includes a series of steps. First, some simple quality control checks need to be performed to ensure that the raw data is of good quality before analyzing the RNA-Seq data [8]. FastQC is a computational tool that provides a QC report which can spot problems originating either in the sequencer or in the starting library material [9]. Second, the R and Bioconductor (a set of packages that run in R) programs will be installed. R and Bioconductor will be used to perform most of the analyses [10, 11]. R is a free, very powerful statistics environment [10]. The sequence files will be read and the sequence reads will be mapped to a reference genome. Gene expression values will be estimated by calculating either the raw read count number or Reads Per Kilobase of transcript per Million reads mapped (RPKM). Statistical analyses will be performed to discover DEGs.

1. For the quality control checks, download an appropriate version of FastQC from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (*see* **Note 12**).
2. Install FastQC as instructed (*see* **Note 13**).
3. Perform basic operations in FastQC, including opening a sequence file, evaluating results, and saving a report (*see* **Note 14**).
4. Go to R project website (http://www.r-project.org/), download and install an appropriate R version (R Version 3.1.2 and up is recommended).
5. Start R on your computer.
6. After starting R, paste or type following commands to install Bioconductor (if the Bioconductor program has not been installed before). The installation will take a few minutes.

```
source("http://bioconductor.org/biocLite.R")
biocLite()
```

7. Set working director to your local folder. In the following command, change "path to your local folder" to your specific working directory. For example, I set my working directory as *setwd("/Users/ezeng/Documents/Teaching/RNA-Seq")*. Note that my data subdirectory is "*/Users/ezeng/Documents/Teaching/RNA-Seq/*data." The data subdirectory contains RNA-Seq FASTQ files from the study of human estrogen receptors, as well as the corresponding human reference genome sequence (FASTA) and annotation (GFF) file (*see* **Note 15**).

```
setwd("path to your local folder")
```

8. Install and load *QuasR* [12]. *QuasR* is a versatile NGS mapping and post-processing pipeline for RNA-Seq data analysis.

It uses *Rbowtie* for ungapped alignments and *SpliceMap* for spliced alignments. Install and load *QuasR* package.

```
biocLite("QuasR")
library(QuasR)
```

9. To read sequence files and map sequence reads to the reference genome, first read sequence file information. The FASTQ files are organized in the provided samples.txt file in data subdirectory (*see* **Note 16**). To import samples.txt, we run the following commands from R.

```
samples <- read.delim("data/samples.txt")
```

10. Set environment.

```
write.table(samples[,1:2], "data/QuasR_samples.txt",
    row.names=FALSE, quote=FALSE, sep="\t")
sampleFile <- "./data/QuasR_samples.txt"
genomeFile <- "./data/Homo_sapiens.GRCh38.dna.top-
    level.fa"
# Note: all output data will be written to subdirec-
    tory 'results'
dir.create("results")
# Defines location where to write results
results <- "./results"
# Defines number of CPU cores to use
cl <- makeCluster(1)
```

11. Use single command to index reference, align all samples, and generate BAM files (*see* **Note 17**).

```
proj <- qAlign(sampleFile, genome=genomeFile, max-
    Hits=1, splicedAlignment=FALSE, alignmentsDir=
    results, clObj=cl, cacheDir=results)
```

12. Get alignment summary report.

```
alignstats <- alignmentStats(proj)
alignstats
```

13. Enumerate the number of reads in each FASTQ file and how many of them aligned to the reference. For *QuasR* this step can be omitted because the *qAlign* function generates this information automatically.

```
biocLite("ShortRead")
biocLite("Rsamtools")
library(ShortRead)
library(Rsamtools)
Nreads <- countLines(dirPath="./data", pattern=".
    fastq$")/4
bfl <- BamFileList(alignments(proj)$genome$FileName,
    yieldSize=50000, index=character())
```

```
Nalign <- countBam(bfl)
read_statsDF <- data.frame(FileName=names(Nreads), Nreads=Nreads, Nalign=Nalign$records, Perc_Aligned=Nalign$records/Nreads*100)
write.table(read_statsDF, "results/read_statsDF.xls", row.names=FALSE, quote=FALSE, sep="\t")
```

14. To estimate the gene expression values, calculate either raw read count number or RPKM. To do this, get annotation data from GFF.

```
biocLite("rtracklayer")
biocLite("GenomicRanges")
library(rtracklayer)
library(GenomicRanges)
gff <- import.gff("./data/ref_GRCh38_top_level.gff3", asRangedData=FALSE)
seqlengths(gff) <- end(ranges(gff[which(elementMetadata(gff)[,"type"]=="chromosome"),]))
subgene_index <- which(elementMetadata(gff)[,"type"] == "exon")
gffsub <- gff[subgene_index,] # Returns only gene ranges
ids <- gsub("Parent=|\\..*", "", elementMetadata(gffsub)$group)
gffsub <- split(gffsub, ids) # Coerce to GRangesList
```

15. Store annotation rangers in *TranscriptDb* databases, which make many operations more robust and convenient.

```
biocLite("GenomicFeatures")
library(GenomicFeatures)
txdb <- makeTranscriptDbFromGFF(file="data/ref_GRCh38_top_level.gff3",
format="gff3",
dataSource="NCBI",
species="Homo sapiens")
saveDb(txdb, file="./data/GRCh38.sqlite")
txdb <- loadDb("./data/GRCh38.sqlite")
eByg <- exonsBy(txdb, by="gene")
```

16. Read counting with *qCount* from *QuasR*.

```
countDF <- qCount(proj, txdb, reportLevel="gene", orientation="any")
write.table(countDF, "results/countDFgene.xls", col.names=NA, quote=FALSE, sep="\t")
write.table(countDF[,2:5], "./results/countDF", quote=FALSE, sep="\t", col.names = NA)
```

17. Perform a simple RPKM normalization (*see* **Note 18** for an alternative way to calculate RPKM).

```
returnRPKM <- function(counts, gffsub) {
geneLengthsInKB <- sum(width(reduce(gffsub)))/1000
millionsMapped <- sum(counts)/1e+06
rpm <- counts/millionsMapped
rpkm <- rpm/geneLengthsInKB
return(rpkm)
}
countDFrpkm <- apply(countDF, 2, function(x)
    returnRPKM(counts=x, gffsub=eByg))
```

18. Check the sample reproducibility by computing a correlating matrix and plotting it as a tree (*see* **Note 19**).

```
biocLite("ape")
library(ape)
d <- cor(rpkmDFgene, method="spearman")
hc <- hclust(dist(1-d))
plot.phylo(as.phylo(hc), type="p", edge.col=4, edge.
    width=3, show.node.label=TRUE, no.margin=TRUE)
```

19. To perform statistical analyses to discover differentially expressed genes (DEGs), define the *colAg()* function (*see* **Note 20**).

```
colAg <- function(myMA=myMA, group=c(1,1,1,2,2,2,3,
    3,4,4), myfct=mean, …) {
myList <- tapply(colnames(myMA), group, list)
names(myList) <- sapply(myList, paste, collapse=
    "_")
myMAmean <- sapply(myList, function(x) apply(myMA
    [, x,
drop=FALSE], 1, myfct, …))
return(myMAmean)
}
```

20. Compute mean values for replicates using function *colAg()* (*see* **Note 21**).

```
countDFrpkm_mean <- colAg(myMA=rpkmDFgene, group=
    c(1,1,2,2), myfct=mean)
```

21. Calculate $\log_2$ fold changes.

```
countDFrpkm_mean <- cbind(countDFrpkm_mean, log2ratio=
    log2(countDFrpkm_mean[,2]/countDFrpkm_mean[,1]))
countDFrpkm_mean <- countDFrpkm_mean[is.
    finite(countDFrpkm_mean[,3]),]
degs2fold <- countDFrpkm_mean[countDFrpkm_mean[,3]
    >= 1 | countDFrpkm_mean[,3] <= -1,]
write.table(degs2fold, "./results/degs2fold.xls",
    quote=FALSE, sep="\t", col.names = NA)
degs2fold <- read.table("./results/degs2fold.xls")
```

22. Perform statistical analysis with DESeq library (*see* **Note 22**). Note that DESeq is expected to use raw count data [13].

```
biocLite("DESeq")
library(DESeq)
countDF <- read.table("./results/countDF")
conds <- samples$Factor
# Creates object of class CountDataSet derived from
   eSet class
cds <- newCountDataSet(countDF, conds)
# Estimates library size factors from count data.
cds <- estimateSizeFactors(cds)
# Estimates the variance within replicates
cds <- estimateDispersions(cds)
# Calls DEGs with nbinomTest
res <- nbinomTest(cds, "Control", "Treatment")
res <- na.omit(res)
res2fold <- res[res$log2FoldChange >= 1 |
   res$log2FoldChange <= -1,]
res2foldpadj <- res2fold[res2fold$padj <= 0.05,]
```

23. Perform statistical analysis with edgeR library (*see* **Note 22**). Note that edgeR is also expected to use raw count data [14].

```
biocLite("edgeR")
library(edgeR)
countDF <- read.table("./results/countDF")
# Constructs DGEList object
y <- DGEList(counts=countDF, group=conds)
# Estimates common dispersion
y <- estimateCommonDisp(y)
# Estimates tagwise dispersion
y <- estimateTagwiseDisp(y)
# Computes exact test for the negative binomial
   distribution.
et <- exactTest(y, pair=c("Control", "Treatment"))
topTags(et, n=4)
edge <- as.data.frame(topTags(et, n=50000))
edge2fold <- edge[edge$logFC >= 1 | edge$logFC <=
   -1,]
edge2foldpadj <- edge2fold[edge2fold$FDR <= 0.05,]
```

24. Perform statistical analysis with edgeR using generalized linear models (glms) (*see* **Note 22**).

```
library(edgeR)
countDF <- read.table("./results/countDF")
```

```
# Constructs DGEList object
y <- DGEList(counts=countDF, group=conds)
# Filtering and normalization
keep <- rowSums(cpm(y)>1) >= 2; y <- y[keep,]
y <- calcNormFactors(y)
# Design matrix
design <- model.matrix(~0+group, data=y$samples);
   colnames(design) <- levels(y$samples$group)
# Estimates common dispersions
y <- estimateGLMCommonDisp(y, design, verbose=TRUE)
# Estimates trended dispersions
y <- estimateGLMTrendedDisp(y, design)
# Estimates tagwise dispersions
y <- estimateGLMTagwiseDisp(y, design)
# Fit the negative binomial GLM for each tag
fit <- glmFit(y, design)
# Contrast matrix is optional
contrasts <- makeContrasts(contrasts="AP3-TRL",
   levels=design)
# Takes DGEGLM object and carries out the likelihood
   ratio test
lrt <- glmLRT(fit, contrast=contrasts[,1])
edgeglm <- as.data.frame(topTags(lrt, n=length
   (rownames(y))))
# Filter on fold change and FDR
edgeglm2fold <- edgeglm[edgeglm$logFC >= 1 |
   edgeglm$logFC <= -1,]
edgeglm2foldpadj <- edgeglm2fold[edgeglm2fold$FDR
   <= 0.05,]
```

25. Heatmap of top-ranking DEGs (*see* **Note 23**).

```
biocLite("lattice")
biocLite("gplots")
library(lattice)
library(gplots)
y <- countDFrpkm[rownames(edgeglm2foldpadj)[1:20],]
colnames(y) <- targets$Factor
y <- t(scale(t(as.matrix(y))))
y <- y[order(y[,1]),]
levelplot(t(y), height=0.2, col.regions=colorpanel
   (40, "darkblue", "yellow", "white"), main=
   "Expression Values (DEG Filter: FDR 5 %, FC > 2)",
   colorkey=list(space="top"), xlab="", ylab="Gene
   ID")
```

4 Notes

1. Although it is possible to start with 100 ng total RNA or even less, a smaller quantity of starting material will yield suboptimal results, e.g., inefficient adapter ligation, lower library yield, and inaccurate quantification.
2. This heating step denatures the RNA and disrupts secondary structures.
3. Allow beads to fully pellet against magnetic stand. Do not allow the beads to dry.
4. Take care to remove all supernatant; fragmentation will be affected if there is contamination with residual washing buffer.
5. A shorter incubation time will result in longer fragments. Fragment size can be checked by running 1 μL of isolated RNA on Agilent BioAnalyzer.
6. The 42 °C incubation is for reverse transcription. The reverse transcriptase is deactivated during the 70 °C incubation.
7. If multiple samples will be sequenced in the same lane, use adapters with different index. Take care to avoid cross contamination.
8. Library size (including ds cDNA insert and adapters) can be adjusted by changing the volume ratio of bead:sample. Increased bead to DNA ratio recovers more shorter fragments, while keeping the long fragments. When only longer fragments are desired, user should try lower the ratio, thus to remove short fragments. However, lower ratios usually result in lower yields.
9. Too many PCR cycles may introduce bias (certain sequences get more representation in the RNA-Seq data) and higher duplication rate (same sequence get sequenced multiple times).
10. Bioanalyzer electropherograms of libraries described in this protocol usually show a peak starting from 200 bp to 500 bp, with a summit at around 260–280 bp. Pay attention to any adapter/dimer peaks, which show up approximately 60 bp or 120 bp if they are present. Adapters have negative effects for library quantification and cluster generation. If necessary, perform one more round of purification to remove adapters. Average library size can be estimated by Bioanalyzer software (refer to the manufacturer's documents).
11. It is highly recommended to have sufficient index diversity in a pooled library. Low diversity in the index sequences would result in unbalanced signals and low base-calling quality, which makes de-multiplexing difficult. Illumina provides software (Illumina Experiment Manager, http://support.illumina.com/sequencing/sequencing_software/experiment_manager/

downloads.html) to help check index compatibility for pooling.

12. Based on your operating system, there are Windows, Linux, and MAC versions available for downloading. Users can also download source code and build FastQC from scratch.
13. Detailed installation and setup instructions are available on the website http://www.bioinformatics.babraham.ac.uk/projects/fastqc/INSTALL.txt. FastQC is a java application. In order to run, your system must have a suitable Java Runtime Environment (JRE) installed.
14. Detailed information about basic FastQC operations can be found on http://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/2%20Basic%20Operations/. Documentation about analysis modules is available on http://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/3%20Analysis%20Modules/. It is important to notice that although the analysis results appear to give a pass/fail result, these evaluations must be taken in the context of what you expect from your library. A "normal" sample as far as FastQC is concerned is random and diverse. Users should treat the summary evaluations as pointers to their own concentration, if the experiments are expected to produce libraries that are biased in particular ways. An example report of good Illumina data can be found at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/good_sequence_short_fastqc.html, and an example report of bad Illumina data can be found at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/bad_sequence_fastqc.html. It is recommended to perform additional data trimming and filtering if needed.
15. Download human reference genome sequence (FASTA) from ftp://ftp.ensembl.org/pub/release-78/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.toplevel.fa.gz, unzip it and store it to the data subdirectory. Download the annotation (GFF) file from ftp://ftp.ncbi.nih.gov/genomes/Homo_sapiens/GFF/ref_GRCh38_top_level.gff3.gz, unzip it, and store it to the data subdirectory.
16. The file samples.txt is a tab-delimited file that records information about experiments. An example of the content in samples.txt is as following:

FileName	SampleName	Factor
Seq1.fastq	Sample1	Control
Seq2.fastq	Sample2	Control
Seq3.fastq	Sample3	Treatment
Seq4.fastq	Sample4	Treatment

17. In this command, *splicedAlignment* should be set to *TRUE* when reads are >=*50 nt* long. In this example, the read length is short and less than 50 nt, so *splicedAlignment* was set to *FALSE*.

18. An alternative way to calculate RPKM, you will see same results stored in *countDFrpkm* and *rpkmDFgene*

```
rpkmDFgene <- t(t(countDF[,-1]/countDF[,1] *1000)/
   colSums(countDF[,-1]) *1e6)
```

19. The *plotMDS* function from *edgeR* is a more robust method for this task.

20. The *colAg()* function is a convenience function for applying a variety of computations on any combination of column aggregates in a matrix or data frame.
    How to run the function:

```
myMA <- matrix(rnorm(100000), 10000, 10, dimnames=
   list(1:10000, paste("C", 1:10, sep="")))
```

```
colAg(myMA=myMA, group=c(1,1,1,2,2,2,3,3,4,4), myfct=
   mean)[1:4,]
```

21. In this example, four samples are assumed to be analyzed, of which, two are samples for controls, and the other two are group with treatment (the same as example samples.txt in **Note 16**). Users need change the code accordingly, based on their own experimental designs. For example, if the experiment has three groups, each having three replicates, then the command should like this:

```
countDFrpkm_mean <- colAg(myMA=rpkmDFgene, group=
   c(1,1,1,2,2,2,3,3,3), myfct=mean)
```

22. There are different ways to discover DEGs using statistical analysis. **Steps 19**, **20**, and **21** include commands to discover DEGs using three methods. Users can select all of the three methods, and compare the sets of three DEGs using visualization tool such as Venn diagram. Note that the final DEGs as obtained from **steps 19**, **20**, and **21** require twofold change or larger and an adjusted p-value less than 0.05. Users may change these parameters accordingly.

23. These commands generate a heatmap of top 20 DEGs resulting from **step 21**. Users can change related parameters to generate heatmaps resulting from **steps 19** and **20** as well.

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Chapter 10

DNA Microarray Analysis of Estrogen-Responsive Genes

Kathleen M. Eyster

Abstract

DNA microarray is a powerful, non-biased discovery technology that allows the analysis of the expression of thousands of genes at a time. The technology can be used for the identification of differential gene expression, genetic mutations associated with diseases, DNA methylation, single-nucleotide polymorphisms, and microRNA expression, to name a few. This chapter describes microarray technology for the analysis of differential gene expression in response to estrogen treatment.

Key words DNA microarray, Gene expression, Estrogen

1 Introduction

DNA microarray is a powerful, non-biased discovery technology that has been adapted to many applications. DNA microarray allows the analysis of the expression of thousands of genes at a time. In addition to analysis of differential gene expression [1–3], microarray technology has been applied to the identification of genetic mutations associated with diseases [4–6], DNA methylation [7, 8], single-nucleotide polymorphisms [9, 10], and microRNA expression [11, 12], and others [13].

This chapter describes microarray technology for the analysis of differential gene expression in response to estrogen treatment. In this application, DNA sequences are spotted on treated microscope slides to form the arrays. Total RNA extracted from vehicle-treated control and from estrogen-treated cells or tissues is used as the starting material. The RNA is reverse transcribed to complementary DNA (cDNA) and the second strand of cDNA is synthesized. The double-stranded cDNA is purified and used as a template for the synthesis of antisense RNA (aRNA, also called complementary RNA, cRNA). The aRNA is labeled with the incorporation of biotin-UTP into the aRNA during aRNA synthesis. The aRNA is hybridized with the microarray slide overnight. Post-hybridization processing steps incorporate washing and incubation of the

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microarrays with streptavidin-Alexa fluor 647 to complete the labeling of the samples on the arrays. The washed microarrays are scanned and pixel intensity is translated to gene expression. Data are associated with gene names, and statistical comparisons are made between controls and treated samples.

2 Materials

1. RNAlater (Ambion) or similar RNA preservation reagent.
2. Four-week-old female rats.
3. Vehicle (2-hydroxypropyl-β-cyclodextrin) and estrogenic compounds (e.g., ethinylestradiol, 0.15 mg/kg) for gavage treatment.
4. Dissecting tools: Scissors, scalpels, forceps.
5. Tri reagent (Molecular Research Center).
6. 2 mL microfuge tubes.
7. Polytron homogenizer with 7 mm probe (Kinematica).
8. Bromochloropropane.
9. Sodium acetate, 3 M.
10. RNase Zap (Ambion) or RNase Away (Life Technologies).
11. Diethylpyrocarbonate (DEPC)-treated water: Mix 999 mL purified water (18.2 MΩ resistivity) with 1 mL DEPC. Mix on a stir plate overnight. Autoclave the water to destroy the toxicity of the DEPC. Store at room temperature.
12. Silica gel membrane RNA purification spin columns such as the RNeasy columns (Qiagen) or equivalent. The RNeasy Kit contains silica gel columns, binding and washing buffers, and nuclease-free water.
13. RLT buffer: Add 10 μL β-mercaptoethanol to 1 mL of the RNeasy lysis buffer/binding buffer RLT before use.
14. RPE buffer: Add 44 mL ethanol to 11 mL washing buffer RPE concentrate before use.
15. 100 % ethanol.
16. RNase-free DNase (Qiagen) stock solution: Add 550 μL nuclease-free water to the vial of lyophilized DNase and mix gently.
17. RNase-free DNase (Qiagen) working solution: Add 10 μL DNase stock solution to 70 μL RNase-free DNase dilution buffer for each sample and mix gently.
18. Agilent Bioanalyzer and Nano 6000 LabChip and reagents for RNA analysis.
19. Gel matrix for the Agilent RNA 6000 Nano LabChip: Mount a spin filter cartridge in a microfuge tube and add 550 μL of

the gel matrix to the cartridge. Centrifuge at 1500×*g* for 10 min at room temperature. Store the gel in 65 µL aliquots at 4 °C for up to 1 month.

20. MessageAmp II-Biotin *Enhanced* Kit (Ambion): Contains oligo dT primer, ArrayScript reverse transcriptase, RNase inhibitor, 10× first-strand buffer, dNTP mix, 10× second-strand buffer, DNA polymerase, RNase H, T7 enzyme mix, T7 10× reaction buffer, biotin-NTP mix, nuclease-free water, cDNA filter cartridges, aRNA filter cartridges.
21. Reverse transcription master mix: For each sample/each microarray, mix 1 µL nuclease-free water, 1 µL T7 oligo dT primer, 2 µL 10× first-strand buffer, 4 µL dNTP mix, 1 µL RNase inhibitor, and 1 µL ArrayScript reverse transcriptase. Increase the volume by 5 % for pipetting overage.
22. Second-strand master mix: For each sample/each microarray, mix 63 µL nuclease-free water, 10 µL second strand buffer, 4 µL dNTP mix, 2 µL DNA polymerase, and 1 µL RNase H plus 5 % volume overage.
23. Wash buffer for cDNA and aRNA spin column purification: add 24 mL ethanol to the entire bottle of wash buffer before using.
24. In vitro transcription (IVT) master mix: For each sample/each microarray, mix 4 µL T7 10× reaction buffer, 4 µL T7 enzyme mix, and 12 µL Biotin-NTP mix, plus 5 % volume overage. (The Biotin-NTP mix contains ATP, GTP, CTP, and biotin-11-UTP.)
25. DNA microarrays spotted on treated microscope slides, such as those from Microarrays, Inc., Phalanx Biotech, or CodeLink.
26. Adhesive hybridization chamber (Microarrays, Inc.).
27. Prehybridization buffer: 5× SSC, 0.1 % SDS, 0.1 % bovine serum albumin (BSA).
28. Hybridization buffer: 40–70 % deionized formamide, 10× SSC, 0.2 % SDS, 0.02 % sheared salmon sperm DNA.
29. Post-hybridization wash buffer #1: 0.75 × TNT buffer (75mM Tris–HCl, pH 7.6, 112.5 mM NaCl, 0.0375 % Tween-20).
30. Streptavidin Alexa 647 fluor: for stock solution, dissolve 1 mg Streptavidin Alexa 647 in 1 mL 1 × PBS, pH 7.4. Aliquot the stock solution and freeze at –80 °C. For working solution, dilute 6.8 µL of stock solution in 3.393 mL of TNB buffer for each microarray. TNB buffer: 100 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.5 % NEN Blocking reagent, filtered through 0.88 micron filter.
31. Post-hybridization wash buffer #2: 1 × TNT buffer (100 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05 % Tween-20).
32. Post-hybridization wash buffer #3: 0.1× SSC, 0.05 % Tween-20 at room temperature.

3 Methods

3.1 Tissue Procurement

All experiments utilizing animals must have prior approval by the appropriate institutional ethics committee for animal usage, such as the Institutional Animal Care and Use Committee.

1. Ovariectomize 4-week-old female rats (e.g., Sprague Dawley or strain of choice); allow 2 weeks for recovery of the animals from surgery.
2. Treat the animals with vehicle or ethinylestradiol for 3 weeks (*see* **Note 1**).
3. Euthanize the animals and rapidly dissect the estrogen-responsive tissues of interest (e.g., uterus, mesenteric arteries, liver) (*see* **Note 2**).
4. Place the tissues in the RNA preservation reagent, RNAlater, and store at −80 °C until processed for RNA extraction, or proceed directly to RNA extraction.

3.2 RNA Hygiene and RNA Extraction

High-quality purified RNA is critical for applications such as DNA microarray or real-time RT-PCR. Therefore, care must be taken to prevent degradation of RNA by RNases and to remove contaminating substances such as genomic DNA from the RNA.

1. Clean the surfaces of all instruments, racks, bench tops, and any other materials that will be used in the extraction of RNA with an RNase degradation reagent such as RNase Zap. Spray surfaces with RNase Zap and rinse with DEPC-treated water.
2. Wear gloves at all times and periodically change gloves and/or clean gloves with RNase Zap.
3. Before using the Polytron homogenizer, soak the probe in RNase Zap and then run the probe in a graduated cylinder (that has been cleaned with RNase Zap) full of DEPC-treated water. Dry the probe before homogenizing each sample.
4. Use nuclease-free microfuge tubes and pipette tips.
5. To extract total RNA from tissue stored in RNAlater, blot the reagent from the surface of the tissue. Weigh the tissue; the capacity of the RNA purification columns is approximately 30 mg tissue weight.
6. Mince the tissue into fine pieces with two scalpels under Tri reagent.
7. Place 1 mL of Tri reagent and the minced tissue in a 2 mL microfuge tube or a 12×75 mm nuclease-free test tube (*see* **Notes 3** and **4**).
8. Homogenize the tissue using the 7 mm probe for 10 s three times with 30-s rest periods between each homogenization run (*see* **Notes 5**–**7**).

9. Clean the homogenizer probe carefully between each sample. Rinse the probe with DEPC-treated water then repeat **step 3** of Subheading 3.2.
10. When all of the samples have been homogenized, immediately clean the homogenizer probe very thoroughly. Tri reagent is corrosive and will destroy the probe if it is allowed to remain on the probe.
11. Add 200 µL bromochloropropane and 60 µL 3 M sodium acetate to each homogenized sample.
12. Shake the samples well to mix; the clear pink Tri reagent will turn milky. Incubate on ice for 15 min.
13. Centrifuge the samples for 5 min at 8000 × *g* to separate the phases.
14. Transfer the top (clear, aqueous) layer containing the RNA to a nuclease-free 12 × 75 test tube. Avoid the layer of genomic DNA at the interface between the two layers (*see* **Note 8**).
15. Add 1 mL RLT buffer to the aqueous phase containing the RNA (approximately 450 µL volume).
16. Add 1.2 mL 100 % ethanol to the sample and mix.
17. Transfer a 700 µL aliquot of the sample to a silica gel membrane spin column (RNeasy) that has been placed in a microfuge collection tube and centrifuge for 30 s at 10,000 × *g*. Discard the flow through and repeat until all of the sample has been passed through the column (*see* **Note 9**).
18. Wash the column with 350 µL of RW1 wash buffer from the extraction kit.
19. Perform an on-column DNase treatment: add 80 µL of RNase-free DNase working solution directly to the gel membrane of the column.
20. Incubate at room temperature for 15 min.
21. Add 350 µL of wash buffer RW1 to the column; centrifuge the column for 30 s at 10,000 × *g*. Discard the flow through.
22. Add 500 µL RPE buffer to the column and centrifuge the column for 30 s at 10,000 × *g*; discard the flow through.
23. Repeat the wash step with 500 µL RPE buffer.
24. Dry the membrane by centrifuging the column at 14,000 × *g* for 2 min without adding any buffer.
25. Move the column to a new, dry, well-labeled 1.5 mL microfuge collection tube.
26. Add 50 µL nuclease-free water (not DEPC-treated water) to the center of the membrane.
27. Incubate for 10 min at room temperature.

28. Elute the total RNA by centrifuging the column for 1 min at 10,000 × *g*. Save the flow-through; it contains the RNA.
29. Repeat **steps 26** and **27** of Subheading 3.2, eluting the second aliquot of RNA into the same collection tube (*see* **Note 10**).
30. Discard the column.
31. Store the purified RNA at −80 °C until used for DNA microarray.

3.3 Analysis and Quantitation of RNA

1. Assess the quality and quantity of the RNA using the Agilent RNA 6000 Nano LabChip (or the RNA 6000 Pico Chip if concentration is expected to be low) (*see* **Note 11**).
2. Remove kit reagents from storage at 4 °C 30 min before use and place them in an enclosed box to allow them to come to room temperature in the dark: dye concentrate, marker mixture, and one 65 μL aliquot of the gel matrix (from **item 18** of Subheading 2). Make sure that the water bath is turned on and set at 70 °C.
3. Mix the dye concentrate by vortexing, and then centrifuge for 5–10 s at 10,000 × *g*.
4. Mix 1 μL of dye concentrate with the aliquot of gel matrix and vortex well. Centrifuge for 10 min at 13,000 × *g*.
5. Incubate the RNA ladder and samples at 70 °C for 2 min to denature and chill on ice.
6. Pipet the gel matrix into the specified wells on the chip and pressurize. Pipet the marker mixture, RNA ladder, and samples according to the manufacturer's directions.
7. Use the vortex adaptor to vortex the chip at 2400 rpm for 1 min.
8. Read the chip in the Agilent Bioanalyzer and record the RNA concentration and RNA integrity number for each sample.

3.4 Synthesis and Labeling of aRNA

The following reactions will result in the synthesis of enough antisense RNA (aRNA, also called complementary or cRNA) for one microarray from each sample processed.

1. The manufacturer of your microarray platform will indicate the ideal starting RNA concentration for processing samples for their arrays. This is typically 0.2–2.0 μg total RNA. For vehicle- and estrogen-treated samples, calculate the volume containing the appropriate quantity of total RNA (*see* **Note 12**). If the volume is greater than 10 μL, reduce the volume to 10 μL using a vacuum concentrator (e.g., SpeedVac). Add nuclease-free water to raise the volume to 10 μL if the starting volume is less than 10 μL.
2. Add 10 μL reverse transcription master mix to each sample containing 10 μL of total RNA.

3. Place the samples in a heat block or thermocycler at 42 °C for 2 h. Remove from heat and place on ice. This reaction synthesizes the first strand of cDNA.
4. Add 80 μL of second-strand master mix to each reaction tube.
5. Incubate at 16 °C for 2 h. Use a thermocycler or heat block in which the temperature does not fluctuate.
6. Place the samples on ice. The sample now contains double-stranded cDNA.
7. Heat nuclease-free water to 55 °C (24 μL per sample) in preparation for cDNA purification.
8. To purify the double-stranded cDNA on the cDNA filter cartridges from the kit, add 250 μL cDNA binding buffer to each 100 μL sample of double-stranded cDNA.
9. Mix by pipetting, and then transfer each sample of cDNA onto a spin column filter cartridge.
10. Centrifuge at 10,000 × *g* for 1 min. Discard the flow-through.
11. Pipet 500 μL of wash buffer onto each filter cartridge and centrifuge at 10,000 × *g* for 1 min.
12. Discard the flow-through.
13. Centrifuge the filter cartridge without adding buffer at 10,000 × *g* for 1 min.
14. Transfer the cartridge to a new well-labeled collection tube.
15. Add 22 μL of 55 °C nuclease-free water (preheated in **step 7** of Subheading 3.4) to the center of each spin column.
16. Incubate at room temperature for 2 min.
17. Centrifuge the cartridge at 10,000 × *g* for 1 min. This step will deposit the purified double-stranded cDNA in the eluate.
18. Add 20 μL IVT Master Mix to the 20 μL of purified double-stranded cDNA from **step 17** of Subheading 3.4.
19. Incubate the IVT reaction for 14 h at 37 °C. This reaction synthesizes biotin-labeled aRNA (*see* **Note 13**).
20. Add 60 μL nuclease-free water to each sample to stop the aRNA synthesis reaction.
21. Heat nuclease-free water to 55 °C (200 μL per sample) in preparation for purification of the aRNA.
22. Add 350 μL of aRNA-binding buffer to the aRNA sample.
23. Add 250 μL of molecular grade 100 % ethanol to one sample. Mix by gently pipetting up and down. Do not vortex and do not centrifuge as this will precipitate the aRNA and reduce its recovery. Immediately transfer the entire volume of the sample to an aRNA filter cartridge that has been placed into a collection tube. Perform the entire step of adding ethanol, mixing,

and transfer to a filter cartridge of one sample before moving on to the next sample.

24. When all samples have been mixed with ethanol and transferred to their respective filter cartridges, centrifuge the samples at 10,000 × *g* for 1 min.
25. Discard the flow-through.
26. Wash each filter cartridge with 650 μL Wash buffer; centrifuge at 10,000 × *g* for 1 min.
27. Discard the flow-through and dry the cartridge by centrifuging at 10,000 × *g* for 1 min without the addition of any buffer.
28. Add 200 μL 55 °C nuclease-free water to each cartridge filter. Incubate the samples in the 55 °C heating block for 10 min.
29. Elute the aRNA from the cartridge by centrifugation at 10,000 × *g* for 1.5 min (*see* **Note 14**).
30. Calculate the concentration of the aRNA by reading the absorbance of the sample at 260 nm in a spectrophotometer. To calculate the aRNA concentration, use the equation

$$A_{260} \times \text{dilution factor} \times 40\ \mu\text{g} / \text{mL} \times 0.001\ \text{mL} / \mu\text{L} = \text{mg aRNA} / \mu\text{L}.$$

31. Store the purified aRNA in aliquots at −80 °C or proceed to hybridization.

3.5 Hybridization

1. Calculate the volume of aRNA containing 10 μg of biotinylated aRNA. If this volume is greater than 20 μL, reduce the volume using a vacuum concentrator. If the volume is less than 20 μL, raise the volume with nuclease-free water.
2. Incubate the 20 μL containing 10 μg of biotinylated aRNA with 5 μL of fragmentation buffer for 20 min at 94 °C (*see* **Note 15**).
3. Chill on ice for a minimum of 5 min.
4. Thoroughly mix the 25 μL of fragmented aRNA with 185 μL of hybridization buffer.
5. Denature the aRNA by incubating at 90 °C for 5 min.
6. Chill the samples on ice for 5 min before loading the microarray slides. Load all of the microarray slides within 30 min of completing the denaturation step.
7. Some microarrays require a prehybridization step. Prehybridize the microarrays for 30 min by incubating the slides with gentle rotation in prehybridization buffer that has been heated to 55 °C.
8. Rinse the slides with purified distilled water; to avoid allowing the warm prehybridization buffer from drying on the slide, place the slide holder in the sink and carefully pour the water over the slides.

9. Wash the slides four more times, 1 min each wash, with purified distilled water.
10. Dry the slides by centrifugation using a slide holder that fits into a 96-well plate rotor. Centrifuge for 3 min at 1000 × *g*. The prehybridization should be initiated so that the prehybridization steps and the denaturation of aRNA are completed at the same time.
11. Attach a flexible adhesive cover slip (called a sealed hybridization chamber) to the microarray slide. Line up the adhesive gasket with the edges of the slide and make sure that the gasket is adhered at all edges.
12. Load 200 μL of the hybridization solution containing labeled aRNA through one of the ports in the hybridization chamber.
13. Seal the ports with the seals that are supplied with the chambers.
14. Incubate the microarrays at 42 °C for 14–18 h in a shaking incubator set at 300 rpm or hybridization oven.
15. Place a container of 240 mL 0.75× TNT buffer in a 42 °C water bath during the hybridization incubation to ensure that it reaches temperature. This warmed buffer will be used for the first washing step after the hybridization.

3.6 Post-hybridization Processing

1. Prepare two containers of room temperature 0.75× TNT.
2. Remove the microarray slides from the incubator.
3. Submerge each slide in a container of 0.75× TNT and peel off the flexible cover slip.
4. Place the slide into a rack that is submerged in the second container of 0.75× TNT and continue until the cover slips have been removed from all microarrays.
5. Move the rack of microarray slides into the container of 42 °C 0.75× TNT and incubate at 42 °C for exactly 1 h. This step will remove non-hybridized aRNA.
6. Place the rack of microarray slides into a container containing streptavidin-Alexa fluor 647. Incubate at room temperature for 30 min in the dark. (Alexa 647 does not photobleach as rapidly as many other fluorescent dyes.)
7. Prepare four containers with room temperature 1× TNT buffer. Move the rack of microarray slides from the fluor into the first container and incubate for 5 min.
8. Move the rack from one container to the next, incubating for 5 min in each container of 1× TNT.
9. For a final wash, use 0.1× SSC/0.05 % Tween for 30 s. Gently agitate the rack of slides during this final wash step.
10. Place the rack of slides in a 96-well plate rotor and dry the slides by centrifugation for 3 min at 1000 × *g*.

11. Use an opaque slide box to store the slides until they can be scanned (preferably as soon as possible after completion of the washing and drying of the slides).
12. Use a GenePix Pro 4000B scanner or equivalent to scan the slides. Turn on the scanner and open the GenePix Pro software 15 min before use.
13. If the scans identify regions of high background on the slides, repeat the final washing step in 0.1× SSC/0.05 % Tween for 30 s, dry the slides by centrifugation, and scan the slides again.
14. Analyze the microarray data using a specialized microarray analysis software program such as GeneSpring (Figs. 1 and 2). Freeware programs are also available for microarray analysis. The software performs statistical analyses to identify differentially expressed genes (Fig. 3). Make sure to apply multiple testing correction statistical tests to account for the many comparisons that are performed in this type of experiment.
15. Perform confirmatory experiments using complementary technologies. Real time RT-PCR [14] or in situ hybridization [15, 16] can be used to confirm differential gene expression. Western blot [17, 18], enzyme-linked immunosorbent assay (ELISA) [19, 20], or immunohistochemistry [16, 21, 22] can be used to confirm differential expression of the cognate proteins of differentially expressed genes.
16. Deposit the raw data and the experimental details from each microarray experiment in a public database at the time of publication in compliance with the recommendations of the

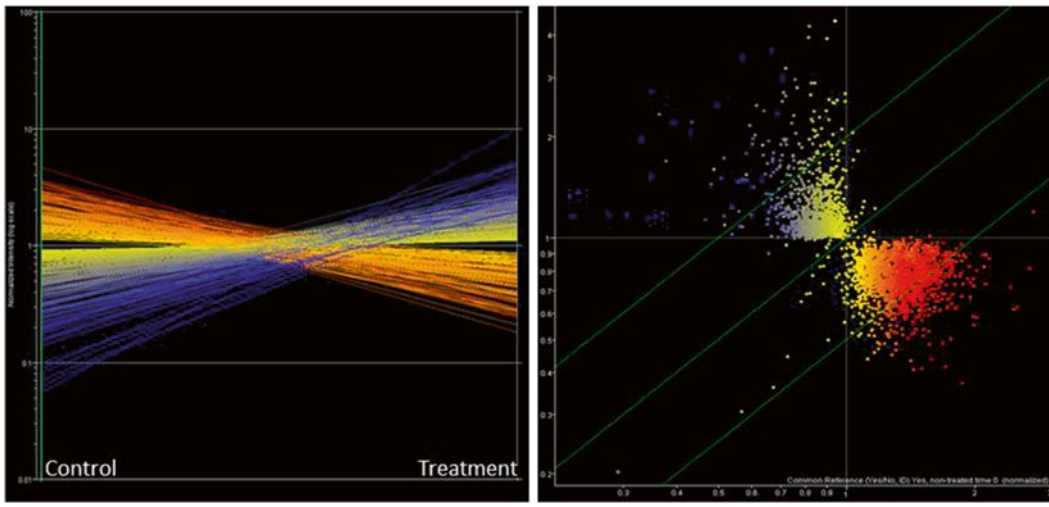

Fig. 1 DNA microarray data can be illustrated in a number of ways. In the *left panel*, each *line* represents one gene and compares expression in control versus treatment. In the *right panel*, each *dot* represents one gene. The location of each *dot* on the matrix is determined by the expression of the gene in control vs. treatment

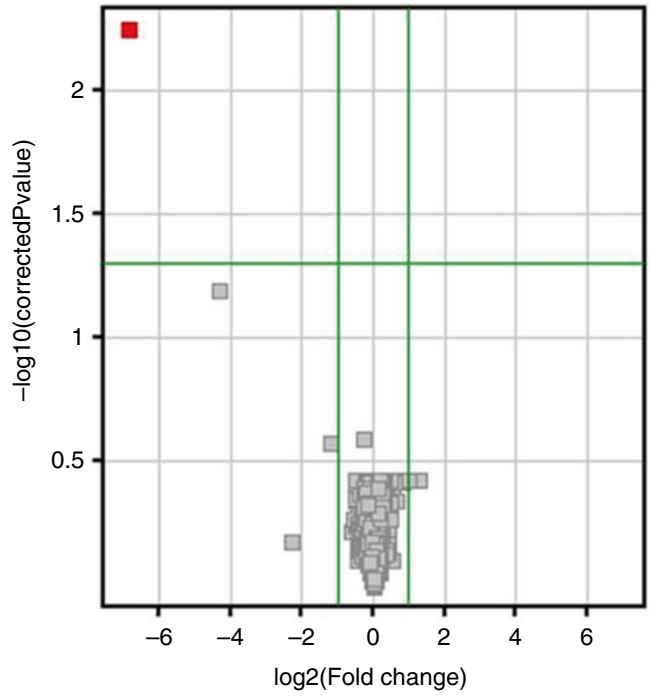

Fig. 2 DNA microarray data can also be represented by a volcano plot. In this data set, only one gene met the cutoff criteria of $p < 0.05$ and greater than two-fold change in expression

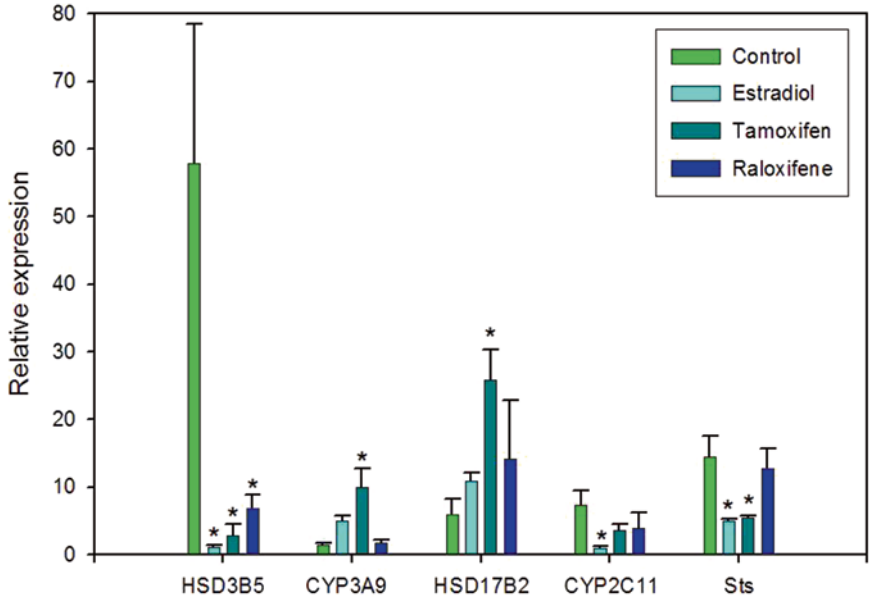

Fig. 3 These microarray data were taken from an experiment in which ovariectomized Sprague-Dawley rats were treated by gavage for 2 days with vehicle (2-hydroxypropyl-beta-cyclodextrin), ethinylestradiol (0.15 mg/kg), tamoxifen (3 mg/kg), or raloxifene (3 mg/kg). Total RNA was extracted from the liver and gene expression signatures were analyzed by DNA microarray. *Asterisks* denote significant difference from control (ANOVA). *HSD* hydroxysteroid dehydrogenase, *CYP* cytochrome P450, *Sts* steroid sulfatase

Minimum Information About a Microarray (MIAME) standards [23]. In the USA, the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo) maintains the Gene Expression Omnibus (GEO) database for deposition of microarray and related data for this purpose.

4 Notes

1. Treatment of the animals for 3 weeks is designed to mimic the type of long-term treatment that is administered to women.
2. Be careful not to place too large a piece of tissue into too small a volume of RNAlater as it is possible to overwhelm the RNA protection capacity of the reagent.
3. The 7 mm probe fits readily into these sizes of tubes but check your tubes before beginning. The bottom shape of some microfuge tubes may not be compatible with the probe. Also use caution as the 2 mL microfuge tubes are near capacity when the probe is introduced into 1 mL of volume.
4. If samples are especially small, homogenize the sample in 600 μL of Tri reagent, then rinse the probe in 400 μL of fresh Tri reagent and combine the two aliquots. The rinsing step rescues the drop of Tri reagent containing RNA that gets caught in the end of the probe and can substantially increase the recovery of RNA from small samples.
5. For soft tissue such as liver, three homogenization pulses will thoroughly disperse the tissue. For tissue with more muscle or connective tissue, more pulses of homogenization may be necessary to completely disperse the tissue. For especially difficult tissues, soak the minced tissue in Tri reagent for 1 h before beginning the homogenization step.
6. The RLT buffer in the RNeasy kits is a lysis buffer that can be used in place of Tri reagent for the homogenization step. Another alternative for sample homogenization is the QIAShredder column (Qiagen) which performs a homogenization step on the columns.
7. If using cultured cells for RNA extraction, remove the culture medium from the cells and rinse the cells with phosphate buffered saline. Add 1 mL Tri reagent to the cells in a T25 flask; the cells essentially "melt" in Tri reagent. If necessary, scrape the cells and transfer the Tri reagent containing the cell debris into vials. If pooling cells from several flasks, the milliliter of Tri reagent can be transferred from one flask to the next to keep the volume of Tri reagent at just 1 mL. Store the Tri reagent containing cellular material at −80 °C or proceed directly to RNA extraction.

8. The protein from the sample can be extracted from the bottom layer after this centrifugation step [24]. However, some proteins do not survive the protein extraction protocol well.
9. The advantage of using the RLT lysis buffer for the homogenization step (as in **Note 6** above) is that the sample can be placed directly onto the RNA purification column without the intervening centrifugation step. The disadvantage is that all of the genomic DNA and protein are still in the sample and go onto the column with the RNA. The columns are designed to bind the RNA while the DNA and protein pass through; however, the more genomic DNA that remains in the sample, the more likely it is that some will contaminate the RNA.
10. We observe better recovery of RNA if we elute twice with 50 μL volume each time than if we elute with a smaller volume. If the RNA is too dilute in this elution volume, it can be concentrated with a vacuum device such as the SpeedVac concentrator. We have found the silica gel membrane columns to yield more consistent RNA samples than the phenol/chloroform/isoamyl alcohol method [24]. However, not all of the silica gel columns are of equal quality and cheaper columns may compromise the quantity and quality of recovered RNA.
11. The quantity and quality of RNA can be assessed by spectrophotometry and agarose gel electrophoresis [24]. However, the Agilent Bioanalyzer has the advantage of producing both the quantity measurement and image of RNA bands while consuming only 1 μL of sample. At the same time, the Bioanalyzer provides an expression of the quality of the RNA sample called the RNA integrity number (RIN). Samples with an RIN number less than 8 should not be used for DNA microarray analysis.
12. If the quantity of total RNA available for the reaction is less than 0.2 μg or if you have reason to believe that a sample may not amplify efficiently, then carry out two rounds of aRNA amplification. Use the MessageAmp II kit to synthesize aRNA using the same reactions as described in Subheading 3.4 except do not incorporate biotin label into the aRNA in the final synthesis step. (Biotinylated aRNA cannot be used to synthesize cDNA.) Prime the single-stranded aRNA from the first reaction with random primers for synthesis of a second round of first-strand cDNA. Synthesize the second strand of cDNA using T7 oligo(dT) as the primer, and use the double-stranded cDNA for the synthesis of aRNA with the incorporation of biotin-11-UTP as described in Subheading 3.4.
13. In the initial reverse transcription reaction, the T7 oligo dT primer contains oligo (dT) sequence plus the T7 promoter sequence. The oligo (dT) sequence binds to the poly-A tail of messenger RNAs in the sample. During the reverse transcription

reaction, the T7 promoter sequence is added to each new cDNA transcript so it is present in the double-stranded cDNA when the second strand reaction is complete. The T7 enzyme in the IVT reaction is an RNA polymerase that will transcribe all transcripts that have the T7 promoter at its 5′ end, so it will transcribe the double-stranded cDNA that was synthesized in the reactions described here.

14. The synthesis of biotin-labeled aRNA from total RNA is a fairly long process. The process can be stopped and the samples frozen at −80 °C for continuation later after the second-strand synthesis reaction (**step 6** of Subheading 3.4), after purification of double-stranded cDNA (**step 17** of Subheading 3.4), or after purification of aRNA (**step 30** of Subheading 3.4).
15. The aRNA is fragmented before the hybridization step to reduce secondary and tertiary RNA structure which has been shown to interfere with RNA hybridization to probes on the microarray. The fragmentation is carried out by metal-induced hydrolysis.

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Chapter 11

Shotgun Proteomics Analysis of Estrogen Effects in the Uterus Using Two-Dimensional Liquid Chromatography and Tandem Mass Spectrometry

Eduardo A. Callegari

Abstract

Shotgun (gel-free) proteomics is a useful approach to perform identification and relative quantification of protein in complex mixtures such as tissue homogenates, biological fluids, cell lysates, and extracellular proteins. Incorporation of separative and analytical techniques such as two-dimensional liquid chromatography at nanoscale (2D-nanoLC) coupled to tandem mass spectrometry (MS/MS analysis) into the shotgun protocol provides an excellent strategy. This chapter describes the application of the shotgun proteomics protocol to evaluate the identity and expression analysis of proteins from rat uterus after estrogen (ethinylestradiol) treatment. The steps of the protocol involve sample preparation (digestion), 2D-nanoLC-MS/MS analysis, and shotgun proteomics analysis including bioinformatics tools for data conversion, organization, and interpretation.

Key words Proteomics, "Shotgun" proteomics, Liquid chromatography, Mass spectrometry, Proteins, Peptides, Rat, Uterus, Estrogen

1 Introduction

The effects of ovarian hormones on the uterus of rats have been well studied. Actions mediated by estrogens contribute to the adult reproductive life through the cycles of proliferation and degeneration of the uterine tissue [1–3, 16]. The large-scale identification of the proteins that are involved directly and/or indirectly with estrogen actions is a useful tool that allows understanding the complexity of such hormonal responses [4]. One of the best approaches to resolve the proteins in complex mixtures present in tissues such as the uterus is the use of "shotgun" proteomics protocols, also known as "gel-free" proteomics [4, 5]. This strategy permits the investigator to perform protein identification, characterization, and relative

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quantification from tissue homogenates, cell lysates, biological fluids, and similar sample types [4–7]. The technique uses a bottom-up strategy in which the proteins are digested using a protease, such as trypsin, that recognizes specific cleavage sites. The tryptic digest peptides that are obtained are separated and analyzed using tandem mass spectrometry, and used to identify the most probable protein/s present in the sample through a combination of specific bioinformatics tools and protein databases [8].

The combination of protein digestion with trypsin in solution, separation of the peptides obtained through two-dimensional liquid chromatography at nanoscale (2D-nanoLC) [9], and analysis of mass by tandem mass spectrometry (MS and MS/MS analysis) allows improvement of the shotgun proteomics protocol by increasing the number of proteins identified from complex mixtures, extends the molecular weight (MW) range, covers the entire range of isoelectric points (pI) from pH 0 to 14, and resolves the protein dynamic range to 10^5. Moreover, it can provide an approximation of the protein relative abundance when it is combined with protocols such as "label free" technology through spectral counting strategy [10, 11]. Also, the gel-free method can be used as a complementary approach to two-dimensional gel electrophoresis (2DGE). An example of the application of shotgun proteomics for the analysis of proteins obtained from uterine tissue of rats ovariectomized with and without treatment with estrogen (ethinylestradiol) is provided in this chapter. The proteins extracted from the uterus were digested in solution using trypsin and the peptides analyzed by a 2D-nanoLC-MS/MS protocol through a 2D-nanoAcquity UPLC with dilution coupled to a quadrupole time-of-flight (Q-Tof) Synapt G1 MS (Waters) [6, 7]. The peptide and fragment masses were submitted against the Swiss Protein database with taxonomy filter for *Rattus norvegicus* database using a combination of bioinformatics tools that included MassLynx v4.1, ProteinLynx Global Server v3.0 (Waters, Milford, MA) and Mascot + Mascot Daemon toolbox v2.5 local license (www.matrixscience.com). The post-mass spectrometry analysis to organize the proteins into different sets and the relative abundance data were performed using a ProteoIQ software v2.7 (www.premierbiosoft.com). A brief summary of the entire protocol is shown in the schematic flow chart of Fig. 1. From a total number of 800 proteins identified, 498 were expressed in common between the control and estrogen-treated groups, 182 proteins were exclusively present in the control, and 162 proteins in the estrogen-treated group (Fig. 2). The 20 most differentially expressed proteins observed, either up- or downregulated by estrogen, have been previously reported in the literature [12–15].

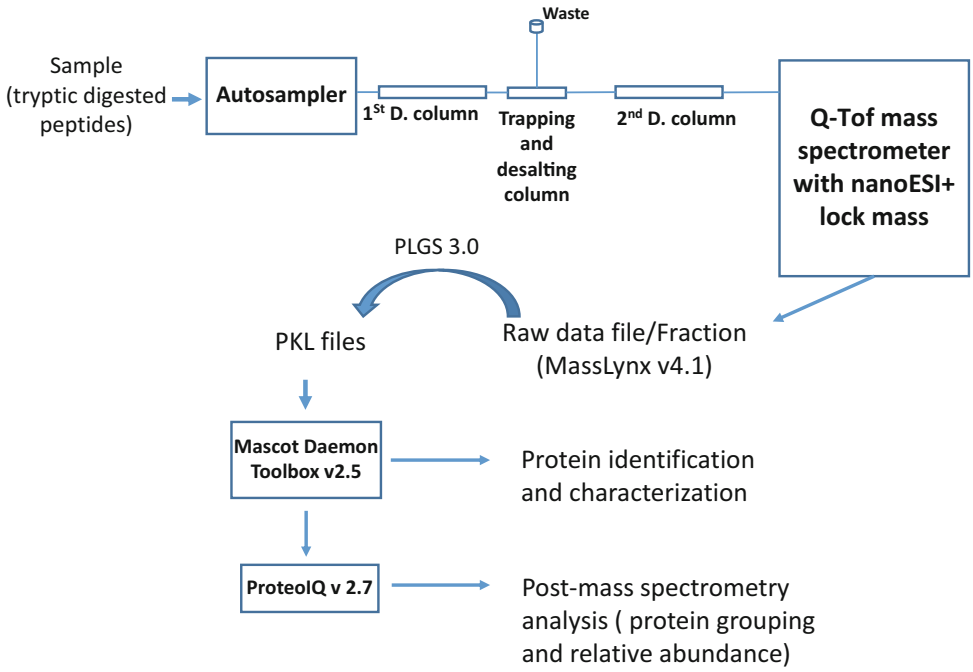

Fig. 1 Schematic representation of the "shotgun" proteomics protocol applied to the sample analysis through the 2D-nanoLC-MS/MS analysis (1st D. = first dimension, 2nd D. = second dimension)

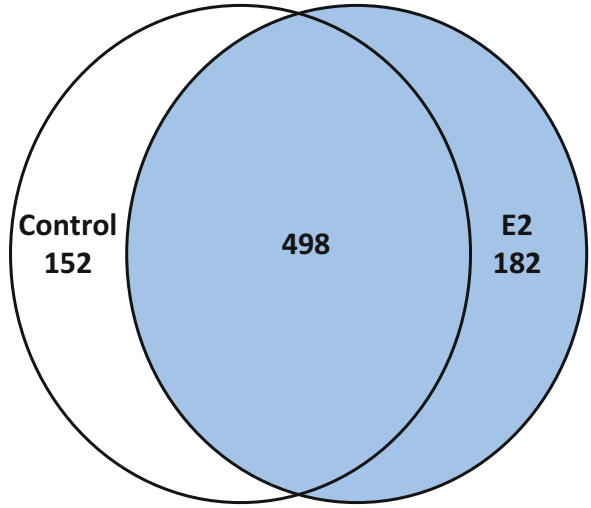

Fig. 2 Venn diagram representing the number of unique proteins identified in the uterus from vehicle control-treated and ethinylestradiol-treated rats, and those proteins common to both groups. The *numbers* in each Venn diagram and/or intersections correspond to the proteins analyzed by 2D-nanoLC-MS/MS analysis

2 Materials

Prepare all solutions using ultrapure water (purified deionized water with a resistivity of 18 MΩ cm at 25 °C), and LC-MS-grade reagents.

1. Extract protein samples from animals or cells that had been treated with vehicle or with estrogen (*see* **Note 1**).
2. Reaction buffer stock solution: 250 mM ammonium bicarbonate. Mix 98.0 mL water and 1.977 g ammonium bicarbonate. Adjust the pH with 0.1 M ammonium hydroxide to pH 8.0. Raise the volume to 100 mL.
3. Reaction buffer working solution (AB): 100 mM ammonium bicarbonate, 5 % acetonitrile.
4. Reducing agent: 50 mM dithiothreitol (DTT). Add 7.7 mg DTT to 1 mL water. Store in aliquots at –20 °C until use.
5. Alkylating agent: 100 mM iodoacetamide. Add 18.5 mg of iodoacetamide to 1 mL water (*see* **Note 2**).
6. Trypsin resuspension buffer: 50 mM acetic acid.
7. Sequencing-grade modified trypsin: 1 μg/μL trypsin. Dissolve 20 μg trypsin in 20 μL trypsin resuspension buffer. Keep on ice until use (*see* **Note 3**).
8. Sample injection and mobile-phase buffer stock solution: 200 mM ammonium formate, pH 10. Place 450 mL of purified water in a 500 mL beaker. Using a pipette, add 6.95 mL 28 % ammonium hydroxide and mix well. Carefully add 0.81 mL formic acid and mix well. Adjust the solution to pH 10 using ammonium hydroxide or formic acid in water. Add water to bring the volume to 500 mL (*see* **Note 4**).
9. Sample injection buffer working solution: 100 mM ammonium formate, pH 10. Mix one volume of 200 mM ammonium formate, pH 10, with one volume of purified water.
10. First-dimension mobile phases for liquid chromatography, solution A1: 20 mM ammonium formate, pH 10. Prepare 400 mL solution A by mixing 360 mL of purified water with 40 mL 200 mM ammonium formate, pH 10. Degas the solvent with an ultrasonic liquid processor (sonicator) for 5 min or with Argon gas.
11. First-dimension mobile phases for liquid chromatography, solution B1: 100 % acetonitrile. Degas the solvent with a sonicator for 5 min or with Argon gas.
12. Second-dimension mobile phases for liquid chromatography, solution A2: 0.1 % formic acid in water. Add 400 μL formic acid to 400 mL water. Degas the solution for 5 min in a sonicator.

13. Second-dimension mobile phases for liquid chromatography, solution B2: Acetonitrile with 0.1 % formic acid. Add 400 μL formic acid to 400 mL acetonitrile. Degas the solution for 5 min in a sonicator.
14. First-dimension column 1: XBridge BEH130 C18, 5 μm, 300 μm × 50 mm NanoEase reversed phase column (Waters).
15. First-dimension column 2 for trapping and desalting online: 180 μm × 20 mm, 5 μm Symmetry C18 nanoAcquity UPLC reversed phase trap column (Waters).
16. Second-dimension column: BEH130C18 1.7 μm, 100 μm × 100 mm nanoAcquity UPLC reversed phase column (Waters).
17. Liquid chromatography instrumentation: 2D-nanoAcquity Ultra Performance Liquid Chromatography (UPLC) with dilution (Waters).
18. Mass spectrometry and liquid chromatography standards for MS calibration and LC analysis: Human [Glu^1]-fibrinopeptide B human; phosphorylase B (rabbit), alcohol dehydrogenase (ADH, *Saccharomyces cerevisiae*), enolase 1 (*Saccharomyces cerevisiae*), and bovine serum albumin (BSA, *Bos taurus*) (*see* **Note 5**).
19. Mass spectrometry (MS) instrumentation: Q-Tof Synapt G1 MS with nano electrospray ionization and lock mass (Waters) (*see* **Note 6**).
20. Bioinformatics tools: MassLynx v4.1 (LC-MS control and operation, MS, and MS/MS data acquisition) (Waters) (*see* **Note 7**).
21. Bioinformatics tools: ProteinLynx Global Server v3.0 (PLGS 3.0) (Waters) (raw file processing, centering, smoothing, and de-isotoping, and generation of list of masses: peak list files = PKL files (*see* **Note 7**)).
22. Bioinformatics tools: Mascot server v2.5 and Mascot Daemon toolbox v2.4 (data searching against databases) in MS/MS Ion Search Mode local license (www.matrixscience.com).
23. Bioinformatics tools: ProteoIQ v2.7 software (www.premier-biosoft.com) (post-mass spectrometry data analysis).
24. Digital dry bath incubator.
25. Refrigerated microcentrifuge.
26. Centrifugal vacuum concentrator such as the SP1010 SpeedVac (Thermo) with variable range of vacuum.
27. Sonicator.

3 Methods

Perform all procedures at room temperature unless otherwise specified.

3.1 Sample Preparation and Protein Digestion

1. For lyophilized proteins, dissolve the samples in reaction buffer (100 mM ammonium bicarbonate/5 % acetonitrile) to obtain a protein solution of 1 μg/μL. If the proteins are already in solution in another buffer (such as 20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 5 mM EGTA, 0.25 M sucrose, 50 mM β-mercaptoethanol and 10 μg/μL phenylmethylsulfonyl fluoride), dilute the sample by addition of reaction buffer to reach a final concentration of 100 mM ammonium bicarbonate/5 % acetonitrile as well as 1 μg/μL protein. Titrate the pH of the solution to 8.0 and place on ice until the next step (*see* **Notes 8** and **9**).
2. Perform the reduction of the sample in solution by adding 1/10 (v/v) of 50 mM DTT, and incubate at 65 °C for 5 min (*see* **Note 10**).
3. Cool, centrifuge (10,000 × *g* for 10 s), and return the sample to ice.
4. Place the sample in a rack at room temperature and add 1/10 (v/v) 100 mM iodoacetamide. Incubate at room temperature for 30 min and protect the samples from direct exposure to light with aluminum foil (*see* **Note 11**).
5. Place the sample on ice for at least 20 min.
6. Add 1 μg/μL trypsin solution 1:40 ratio of μg trypsin:μg of sample protein (*see* **Note 12**).
7. Incubate at 37 °C overnight or at least 15 h.
8. Centrifuge the sample (10,000 × *g* for 10 s).
9. Add 0.5 % acetic acid and titrate to pH 2.0 to halt the digestion (a pH tape can be used to follow the change in pH).
10. Place the tube on dry ice and concentrate in SpeedVac until almost dry. SpeedVac conditions: temperature: Off, vacuum: 24 Torr/min, vacuum ramp: level 4, time: 35 min, auto run mode (*see* **Note 13**) (Fig. 3).

3.2 Peptide Analysis by 2D-nanoLC and Tandem Mass Spectrometry

1. Dissolve the tryptic-digested peptides obtained from the digestion in Subheading 3.1 above in sample injection buffer (100 mM ammonium formate pH = 10) to a final concentration of 1 μg/μL of digested peptide.
2. Place the peptides in a total recovery vial and inject 1 μg of digested peptide into the 2D-nanoLC system using the autosampler (*see* **Notes 14–16**).
3. Set up the conditions for the 2D-nanoLC-MS/MS using a 10 fraction + flushing protocol. Perform the first dimension on an XBridge BEH130 C18, 5 μm, 300 μm × 50 mm NanoEase Column using solvents A1 and B1. Set the flow in the first dimension at 2 μL/min and program 11 step gradients (dilution method) for 20 min each.

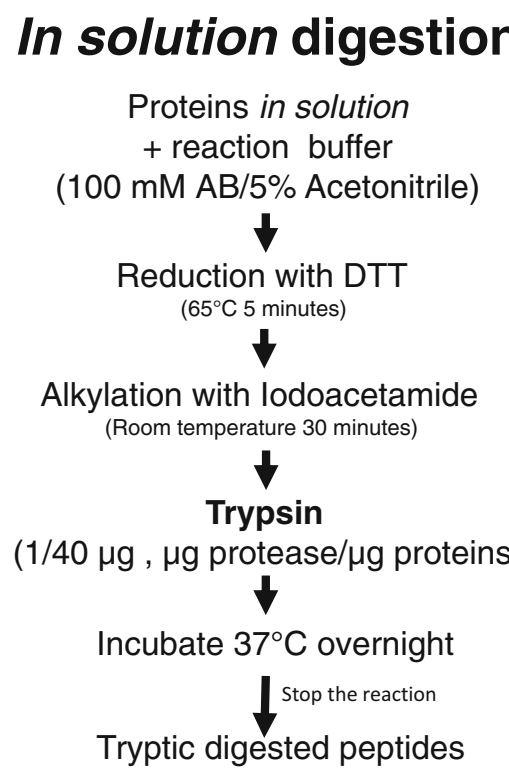

Fig. 3 Schematic representation of the in-solution digestion protocol for preparation of digested peptide sample for "shotgun" proteomics analysis

4. Include a second column for trapping and desalting online. Set the flow at 20 µL/min through the 180 µm × 20 mm, 5 µm Symmetry C18 nanoAcquity UPLC trap column. Use column buffers 99 % A2 to 1 % B2 for 20 min.
5. After the peptides are desalted and concentrated, separate them online in the second dimension through a BEH130C18 1.7 µm, 100 µm × 100 mm nanoAcquity UPLC column using a flow rate of 400 nL/min for 60 min through the following gradient program: begin with the standard solvent gradient of 0–2 min, 3 % B2 isocratic, 2–40 min, 3–85 % B2 linear, and return to the original gradient of 3 % B2 until the 60-min separation is complete. For specific information on the plumbing and gradients corresponding to each dimension, *see* Fig. 4, Tables 1 and 2, respectively.
6. Acquire the data coming from each fraction with MassLynx v4.1 program through the data depend acquisition (DDA) mode and uses the following parameters: MS and MS/MS range = 400–2000 and 50–2000 m/z, respectively, with a time scanning of 1 s and 0.02 s for inter-scan delay, total acquisition time = 59 min (*see* **Notes 7** and **17**).

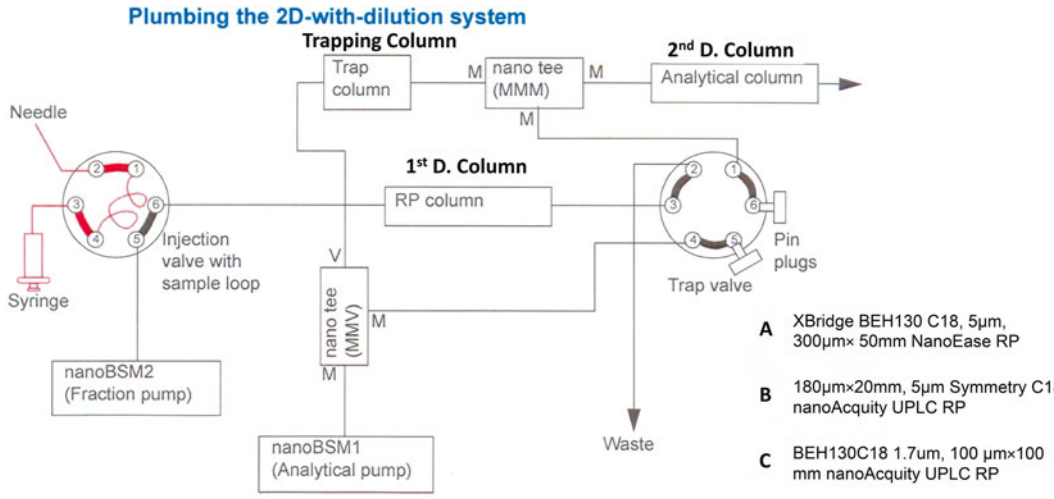

Fig. 4 Schematic representation of the plumbing for 2D with dilution system configuration. An Xbridge BEH130, 5 µm, 300 µm × 50 mm NanoEase RP (pH = 10) column was used as the first dimension. A 180 µm × 20 mm, 5 µm Symmetry nanoAcquity UPLC and BEH130, 5 µm, 100 µm × 100 mm nanoAcquity UPLC (pH = 2) columns were used in the second dimension for the trapping and desalting and analytical, respectively (©Waters Corporation. Used with permission)

Table 1
First-dimension separation gradient setup using a protocol for 10 fractions + flushing

2D-nanoLC with dilution (1st. D. gradients)

Method: 10 Fractions with Dilution				
Time (minutes)	Flow (uL/min)	% Solvent A1	% Solvent B1	Curve
Initial	2	97	3	
0.5	2	97	3	6
1	2	% table	% table	6
5	2	% table	% table	6
5.5	2	97	3	6
20.5	2	97	3	6

% Table (Cycles of Gradient)				
Fraction	Time (min.)	% Solvent A1	% Solvent B1	Flow
1	1	91.8	8.2	2 uL/min
1	5	91.8	8.2	2 uL/min
2	1	88.3	11.7	2 uL/min
2	5	88.3	11.7	2 uL/min
3	1	87	13	2 uL/min
3	5	87	13	2 uL/min
4	1	85.5	14.5	2 uL/min
4	5	85.5	14.5	2 uL/min
5	1	84.1	15.9	2 uL/min
5	5	84.1	15.9	2 uL/min
6	1	82.6	17.4	2 uL/min
6	5	82.6	17.4	2 uL/min
7	1	81.1	18.9	2 uL/min
7	5	81.1	18.9	2 uL/min
8	1	79.2	20.8	2 uL/min
8	5	79.2	20.8	2 uL/min
9	1	76.4	23.6	2 uL/min
9	5	76.4	23.6	2 uL/min
10	1	55	45	2 uL/min
10	5	55	45	2 uL/min
Flush	1	35	65	2 uL/min
Flush	5	35	65	2 uL/min

Solvent A1 and B1 are 20 mM ammonium formate and acetonitrile 0.1 %, respectively. The gradient step in each fraction was run for 5 min (©Waters Corporation. Used with permission)

Table 2
Second-dimension separation gradient setup using a protocol for 10 fractions + flushing

Time (min)	Flow (nl/min)	%A	%B	Curve
0	400	99	1	6
1	400	99	1	6
30	400	50	50	6
31	400	15	85	6
35	400	15	85	6
36	400	99	1	6

Solvent A2 and B2 are Water/0.1 % formic acid and acetonitrile 0.1 % formic acid, respectively. The gradient step in each fraction is similar in the second dimension, and the standard solvent gradient used is 0–2 min, 3 % B2 isocratic; 2–40 min, 3–85 % B2 linear, at a flow rate of 400 nL/min for 60 min run for 5 min (©Waters Corporation. Used with permission)

3.3 Bioinformatics Analysis of Data

1. Transfer the raw files generated from each fraction onto ProteinLynx Global Server v3.0 (PLGS 3.0) and convert the spectrum into a list of masses (peak list or PKL files). Use the same processing parameters as the mass spectrometer conditions for the data acquisition (de-isotoping, background correction, centering and smoothing) (*see* **Notes 7**, **18** and **19**).
2. Move the PKL files from PLGS 3.0 to a Mascot Daemon toolbox (v2.5) to perform a search against NCBInr and Swiss Protein databases using a taxonomy filter for *Rattus norvegicus*.
3. Browse all of the PKLs corresponding to each fraction from the sample into Mascot Daemon toolbox v2.5 using the following parameter set up for the search: (a) MS/MS ion search mode, (b) enzyme = trypsin, (c) missed cleavage = 1, (d) fixed modifications = carbamidomethyl (C), (e) variable modifications = deamidation (N and Q), and oxidation (M), (f) peptide and MS/MS tolerance = 50 ppm and 0.3 Da, respectively, (g) monoisotopic, (h) data format = micromass (.PKL), (i) instrument = ESI-QUAD-ToF, and error-tolerant mode. Before submitting the search, select "merge" to combine the partial data into one unique report of the most probable proteins identified in the sample (*see* **Note 20**).
4. Export the data obtained from Mascot (Fig. 5) as Mascot.dat file format to ProteoIQ software to perform a post-mass spectrometry analysis of the proteins identified as well as their relative abundance (*see* **Notes 21** and **22**) (Figs. 2 and 6).

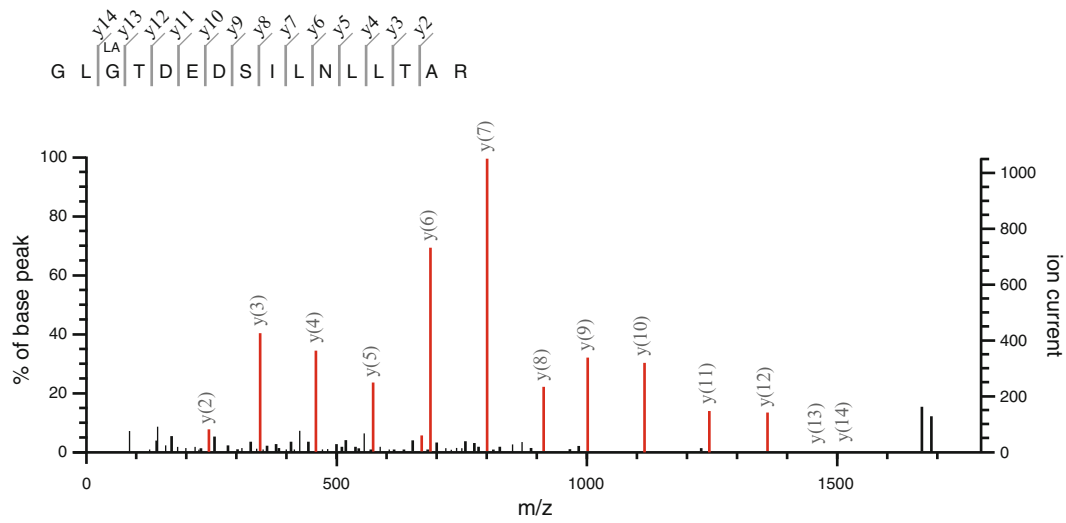

Fig. 5 MS/MS fragmentation map of GLGTDEDSILNLLTAR found in Annexin A5 [*Rattus norvegicus*]. Peptide mass 1686.8818, and charge state = +2

4 Notes

1. The samples that were used in this example are came from protein homogenates extracted from the uterus of vehicle-treated control and ethinylestradiol-treated female rats that had been ovariectomized 2 weeks prior to treatment. The proteins were originally in a buffer containing 20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 5 mM EGTA, 0.25 M sucrose, 50 mM β-mercaptoethanol, and 10 μg/μL phenylmethylsulfonyl fluoride (PMSF). However, to avoid the interference that the β-mercaptoethanol and PMSF could cause to the trypsin activity, reaction buffer was added to the sample to adjust the concentrations of PMSF and β-mercaptoethanol prior to the digestion (*see* **Notes 8** and **9**).
2. The iodoacetamide solution should be covered with aluminum foil to protect it from light as it is light sensitive. The iodoacetamide must be prepared fresh.
3. The trypsin powder must be stored at −20 °C. In solution it should be stored at −80 °C. It can be frozen and thawed for no more than two cycles.
4. Work in a fume hood and add carefully to avoid inhaling potential formic acid released from the reaction between ammonium hydroxide and formic acid.
5. Human [Glu^1]-fibrinopeptide B human is used for mass calibration in MS and MS/MS modes at the mass spectrometer. Phosphorylase B (rabbit), alcohol dehydrogenase (ADH, *Saccharomyces cerevisiae*), enolase 1 (*Saccharomyces cerevisiae*),

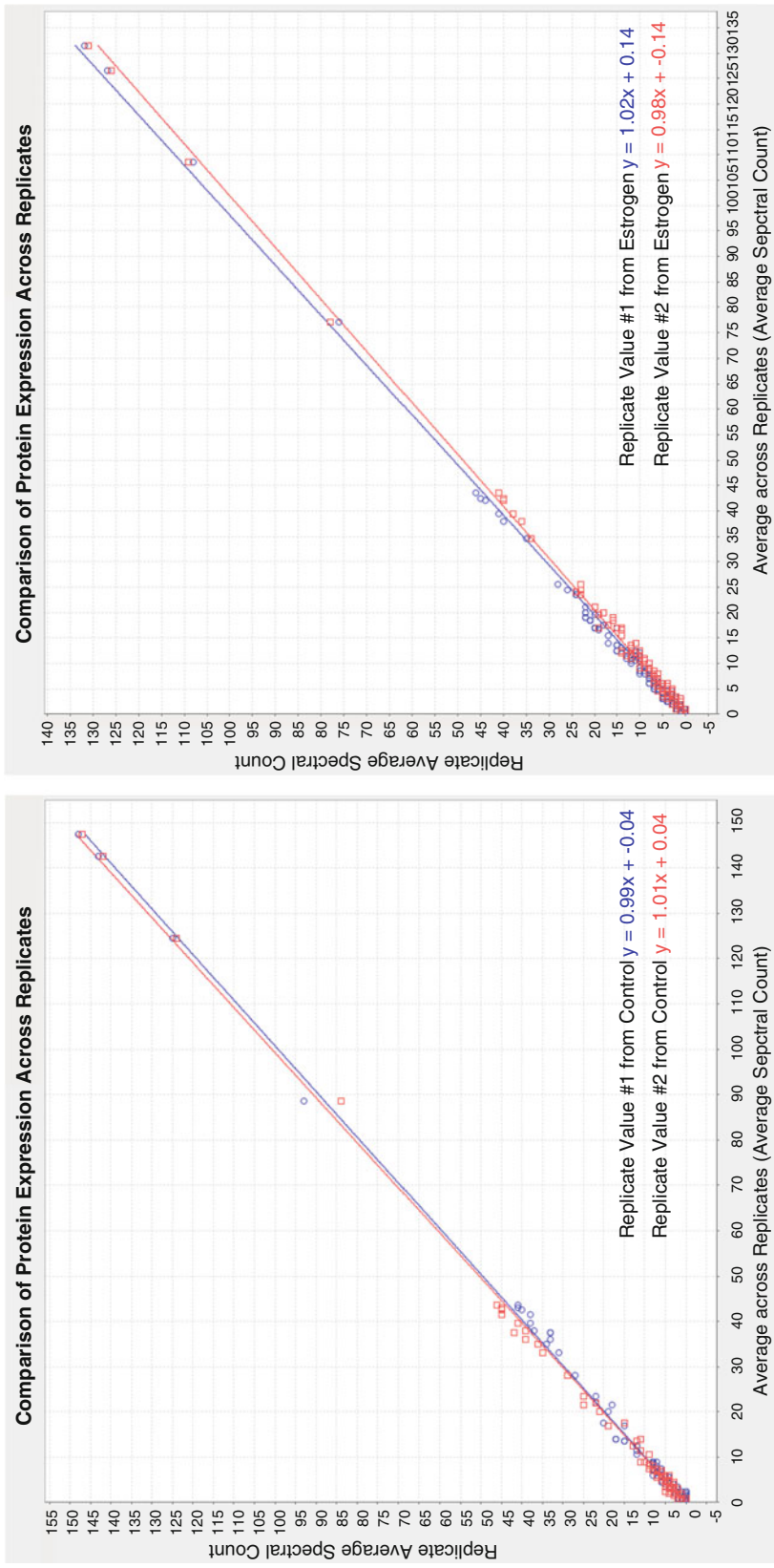

Fig. 6 Replicate scatterplot analysis showing the comparison of protein expression across sample control and estrogen replicates through the average of spectral count. "*y*"- and "*x*"-axes represent the replicate average spectral count, and the average across replicates (average spectral count), respectively

and BSA (*Bos taurus*) digestion standards are used for quality control of the chromatography step (retention time, peak shapes and areas, and separation reproducibility prior the sample analysis), as well as to evaluate the sensitivity and mass accuracy of the mass spectrometer prior the analysis.

6. The MS used in this protocol is purchased from a specific vendor, but the procedures could be carried out on any Q-Tof mass spectrometer.
7. The tools MassLynx 4.1 and ProteinLynx 3.0 (PLGS 3.0), as well as the processing parameters used for the data acquisition and mass list extraction mentioned above are related to the vendor of the mass spectrometer; however, these tools can be used with other instruments.
8. The sample preparation step involves the resuspension or dilution of the proteins present in the samples, depending on whether they are lyophilized or in solution. The reaction buffer used for the protein digestion is ammonium bicarbonate + acetonitrile, which provides the proper environment for the protease action. If the proteins are lyophilized, the reaction buffer should be used directly to dissolve it. However, if the proteins are in solution with an original extraction buffer, a volume of reaction buffer stock solution should be added to reach a final concentration of 100 mM ammonium bicarbonate/5 % acetonitrile.
9. If the protein sample contains β-mercaptoethanol or protease inhibitors such as PMSF, they need to be diluted so they do not inhibit the ability of the protease (e.g., trypsin) to digest proteins. The current sample contained 10 μg/μL PMSF and 50 mM β-mercaptoethanol in order to inhibit endogenous serine protease activities. PMSF will not affect trypsin activity at a concentration of 0.17 μg/μL, so dilution of the current sample to this concentration with reaction buffer was necessary to avoid potential inhibition of the trypsin activity during protein digestion [17, 18].
10. The proteins in solution are reduced with dithiothreitol (DTT) to reduce the disulfide bonds between cysteines located in the loop of the molecule. This action allows the molecule to expand and exposes the cleavage sites to be recognized by the protease. In general, the DTT is prepared fresh; however, in the case of having many samples from different batches, DTT could be stored in aliquots at −20 °C, avoiding more than one cycle of thaw.
11. Immediately after the reduction, an alkylating agent such as iodoacetamide is added to block the sulfhydryl groups to prevent the bonds from reforming. This reagent must be protected from the action of light before and during the process of alkylation of the samples by covering the tubes with aluminum foil.

12. Trypsin is a universal protease utilized for protein digestion that recognizes cleavage sites at lysine (K) and arginine (R) when they are present in the protein, except when those amino acids are surrounded by an amino acid such as proline that can produce an impediment to trypsin recognition and cleavage. Hydrolysis mediated by trypsin converts the amine bonds of the C-terminus side of the R or K residues to a strong basic site, and moderates the N-terminus. The combination of those kinds of peptides and the acidic conditions used with the electrospray ionization (ESI) produces protonation at both C-termini, either at the side chain of K or R [19], thus becoming more effective positive ions.
13. Concentrate the samples to almost, but not completely dry, to make it easier to resuspend the peptides in the sample injection buffer.
14. The volume of tryptic-digested peptides to be injected is 1 μL (1 μg/μL) in sample injection buffer as the initial conditions. If the initial analysis indicates that the peptide concentration is too low, a second run can be performed with a higher concentration of peptides.
15. The volume of the sample loop used at the injection valve is 5 μL and the partial loop method allows injection of volumes between 0.2 and 4.9 μL.
16. The 2D-nanoLC with dilution is a new technology that permits the performance of orthogonal separation using two reversed-phase columns working at high and low pH respectively. The tryptic peptide sample is solubilized in 100 mM ammonium formate buffer (AF) (sample injection buffer, pH 10, to improve the peptide bound to the stationary phase of the first column, and loaded into the first dimension column = pH 10) in which the unwanted solutes are washed and thus removed. Setting a gradient for the first dimension with 20 mM AF buffer and 100 % acetonitrile as mobile phases (solutions A1 and B1, respectively), the pH from the first-dimension column can be decreased, releasing peptides into the second-dimension column according to the pH of each peptide. The second-dimension column works as an analytical column, resolving the peptide mixtures coming from the first dimension column, and infusing them into the ESI of the mass spectrometer. A total number of 10 fractions + 3 flushing washes are set up for running each sample. Throughout the ten fractions, the gradient changes by increasing the percentage of the mobile phase corresponding to the organic solvent (acetonitrile) and reducing the percentage of buffer. The flushing permits cleaning the column for the next sample to be injected and avoids potential carryover of sample.

17. The data acquired for each fraction are saved in an individual raw data file. PKL is a format from Waters and consists of a base text file with specific syntax and order of the data (precursor mass, intensity, charge state and fragment masses) corresponding to each precursor or parent fragmented.
18. MassLynx v4.1, Mascot Distiller or similar programs can be used to extract the list of masses to be exported in different formats (PKL was used specifically in this case because it is the Waters format).
19. After finishing the data acquisition, the list of masses is extracted using the tool ProteinLynx Global Server v3.0 Expression Analysis and exported as a PKL format. The processing parameters applied during the extraction of the list of masses must match the conditions present in the mass spectrometer during the data acquisition. For the programs described herein, the following list describes the parameters that should be matched. Set the processing parameters as in the following example (also *see* **Note 7**):

Mass accuracy	
Electrospray survey	
Attribute	*Value*
Perform lock spray calibration	Yes
Lock spray lock mass	785.8427 Da/e
Lock spray scan	10
Lock mass tolerance	0.1 Da
MSMS	
Attribute	*Value*
Perform lock spray calibration	Yes
Lock spray lock mass	785.8427 Da/e
Lock spray scan	10
Lock mass tolerance	0.1 Da
Noise reduction	
Electrospray survey	
Attribute	*Value*
Background subtraction type	Normal
Background threshold	35 %
Background polynomial	5
Perform smoothing	Yes
Smoothing type	Savitzky-Golay

(continued)

(continued)

Smoothing iterations	2
Smoothing window	3 channels
MSMS	
Attribute	*Value*
Background subtraction type	Normal
Background threshold	35 %
Background polynomial	5
Perform smoothing	Yes
Smoothing type	Savitzky-Golay
Smoothing iterations	2
Smoothing window	3 channels
Peptide filter	
De-isotoping and centroiding	
Electrospray survey	
Attribute	*Value*
Perform de-isotoping	Yes
De-isotoping type	Fast
Iterations	30
Automatic thresholds	No
Threshold	3 %
Minimum peak width	4 channels
Centroid top	80 %
TOF resolution	10,000
NP multiplier	0.7
MSMS	
Attribute	*Value*
Perform de-isotoping	Yes
De-isotoping type	Fast
Iterations	30
Automatic thresholds	No
Threshold	3 %
Minimum peak width	4 channels
Centroid top	80 %
TOF resolution	10,000
NP multiplier	0.7

20. All of the PKL files corresponding to the 10 fractions and flushes are imported into the Mascot Daemon toolbox v2.5 to perform searches against the databases. Each submission is searched as an individual event for each fraction coming from the sample, and all of the data are merged into one unique protein list. The explanation for each parameter set at Mascot server is the following: (a) MS/MS ion search mode = the data is acquired using tandem mass spectrometry (the mass of the precursor or parent peptides is determining followed by fragmentation of the peptide and the determination of the fragment masses (MS/MS analysis); (b) enzyme: trypsin: the proteins in the sample are digested with this particular protease; (c) missed cleavage = 1, means the probability that the enzyme fails in recognizing one site for cleavage of the proteins during the digestion; (d) fixed modifications: carbamidomethyl (C): the proteins are reduced and alkylated prior to digestion with the purpose of breaing the disulfide bridge between cysteines, making the recognition sites more available to the enzyme to perform cleavage (DTT and iodoacetamide are used during the reduction and alkylation step, respectively, generating a fixed modification transduced in the addition of a carbamidomethyl group modification at the cysteine group, which produced a shift of 57 Da at the total peptide mass); (e) variable modifications: deamidation (*N* and *Q*), and oxidation (*M*), are variable because they may formed after the reduction and alkylation of the proteins and their presence could produce the additions of mass (oxidation in *M* = 15.9949 Da, and deamidation in *N* or *Q* = 0.9840 Da); (f) peptide and MS/MS tolerance = 50 ppm and 0.3 Da, respectively, represent the potential error permitted in the differences of masses between the experimental (at the mass spec) and theoretical from the database; (g) monoisotopic: this parameter indicate that the mass measured is related to ^{12}C); (h) data format: micromass (.PKL) corresponds to the type and brand of instrument used; (i) Instrument = ESI-QUAD-ToF, means that the mass spectrometer used is hybrid Q-ToF; (j) error-tolerant mode = allows the search to be more flexible in terms of the potential modifications detection in the peptides that were not selected prior at the fixed and/or variable mode.
21. This tool allows accurate and organized data management for statistical analysis between replicates or samples and the normalization, as well as the relative quantification through label free using spectral counts according to the criteria from the Paris Proteomics Guidelines. Moreover, data can be exported in different formats such as Venn diagrams, heat maps (absolute normalization or log 2 values), scatter, bar, and pie charts, also introducing gene ontology (GO) and pathway (KEGG) analysis.

22. The data organization as well as relative quantification through a "label-free" protocol was performed using ProteoIQ software v2.7. The protein list coming from Mascot can be exported as a Mascot.Dat File (format compatible with ProteoIQ) into the ProteoIQ. Also, the database to be used in the program mentioned earlier must be the same as it was for the Mascot search.

Acknowledgements

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Chapter 12

Assessment of Protein Expression by Proximity Ligation Assay in the Nonhuman Primate Endometrium, Placenta, and Fetal Adrenal in Response to Estrogen

Thomas W. Bonagura, Jeffery S. Babischkin, Gerald J. Pepe, and Eugene D. Albrecht

Abstract

In the field of protein biology, immunology-based techniques have been evolving for detection and quantification of protein levels, protein-protein interaction, and protein modifications in cells and tissues. The proximity ligation assay (PLA), a method of detection that combines immunologic and PCR-based approaches, was developed to overcome some of the drawbacks that are inherent to other detection methods. The PLA allows for very sensitive and discretely quantifiable measures of unmodified, native protein levels, and protein-protein interaction/modification complexes in situ in both fixed tissues and cultured cells. We describe herein the PLA method and its applicability to quantify the effects of estrogen on expression of angioregulatory factors, e.g., angiopoietin-1 (Ang-1) in the endometrium, vascular endothelial growth factor (VEGF) in the placenta, and melanocortin 2 receptor (MC2R)/accessory protein (MRAP) in the fetal adrenal of the nonhuman primate.

Key words Proximity ligation assay, Immunocytochemistry, Immunofluorescence, Estrogen, Endometrium, Placenta, Adrenal

1 Introduction

Detection of proteins by standard immunohistochemistry techniques using specific antibodies to epitopes on a target protein has tremendous ability to provide qualitative images and data. However, the ability to quantify or make definitive statements on the in situ nature of protein—a single protein, a modified protein, or part of a multiprotein interaction complex—has been hampered by both selectivity and sensitivity of available techniques [1]. Current methods of quantification such as Western immunoblot and sandwich ELISA involve antibody labeling of immobilized, separated proteins from cellular extracts (Western) or proteins from cellular extracts exposed to an immobilized specific antibody

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(ELISA), procedures which hamper analysis of protein cellular localization and interaction. Conversely, immunostaining techniques that rely on binding of an antibody with a conjugated reporter, such as an enzyme to generate a color product—3,3′-diaminobenzidine (DAB)—or a fluorophore, provide valuable qualitative information, e.g., location, because they are typically investigated in their in situ environment. However, quantification is compromised by the use of subjective semi-quantitative measures, such as the H-score grading system which is based on the investigator interpreting the staining intensity of a subcellular compartment of a tissue [2]. Significant steps in visualizing and quantifying protein-protein interaction complexes were made using fusion protein constructs and fluorescent labeling technology to develop a set of techniques referred to as protein-complementation assays. Specifically, methods such as fluorescence resonance energy transfer (FRET), bimolecular fluorescence complementation (BiFC), and mammalian protein-protein interaction trap (MAPPIT), rely on overexpression of fusion proteins consisting of the target protein and a reporter construct in a living cell [3]. Other protein-fragment complementation assays have subsequently been developed, primarily using luciferase to increase sensitivity, to address the issue of quantification [4]. These techniques, while powerful, have limitation; for example, overexpression or fusion proteins may not interact or behave the same way as an endogenous protein in its native cellular environment.

Significant improvement of the aforementioned techniques began with development of a novel method of conjugation of small oligonucleotides to antibodies to detect cytokine levels. This technology provided the basis for a specific, quantifiable method of protein analysis [5]. This technique served as a template for the evolution of protein detection and analysis: the in situ detection and quantification of protein-protein interaction complexes, protein localization, and protein modifications at the single molecule level via the proximity ligation assay (PLA) [6]. For in situ evaluation of protein complexes by PLA (Fig. 1a), two specific primary antibodies, each raised in a different species, are used to label two potentially interacting proteins [7]. Primary antibodies bind their respective proteins and proximity probes with the conjugated nucleotide tags are then added. Proximity ligation probes are simply secondary antibodies against the primary antibody; hence, the requirement that antibodies must be raised against two different species. The oligonucleotides attached to the probes have been modified to contain complementary sequences that allow them to be circularized by the addition of complementary oligonucleotides, DNA ligase, and DNA polymerase into a template for rolling circle PCR amplification (Fig. 1b) [6, 7]. The oligonucleotides only become in close enough “proximity” to be ligated if the target proteins interact. The oligonucleotides are designated as PLUS

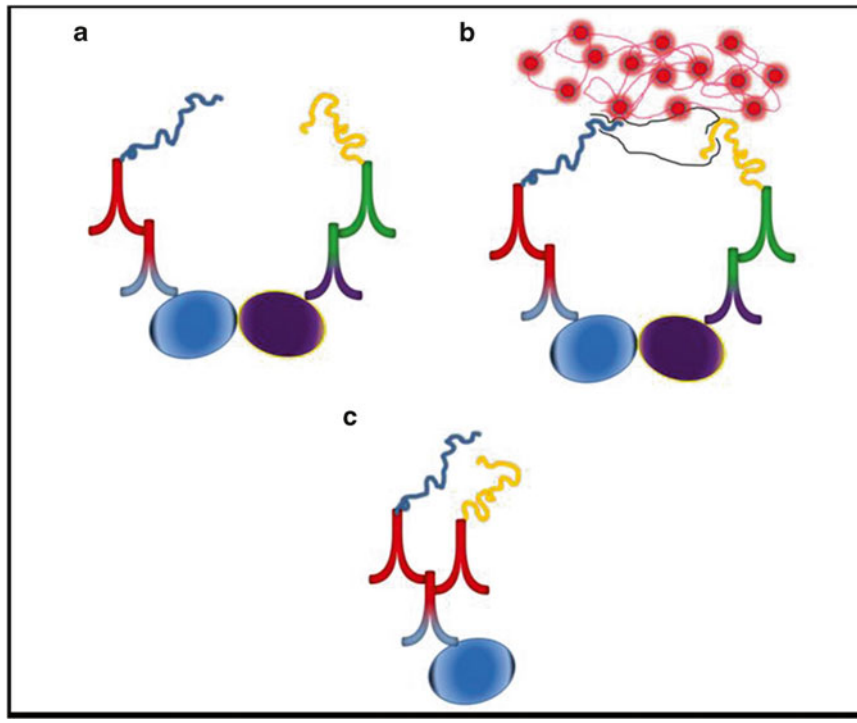

Fig. 1 Schematic of proximity ligation assay. (**a**) Dual recognition of interacting proteins shown in *light blue* and *purple*, respectively. Primary antibodies to each protein need to be raised in different species and are represented by the *red* and *green* heavy chain regions. Subsequently, two secondary antibodies, the PLA probes, each against one of the species, are added. Attached to each PLA probe is a conjugated oligonucleotide tag, noted as PLA probe PLUS and PLA probe MINUS and shown in *blue* and in *yellow*. (**b**) Ligation, amplification and detection. Oligonucleotides (shown in *black*) complementary to the PLA probe oligonucleotide tags are added with DNA ligase (not shown). If the two protein targets are in close enough proximity, ligase will close the oligonucleotides into a closed circle to serve as a template for rolling circle PCR amplification. DNA nucleotides and DNA polymerase (not shown) are then added to generate a concatemeric product (*pink line*) that will be labeled by fluorescently labeled nucleotides (*red circles*) present in nucleotide polymerase mix. (**c**) Recognition of a single protein. In the detection of one protein, two PLA probes are added, both against the lone primary antibody, but one with the PLUS oligonucleotide tag and the other with the MINUS oligonucleotide tag. DNA nucleotides and fluorescently labeled nucleotides are then added as in the recognition assay of two target proteins

and MINUS and the presence of each is required for a successful assay. The resulting amplified chain of DNA is visualized by fluorescent label as a single spot one to two microns in diameter (Fig. 1b). Each fluorescent signal represents a single, endogenous interaction of two proteins that allows for automated counting by a variety of software packages. Unlike other methods currently used to examine protein interactions, the PLA avoids potential artifact generation because it detects endogenous proteins in fixed tissue samples or cultured cells, thereby eliminating the need for overexpression, for example, of fusion proteins.

Ultimately, the study of protein-protein interaction or a single protein, at the localization or expression level, is more definitive

and informative when performed on endogenous proteins in their normal cellular environment [8]. While the PLA was developed for the investigation of protein-protein interaction, the use of the proximity probes also allows for the quantification of a single protein by simply adding two secondary antibodies/probes against the primary antibody, one conjugated with the PLUS and the other with the MINUS oligonucleotide (Fig. 1c). This is referred to as a single recognition method of detection. Further applications/modifications of the PLA are noted in the accompanying method description.

Thus, as reported elsewhere by our laboratory [9, 10] and others [11, 12], we describe herein the methods for detection and quantification of individual proteins, as well as protein-protein interaction, using the PLA. We believe the use of the PLA provides the most definitive assay for sensitive and reliable protein quantification and detection of endogenous in situ proteins and the effects of estrogen on expression of those proteins.

2 Materials

2.1 Buffers and Blocking Reagents

1. Phosphate-buffered saline with 0.01 % Tween-20 (PBST): Dissolve 135.0 g sodium chloride, 104.5 g dibasic potassium phosphate, 20.4 g monobasic potassium phosphate, and 1.5 mL Tween 20 in 15 L deionized, distilled water (ddH_2O). Adjust pH to 7.3 with HCl.
2. Sodium citrate buffer (10 mM): Dissolve 2.1 g sodium citrate in 700 mL ddH_2O. Adjust pH to 6.0 with HCl.
3. Normal horse serum diluted to 5 % in PBST.

2.2 Reagents

1. Xylene.
2. Ethanol, 70, 95, and 100 % (*see* **Note 1**).

2.3 Equipment

1. Large beaker to hold slides in metal slide holder and a hot plate. Alternatively, a nonmetal slide holder and microwave.
2. PAP pen/grease pen.
3. Coplin jars.
4. Humidified chamber.
5. Rocking platform shaker.
6. 37 °C incubator.
7. Cover slips compatible with fluorescence microscopy.
8. Fluorescent microscope with attached digital camera.
9. Imaging and data analysis software.

2.4 Antibodies and Detection Reagents

1. Primary antibody or antibodies derived from different species: Ang-1, VEGF, MC2R, MRAP, and cytokeratin.
2. Duolink In Situ kit specific for fluorescent microscope excitation/emission capabilities and the species of primary antibodies used (Olink Bioscience) (*see* **Note 2**).
3. Alexa Fluor 488 fluorescent labeled secondary antibody (Life Technologies) against a species different from that used in the Duolink In Situ kit.
4. Duolink In Situ 1× wash buffer A: Dissolve 8.8 g sodium chloride, 1.2 g Tris base, and 0.5 mL Tween 20 in 800 mL ddH_2O. Adjust to pH 7.4 with HCl and add ddH_2O to 1000 mL. Filter through 22 μm filter and store at 4 °C. Use at room temperature.
5. Duolink In Situ 1× wash buffer B: Dissolve 5.8 g sodium chloride, 4.2 g Tris base, and 26.0 g Tris–HCl in 500 mL ddH_2O. Adjust to pH 7.5 with HCl and add ddH_2O to 1000 mL. Filter through 22 μm filter and store at 4 °C. Use at room temperature.
6. Duolink In Situ (Olink) mounting media or Vectashield mounting media for fluorescence with DAPI (Vector Labs).

3 Methods (*See* Note 3)

Use standard immunohistochemistry or immunocytochemistry techniques to fix, embed, and section tissue samples on slides (*see* **Note 4**).

3.1 Deparaffinization (*See* Note 5)

Deparaffinize the slides by soaking in sequential baths of xylene and ethanol as follows:

1. Place previously baked slides in slide holder for 30 min in xylene bath.
2. 15 min in second xylene bath.
3. 15 min in third xylene bath.
4. 5 min in 100 % ethanol bath.
5. 5 min in second 100 % ethanol bath.
6. 3 min in 95 % ethanol bath.
7. 3 min in 70 % ethanol bath.
8. Soak in ddH_2O for 2 min—four times.

3.2 Antigen Retrieval (*See* Note 6)

1. Boil sodium citrate buffer in large beaker on hot plate.
2. Add slides in slide holder to boiling buffer for 5 min.

3. Remove beaker from the hot plate and cool slides in the buffer for 20 min.
4. Remove slides to a Coplin jar and wash for 5 min in PBST under gentle agitation on shaker three times (*see* **Note 7**).

3.3 Blocking (See Note 3)

1. Tap off excess PBST and place slides in humidified chamber.
2. Incubate slides in normal horse serum diluted to 5 % in PBST for 1 h at room temperature.
3. Wash for 5 min in PBST—three times.

3.4 Primary Antibody Incubation (See Note 3)

1. Tap off excess PBST and outline tissue section with PAP pen/grease pen (*see* **Note 5**).
2. Dilute antibody in normal horse serum diluted to 5 % in PBST to preferred dilution. If using two primary antibodies make sure that the diluent is the same for both (*see* **Note 8**).
3. Add antibody to tissue sections and incubate in humidified chamber for optimal conditions of antibodies. Take precautions to prevent the slides from drying out (*see* **Note 9**).
4. Tap off antibody solution and wash for 5 min in PBST three times.
5. While washing, place the humidified chamber in a 37 °C incubator.
6. Remove wash buffers A and B from 4 °C and place at room temperature.

3.5 PLA Probes (Secondary Antibody Incubation)

1. Dilute the two PLA probes 1:5 in the same buffer as the primary antibodies (*see* **Note 10**).
2. Place probe mix at room temperature for 20 min.
3. Tap off excess PBST and add PLA probe mix to tissue section.
4. Place slides in the preheated, humidified chamber and incubate at 37 °C for 1 h (*see* **Note 11**).
5. Tap off probe solution and wash in PBST for 5 min two times.
6. Wash in 1× wash buffer A for 5 min two times (*see* **Note 12**).
7. While washing, place the humidified chamber in a 37 °C incubator.

3.6 Ligation of Oligonucleotide Conjugates

1. While washing, prepare the ligation solution by diluting the provided ligation stock solution 1:5 in dd-water and mix. Do not add the ligase (*see* **Note 13**).
2. Immediately before use, add the ligase to the ligation solution at a 1:40 dilution and vortex.
3. Tap off excess PBST and add the ligation solution to the tissue section.

4. Place slides into the preheated humidified chamber and incubate at 37 °C for 30 min.
5. Tap off ligation solution and wash in 1× wash buffer A for 2 min two times.
6. While washing, place the humidified chamber in a 37 °C incubator.

3.7 Rolling Circle Amplification and Detection (See Note 14)

1. While washing, prepare the amplification solution by diluting the provided amplification stock solution 1:5 in ddH_2O and mix. Do not add the polymerase (*see* **Note 13**).
2. Immediately before use, add the polymerase to the amplification solution at a 1:80 dilution and vortex.
3. Tap off excess 1× wash buffer A and add the amplification solution to the tissue section.
4. Place slides into the preheated humidified chamber and incubate at 37 °C for 100 min.

3.8 Final Wash (See Note 15)

1. Tap off excess amplification solution.
2. Wash in 1× wash buffer B for 10 min two times.
3. Wash in 0.01× wash buffer B for 1 min (*see* **Notes 16** and **17**).
4. Shake off excess buffer and let slides dry in the dark at room temperature.
5. When dry, mount slides with a cover slip using a Duolink In Situ mounting media with DAPI (s*ee* **Note 18**).
6. Wait for 15 min before examining slides under a microscope to identify PLA signals (Figs. 2 and 3) (*see* **Note 19**).

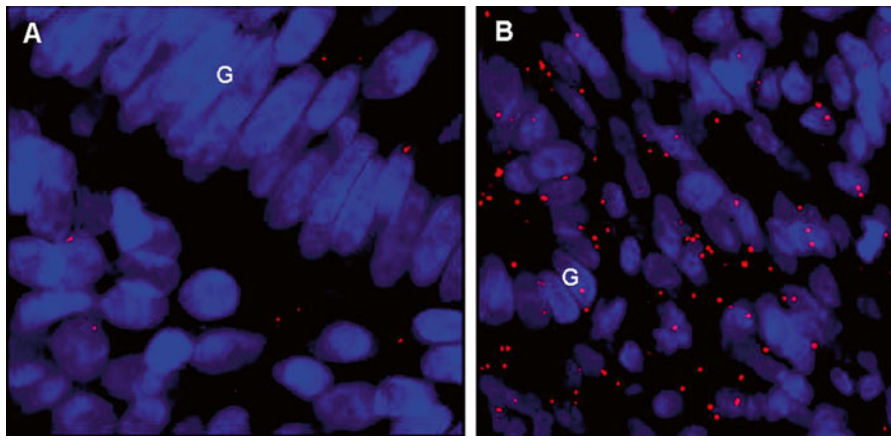

Fig. 2 Angiopoietin-1 protein expression assessed by PLA (*red* signals) in the uterine endometrium during the low estrogen early proliferative (**a**) and elevated estrogen late proliferative (**b**) phases of the baboon menstrual cycle. Nuclei, *blue*. G, glandular epithelium. 400× magnification

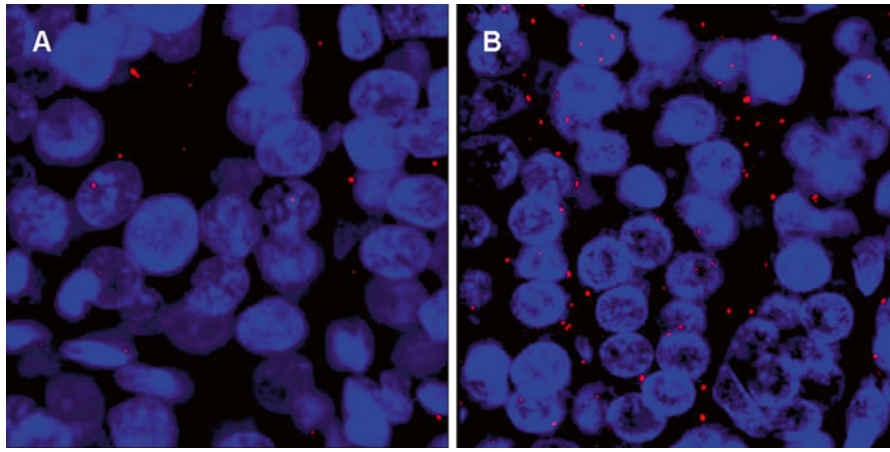

Fig. 3 MC2R-MRAP protein-protein interaction assessed by PLA (*red* signals) in the definitive zone of the fetal adrenal late in gestation in untreated (**a**) and letrozole-treated estrogen-deprived (**b**) baboons. Nuclei, *blue*. 400× magnification

4 Notes

1. Use separate, clean ethanol washes for deparaffinization steps prior to PLA runs to avoid contamination by eosin present in ethanol baths of typical immunohistochemistry stations used in laboratories with a lot of slide staining.
2. The procedure described herein is specific to the reagents purchased through Olink Bioscience for the Duolink In Situ PLA assay. However, all of the reagents and materials can also be produced de novo independently by the user. A detailed protocol and methodology for such is described in early publications, including generation of oligonucleotide-linked antibodies [5, 7, 13].
3. To generalize, everything up to and including the primary antibody incubation remains unchanged, unless noted, from standard immunohistochemistry or immunocytochemistry techniques. Optimization of the primary antibody dilution, antigen retrieval, and blocking should be independently determined before proceeding with the PLA. The unique specializations and characteristics of the PLA assay come into play following the primary antibody incubation. Therefore, the reagents and methods that are successful in your own laboratory can remain essentially unchanged for particular antibodies and tissues.
4. The PLA procedure can be done on fresh, frozen, and formalin-fixed paraffin embedded tissue sections or cells, adherent cell cultures and cytospin preparations. Our lab has the most experience with FFPE tissue sections, and thus this chapter uses

that preparation as a representative example of the PLA technique. As noted by the manufacturer, primary antibody preference for a particular tissue type or preparation will often play the biggest role in determining the sample type utilized.

5. Slides can be batch processed from this point on until the addition of the primary antibody. From primary antibody addition onward, the procedures described are open droplet reactions on individual slides. Typically, the reaction area is delimited to a minimal size with a PAP/grease pen border drawn around the tissue sample; the procedure can be done without the aid of a delimiting border, but after washing steps, careful and thorough drying of the slide surrounding the tissue must be done to allow a drop to form and hold shape using cohesion properties of water. This area also determines the amount of reagents needed for the reactions. Typically, an area of 1 cm^2 uses a reaction volume of 40 μL.
6. This heat retrieval is the standard method in our laboratory for antigen retrieval from formalin-fixed paraffin-embedded tissues, but we have had success with variations on the heat retrieval including using a microwave for boiling (two times for 5 min with a 1-min rest in between) and changing the cooling time to 25 min. The PLA is also compatible with enzymatic retrieval (e.g., Proteinase K, pepsin, trypsin) for fixed tissue sections. Besides formalin, the PLA assay is also compatible with other methods of fixation generally used for immunohistochemistry (e.g., ethanol, acetone, paraformaldehyde).
7. All washes should be done on the shaker with gentle agitation at room temperature.
8. Primary antibody optimization cannot be underestimated in its importance to a clean PLA assay and interpretable results. Ideally, the primary antibody should have high specificity and little to no background labeling when using an alternative detection method for labeling, such as DAB. For assays to examine the interaction between two proteins, two different primary antibodies derived from different species must be used. Current species recognized by PLA secondary probes are mouse, rabbit, and goat. When using two antibodies in the PLA assay, it is recommended to optimize one antibody for the PLA assay using the single recognition protocol. Optimization for the addition of the second antibody can then be started from the established conditions. While untested in our laboratory, Olink Bioscience (Uppsala, Sweden) is currently providing a product called Duolink In Situ Probemaker which would allow the oligonucleotides currently linked to the secondary antibody probes to be linked to any primary antibody, therefore eliminating the need for a secondary antibody. This chapter focuses on using the secondary antibody probes with their conjugated oligonucleotides.

9. Typically, we use an incubation protocol of 1 h at room temperature followed by 24 h at 4 °C, where care is needed to prevent drying out of the slides. In a proper humidified chamber for immunohistochemistry work, make sure that there is water in the bottom. If using a tray system or small box with a lid and humidity provided by moistened paper towels, simply taping around the seams of the lids to create an airtight seal will ensure that the slides do not dry out.
10. Two PLA probes are used in the PLA assay. As shown (Fig. 2) for the detection and quantification of a single Ang-1 protein in the baboon endometrium using a goat antibody, both probes were antibodies against the primary (i.e., PLA probe anti-goat) and each had a unique oligonucleotide conjugated to it (designated as PLA probe anti-goat MINUS or PLA probe anti-goat PLUS). There are several other applications where two primary antibodies are used which affect the selection of the probes. The primary antibodies must always be raised in different species. To detect and quantify a single protein with high specificity, two primary antibodies can be used, each detecting a separate epitope on a single protein target. This is referred to as a double recognition method. Similarly, to detect protein modifications it is advantageous to use one primary to the protein and one antibody directed at the modification site of the same protein. Finally, as shown (Fig. 3), two antibodies can be used to detect and quantify protein-protein interactions. One goat antibody was directed against the MC2R protein and a second rabbit antibody was directed against the MRAP interacting protein. In all cases, the individual probe must be directed against the species of the primary antibody and contain the complementary oligonucleotide conjugation (for example, PLA probe anti-goat MINUS was used with PLA probe anti-rabbit PLUS). Inclusion of negative controls is vital to ensure successful interpretation. Include the use of negative controls into the making of the probe mixtures. For a no primary antibody control for tissue background labeling, simply use the probe mix as described. Other manipulations may require a separate mixture of probe to be made (for example, use of only one probe). The dilution can also be adjusted if nonspecific background signals are suspected. We have had success with dilutions of 1:15. Additionally, Olink provides an antibody diluent for use with the probes; we have used it successfully as well. If using the supplied diluents, the incubation of probes for 20 min before adding to the slides is not required, as per the manufacturer's protocol directions.
11. Incubation can be extended to a 2 h maximum.
12. Doubling of the wash step here leads to cleaner results in terms of lower background signals.

13. Take the ligase or polymerase into account when calculating the dilution volume. (Example: add 8 μL of stock to 31 μL of ddH_2O, reserving the final 1 μL for the ligase.)
14. These are light-sensitive reagents; minimize exposure to light.
15. Because the fluorescent signal is now incorporated into the amplified nucleotide chain, it is very important to protect the samples from light, not just minimize the exposure. Placing the shaker in a dark room and using ambient light from an open door will provide enough light to work.
16. Dilute 1× wash buffer B with ddH_2O to make a 0.01× concentration.
17. At this point, if so desired, an optional second labeling procedure can be performed on top of the PLA assay for determining cell-type-specific localization of the PLA signals. Typically, we use the PLA Detection Reagents Red to generate red PLA signals and blue (DAPI) nuclear counterstain. For the cell-specific localization of VEGF in the baboon placenta, we have, following the final PLA wash step, incubated our placental tissue sections overnight at 4 °C with a mouse antibody to cytokeratin (a marker of placental trophoblasts) followed the next day by 1-h incubation with a fluorescently labeled secondary antibody (Alexa Flour 488 donkey anti-mouse) in a dimly lit room to protect the PLA signals. Analysis then proceeds under the fluorescent microscope where we can visualize the nuclei (blue), the VEGF PLA signals (red) as well as the location of cytokeratin in the cytoplasm (green) of the trophoblast (Fig. 4).
18. Mount the cover slip carefully to avoid trapping air bubbles underneath. An alternative mounting media for fluorescence may be used as well. A nuclear counterstain should be part of

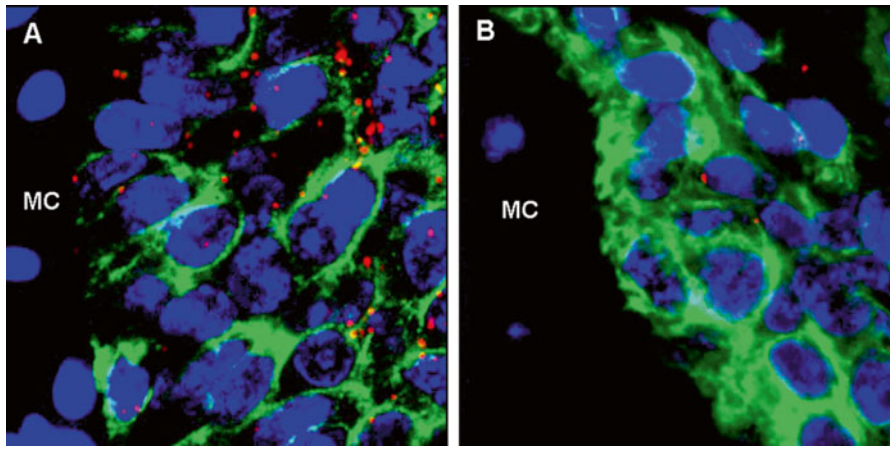

Fig. 4 VEGF protein assessed by PLA (*red* signals) in placenta anchoring villi of baboons untreated (**a**) or treated with estradiol (**b**). Nuclei, *blue*; cytokeratin-positive trophoblast, *green*. MC, mesenchymal core. 400× magnification

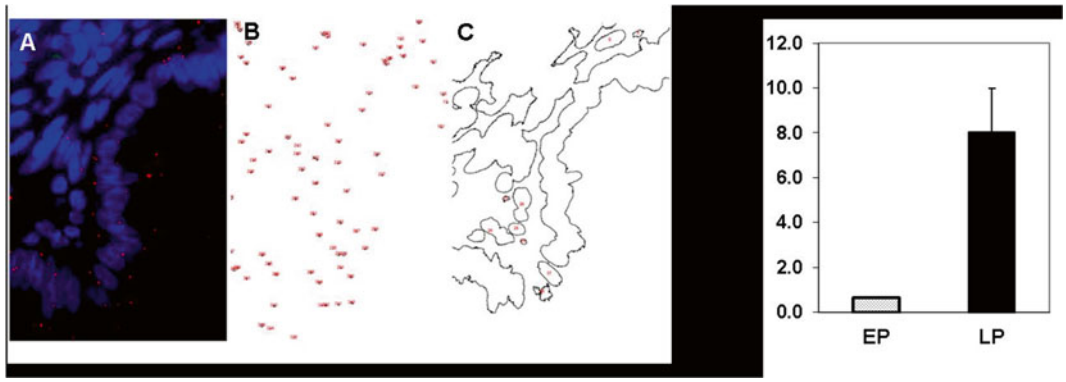

Fig. 5 Representative Image J quantification of angioprotein-1 in the uterine endometrium. (**a**) Color image of area to be quantified. Image must be separated into separate color channels in Image J prior to analysis. Picture will be analyzed according to the parameters set for each color. (**b**) Ang-1 signals (*red spots*) of a certain size and intensity are highlighted and counted. The screen shot shows each signal counted with the outline of the signal and the number assigned to it in *red*. For PLA signals, we quantified the individual number of signals rather than the area of the signal (given as well in the analysis) because the area has no practical meaning as each signal represents one molecular interaction. (**c**) For the nuclei (*blue*), all counted areas are *outlined* and *numbered*. Due to the overlapping nature of the cell nuclei, often a contiguous area was counted as one nucleus. Therefore, we chose to normalize to area of nuclei to represent cell number in an image versus the number of individual nuclei present. (**d**) Angiopoetin-1 protein expression quantified by PLA using Image J software (mean ± SE; PLA signals/nuclear area × 10^4) in the endometrium during the early proliferative (EP, $n = 3$) and late proliferative phases (LP, $n = 3$) of the baboon menstrual cycle. LP values are increased ($P < 0.05$) over EP

the mounting media with DAPI or Hoechst 33342 being the best choices because the blue signal is not offered as part of the detection reagent options with the Duolink In Situ PLA reagents. Our laboratory uses Vectashield mounting media for fluorescence with DAPI. Currently, there are multiple fluoroprobes available as part of the detection reagent kit, as well as a bright field option for PLA detection using a NovaRed to generate brown signals. A nuclear counterstain such as hematoxylin would be appropriate.

19. Many software options are available for image acquisition, processing and quantification purposes, including quantitative software from Olink Bioscience directly or even freeware such as Image J (available for download from the National Institutes of Health). The use of Image J is shown (Fig. 5) for the analysis of Ang-1in baboon uterine endometrium.

Acknowledgements

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Chapter 13

Colocalization of Estrogen Receptors with the Fluorescent Tamoxifen Derivative, FLTX1, Analyzed by Confocal Microscopy

Araceli Morales, Raquel Marín, Jorge Marrero-Alonso, Alicia Boto, and Mario Díaz

Abstract

Tamoxifen is a selective estrogen receptor modulator that competitively binds the ligand-binding domain of estrogen receptors. Binding of tamoxifen displaces its cognate ligand, 17β-estradiol, thereby hampering the activation of estrogen receptors. Cellular labeling of ER is typically carried out using specific antibodies which require permeabilization of cells, incubation with secondary antibodies, and are expensive and time consuming. In this article, we describe the usefulness of FLTX1, a novel fluorescent tamoxifen derivative, which allows the labeling of estrogen receptors in immunocytochemistry and immunohistochemistry studies, both under permeabilized and non-permeabilized conditions. Further, besides labeling canonical estrogen receptors, this novel fluorescent probe is also suitable for the identification of unconventional targets such membrane estrogen receptors as well as other noncanonical targets, some of which are likely responsible for the number of undesired side effects reported during long-term tamoxifen treatments.

Key words Estrogenreceptors, Fluorescent tamoxifen derivative, Confocal microscopy, FLTX1, Membrane estrogen receptors, Intracellular estrogen receptors

1 Introduction

Tamoxifen is a selective modulator of estrogen receptors (SERMs) widely used in the treatment of breast cancer [1, 2]. Tamoxifen is also used as coadjuvant therapy after breast surgery to prevent contralateral breast cancer. Chemically, tamoxifen is a triphenylethylene derivative endowed with a dialkylaminoethoxy side chain that competitively binds estrogen receptors in breast cells, and prevents the transcriptional activation of estrogen-dependent genes controlling the proliferation of mammary gland cells [2]. Although it was first introduced in the late 1970s, it is still the first pharmacological strategy for hormone-sensitive breast cancers. In spite of its proven beneficial effects in treating estrogen

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receptor positive breast cancers, several side effects, some of them life-threatening, appear exacerbated in patients receiving chronic therapies [3, 4]. It is thought that these effects are due, at least in part, to the existence of both canonical and noncanonical molecular targets, whose identification has been hampered by the lack of appropriate molecular tools. In recent years, we have designed and developed a novel fluorescent tamoxifen derivative, FLTX1, that retains most pharmacological properties of tamoxifen (specific ER binding, antagonism of ER-mediated transcriptional activation and cell proliferation), but lacks any of the uterotrophic actions of tamoxifen in the uterus [5]. In addition to its pharmacological properties, FLTX1 is endowed with especial photonic and fluorescent properties. When dissolved in an appropriate gain medium and excited with a picosecond pulsed laser beam, it behaves as a laser dye to emit amplified laser spontaneous emission with an efficiency higher that reported for any laser dye commercially available [6]. In this chapter we provide protocols for the identification of classical targets such as ERs, and also for some noncanonical antiestrogen-binding sites known to be targets of tamoxifen, by immunofluorescence using FLTX1.

2 Materials

Prepare all solutions using ultrapure deionized water (resistivity 18.2 MΩ) and use analytical grade reagents (dimethylsulfoxide, ethanol, and acetone). Prepare and store all reagents at room temperature unless indicated otherwise.

1. Tamoxifen.
2. Ethyl chloroformate.
3. Dichloroethane.
4. 4-Chloro-7-nitro-1,2,3-benzoxadiazole.
5. Methanol.
6. Acetone or dimethylsulfoxide (DMSO).
7. Phosphate-buffered saline (PBS): 1.37 M NaCl, 27 mMKCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4. To prepare 10× stock, dissolve in 800 mL distilled H_2O: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4. Adjust pH to 7.4 with HCl. Adjust volume to 1 L with additional distilled H_2O. Store at 4 °C.
8. 1× PBS: Dilute 1 volume 10× PBS with 9 volumes of ultrapure water. Prepare fresh 1× PBS weekly by diluting 1 volume PBS stock solution with 9 volumes of ultrapure deionized water. Store at room temperature.

9. Citrate buffer: 10 mM citric acid, 0.05 % Tween 20, pH 6.0. To prepare, mix 1.92 g of anhydrous citric acid in 800 mL of purified water. Adjust the pH to 6.0 with 1 N NaOH, then add 0.5 mL of Tween 20. Adjust volume to 1 L with purified water. Store at room temperature for up to 3 months; for extended storage, keep at 4 °C.
10. ERα antibody: Rabbit anti-ERα (sc-542, Santa Cruz Biotechnology) and Alexa Fluor® 514 goat anti-rabbit IgG secondary antibody (A-31558, Life Technologies) are used to label ERα.
11. ERβ antibody: Goat anti-ERβ (sc-6821, Santa Cruz Biotechnology), donkey anti-goat IgG biotin conjugate, and Alexa Fluor® 514 streptavidin conjugate (S-32353, Life Technologies) are used to label ERβ.
12. Propidium iodide: 3 μM propidium iodide dihydrochloride in PBS. Store at 4 °C.
13. DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine): 300 nM DAPI in PBS. Store at 4 °C.
14. Tamoxifen (Tx, purity = 99 %). Prepare Tx stock solution at 10–30 mM; store at −20 °C. Final concentrations should be prepared by dilution in ethanol shortly before starting the experiments, but can be stored at 4 °C and used for a week.
15. 17β-Estradiol (E2, purity = 98 %). Prepare E2 stock solution at 10-30 mM and store at −20 °C. Final concentrations should be prepared by dilution in ethanol shortly before starting the experiments, but can be stored at 4 °C and used for a week.
16. FLTX1 (*N*-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)demethyl-tamoxifen): To prepare a 30 mM stock solution, add 1 mL acetone to 15.6 mg FLTX1. Store the stock solution at −20 °C. The stock is stable for 6–8 weeks (*see* **Note 1**).
17. DMEM culture medium: Dulbecco's modified Eagle's medium (DMEM) with/without phenol red supplemented with 10 % heat-inactivated (at 50 °C for 30 min) fetal bovine serum (FBS), 60 U/mL penicillin-G, 100 μg/mL streptomycin and 3.7 mg/mL $NaHCO_2$.
18. Plasma-treated tissue culture flasks and 8-well chamber slides for cell culture.
19. 0.25 % trypsin-EDTA solution.
20. Non-permeabilizing cell fixative: 2 % paraformaldehyde, 0.1 % glutaraldehyde, 150 mM sucrose.
21. Permeabilizing cell fixative solution: 0.5 % Nonidet P-40.
22. Image-iT™ FX signal enhancer (Life Technologies).

23. Mounting solution: 1 volume PBS to 1 volume glycerol (1:1), containing 10 % (w/v) 1,4-Diazabicyclo[2.2.2]octane (DABCO).
24. Cover slips, fingernail polish.
25. ERα agonist propyl pyrazoletriol (PPT) and ERβ agonist diarylpropionitrile (DPN): Prepare stock solutions at 10 mM in DMSO and store at −20 °C. Dilute in ethanol to obtain the desired final concentrations. Dilutions can be stored at 4 °C and used for several weeks.
26. Selective estrogen receptor modulators (SERM) endoxifen and 4-OH-tamoxifen. Prepare as with tamoxifen.
27. 4 % paraformaldehyde diluted in PBS.
28. 70, 80, 90, 96, 100 % ethanol, diluted in PBS.
29. Gelatin-treated microscope slides.

3 Methods

3.1 Synthesis of FLTX1

1. FLTX1 is not commercially available, but it can be either synthesized from tamoxifen or obtained upon request to the authors. Synthesis of FLTX1 is carried out following the procedure described in detail in [5]. Briefly, FLTX1 is formed in two sequential steps, consisting of the demethylation of the tamoxifen side chain, followed by introduction of the NBD group (7-nitro-1,2,3-benzoxadiazole).
2. Treat tamoxifen with commercial ethyl chloroformate and reflux in dichloroethane (shown in step A, Fig. 1).
3. Treat the resulting *N*-demethyltamoxifen with 4-chloro-7-nitro-1,2,3-benzoxadiazole (NBD-Cl) in methanol (shown in step B, Fig. 1) to yield FLTX1. FLTX1 fluorescence spectra show maximal excitation and emission peaks centered at 476 nm and 527 nm, respectively.
4. Store FLTX1 at −20 °C; it is stable for several months.

3.2 Preparation of Cells

1. The following cell culture procedures can be adapted to different ER-positive cell lines. We have used MCF7, T47D, SN56, and HT22 established cell lines from different origins, from epithelial to neuronal. This protocol describes the use of the estrogen receptor-positive breast cancer cell line, MCF7.
2. Maintain cell culture stocks in DMEM culture medium at 37 °C under 95 % air/5 % CO_2 atmosphere. Use plasma-treated tissue culture flasks made of high-quality polystyrene.
3. At 90 % confluency, trypsinize cells with trypsin-EDTA solution. Typically, MCF7 cells require at least 3–5 min exposure to trypsin solution at room temperature whereas many other

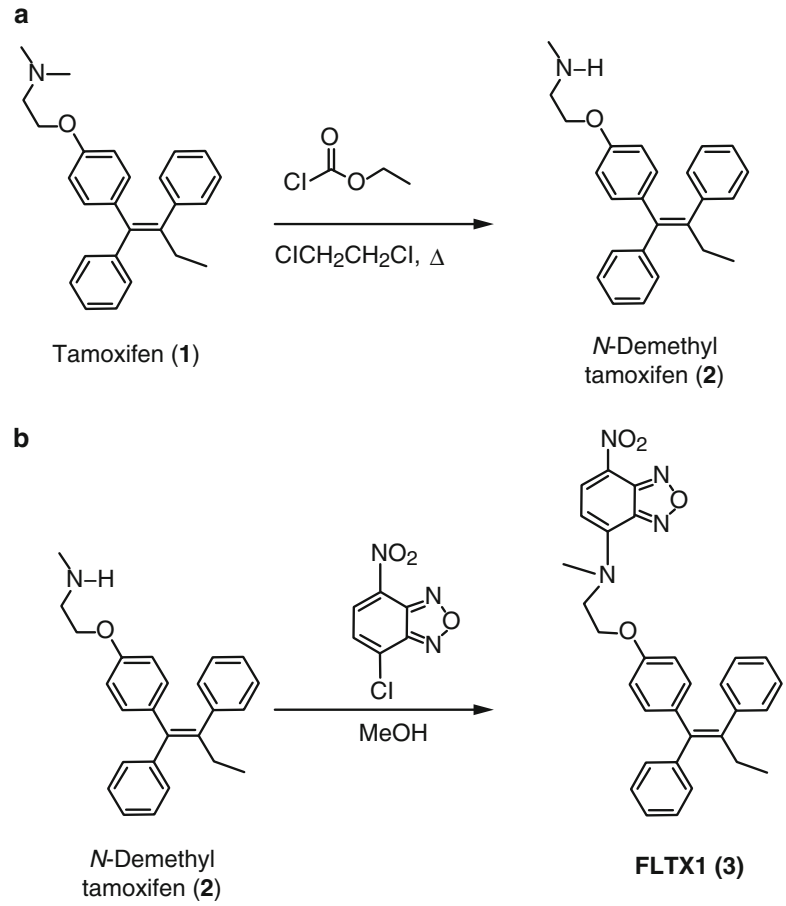

Fig. 1 Synthesis of *N*-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)demethyltamoxifen (FLTX1). In the first reaction, tamoxifen is *N*-demethylated to form *N*-demethyltamoxifen (**a**), then *N*-(7-nitrobenzo[c][1,2,5]oxadiazol) (NBD) is covalently bound in the presence of methanol (**b**)

cell lines require less time (1–2 min). It is convenient to follow the progress of trypsinization using a phase contrast microscope until becoming familiar with the process (*see* **Note 2**).

4. Cell fixation/permeabilization. Grow ER-positive MCF7 cells on 8-well chamber slides until 80 % confluency.
5. For experiments in which penetration of the probe into cells is not recommend (non-permeabilizing conditions), which allow labeling of plasma membrane targets, incubate slides with non-permeabilizing cell fixative solution for 30 min at room temperature.
6. For experiments that require intracellular penetration of the fluorescent probe to allow labeling of cytoplasmic or nuclear targets (permeabilizing conditions), incubate the cells in permeabilizing cell fixative solution for 2 min at room temperature.

3.3 FLTX1 Staining

1. After fixation, wash the cells twice with PBS and incubate with Image-iT™ FX signal enhancer for 30 min at room temperature to block nonspecific interactions of FLTX1.
2. Expose the cells to FLTX1 (50–100 μM in acetone, DMSO or ethanol) for 2 h at room temperature.
3. Wash the cells five times with PBS.
4. Mount the cell chamber slides in mounting solution. Apply cover slips and seal with fingernail polish. Figure 2 illustrates typical FLTX1 staining of MCF7 cells under non-permeabilizing (a) and permeabilizing conditions (c).

3.4 Cell Double Staining with FLTX1 and ER

This protocol allows colocalization analysis of FLTX1 with ERα or ERβ.

1. Culture the cells on 8-well chamber slides.
2. Fix the cells under permeabilizing conditions (for nuclear and intracellular staining) or non-permeabilizing conditions (for plasma membrane staining).
3. Wash the cells three times with PBS.
4. Incubate the slides with polyclonal anti-ERα diluted 1:100 in PBS in the presence of 0.1 % normal goat serum.

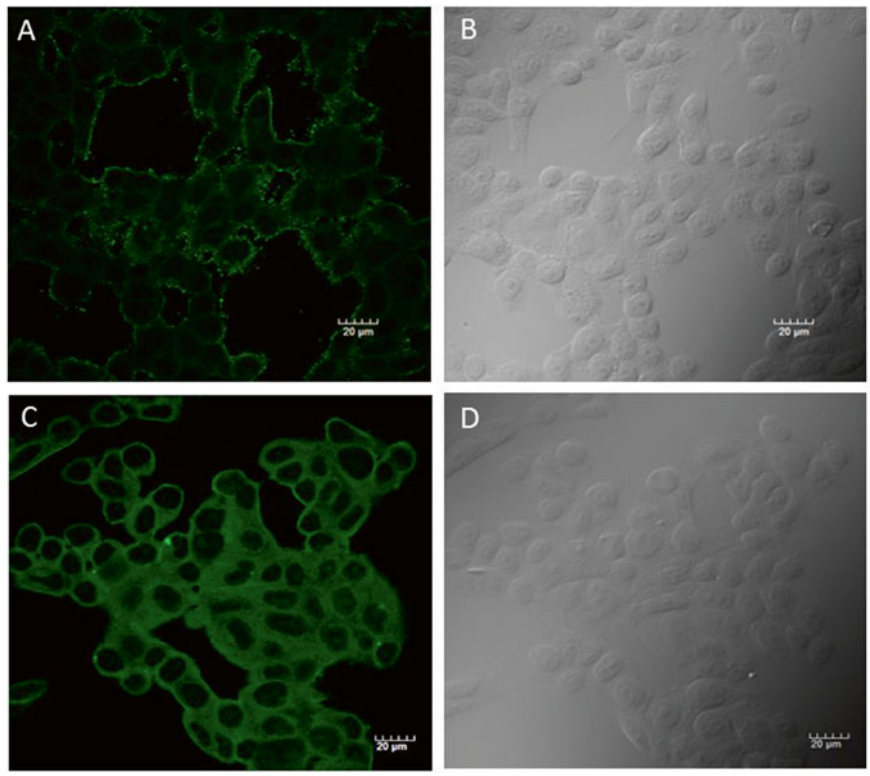

Fig. 2 FLTX1 staining under non-permeabilizing (**a**) and permeabilizing (**c**) conditions. Corresponding transmission images are shown in **b** and **d**. Concentration of FLTX1 used was 50 μM in 100 % DMSO

5. Use polyclonal anti-ERβ diluted 1:50 in PBS in the presence of 0.1 % normal goat serum for ERβ labeling. Allow incubation with anti-ERα or anti-ERβ antibodies overnight at 4 °C under gentle agitation.
6. Remove primary antibody.
7. Wash slides five times with PBS.
8. Incubate with a corresponding Alexa 514-coupled secondary antibody diluted 1:500 in PBS in the presence of 0.1 % normal goat serum.
9. Incubate cells with 50 μM FLTX1 as in Subheading 3.3 and mount slides for visualization in with mounting medium.

3.5 Cellular Nuclei Staining

If staining of the nuclei is required, carry out the following steps after the staining of ERα or ERβ and FLTX1.

1. Expose the cells to DAPI for 5 min at room temperature.
2. Wash the cells immediately with PBS three times.
3. Mount slides for visualization using mounting solution. Figure 3 illustrates a typical FLTX1-ERα-DAPI colocalization experiment in MCF7 cells.

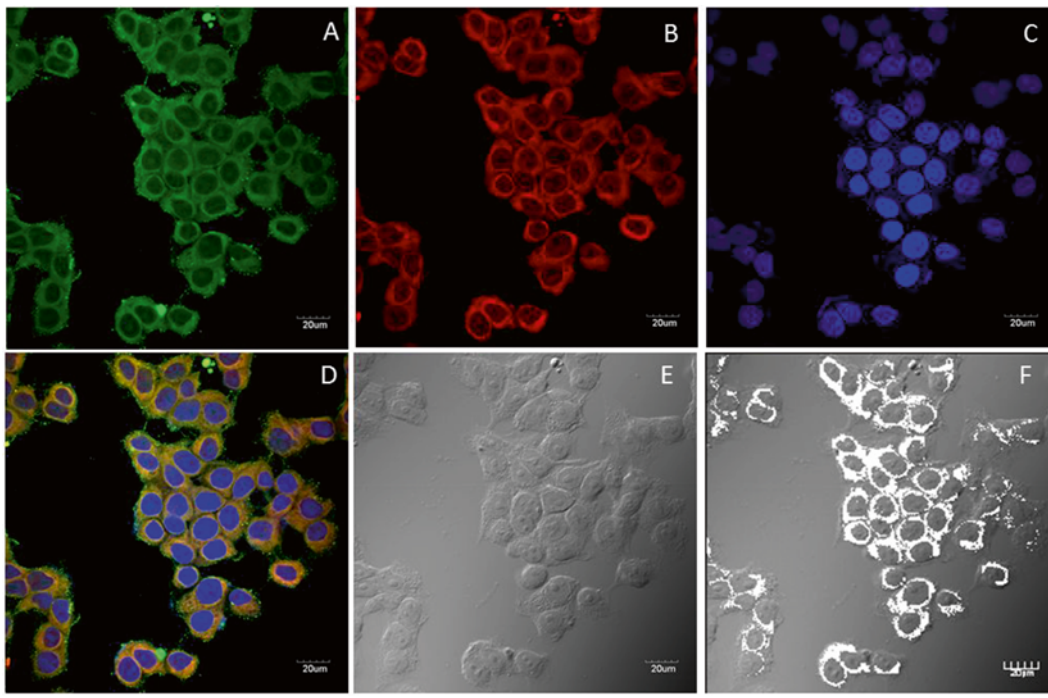

Fig. 3 FLTX1 labeling (**a**) colocalizes with estrogen receptor alpha (ERα) (**b**) in unstimulated MCF7 cells. Nuclei stained with DAPI are shown in **c**. (**d**) Merged image of FLTX1, ERa, and DAPI staining, resulting in a *yellowish* color, mostly located in the cytoplasm, while the nuclei are distinctively colored in *blue*. Transmission image is shown in (**e**) and overlapped with *white spots* indicating colocalization (**f**)

3.6 Competition Experiments

Competition experiments allow an analysis of the binding site(s) for FLTX1 in a given cell or tissue type. That is, the nature of a binding site for FLTX1 in a particular cell or tissue type can be identified by the ability of one agent versus another to compete with FLTX1 for binding to that site. This also allows the discrimination of canonical ER from other noncanonical antiestrogen-binding sites that are capable of binding tamoxifen but are unrelated to ERα and ERβ.

1. For competition assays, grow MCF7 cells (or cells of your choice) on 8-well chamber slides. Allow the cells to reach 60-80 % confluency.
2. Proceed with the permeabilizing or non-permeabilizing protocol as indicated in Subheading 3.2, **steps 5** and **6**, depending on the location of the expected target.
3. After fixation, wash the cells twice with PBS.
4. Incubate with Image-iT™ FX signal enhancer for 30 min at room temperature to block nonspecific interactions as in Subheading 3.3, **step 1**.
5. Wash fixed cells twice with 1× PBS.
6. Wash the cells twice with the solvent that was used to dissolve the competitor ethanol or DMSO (depending on the competitor solvent).
7. Expose the cells to the desired competitor at concentrations of 1–100 μM for 30 min at room temperature. Typical competition experiments may include 17β-estradiol, the ERα agonist PPT, the ERβ agonist DPN [7], or SERMs such as Tx, 4-OH-tamoxifen, or endoxifen [8] (*see* **Note 3**).
8. After the cells have been exposed to competitors, incubate for 2 h at room temperature in the presence of 50 μM FLTX1 plus the appropriate competitor. FLTX1 vehicle must be the solvent used to prepare the competitor (typically ethanol or DMSO).
9. Wash with PBS five times.
10. Mount the slides in with mounting solution for confocal microscopy.

3.7 Preparation and Labeling of Uterine Tissue Sections

1. For uterine histological and confocal microscope studies, fix mouse uterus with 4 % paraformaldehyde diluted in PBS overnight at 4 °C.
2. Dehydrate the tissue in successive ethanol baths at 70, 80, 90, 96, and 100 % ethanol in PBS, for 30 min in each ethanol bath, at room temperature.
3. Wash uterine tissues four times in melted paraffin 56–58 °C.
4. Embed tissue in paraffin blocks for microtome sectioning.
5. Keep paraffin-tissue blocks at 4 °C until use, and cut crosswise with a microtome in 10 μm thick sections.

6. Mount sections onto gelatin-treated microscope slides.
7. For immunohistochemistry, deparaffinize the uterine sections in xylol for 5 min at room temperature.
8. Rehydrate the tissue sections by subsequent ethanol baths (100, 96, 90, 80, and 70 % ethanol in PBS) for 5 min each ethanol concentration at room temperature.
9. For antigen retrieval, incubate tissues in citrate buffer by immersing the slides in a steamer in a glass dish containing sodium citrate buffer at 95 °C for 20 min.
10. Remove the slides from the steamer and allow to cool for an additional 20 min.
11. Rinse the slides twice in PBS, and immediately process for fluorescent staining.
12. Optional step: For colocalization studies, sections may be incubated at this stage with polyclonal anti-ERα antibody diluted 1:100 in PBS, pH 7.4, with 0.1 % Tween-20 and 0.1 % normal goat serum, to label ERα. Incubate tissue sections with the antibody overnight at 4 °C and then wash preparations with PBS.
13. Incubate tissues on slides with FLTX1 (100 μM) diluted in 100 % acetone, overnight at 4 °C, covered with a cover slip to avoid excessive evaporation.
14. Wash the sections three times in PBS.
15. Following treatments, propidium iodide may be used to stain nuclei in uterine sections. For this purpose, incubate the preparations in the presence of propidium iodide for 5 min at room temperature.
16. Rinse three times in PBS.
17. Wash slides three times in PBS, pH 7.4, and mount for microscope visualization in 1 % DABCO 33-LV and PBS: glycerol (1:1).

3.8 Analysis of Fluorescent Signals

1. Visualize fluorescent signals by confocal microscopy (*see* **Note 4**). FLTX1 and Alexa Fluor 514-coupled secondary antibody should be excited using, respectively, the 458 nm and 514 nm spectral lines of the argon-ion laser. DAPI and propidium iodide nuclear probes are excited using a violet 405 nm diode laser and a helium-neon 543 nm laser, respectively.
2. For colocalization experiments, scan the cells sequentially with the individual lasers to detect fluorescence in each photomultiplier channel to coincide with laser illumination. This will allow accurate colocalization analyses and eliminate spectral bleed-through artifacts.

3. Using the confocal microscope software, determine the degree of fluorophore colocalization of cell images from the composite image derived from independent channel single-wavelength acquisitions.
4. Represent the graphical 2D display for colocalization analysis by a scatterplot or fluorogram which correlates the relationship between FLTX1 and ERα (or ERβ). Intensity pairs generate a distribution pattern that enables identification of FLTX1-ERα (or FLTX1-ERβ) colocalization.
5. Colocalization values are estimated analyzing only the signal arising from pixels within the selected region of interest (*see* **Note 5**). A colocalization binary threshold mask is obtained by overlapping pixel maps in the cells, and can be superimposed on the image (Fig. 3f, colocalization threshold mask merged with the differential interference contrast image).
6. For competition assays, use the same confocal configuration, that is, laser power (4–5.5 %), gain (1), photomultiplier voltage, and so forth, to acquire images. Quantitative values for competitive assays may be obtained by measuring pixel intensity values in regions of interest for each experimental condition and referred to the percentage of pixel intensity in cells exposed to FLTX1 alone.

4 Notes

1. FLTX1 is also readily dissolved in DMSO; it may be more stable in DMSO than in acetone. FLTX1 is less soluble in ethanol. To dissolve FLTX1 in ethanol, vortex the solution for approximately 30 s, then sonicate twice for 10 s on ice. After that, heat the solution at 30 °C for 5 min to completely dissolve the compound.
2. Do not use cells beyond 9–10 passages, as they may change their ER phenotype.
3. Non-canonical targets may also be studied by competition with appropriate cognate ligands. For instance, we have used the same protocol in Subheading 3.5 to analyze the competition by nifedipine (a blocker of L-type calcium channels) of FLTX1 binding in neuronal cell lines.
4. The confocal microscope that we use is an Olympus FluoView FV1000 equipped with a 60× objective (oil Plan Apo, NA = 1.35); equivalent confocal microscopes with the same lasers will also work for these analyses.
5. To set the threshold levels of signal that will be included in the analysis, a region of interest must be selected in the scatterplot to determine the background signal that will be subtracted.

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Chapter 14

Live-Cell Imaging of the Estrogen Receptor by Total Internal Reflection Fluorescence Microscopy

Kassandra Kisler* and Reymundo Dominguez*

Abstract

Trafficking studies of plasma membrane-localized intracellular estrogen receptors have mainly relied on biochemical and histological techniques to locate the receptor before and after estradiol stimulation. More often than not these experiments were performed using postmortem, lysed, or fixed tissue samples, whose tissue or cellular structure is typically severely altered or at times completely lost, making the definitive localization of estrogen receptors difficult to ascertain. To overcome this limitation we began using total internal reflection fluorescence microscopy (TIRFM) to study the trafficking of plasma membrane estrogen receptors. This real-time imaging approach, described in this chapter, permits observation of live, intact cells while allowing visualization of the steps (in time and spatial distribution) involved in receptor activation by estradiol and movements on and near the membrane. TIRFM yields high-contrast real-time images of fluorescently labeled E6BSA molecules on and just below the cell surface and is ideal for studying estrogen receptor trafficking in living cells.

Key words Estrogen action, Estrogen receptor alpha, ERα, Membrane-initiated estradiol signaling, Plasma membrane estrogen receptors, Total internal reflection fluorescence microscopy, TIRFM, Trafficking, Endocytosis, E6BSA-FITC

1 Introduction

In the mammalian brain the sex steroid hormone estradiol (17β-estradiol, E2), the most predominant and active form of estrogen, controls development and regulates neurons that endogenously express estrogen receptors. In estrogen receptor (ER) expressing neurons it is well known that estradiol is capable of regulating its actions by a classic steroid hormone receptor mechanism [1]. For example, estradiol crosses the plasma membrane lipid bilayer to bind to intracellular ER proteins, of which there are two main isoforms, ERα and ERβ [2, 3]. Estradiol binding activates intracellular receptors to form ER dimers that translocate to the cell

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nucleus and bind to estrogen-responsive genes. Activation of the genomic pathway ultimately leads to gene transcription, translation, and protein production [4].

The genomic expression of several neurotransmitter and growth hormone receptors are regulated by estradiol and estrogen receptor activation [5]. In addition, estradiol regulates neurotransmitter receptor activity as well as signal transduction pathways by a mechanism that occurs too quickly (seconds to minutes) to involve the genomic expression of proteins, and that persists even in the presence of protein synthesis inhibitors [6, 7]. Data strongly suggest these rapid actions are regulated by a subpopulation of intracellular ER proteins localized to the plasma membrane [8]. Our western immunoblot and PCR studies show that hypothalamic, cortical, and hippocampal neurons endogenously express full-length 66 kDa ERα and a host of other ERα-like proteins, predominantly a 52 kDa ERα splice variant missing the fourth exon (ERαΔ4), that are present on the plasma membrane [9–11].

1.1 Identification of Plasma Membrane ER Localization

Presently, it is largely unclear how intracellular ER proteins become localized and regulated at the plasma membrane of neurons. Sequence analysis of estrogen receptor genes has not been able to identify an integral membrane domain sequence that could explain the localization of receptors on the cell surface [12]. However, evidence strongly suggests that there is a portion of the ER protein structure that extends through the plasma membrane lipid bilayer and directly into the extracellular space [9, 13, 14]. Additionally, studies have shown that estrogen receptors can anchor to the inner leaflet of the plasma membrane via posttranslational palmitoylation [15, 16]. The localization of plasma membrane ER proteins also requires caveolin 1 [17, 18]. Caveolin 1 is a key structural protein involved in the formation of cell membrane caveolae that localize in lipid raft microdomains and are crucial to membrane-initiated estradiol signaling [19].

In past studies we found that estradiol treatment rapidly triggers the trafficking (>5 min) of biotinylated 66 kDa- and 52 kDa-ERα isoforms out of and into the plasma membrane of cortical, hippocampal, and hypothalamic neurons [9–11]. Estradiol-stimulated trafficking of estrogen receptors suggests that the steroid is able to rapidly activate and regulate the level of ERα protein present on the plasma membrane of neurons. We have also visualized the internalization of a commonly used fluorescently labeled membrane-impermeant estradiol receptor ligand, E6BSA-FITC (estradiol conjugated to bovine serum albumin labeled with fluorescein), in primary cortical neurons [11]. The binding of E6BSA-FITC provides additional evidence that an extracellular binding site for estradiol is present on the surface of neuronal plasma membranes. To further investigate the mechanism linked to internalization of the signaling complex we double-treated a subset of cortical neurons with E6BSA-FITC along with the fluorescent

marker for ganglioside GM1, a lipid raft marker associated with vesicular endosomes. Confocal analysis revealed that while GM1 and E6BSA-FITC had somewhat different localization profiles, there were multiple points of colocalization, both on the plasma membrane and within the intracellular space [11].

1.2 Visualizing Plasma Membrane ERα Trafficking in Live Cells

To investigate estrogen receptor trafficking in more detail we have started to use live cells and real-time total internal reflection fluorescence microscopy (TIRFM, Fig. 1). In previous studies, primarily biochemical and histochemical techniques were used to identify and locate estrogen receptors before and after estradiol treatment. Due to the nature of these techniques, the reagents used, and the tissue source it is assumed that some information was lost and that results could have been due to experimental artifacts [20–26]. Using live cells and TIRFM permits us to maintain cellular integrity while teasing apart the sequence of steps (time, speed, direction in the *x*, *y*, and *z* planes) involved in plasma membrane-associated estrogen receptor trafficking before and after its activation by estradiol [10, 27]. This imaging technique, described in this chapter, yields high-contrast, real-time digital images of fluorescent molecules (e.g., E6BSA-FITC) on and just inside the plasma membrane and is ideal for studying receptor protein movements in living cells [28]. Thus cells can be easily grown on top of glass cover slips to image the approach (brightening, moving toward the surface) or retreat (dimming, moving away from the surface) of fluorescent molecules, as they pass into and out of a thin evanescent (decaying) wave illumination field (Fig. 1).

2 Materials

2.1 Fluorescent Materials

1. 10 mL sterile filtered 0.9 % normal saline.
2. 1 mg of fluorescein-labeled β-estradiol-6-(*O*-carboxymethyl) oxime-bovine serum albumin (E6BSA-FITC; Sigma) (*see* **Note 1**).
3. 1 mg bovine serum albumin-fluorescein (BSA-FITC; Sigma) (*see* **Note 2**).
4. Small fluorescent beads with fluorescence properties similar to fluorescein/FITC (TetraSpeck Microspheres, 0.2 μm, Life Technologies).
5. Amicon Ultra-15 Centrifugal Filter Units (Millipore).
6. Sterile 1 mL syringe with 13 mm syringe filter with Supor Membrane, 0.2 μm.

2.2 Glass-Bottom Dishes

Dishes can be purchased or custom made in the lab. Here we provide information for custom-making dishes.

1. 35 mm tissue culture polystyrene dishes (Falcon) (*see* **Note 3**).

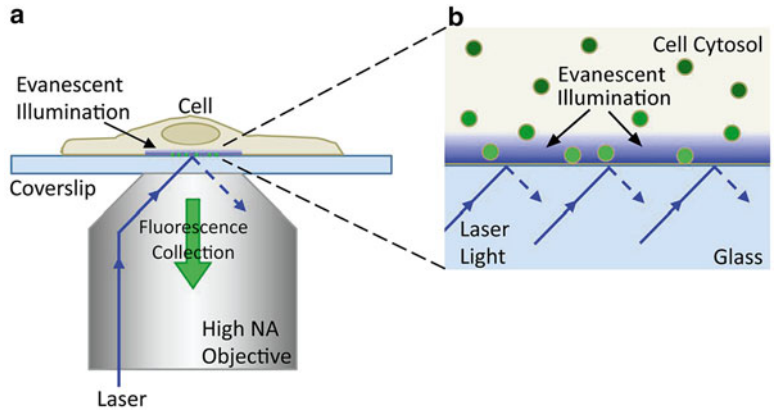

Fig. 1 TIRFM is used to study the trafficking of fluorescent molecules. (**a**) For prismless TIRFM, a laser beam is directed through a high NA objective such that the light is incident at the cover slip/water interface at an oblique angle. At a sufficiently high angle, the incident light undergoes total internal reflection at the interface, generating an evanescent illumination wave that typically penetrates ~100–200 nm into the aqueous medium. Fluorescence generated by this evanescent light is collected by the same objective. (**b**) An expanded view illustrating that only fluorescent molecules near the cover slip interface are illuminated [27]

2. #1 thickness cover slip glass, 25 mm squares, precleaned (*see* **Note 4**).
3. Sylgard 184 Silicone Elastomer (Dow Corning).
4. Autoclaved 200 μL pipette tips.
5. Drill press with ¾″ butterfly drill bit and safety glasses.
6. Self-made wood jig to hold dishes (*see* **Note 5**).
7. Deburring tool (Reed Deb3 RD-04437).
8. 100 % Isopropanol.
9. Dry heat oven.
10. 60 mm Sterile Petri Dishes.

2.3 Cells and Cell Culture Materials

To visualize estrogen receptor trafficking we selected N-38 neurons as a model for membrane-initiated estradiol signaling [10, 29]. N-38 neurons adhere tightly to glass cover slips without the need of a substratum. In general, the adhesion of cells to the glass cover slip is crucial when using the TIRFM technique because the evanescent wave generated at the glass/aqueous interface only penetrates 100–200 nm above the glass and toward the adherent cell surface membrane. Use adherent cells appropriate to your experimental design. Some cell types may require a coating or substratum on the glass for sufficient membrane adhesion.

1. Stock of N-38 neurons (Cellutions Biosystems).
2. Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate.

3. Penicillin/streptomycin (pen-strep, 100×): Stock solution is 10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin.
4. Heat inactivated fetal bovine serum (FBS).
5. Phosphate-buffered saline (PBS) with 1 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.2.
6. Insulin-transferrin-sodium selenite (ITS) media supplement.
7. Estrogen receptor (ERα and ERβ) antagonist, ICI 182,780: 1 mM.
8. Ethanol, 200-proof.
9. External solution for imaging: 140 mM NaCl, 3 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM D-GLUCOSE, pH 7.3, osmolarity of 295–310 mmol/kg (*see* **Note 6**).
10. T-25 tissue culture flask, vented.

2.4 TIRF Microscope Setup

There are a variety of commercial and custom-built TIRF microscopes in common usage. We recommend an objective-based or "prismless" system for this application (Fig. 1), and refer the reader to extensive reviews and methods available [30–33], and online resources such as MicroscopyU (http://www.microscopyu.com) and the Microscopy Resource Center (http://www.olympusmicro.com) for more information regarding the theory and practice of TIRFM. Herein we describe the components of a specific single-color custom-built laser TIRFM system. However, all prismless TRIFM systems will have similar components regardless of manufacture. Also, experimental capabilities can be expanded to multiple colors with the addition of appropriate light sources, filters, color splitters, and so forth, to the system.

1. Inverted microscope (Olympus IX70), equipped with a dichroic suitable for reflecting the excitation wavelengths, and transmitting fluorophore emission light (XF2046, Omega) (*see* **Note 7**), and an emission filter (HQ 515/30 m, Chroma). The microscope should also have bright-field capabilities (*see* **Note 8**).
2. Laser light source to excite fluorophores (473 nm for the fluorophore described here), coupled to the microscope such that the angle of incidence at the cover slip can be changed. Lasers should include shutters (Uniblitz) or similar to control light output.
3. High Numerical Aperture (NA) objective (α Plan-FLUAR 100× oil, NA 1.45, Zeiss) and appropriate low-fluorescence immersion oil (*see* **Note 9**).
4. High-sensitivity camera (Cascade 512B EMCCD camera, Roper) and acquisition and control software (Metamorph 4.6, Universal Imaging) (*see* **Note 10**).

3 Methods

3.1 Preparation of E6BSA-FITC and BSA-FITC Chemicals

Estradiol is known to become detached and free from E6BSA preparations, so to reduce the level of free estradiol from our E6BSA-FITC preparation we use a modified high-speed centrifugation protocol [34]. Control BSA-FITC is prepared in the same manner.

1. Take care to prevent the photobleaching of FITC during processing and storing.
2. Add 1 mL of 0.9 % sterile saline to 1 mg of E6BSA-FITC to make stock solution. Vortex until powder is dissolved completely.
3. Prewash two Amicon Ultra-15 Centrifugal Filter Units by adding 500 μL of 0.9 % sterile saline and centrifuging the units in a swing-bucket rotor at 4000 × *g* for 15 min at 4 °C.
4. Add 500 μL of the 1 mg/mL E6BSA-FITC stock solution to each one of the filter units and centrifuge them in a swing-bucket rotor at 4000 × *g* for 25 min at 4 °C.
5. Wash the filter retentate containing E6BSA-FITC three times by adding 500 μL of 0.9 % sterile saline and centrifuging each unit in a swing-bucket rotor at 4000 × *g* at 4 °C for 15 min.
6. Calculate and replace the volume of 0.9 % saline needed to return the solution to 1 mg/mL E6BSA-FITC (*see* **Note 11**).
7. Filter each E6BSA-FITC sample through a 0.2 μm syringe filter to sterilize, make small aliquots, and store at –20 °C until use.
8. Prepare a BSA-FITC stock using the same filter purification steps.

3.2 Glass-Bottom Dish Preparation

1. Use sterile gloves when handling dish lids and wear safety glasses when operating the drill.
2. Place the lid from a Falcon 35 mm tissue culture polystyrene dish into the wood jig (*see* **Note 12**).
3. With the jig and a ¾″ butterfly drill bit in place, slowly drill a hole at the exact center through the dish lid.
4. Use a deburring tool to remove the rough edges left over from drilling the polystyrene (*see* **Note 13**).
5. After deburring wash the lids with 100 % isopropanol alcohol and air-dry.
6. Mix Sylgard 184 according to the manufacturer's directions.
7. Place dish upside-down. Use a 200 μL tip to draw a small bead of Sylgard 184 around the outer surface of the hole, then place a 25 mm square of #1 thickness glass cover slip to cover the hole. Keep the lid on a flat surface (*see* **Note 14**).

8. Cure Sylgard 184 for 1 h at 60 °C in a dry heat oven. Keep the dish lid on a flat surface during curing (*see* **Note 15**).
9. Immediately after curing expose to UV light for 15 min on each side and then encase each dish inside a sterile 60 mm petri dish for storage (*see* **Note 16**).

3.3 N-38 Cultures and Fluorescence Assays

1. For imaging, seed N-38 neurons at a density of 10^4 cell/cm^2 onto glass-bottom culture dishes (Subheading 3.2) and maintain in DMEM plus 10 % FBS and 1× pen-strep for 2 days. Maintain and grow the cells in a cell culture incubator set at 37 °C with 5 % CO_2 (*see* **Note 17**).
2. On day 3, remove the DMEM + FBS medium, wash the cells twice with PBS, and then maintain the cells in DMEM with ITS (21 mg/L insulin, 19 mg/L transferrin, 25 μg/L sodium selenite) and 1× pen-strep without FBS to starve the cultures of steroid hormones.
3. On day 4, treat the N-38 cultures with DMEM + ITS medium for 30 min with 1 μg/mL E6BSA-FITC or 1 μg/mL BSA-FITC. During each imaging session, add an equivalent volume of 0.9 % saline to a subset of dishes to test as a vehicle control.
4. If desired, antagonize ERα and ERβ activation by pretreating for 1 h with 1 μM ICI 182,780 before cotreatment with E6BSA-FITC. During each imaging session an equivalent volume of 200-proof ethanol is added to a subset of dishes to test as a vehicle control (*see* **Note 18**).
5. After treatments, wash the cells and replace the media with warmed External Solution for imaging.

3.4 Total Internal Reflection Fluorescence Microscope Preparation

1. Turn on all equipment at least 30 min prior to imaging to allow the laser, camera, and microscope to stabilize (*see* **Note 19**).
2. Dilute fluorescent beads in sterile water (1:1000 for TetraSpec beads) and add to the center of a glass-bottom dish. Allow 2–3 min for some of the beads to reach and settle on the bottom of the dish.
3. Add oil to the objective and place the dish on the microscope and focus at the water-cover slip interface to observe the beads under laser illumination.
4. Check that the laser is aligned for total internal reflection (TIR) illumination [35]. In TIRFM, only the beads closest to the cover slip surface will be visible, and often nearly motionless. If the laser is not aligned for TIRFM, then beads at many depths will be visible, and will mostly likely be moving around.
5. Adjust the incident angle of the laser until only beads near the cover slip surface are visible to achieve TIR illumination.

3.5 TIRFM Image Acquisition

1. Place treated cells (*see* Subheading 3.3) on the microscope and focus on the cells. This is most easily done in bright-field or epifluorescence. Identify a cell of interest that has a significant portion of its basal surface close to the cover slip (*see* **Note 20**).
2. Switch to TIR illumination, and focus at the bottom of the cell. Adjust image acquisition settings to obtain sufficient signal-to-noise (*see* **Note 21**).
3. Acquire a series of images. For our experiments, we acquire sets of 500 frames at 4 Hz (250 ms/frame) exposure time. Use the same acquisition settings for all experiments (*see* **Note 22**).
4. Take a bright-field or epifluorescence image to record cell shape, refocusing if necessary to obtain the best image.

3.6 Particle Analysis and Tracking

Perform analysis of images using ImageJ software (http://imagej.nih.gov/ij/), with publicly available MTrackJ plug-in from the ImageJ website http://www.imagescience.org/meijering/software/mtrackj/ [36]. The MTrackJ plug-in is used to track moving objects in image sequences and keep basic traced path and measurement statistics (*see* **Note 23**).

1. Open images in ImageJ software. If you choose, create subfolders for processed images.
2. For particle analysis, choose a single image from the beginning of the experiment. Threshold the image using a built-in algorithm (we use "intermodes").
3. Find fluorescent puncta using built-in routines in ImageJ. Adjust settings to exclude artifact pixels if necessary. Assess FITC puncta number, the area of FITC puncta coverage, and FITC puncta size (Fig. 2) (*see* **Note 24**). Note the edges of the cell from a bright-field or epifluorescence image so that results can be expressed in terms of puncta per cell, and extracellular puncta can be excluded.
4. For particle tracking during image sequences, use the MTrackJ plug-in. For convenience, we broke up the image sequences into 100 frame (25 s) subsets (Fig. 3).
5. Set MTrackJ for single frame steps and local cursor snapping during tracking with a snap range of 13×13 pixels and bright centroid snap feature.
6. Track the motion of each fluorescent puncta for the duration that it is visible in the image sequence. Repeat for each puncta.

4 Notes

1. The E6BSA-FITC from Sigma-Aldrich (USA) ranges in estradiol concentration. This range is dependent on the amount of hormone attached per molecule of BSA (5–10 mol estradiol

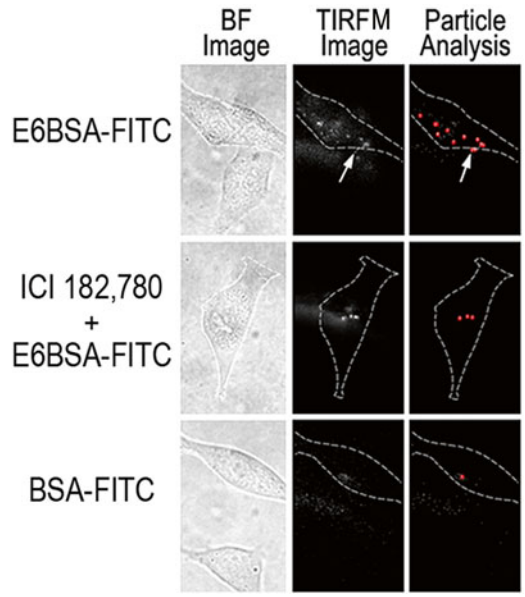

Fig. 2 TIRFM particle analysis of E6BSA-FITC molecules on the membrane surface of hypothalamic N-38 neurons. Transmitted light bright field (BF) and TIRFM digital images were taken of N-38 neurons treated with 1 μg/mL E6BSA-FITC or BSA-FITC for 30 min. Subsets of the cultures were pretreated with 1 μM ICI 182,780 for 1 h prior to fluorescent ER ligand treatment. Imaging of cells took place during the period of 35–50 min after the start of treatment with the ligand. The *dashed line* is used to outline the cell body perimeter. Fluorescent puncta not found within the perimeter (*arrow*) should not be computed in the particle analysis [27]

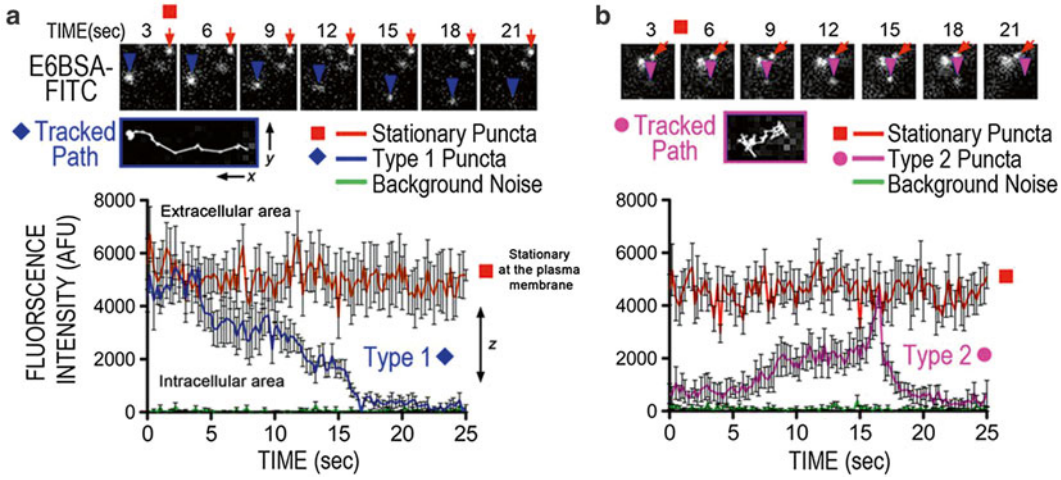

Fig. 3 TIRFM particle tracking of E6BSA-FITC at the plasma membrane of hypothalamic N-38 neurons. (**a** and **b**) Frame-by-frame tracking (four frames per second) of E6BSA-FITC fluorescent puncta intensity and position revealed different types of fluorescent molecule movements. E6BSA-FITC puncta were mostly stationary (*orange squares*), however a few initially stationary fluorescent puncta (**a**; Type 1; *blue diamonds*) suddenly moved long distances along a directed path (also see *Tracked Path inset*) only to dim away gradually. (**b**) Other puncta (Type 2; *magenta circles*) rapidly appeared, moved randomly a short distance (also see *Tracked Path inset*) and then fluorescence rapidly (~2.5 s) disappeared. *AFU* arbitrary fluorescence units [27]

per mol of BSA; 3–4 mol FITC per mol BSA). It is likely that similar variability would be present in this reagent from other suppliers.

2. BSA-FITC (>7 mol FITC per mol BSA) should also be prepared. We use BSA-FITC as a control to determine if either BSA or FITC molecule causes binding and internalization artifacts.
3. Glass-bottom culture dishes with #1 glass cover slips may be purchased through the MatTek Corporation.
4. Check that the glass used is compatible with the objective used (Subheading 2.4) (*see* **Note 8**). Some of the higher NA objectives (above NA ~ 1.52) require special cover slip materials.
5. A custom made wood jig is used to hold the polystyrene lid in place and keep it from spinning while the hole is drilled. Our jig consists of a plywood platform affixed on top of the drill press platform to which we have added small blocks on pivots (wood screws) spaced to wedge the lid (diameter ~44 mm) into place before drilling.
6. Cell culture media usually have components that fluoresce under the illumination used for fluorescence imaging, typically generating a lot of background light. Imaging in External Solution results in lower background fluorescence. Dye-free media is another option, but may still contain some fluorescent moieties.
7. This particular dichroic has two pass bands, for imaging in two colors. This type of dichroic is not required for single-color imaging.
8. In addition to bright-field, epifluorescence illumination capabilities can make it easier to identify cells of interest quickly.
9. An objective with NA (numerical aperture) of 1.4 is the absolute minimum NA that can produce TIR in a biological sample. Objectives with NA of 1.45 or higher are better suited for TIRFM.
10. For live cell imaging, speed and sensitivity are often of the essence. A camera with frame-transfer capabilities significantly reduces the acquisition down time between frames in an image series. We recommend using the camera in frame-transfer mode. Electron multiplication gain (EM) CCDs are designed for low light applications and typically have a better signal-to-noise characteristics then standard camera CCDs.
11. Measure the total volume of filter wash through by careful hand pipetting, taking into account that 1.5 mL of the volume is from the wash. After the subtraction of 1.5 mL from the total amount of wash collected, the extra amount can be calculated. This value in volume of saline is then added back to the retentate to bring it back to 1 mg/mL. If accurate this means

that when removing the E6BSA-FITC from the filter unit (use a 200 μL tip and p200 pipettor) the total volume should be approximately 500 μL; adjust by the pipettor by hand accordingly. If the recovered volume is <500 μL you can adjust by adding saline to bring the volume up. If the recovered volume is >500 μL you must determine the new concentration (using $c_i \cdot v_i = c_f \cdot v_f$, where c = concentration and v = volume).

12. We use the lids of 35 mm culture dishes, which have a short lip. The lower side lip wall enables use of the dishes for electrophysiology experiments as well. For pure imaging experiments, dishes can be made from either the dish or lid.
13. When deburring the hole in the lid keep in mind that a flat lid surface will be helpful later when adding the glass cover slip.
14. Make sure the Sylgard has made a continuous seal between the outer lid surface and the glass. Examine each dish by eye. If the seal between the lid and glass is not complete do not use because the dish will leak.
15. Sylgard can also be cured at lower temperatures for longer periods, or even by keeping the dishes at room temperature for 24 h.
16. Dishes are housed in larger 60 mm petri dishes for cell culture incubation and transport.
17. Here we provide the items required for culturing N-38 neuronal cells [37]. Follow the manufacturer's guidelines for culturing. In short, take frozen N-38 stock cells from cyrostorage and place into T-25 cell culture flasks containing DMEM with 10 % FBS and pen-strep until the cells become seeded to bottom of the flask (~1 h). Remove the medium and replace with fresh DMEM with 10 % FBS and pen-strep. Maintain and grow the cells in a cell culture incubator set at 37 °C with 5 % CO_2. Grow cells to 80–90 % confluency and reseed into new T-25 flasks five times before plating to glass bottom dishes for imaging experiments. Split N-38 cells using trypsin and following standard cell culture methods [10, 27].
18. Make stock ICI 182,780 solution at 1 mM using 200 proof ethanol.
19. Thermal stabilization is very important. Thermal differences can result in focal drift during an experiment. If there is a lot of focal drift in the system, try giving it more time to stabilize, adding an objective heater, or determining if there is a problem with the focusing mechanism of the microscope.
20. Under the conditions described, the best data is often obtained within approximately the first 20 min of placing the cells on the microscope. Kinetics can be highly temperature dependent, and without a temperature regulation system the cells slowly cool to room temperature.

21. The goal here is to obtain sufficient signal to make analysis possible, but not to use so long an exposure time that fast events are missed or camera pixels are saturated. Similarly, if the laser power is too high, the fluorescence can be rapidly bleached away. It might take a couple of trials to work out the ideal combination of exposure time, gain, and laser power settings.
22. Because the apical surface of the cell is more exposed to the bulk medium, it is likely responsible for a large part of E6BSA-FITC binding. Thus, the E6BSA-FITC observed in TIRFM at the basal surface of the cell has become either internalized and moved to the basal membrane, or has traveled within the thin aqueous layer between the glass and the basal cell membrane to a receptor.
23. "Fiji" (http://fiji.sc/Fiji) is a variant of ImageJ that comes with many image analysis plug-ins preinstalled, and is sometimes more convenient to work with then the more basic ImageJ.
24. With our system, the pixel scaling was 165 nm/pixel. At this scale, particle sizes of less then 2 pixels^2 we considered artifacts and excluded from the analyses.

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Chapter 15

In Situ Hybridization of Estrogen Receptors α and β and GPER in the Human Testis

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Abstract

In situ hybridization (ISH) is an excellent method for detecting RNA in histological sections, both to detect gene expression and to assign gene expression to a distinct cell population. Therefore, ISH may be used in basic cell biology to detect the expression of certain genes within a tissue containing various cell populations. Here, we describe the detection and cellular localization of three estrogen receptors, both isoforms of the genomic estrogen receptor (ERα and ERβ) as well as the membrane-bound G-protein-coupled estrogen receptor 1 (GPER) in the human testis.

Key words In situ hybridization, Testis, Estrogenreceptors, Molecular biology, Gene expression, Cellular localization

1 Introduction

The basis for the modern in situ hybridization (ISH) technique is the ability of nucleic acids to hybridize with each other. The reassociation of DNA strands by Schildkraut and coworkers [1] as well as the hybridization of single-stranded DNA with complementary RNA [2] were known by the early 1960s. Hybridization occurs between DNA and DNA, and DNA and RNA, as well as RNA and RNA, the latter replacing the technique of Northern Blotting [3]. For Northern Blotting, RNA is extracted from the tissue, separated by electrophoresis due to molecular size, and transferred to a nitrocellulose membrane, and then bound to hybridization probes to detect specific RNA. Cellular localization by Northern Blot is therefore not possible. In ISH, target RNA remains within the tissue and is detected on site by complementary RNA (cRNA) probes which can be generated or purchased.

In the testis, ISH has been widely used for the detection of stage-specific gene expression pattern [4, 5] in germ cells as well as for the detection of cell-specific gene expression in Sertoli cells [6]. As the question of estrogen receptor expression is controversial in

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the literature, the expression pattern and cellular localization of all three known estrogen receptors (ERα, ERβ, and GPER) were assessed by ISH within human testicular biopsy material [7]. Spermatogenesis is a complex process of developing germ cell stages (i.e., spermatogonia, spermatocytes, spermatids, and spermatozoa) which need to be supported by surrounding somatic Sertoli cells. Between the seminiferous tubules containing the different germ cell stages and the Sertoli cells, interstitial tissue contains the steroid hormone-producing Leydig cells, blood vessels, immune cells, and nerve fibers [8]. This rather complex structure aggravates the application of the ISH technique in the testis and requires a perfect morphological preservation of testicular biopsies. Therefore, we recommend a fixation in Bouin's solution with subsequent paraffin embedding [9]. In this manner cell types may be easily distinguished, but nucleic acid quality in respect to fragmentation and DNA/RNA yield is poor compared to cryopreserved material. As cellular localization of estrogen receptors in the testicular cell populations was the underlying concept of the performed study, we had to deal with this issue by producing rather short, specific cRNA probes and establishing a sufficient protocol to overcome the problems mentioned. Thus this chapter describes the protocols for ISH of ERα, ERβ, and GPER in the testes.

2 Materials

Use sterile glassware for all solutions, buffers, and alcohol series. All solutions must be prepared with highly purified water (resistivity of 18.2 MΩ) that has been sterilized and treated with diethylpyrocarbonate (DEPC).

2.1 Media

1. Agar culture media (solid) with ampicillin: Add ten capsules LB-agar medium to 250 mL sterile purified water and autoclave for 20 min at 121 °C and 1.1 bar. Let cool for 2 h in 60 °C water bath. Add 250 μL ampicillin sodium salt (100 mg/μL) and mix carefully to avoid air bubbles. Spread out the LB-agar medium in eight to nine plastic culture dishes (⌀ 90 mm), let cool, and store at 4 °C (*see* **Note 1**).
2. Agar culture medium (liquid) without ampicillin: Add six capsules LB-agar medium to 250 mL sterile purified water and autoclave as described above. After medium is cooled to room temperature, store at 4 °C.

2.2 Stock Solutions

Stock solutions may be prepared days to weeks before ISH procedure and stored for a long term.

1. 20× SSC (sodium chloride-sodium citrate) buffer: Dissolve 88.23 g sodium citrate ($C_6H_5O_7Na_3 \times 2H_2O$) and 175.29 g

NaCl in 1 L sterile purified water. Adjust to pH 7.0. Add 1 mL DEPC and shake well. Incubate overnight at 37 °C and autoclave (*see* **Note 2**).

2. 10× TNMT (Tris, NaCl, MgCl, and Tween) buffer: Dissolve 121.1 g Tris–HCl, 58.4 g NaCl and 4.17 g $MgCl_2$ in 1 L sterile purified water. Adjust to pH 7.5 by using 25 % HCl. Add 1 mL DEPC and mix well. Incubate overnight at 37 °C and then autoclave. After cooling, add 5 mL Triton-X-100 and mix.
3. 5× NTB (nitro-blue tetrazolium chloride) buffer: Dissolve 60.5 g Tris–HCl and 29.2 g NaCl in 1 L sterile purified water. Adjust to pH 7.5 by using 25 % HCl. Add 1 mL DEPC and mix well. Incubate overnight at 37 °C and then autoclave.
4. 3 % BSA (bovine serum albumin) blocking buffer: Dissolve 6 g BSA in 200 mL 1× TNMT buffer (*see* **Note 3**). Aliquot and freeze until use.
5. 50 % dextran sulfate: Dissolve 5 mg dextran sulfate in 5 mL DEPC water. Let stand at 4 °C until completely dissolved. Add DEPC water to a final volume of 10 mL and aliquot.
6. Preparation of DEPC-treated water: Add 1 mL DEPC (diethylpyrocarbonate) to 1 L sterile purified water (resistivity of 18.2 MΩ). Mix well, incubate overnight at 37 °C, and then autoclave. DEPC is active and toxic prior to autoclaving, but autoclaved DEPC is inactivated and not toxic. DEPC water may be stored at room temperature.

2.3 Working Solutions for In Situ Hybridization

Prepare working solutions directly before starting the ISH procedure.

1. 1× PBS-M (phosphate-buffered saline—$MgCl_2$) buffer: Dissolve one PBS tablet in 400 mL DEPC-treated water and add 1 mL 1 M $MgCl_2$ solution (81.4 g $MgCl_2$ in 400 mL DEPC water). Store at 4 °C (*see* **Note 4**).
2. SSC working solution: Use sterile DEPC water for dilution steps. For 100 mL 4× SSC mix 20 mL 20× SSC with 80 mL sterile DEPC water. For 2× SSC, mix 50 mL 20× SSC with 450 mL sterile DEPC water. For 0.2× SSC, mix 20 mL 20× SSC with 180 mL sterile DEPC water.
3. Hybridization buffer: Mix 20 µL 20× SSC, 4 µL Denhardt's reagent, 100 µL deionized formamide (working solution 50 %), 40 µL 50 % dextran sulfate with 26 µL DEPC water.
4. 1x TNMT buffer: Dilute 20 mL 10× TNMT, with 180 mL sterile purified water.
5. 20 % glycerol in DEPC water (pre-hybridization): Mix 10 mL glycerol with 50 mL sterile DEPC water.
6. 1× NTB buffer: Dilute 20 mL 5× NTB, with 75 mL sterile purified water. Add 5 mL 1 M $MgCl_2$ solution.

2.4 Other Components for cRNA Probe Preparation

1. Ampicillin sodium salt stock solution, 100 mg/mL: Dissolve 100 mg ampicillin sodium salt in 1 mL sterile purified water. Aliquot to 250 µL and store at −20 °C.
2. pCR™II TOPO® vector (Invitrogen, https://www.lifetechnologies.com/order/catalog/product/K465040?ICID=search-product).
3. Competent cells (such as One Shot ® TOP 10 competent *E. coli* (Invitrogen)).
4. X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) for blue/white screening: Dissolve 20 mg X-Gal in 1 mL *N*,*N*-dimethylformamide (*see* **Note 5**). Store at −20 °C.
5. S.O.C. medium (Super Optimal broth with Catabolite repression; S.O.B. medium supplemented with 20 mM glucose, Invitrogen), included with vector/competent cells.
6. 0.5 M EDTA solution.
7. 8 M LiCl solution.
8. Glass fiber column kit for plasmid purification such as the QIAprep Spin Miniprep kit (Qiagen).
9. Standard UV spectrophotometer.
10. 5× transcription buffer, 100× DTT, DIG-RNA labelling mix (Roche).
11. RNA polymerase, 0.5 M EDTA, 8 M LiCl, 70.7 µL 96 % ethanol (ice cold).
12. General laboratory equipment: Microfuge, chemical fume hood, water baths, heat plates, laboratory with safety category S1.

2.5 Other Components for In Situ Hybridization

1. 0.2 N HCl: Add 5.2 mL HCl (25 %) to 200 mL DEPC water.
2. Proteinase K stock solution: Dissolve 20 mg proteinase K in 5 mL sterile DEPC water to achieve a concentration of 10 µg/mL. Aliquot to 20 µL and store at −20 °C.
3. 0.2 % glycine solution: Add 100 mg glycine to 50 mL 1× PBS-M buffer.
4. 4 % paraformaldehyde solution: Dissolve 20 g paraformaldehyde in 500 mL 1× PBSM buffer. Perform weighing and preparing of solution under a fume hood! Heat while stirring on a magnetic stirrer to approximately 70 °C. Add 4 N NaOH dropwise until the solution starts to clear. Adjust to pH 7.0, aliquot to 50 mL and store at −20 °C.
5. 0.25 % acetic acid in 10 mM triethanolamine solution: Add 250 µL concentrated acetic acid (100 %) and 160 µL 10 mM triethanolamine to 99.59 mL DEPC water (*see* **Note 6**).
6. Coated glass slides (e.g., SuperFrost®, Menzel, ThermoFisher Scientific).

7. Probe preparation: Add 2 μL Salmon sperm DNA (working solution 40 μg/mL) and 4 μL yeast t-RNA to 4 μL DIG-labelled RNA-probe (*see* **Note 7**).
8. Humidified incubation chamber: For hybridization, drench laboratory tissues with a mixture of 100 mL formamide, 20 mL 20× SSC, and 80 mL DEPC and put into incubation trays (*see* **Note 8**).
9. Labelling: Anti-Digoxigenin-AP, Fab fragments (Roche): anti-DIG-antibody conjugated with alkaline phosphatase (Fab fragment); dilution 1:500–1:1000 in 3 % BSA in 1× TNMT buffer.
10. 1 M levamisol: Solve 2.4 g levamisol (use respiratory protection device during weighing procedure!) in 10 mL 1× NTB buffer and aliquot.
11. Staining: NTB/BCIP solution (purchased ready to use).
12. Mounting medium: Kaiser's glycerol gelatin (Millipore).
13. Descending alcohol series: 3× xylene (≥99 % purity), 2× absolute ethanol, 1× 96 % ethanol, 1× 70 % ethanol, 1× DEPC water, and 1× 0.2 N HCl in DEPC.
14. 0.25 % acetic acid in 10 nM triethanolamine.
15. 0.2 μm syringe filter.

3 Methods

3.1 Preparation of PCR Product for RNA Probe Synthesis

For RNA probe synthesis, a PCR product of the gene to be detected is required. Three factors have to be kept in mind regarding primer design: penetration capacity of probes, probe specificity, and signal intensity. Probe specificity and signal intensity can be enhanced with longer probes as the amount of labelled UTP increases with length of sequence. However, long probes show decreased tissue penetration behavior resulting in a low staining signal. On the other hand, shorter probes may penetrate easily into the tissue, but specificity may be low and therefore unspecific (background) signal intensity may be increased. RNA probes have been reported at 100–1000 bp long; in our lab we prefer probes that are 150–350 bp. This probe length yields the best results in human testis tissue fixed in Bouin's solution and embedded in paraffin wax. For the actual study, we used primer pairs to create a 183 bp amplicon for ERα (3′-TCCTACCAGACCCTTCAGTG-5′ as forward, 3′-CAGACGAGACCAATCATCAG-5′ as reverse primer, NCBI RefSec accession number NM_000125.2), a 329 bp amplicon for ERβ (3′-AGAGTCCCTGGTGTGAAGCAAG-5′ as forward and 3′-TCCCACTTCGTAACACTTCCG-5′ as reverse primer, NCBI RefSec accession number NM_001040275), and a 310 bp amplicon for detection of GPER (3′-CAGTACGTGATCGGCCTGTT-5′ as forward and 3′-TGTAGCGGTCGAAGCTCA-5′ as reverse

primer, NCBI RefSec accession no. NM_001505.2). RNA extraction, cDNA synthesis and reverse transcription of RNA from testis tissue as well as RT-PCR was performed as described [7]. It is most important to use PCR products not older than 7 days that have been stored immediately at –20 °C after RT-PCR reaction. If the RT-PCR shows a clear specific band on a gel, the product does not have to be purified. If there is more than one discrete band, you may gel-purify your PCR product before subcloning.

3.2 Ligation of PCR Product into the Vector and Transformation of Competent Cells

1. Ligate the PCR product into pCR™II TOPO® vector following the manufacturer's recommendations. Mix 0.5–4 μL PCR product with 1 μL salt solution and add sterile purified water to a total volume of 5 μL. Add 1 μL pCR™II TOPO® vector, mix gently and let incubate at room temperature for 5–30 min (*see* **Note 9**). Do not mix by vortexing or pipetting up and down!
2. The following steps of bacterial transformation and plasmid preparation have to be performed in a laboratory with safety category S1.
3. Preparatory steps required before transforming the competent cells: Remove the LB-agar plates from the refrigerator and prewarm to 37 °C. Prepare a 42 °C non-shaking water bath, ice, and a 37 °C shaking incubator.
4. Under the laminar flow cabinet, spread 40 μL X-Gal on an agar plate with a glass spatula (flame prior to use with 70 % ethanol) and let dry.
5. On ice, thaw one vial of competent cells for each transformation reaction for 5 min.
6. Add 2 μL of the ligated vector into each vial of competent cells and mix very gently by flicking the tube slowly. Do not mix by vortexing or pipetting up and down!
7. Incubate on ice for 5–20 min.
8. Heat shock the cells for 30 s at 42 °C without shaking and afterwards immediately transfer the tubes back on ice.
9. Add 250 μL S.O.C. medium and incubate for 1 h at 37 °C in a shaking incubator (approximately 200 rpm).
10. Spread cells in differing volumes (e.g., 40, 55, 70 μL) on the prepared selective plates and incubate at 37 °C in a non-shaking incubator overnight.
11. After 24-h incubation, pick white colonies using a flamed wire eyelet and transfer each colony to 6 mL liquid LB agar medium with 6 μL ampicillin for subsequent plasmid extraction (Fig. 1).
12. Incubate overnight at 37 °C in a shaking incubator at approximately 225 rpm.

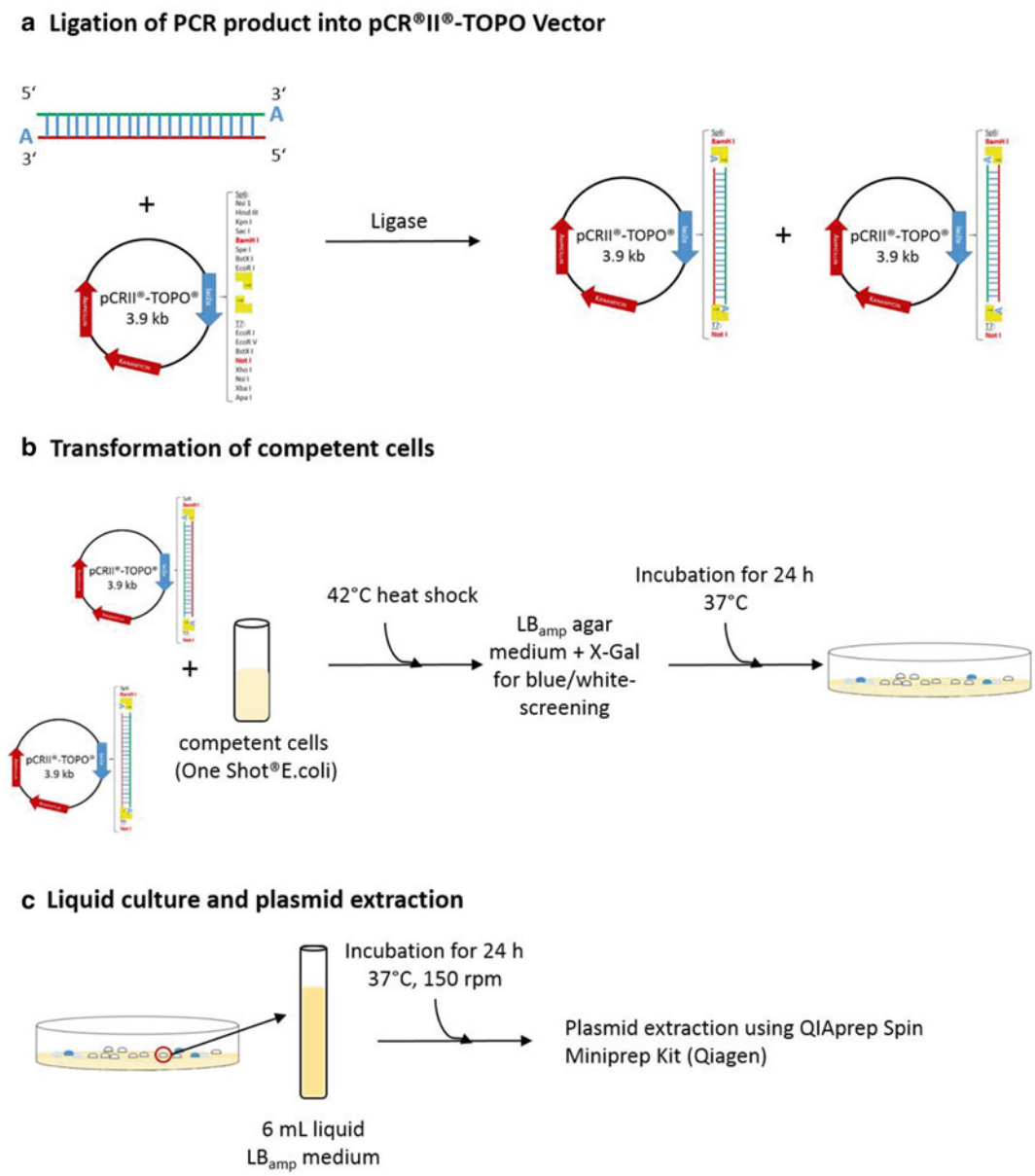

Fig. 1 Diagram of the steps of ligation and transformation of competent *E. coli* using the pCR II-TOPO vector, and plasmid extraction

3.3 Plasmid Purification and Digestion

1. Perform plasmid extraction using a column purification kit such as the QIAprep Spin Miniprep kit (Qiagen) as recommended by the manufacturer. Work under a laminar flow cabinet and make sure that all bacterially contaminated waste is collected to be autoclaved after the process. Perform all centrifugation steps in a microfuge at 4 °C.

2. Transfer the liquid LB media to 2 mL microfuge tubes and centrifuge at 8000 × *g* for 2 min to pellet the bacteria. Discard the supernatant.
3. Repeat **step 2** until all of the liquid culture has been centrifuged. Remove and discard the remaining supernatant with a pipette.
4. Add 250 μL buffer P1 (from the purification kit, stored at 4 °C) and resuspend the bacteria by vortexing.
5. Add 250 mL buffer 2 (lysis buffer from the kit) and mix by flicking the tubes four to six times (*see* **Note 10**).
6. After approximately 3 min, stop lysis by adding 350 μL buffer N3, and mix again by carefully flicking the vial.
7. Centrifuge for 10 min at 18,000 × *g*. Place the provided spin columns into tubes and transfer the supernatant onto the column membrane.
8. Centrifuge at 13,000 × *g* for 1 min (*see* **Note 11**). Discard the flow-through. The plasmid is now bound to the silica membrane.
9. Add 750 μL buffer PE to wash the membrane. Centrifuge at 13,000 × *g* for 1 min. Discard the flow-through and centrifuge for a second time to dry the membrane completely.
10. Discard the tube and place the columns in new 1.7 mL microfuge tubes.
11. Add 50 μL buffer EB directly onto the silica membrane without touching it and incubate for 1 min at room temperature.
12. Centrifuge at 13,000 × *g* for 1 min to elute the plasmid from the membrane. The flow-through contains the plasmid and can be stored at –20 °C.
13. Calculate plasmid DNA content by analysis of absorbance at 260 and 290 wavelengths with a standard spectrophotometer.
14. Perform a double digest to check if the PCR product is correctly inserted. Choose restriction enzymes based on the vector map and the inserted PCR product (*see* **Note 12**). We used NotI and BamHI for double digest. For a 20 μL preparation, mix 200 ng of plasmid with 2 μL buffer (choose one buffer that fits both restriction enzymes with at least 75 %), 1 μL each of enzymes, and sterile purified water to a final volume of 20 μL.
15. Mix carefully and incubate for 4–6 h or overnight in a 37 °C water bath.
16. Separate the products of the double digest on a 2 % agarose gel using SybrGreen I as dye. Perform gel electrophoresis at 125 V for approximately 40 min.
17. Visualize the gel under UV light. A band should be visible at about 3 kb (the length of the vector) and a second band at the expected length of the insert.

18. Send plasmid DNA out for sequencing.
19. After successful sequencing of the insert, a single digestion is performed to obtain the template for in vitro transcription. For a single digest, prepare two mixes each containing 5 μL plasmid DNA, 2 μL buffer (*see* Subheading 3.3, **step 14**), 1 μL restriction enzyme (one mix with NotI, one mix with BamHI) and sterile purified water to a final volume of 20 μL. It is important to add the enzymes as the last component.
20. Centrifuge briefly after mixing and incubate for 6 h at 37 °C.
21. Stop the enzyme reaction by incubation at 65 °C for 20 min in a water bath.
22. After heat inactivation, centrifuge again and analyze the single digest on a 1 % agarose gel.
23. Electrophorese the gel for 60 min at 95 V to obtain a fine separation.
24. Visualize the gel under UV light. After a correct single digest, a single band should be visible at approximately 4000 bp length. This indicates that the vector ring is present as a linear double strand and ready for the in vitro transcription procedure.

3.4 In Vitro Transcription

1. Prepare a mix for antisense (AS) and sense (S) probes by mixing 7 μL DEPC water, 4 μL 5× transcription buffer, 2 μL 100× DTT, 2 μL DIG-RNA labelling mix (Roche), 3 μL single digest, and 1 μL RNA polymerase (*see* **Note 13**). Mix gently.
2. Centrifuge briefly to remove droplets from the vial wall.
3. Incubate for at least 2 h at 37 °C. A DNAse digestion step may be added at this point, but is not routinely performed in our lab.
4. To stop the reaction, add 0.5 μL 0.5 M EDTA solution. Further add 1.2 μL 8 M LiCl solution and 70.7 μL 96 % ethanol (ice cold) for precipitation of the probe and to avoid contamination of the probe with excess labeled nucleotides. Mix carefully.
5. Incubate for at least 1 h at −80 °C (may be stored at that temperature overnight).
6. Centrifuge probes for 20 min at 13,000 × *g* at 4 °C. After centrifugation, localize the pellet and mark its location in the tube with a permanent pen.
7. Remove the supernatant and add 200 μL 70 % ethanol (prepared with DEPC water) to wash the pellet.
8. Centrifuge again for 15 min at 13,000 × *g* and 4 °C.
9. Discard the supernatant and place tubes in a 37 °C incubator to let the pellet dry. This might take 10–20 min; examine the pellet with a magnifier to make sure that the pellet has dried completely.

10. Add 50 µL DEPC water and resuspend the pellet by incubating for 10–15 min in a 70 °C water bath. Visualize resuspension of the pellet with a magnifier.
11. Centrifuge each probe preparation briefly to remove droplets from the vial wall and store at –20 °C.

3.5 Preparation of Histological Sections

1. Wipe a water bath with 70 % ethanol, fill the water bath with distilled water, and preheat to 42 °C.
2. Trim formalin-fixed paraffin-embedded testicular tissue with a sterile surgical blade and cut into 5 µm thick sections using a common microtome (*see* **Note 14**).
3. Place sections in the water bath to stretch and mount on coated glass slides.
4. Place the slides in a 60 °C incubator overnight to dry completely.

3.6 In Situ Hybridization: Preparation of Sections and Hybridization Reaction

It is most important that all steps of ISH on the first day are performed under sterile conditions to avoid RNases! Use only DEPC water for ethanol dilutions and buffers as described above. Contamination with RNases in this stage of the experiment will lead to false-negative results or blur the results. Perform all processes at room temperature unless stated otherwise.

1. Prepare a descending alcohol series: 3× xylene (>99 % purity), 2× absolute ethanol, 1× 96 % ethanol, 1× 70 % ethanol, 1× DEPC water, and 1× 0.2 N HCl in DEPC.
2. Preheat a water bath, 100 mL 2× SSC, and a glass cuvette with xylene (≥99 °C purity) to 60 °C using an incubator. Preheat heating block to 80 °C and a heating plate to 70 °C.
3. Place the histological sections in a sterile glass tray.
4. To remove paraffin from the histological sections, process the slides through a descending xylene/ethanol series (*see* **Note 15**):
   (a) Xylene (≥99 % purity), 60 °C: 5 min
   (b) Xylene: 2 × 5 min
   (c) Ethanol (100 %): 2 × 5 min
   (d) Ethanol (96 %): only dip briefly
   (e) Ethanol (70 %): 5 min
   (f) DEPC water: only dip briefly
   (g) 0.2 N HCl: 20 min
5. Incubate the sections for 15 min in 4× SSC at 70 °C to equilibrate.
6. Wash the sections briefly in 1× PBS-M buffer.
7. Place the sections in a humidified incubation chamber and apply proteinase K solution (10 µg/mL) for 15 min at 37 °C

Table 1
Troubleshooting tips for the ISH procedure

Step of ISH procedure		Improvement possibilities
Preparation of sections prior to hybridization procedure		
Proteinase K digestion	Digestion of proteins to retrieve nucleic acids for probe binding	*Higher concentrations* will lead to a higher nucleic acid retrieval and enhance probe binding, but decrease morphology *Lower concentrations* will result in better morphology, but decreased probe binding efficiency
Hybridization procedure		
Probe length	Detection of complementary mRNA sequences within the histological section	*Longer probes* result in higher specificity and also higher signal intensity due to greater number of DIG-labeled nucleotides per probe; penetration capacity is lower with longer probes *Shorter probes* show higher tissue penetration ability, but lower signal intensity and specificity
Probe concentration and incubation time		*Higher concentration* and *longer incubation* are recommended for rare transcripts and difficult tissue penetration *Lower concentration* and *shorter incubation* are possible for abundant transcripts and good tissue penetration
Post-hybridization steps		
Stringent washing	Removal of unbound and incomplete bound probes	*High temperature* and *low SSC concentration* = higher stringency; removes more nonspecific staining signal but may possibly remove or weaken specific signals *Lower temperature* and *high SSC concentration* = lower stringency; low stringent washing may result in nonspecific staining signals

to digest the proteins and to retrieve nucleic acid. The proteinase K digestion step is very variable and depends on the tissue and probe used (*see* **Note 16** and Table 1).

8. To stop proteinase K digestion, incubate slides with 0.2 % glycine solution for 5 min.
9. Wash the slides briefly in PBS-M buffer.
10. Post-fix by immersing the slides in 4 % paraformaldehyde for 10 min.
11. Stop the post-fixation step by washing twice with 1× PBS-M for 5 min each.
12. Incubate the sections with 0.25 % acetic acid in 10 nM triethanolamine solution for 10 min (*see* **Note 17**).
13. Wash two times with 1× PBS-M for 5 min each.

14. Prior to hybridization, incubate the sections in 20 % glycerol solution for 60 min at room temperature.
15. Equilibrate the sections in 2× SSC for 5 min.
16. Prepare the hybridization buffer (Subheading 2.3, **item 3**) separately from the probe.
17. Prepare the sense and antisense DIG-labeled probe (Subheadings 2.5, **item 6** and 3.5) (*see* **Note 7**) to obtain a probe concentration of 1:50 (*see* **Note 18**).
18. Heat the probe to 80 °C in a heat block for 10–12 min to denature it, then immediately place on ice.
19. Similarly, place the sections on a heat plate at 70 °C and afterwards store it on a cold pack. Surround the sections with a Delimiting Pen (Dako) or wax pencil to prevent spread of hybridization buffer and probe.
20. Mix 10 µL probe preparation with 190 µL hybridization buffer and apply at least 50 µL per section.
21. Place slides in the humidified incubation chamber (Subheading 2.5, **item 8**) and place the chamber on a heat plate for hybridization. Determine the hybridization temperature empirically, start with a low (37 °C) and high (45 °C) temperature for at least 4 h (for abundant transcripts) and up to 40 h (for rare transcripts) in the humidified incubation chamber.

3.7 In Situ Hybridization: Washing and Immunohistochemistry

The processes on day 2 do not have to be performed under sterile conditions, but evertheless buffers and other reagents are prepared with sterile purified water.

1. Before starting, preheat a water bath to a temperature +10 °C of the hybridization temperature (e.g., if hybridization was performed at 45 °C, preheat water bath to 55 °C) and prepare 500 mL 2× SSC and 200 mL 0.2× SSC (*see* **Note 19**).
2. Remove the slides from the humidified incubation chamber and place them into a glass cuvette. Wash the slides as follows:
   (a) 2× SSC, room temperature: 2 × 5 min
   (b) 2× SSC, HT + 10 °C: 4 × 5 min
   (c) 0.2× SSC, HT + 10 °C: 2 × 30 min
   (d) 2× SSC, room temperature: 5 min
   (e) 1× TNMT, room temperature: 10 min
3. The following steps carry out the immunohistochemical detection of DIG-labelling. To block nonspecific binding sites, incubate washed slides with 3 % BSA in 1× TNMT buffer for 30 min to 1 h (*see* **Note 20**).
4. Mix 666 µL 1× TNMT buffer and 333 µL 3 % BSA in 1× TNMT buffer with 1 µL Anti-Digoxigenin-Fab antibody

(Roche) to obtain a dilution of 1:1000 and apply to the sections.

5. Incubate overnight at 4 °C in a humidified incubation chamber.

3.8 In Situ Hybridization: Signal Detection

1. Remove the antibody by washing with 1× TNMT buffer for 2× 10 min.
2. Wash the sections in 1× NTB buffer for 5 min to equilibrate.
3. Wash 5 min 1× NTB buffer + 1 mL 1 M levamisol to block endogenous alkaline phosphatases. Keep the 1× NTB + levamisol buffer for later application.
4. Use the humidified incubation chamber with exclusion of light for staining of the slides.
5. Fill a 2 mL syringe with the ready-to-use NTB/BCIP-solution (*see* **Note 21**) and attach a 0.2 μm syringe filter.
6. Apply the staining solution to the slides through the filter.
7. Incubate with staining reagent for 1–4 h. Visually examine progress of the staining reaction periodically.
8. Block the staining reaction by washing the slides in 1× NTB + levamisol buffer for 5 min in a glass cuvette.
9. Wash the slides distilled water for 5 min.
10. Mount cover slips on the slides using Kaiser's glycerol gelatin.
11. Examples of good and bad ISH results may be seen in Fig. 2 and troubleshooting tips to improve ISH procedure and enhance results are shown in Table 1.

4 Notes

1. The agar plates should be poured under a laminar flow cabinet to avoid any contamination. The agar plates may be kept for up to 4 weeks in the refrigerator.
2. To save DEPC (as it is toxic), it is possible to use 20× SSC buffer in DEPC only on the first day of ISH and use 20× SSC buffer in conventional purified water on the other days of the procedure. For this it is absolutely necessary to use sterile purified water, reagents and glassware and to work very properly.
3. If BSA does not dissolve in TNMT buffer, incubate overnight at 4 °C in the refrigerator. The next day, the BSA will dissolve much more easily.
4. Always prepare PBSM buffer as freshly as possible. Store only for short periods at 4 °C.

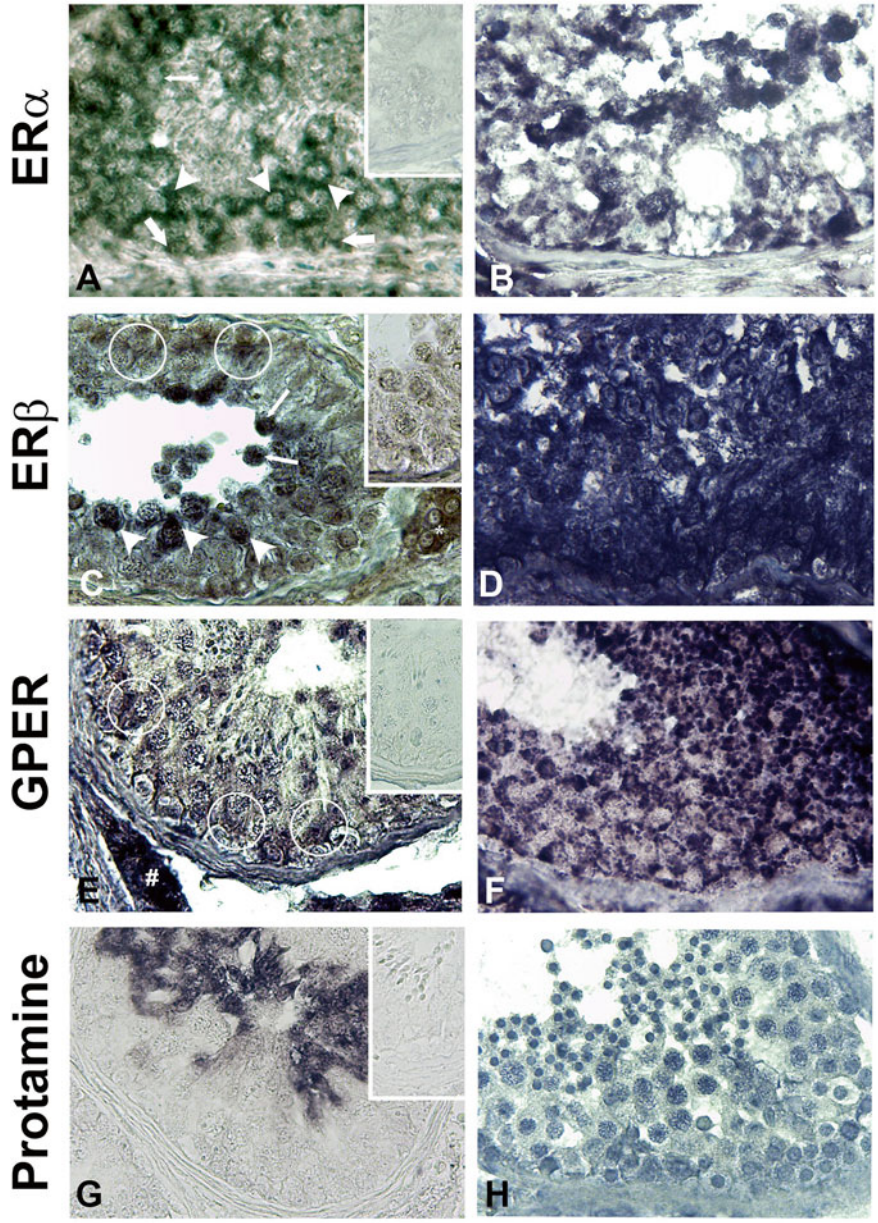

Fig. 2 Examples of good and bad ISH results. NTB/BCIP staining, primary magnification ×40. *Inset*: Negative control with respective sense probe, primary magnification ×40. (**a** and **b**) ERα staining: (**a**) shows a distinct staining signal in spermatogonia (*bold arrows*), pachytene spermatocytes (*arrowheads*), and early round spermatids (*thin arrows*), whereas (**b**) shows an excessive proteinase K digestion with a typical "perforated" section. (**c** and **d**) ERβ staining: (**c**) shows the correct staining for ERβ antisense probe in pachytene spermatocytes (*arrowheads*), early round spermatids (*thin arrows*), and Sertoli cells (*circles*). A nonspecific staining signal in Leydig cells (also present in negative control) is indicated by an *asterisk* in the *lower right* of the image. In (**d**), an excessive staining signal can be seen. Reasons for this might be far too much probe, too high probe concentration, or too long development time. (**e** and **f**) GPER staining: in (**e**), a specific staining in Sertoli cells (*circles*) and Leydig cells (*hash*) can be seen. In contrast to that, (**f**) shows the result of a poor de-paraffinization procedure. This leads to a floe-like morphology in ISH and a blurry picture. (**g** and **h**) Probes against protamine

5. Dimethylformamide is very toxic and must be pipetted under a fume hood! X-Gal is sensitive to light and has to be protected by using aluminum foil when removed from the freezer.
6. Alternatively, 0.1 % active DEPC can also be used for signal enhancement. As results are comparable and active DEPC is toxic, we use 0.25 % acetic acid solution.
7. Salmon sperm DNA and yeast t-RNA may also be added directly to the hybridization buffer. We prefer to prepare a "probe solution" as salmon sperm DNA and yeast t-RNA are the most costly reagents.
8. Any tightly closing plastic ware can be used here.
9. Usually, we use 2 μL RT-PCR product for the ligation process. If the gel electrophoresis shows a weak band, up to 4 μL may be used. As stated in the TOPO® vector manual, the ligation time can be varied from 30 s to 30 min; 5 min has shown to work very well for us.
10. It is extremely important NOT TO VORTEX the samples after addition of buffer 2 (lysis buffer). We start a lab timer at the moment the buffer is added to the first tube as the buffer should only interact for a maximum of 5 min with the bacteria.
11. Make sure the centrifuge reaches 13,000 × *g* for a full minute.
12. The restriction enzymes used depend on the vector used; for appropriate enzymes refer to the vector card. Always check that the chosen restriction enzymes do not cut within the inserted PCR product by using, for example, http://tools.neb.com/NEBcutter2/. The required buffer depends on the enzymes used and may be seen in the product specifications provided by the manufacturer. We used BamHI and NotI as the appropriate restriction enzymes for our probes. Although we prefer an incubation time of about 5 h, it is also possible to perform the incubation step overnight. In this case, reduce the amount of enzyme to 0.5 μL each per reaction volume and add sterile purified water to a final volume of 20 μL. The digested plasmids may be stored at −20 °C. Sequencing of the insert in both directions (SP6 and T7) is important, as both directions are used for probe design later on. Whereas only the antisense probe is complementary to the RNA within the section, the

Fig. 2 (continued) 1 (a transition protein showing DNA condensation, which is expressed stage-dependently in spermatids) are used as positive controls in our lab [5]. (**g**) Shows a correct staining signal in spermatids, whereas in (**h**), a nuclear staining can be seen. cRNA probes are used to detect mRNAs, which are transferred from the nucleus in to the cytoplasm to be translated into protein. A nuclear staining is therefore nonspecific and requires revision of probes and protocol used. Images (**a**, **c** and **e**) are adapted from [7], published by Springer Heidelberg and are used with kind permission of Springer Science + Business Media

sense probe is used as a powerful internal negative control. Accurate restriction enzyme application is essential for correct double and single digest and therefore probe template design.

13. It is important to use the correct RNA polymerase for the single digest preparation; if an enzyme "cutsoff" the T7 site, SP6 RNA polymerase is added and the other way around. In our study, we used NotI cutting of the T7 promotor region, so SP6 RNA polymerase has to be added. BamHI cuts off the SP6-binding site, so T7 polymerase is used for in vitro transcription mix.
14. Make sure that the samples are prepared and stored under RNase-free conditions. We use Bouin's solution (formalin and picric acid) for fixation of testicular biopsies [9], which grants a clear morphological preservation but might lead to a diminished RNA quality due to formalin-protein linkage and nucleic acid fragmentation. Always prepare sections freshly, preferably the day before starting ISH.
15. De-paraffinization is very important for ISH procedure, as remaining paraffin within the tissue leads to an insufficient proteinase K digestion and therefore a worse hybridization reaction. Sections, which have not been de-paraffinizated properly show a "blurry" morphology (Fig. 2b).
16. After de-paraffinization, proteinase K digestion is a very important step in ISH as proteinase K frees the nucleic acids from surrounding proteins, which have been linked by formalin fixation. This is necessary for the binding of probes to the RNA. This step is also somewhat tricky, as proteinase K digestion leads to a diminished tissue preservation and therefore worse morphology (Fig. 2c–d). To strike a balance between both aspects, enzyme concentration and incubation time have to be determined empirically. Concentrations of proteinase K between 5 and 50 μg/mL are usually used for paraffin embedded tissue, and sometimes even 20–100 μg/mL are recommended. Incubation time can be varied from 5 to 30 min. We have had good experience with a rather low proteinase K concentration (15 μg/mL) and a medium long incubation time (15 min) for formalin-fixed paraffin-embedded human testis tissue. For application of ISH with other tissues or other fixations/embedding protocols, proteinase K dilution series must be performed to determine the appropriate concentrations and incubation times.
17. Treatment with acetic acid leads to a diminished background staining and is also thought to inactivate RNases for a more sensitive ISH reaction.
18. We have good experience with probe concentrations between 1:25 and 1:50, but this depends on the probe itself and also on

the tissue used. Perform a dot blot to assure the correct incorporation of DIG-labeled nucleotides by the in vitro transcription process.

19. Stringent washing will remove waste unbound probes and also reduces nonspecific binding. The higher the temperature and the lower the SSC concentration the more stringent is the washing procedure. A low hybridization signal may be enhanced by a less stringent washing, whereas nonspecific hybridization may be removed by a more stringent washing procedure (Table 1).
20. Alternatively, blocking of unspecific binding sites may also be performed with commercially available blocking reagent.
21. As NTB/BCIP solution is toxic, perform all staining steps under a fume hood and wear nitrile gloves!

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Chapter 16

Purification of Histone Lysine Methyltransferase SMYD2 and Co-Crystallization with a Target Peptide from Estrogen Receptor α

Yuanyuan Jiang, Joshua Holcomb, Nicholas Spellmon, and Zhe Yang

Abstract

Methylation of estrogen receptor α by the histone lysine methyltransferase SMYD2 regulates ERα chromatin recruitment and its target gene expression. This protocol describes SMYD2 purification and crystallization of SMYD2 in complex with an ERα peptide. Recombinant SMYD2 is overexpressed in *Escherichia coli* cells. After release from the cells by French Press, SMYD2 is purified to apparent homogeneity with multiple chromatography methods. Nickel affinity column purifies SMYD2 based on specific interaction of its 6×His tag with the bead-immobilized nickel ions. Desalting column is used for protein buffer exchange. Gel filtration column purifies SMYD2 based on molecular size. The entire purification process is monitored and analyzed by SDS-polyacrylamide gel electrophoresis. Crystallization of SMYD2 is performed with the hanging drop vapor diffusion method. Crystals of the SMYD2–ERα peptide complex are obtained by microseeding using seeding bead. This method can give rise to large size of crystals which are suitable for X-ray diffraction data collection. X-ray crystallographic study of the SMYD2–ERα complex can provide structural insight into posttranslational regulation of ERα signaling.

Key words Proteinpurification, Chromatography, Nickel affinity, Gel filtration, Crystallization, Seeding, X-ray crystallography

1 Introduction

X-ray crystallography is the most powerful method for characterizing the three-dimensional structure of biological molecules, such as proteins, to atomic resolution. X-ray crystal structures not only allow for a deeper understanding of protein function, but also can serve as the basis for structure-aided drug discovery against disease. However, structure determination by X-ray crystallography requires milligram quantities of highly purified protein and a crystal formed from the protein. *Escherichia coli* (*E. coli*) is one of the most popular hosts for recombinant protein production since it provides high-level expression and is rapid and easy to use. Expressed proteins are often purified from *E. coli* with multiple chromatography steps in

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order to achieve adequate purity. In this protocol, nickel affinity and gel filtration chromatography are employed to purify the histone lysine methyltransferase SMYD2 into apparent homogeneity. Nickel affinity chromatography separates proteins based on a specific interaction between poly-histidine-tagged proteins and the nickel ions immobilized to a chromatography matrix. Gel filtration, which is also called size exclusion, separates proteins based on the size of the molecules. Crystallization of purified proteins is performed with the hanging-drop vapor diffusion method at room temperature. Vapor diffusion allows for a gradual and gentle increase in protein and precipitant concentration, which slowly creates supersaturation and facilitates the growth of large and well-ordered crystals. However, crystallization remains as the main bottleneck of X-ray crystallography due to the complex nature of proteins. Protein crystallization is essentially a trial and error process that requires large-scale screening of hundreds or even thousands of crystallization conditions. One of the important techniques to facilitating crystallization is crystal seeding, which separates nucleation from growth by introducing crystal seeds to remove the need for spontaneous nucleation [1]. This technique is employed to obtain crystals of SMYD2 in complex with an estrogen receptor α (ERα) peptide.

SMYD2 methylates ERα at lysine 266 [2]. This methylation prevents ERα from binding to the chromatin, thereby negatively affecting ERα target gene expression [2]. In order to understand the molecular basis of this methylation, a viable approach is to determine the structure of the SMYD2–ERα peptide complex. By analyzing this structure, the binding specificity of the SMYD2–ERα interaction can be elucidated at the atomic level [3]. This protocol will describe SMYD2 cocrystallization with an ERα peptide, which is a prerequisite step for X-ray structure determination.

2 Materials

2.1 Stock Solutions

1. 2000× Streptomycin solution (100 mg/mL): Dissolve 1 g streptomycin in 10 mL deionized water. Filter-sterilize with 0.22 μm filter. Store at −20 °C (*see* **Note 1**).
2. 1 M Tris–HCl, pH 8.0 solution (1 L): Dissolve 121.1 g of Tris base in 800 mL deionized water. Adjust the pH to 8.0 with concentrated HCl. Add deionized water to 1 L. Autoclave.
3. 5 M NaCl (1 L): Dissolve 292.2 g NaCl in 800 mL deionized water. Add deionized water to 1 L. Autoclave.
4. 40 % glycerol solution (1 L): Mix 400 mL glycerol with 600 mL deionized water. Autoclave and store at room temperature.
5. 10× ST buffer: 100 mM Tris–HCl, pH 8.0, and 1 M NaCl. Store at room temperature or 4 °C.

6. 0.4 M Isopropyl β-d-1-thiogalactopyranoside (IPTG) (1 mL): Dissolve 953.2 mg IPTG in 1 mL deionized water. Filter-sterilize with 0.22 μm filter. Store at −20 °C.
7. 100 mM Phenylmethylsulfonyl fluoride (PMSF) (100×): Dissolve 17.4 mg PMSF in 1 mL methanol. Store at −20 °C.
8. DNase I (2 mg/mL): Dissolve 5 mg DNase I in a ice-cold solution made of 312.5 μL 80 % glycerol, 18.75 μL 5 M NaCl, and 2.169 mL deionized water. Store at −20 °C.
9. 0.5 M $MgCl_2$ (500 mL): Dissolve 23.8 g $MgCl_2$ in 400 mL deionized water. Add deionized water to 1 L. Filter-sterilize with 0.22 μm filter. Store at room temperature.
10. 0.4 M NaH_2PO_4 stock (1 L): Dissolve 55.2 g NaH_2PO_4 in 1 L deionized water. Autoclave and store at room temperature.
11. 0.4 M Na_2HPO_4 stock (1 L): Dissolve 56.9 g Na_2HPO_4 in 1 L deionized water. Autoclave and store at room temperature.
12. 2 M Imidazole, pH 7.4 (1 L): Dissolve 130.6 g imidazole in 800 mL deionized water. Adjust the pH to 7.4 with concentrated HCl. Add deionized water to 1 L. Filter-sterilize with 0.22 μm filter. Store at room temperature.
13. 0.2 M sodium phosphate buffer, pH 7.4 (500 mL): Mix 95 mL of 0.4 M NaH_2PO_4 with 405 mL of 0.4 M Na_2HPO_4. Add deionized water to 500 mL. Adjust pH if necessary. Filter-sterilize with 0.22 μm filter. Store at room temperature.
14. Luria Broth (LB) medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1 L deionized water. Sterilize by autoclaving.
15. 1 M Dithiothreitol (DTT) stock: Dissolve 1.5 g DTT in 8 mL deionized water. Adjust the volume to 10 mL with deionized water. Store at −20 °C.
16. 5 mM ERα peptide stock: GGRMLKHKRQR (11 aa, residues 261–271). Dissolve 1.367 mg ERα peptide in 200 μL deionized water. Store at −20 °C (*see* **Note 2**).

2.2 Protein Expression

1. Plasmid carrying SMYD2 with N-terminal 6×His-SUMO tag [3].
2. Competent *E. coli* BL21 Codon Plus (DE3) cells.
3. Cell culture dishes.
4. 2.8 L-Fernbatch flasks.
5. Refrigerated incubator shaker.
6. UV/Vis spectrophotometer.
7. Centrifuges.
8. Vortex mixer.
9. French Press (Thermo Scientific) or sonicator.

10. 250 mL centrifuge tubes.
11. 50 mL sterile centrifuge tubes.
12. 0.2 μm filter.

2.3 Protein Purification

1. HisTrap HP column: A ready-to-use Ni^{2+} affinity column for preparative purification of a His-tagged recombinant protein.
2. HiPrep 26/10 Desalting column: For protein buffer exchange.
3. HiLoad 16/60 Superdex 200 prep grade column: Gel filtration (or size exclusion) column designed for preparative protein purification.
4. ÄKTA FPLC Protein Purification System (GE Healthcare) or equivalent.
5. HisTrap column-binding buffer (1 L): 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 20 mM imidazole, 5 % glycerol, 1 mM BME (*see* **Notes 3** and **4**).
6. HisTrap column-elution buffer (1 L): 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 500 mM imidazole, 5 % glycerol, 1 mM BME.
7. Gel filtration buffer (1 L): 10 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 5 % glycerol, 1 mM BME.
8. Fraction collector.
9. 10 mL Test tubes.
10. Gel electrophoresis system.
11. 13 % SDS polyacrylamide gels.
12. Yeast SUMO protease 1 (ULP1): 1 mg/mL.
13. Microcentrifuge tubes.
14. 30K-molecular weight cutoff concentrators.

2.4 Crystallization

1. Protein sample buffer: 10 mM HEPES, pH 7.5, 50 mM NaCl, 5 % glycerol, 2 mM DTT. Store at 4 °C.
2. Well solutions or precipitant solutions: *S1*: 15 % polyethylene glycol (PEG) 8000, 50 mM NaCl, 0.1 M Tris–HCl, pH 8.5 for crystallization of the SMYD2–AdoHcy binary complex (AdoHcy, S-adenosyl-L-homocysteine, cofactor product). *S2*: 0.1 M Tris–HCl, pH 7.5, 20 % PEG3350, 5 % ethanol for cross-seeding of the SMYD2–AdoHcy–ERα ternary complex.
3. Buffer exchange columns such as the Micro Bio-Spin Chromatography Columns (Bio-Rad).
4. 10 mM S-adenosyl-L-homocysteine (AdoHcy) stock.
5. 24-Well crystallization plate.
6. Dow Corning vacuum grease.
7. Siliconized glass cover slides.
8. Cooled crystallization incubator.

9. Seed Bead (Hampton Research).
10. Cat whisker or MicroGripper (MiTeGen).
11. Stereomicroscope (magnification: 5–80×).

3 Methods

3.1 Large-Scale Protein Production (*See* Note 5)

1. Transform recombinant SMYD2, which consists of an N-terminal 6×His-SUMO tag (~12 kDa), into *E. coli* BL21 Codon Plus (DE3) cells [3–6].
2. Prepare a starter cell culture: Take a single colony from a freshly streaked plate or use a freezer glycerol stock. Inoculate in 5 mL LB media supplemented with 50 μg/mL streptomycin. Shake at 250 rpm, 37 °C, overnight.
3. Prepare 500 mL LB media in a 2.8 L-Fernbatch flask. Add streptomycin to a final concentration of 50 μg/mL. Inoculate 5 mL overnight starter culture (from **step 2**, Subheading 3.1) into the media.
4. Shake at 280 rpm and at 37 °C until the optical density at 600 nm (OD600) reaches between 0.4 and 0.6.
5. Pause the shaker and gradually lower the temperature to 15 °C by 5 °C intervals and hold for 10 min each. Hold at 15 °C for 30 min. Add IPTG to a final concentration of 0.1 mM. Shake at 250 rpm, 15 °C, overnight or 18–20 h (*see* **Note 6**).

3.2 Harvest Cells

1. Transfer the cell culture to 250 mL centrifuge tubes. Centrifuge at 4500 × *g*, 4 °C, for 30 min. Collect cell pellet and discard supernatant.
2. Add 20–30 mL ice-cold ST buffer to the cell pellet. Vortex until the cells are completely resuspended.
3. Transfer the cell suspension to 50 mL sterile centrifuge tubes. Centrifuge at 3200 × *g*, 4 °C, for 20 min. Collect cell pellet and discard supernatant.
4. Proceed to the next step for cell lysis or store the cell pellet at −20 °C for later use.

3.3 Lyse Cells

1. Thaw a frozen cell pellet on ice or use the freshly prepared cell pellet (**step 4**, Subheading 3.2).
2. Add 30 mL lysis buffer to the cell pellet.
3. Then add 300 μL 100× PMSF (protease inhibitor) to a final concentration of 100 μg/mL. The volume of the lysis buffer depends on the capacity of the French Press pressure cells (*see* **Note 7**).
4. Vortex to resuspend the cells. *From this step forward, the sample MUST be kept on ice or at 4 °C to avoid protein degradation.*

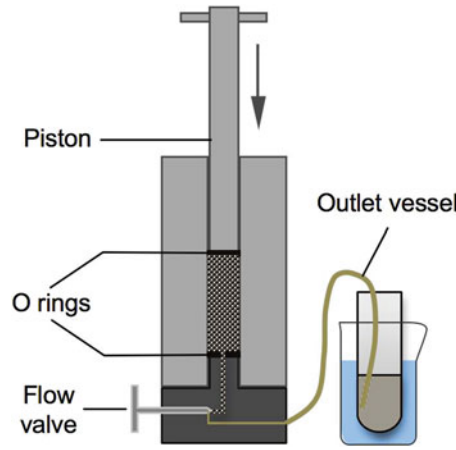

Fig. 1 French Press pressure cell

5. Lyse the cells with the French Press which breaks the plasma membrane with high mechanical pressure (Fig. 1). Collect the cell lysate (*see* **Notes 8** and **9**).
6. Add 150 µL 2 mg/mL DNase I and 300 µL 0.5 M $MgCl_2$ per 30 mL cell lysate. Incubate on ice for 15–20 min (This step can be skipped if sonication is used to lyse the cells) (*see* **Note 9**).
7. Centrifuge at 21,000 × *g*, 4 °C, for 20 min to remove insoluble cell debris.
8. Transfer the supernatant into a clean beaker.
9. Filter the supernatant with a 0.2 µm filter.

3.4 Protein Purification

1. Equilibrate a HisTrap HP column with the column-binding buffer (*see* **Note 10**) (Fig. 2).
2. Load the filtered sample (from **step 9**, Subheading 3.3) onto the column.
3. Wash with the buffer until the A_{280} value reaches a steady baseline.
4. Elute the column with the elution buffer using a linear gradient. Five column volumes are usually sufficient for elution, but a shallow gradient, such as a gradient over 20 column volumes or more, may increase resolution and separation. Collect elution fractions into test tubes (Fig. 3a).
5. Analyze the elution using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
6. Pool the fractions containing a high amount of SMYD2 (Fig. 3a).
7. Add yeast SUMO protease 1 (1 µg per 10 mg of substrate) to the pooled fractions. Incubate at 4 °C overnight. This step is used to cleave off the 6×His-SUMO tag from the protein.

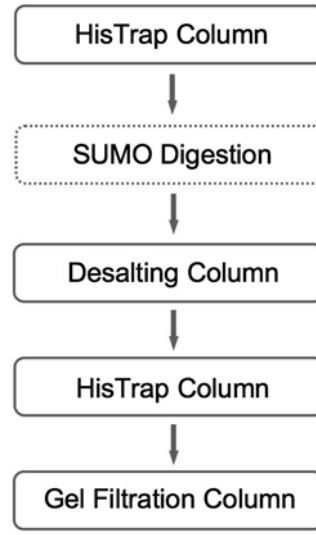

Fig. 2 General procedure of multi-step FPLC protein purification

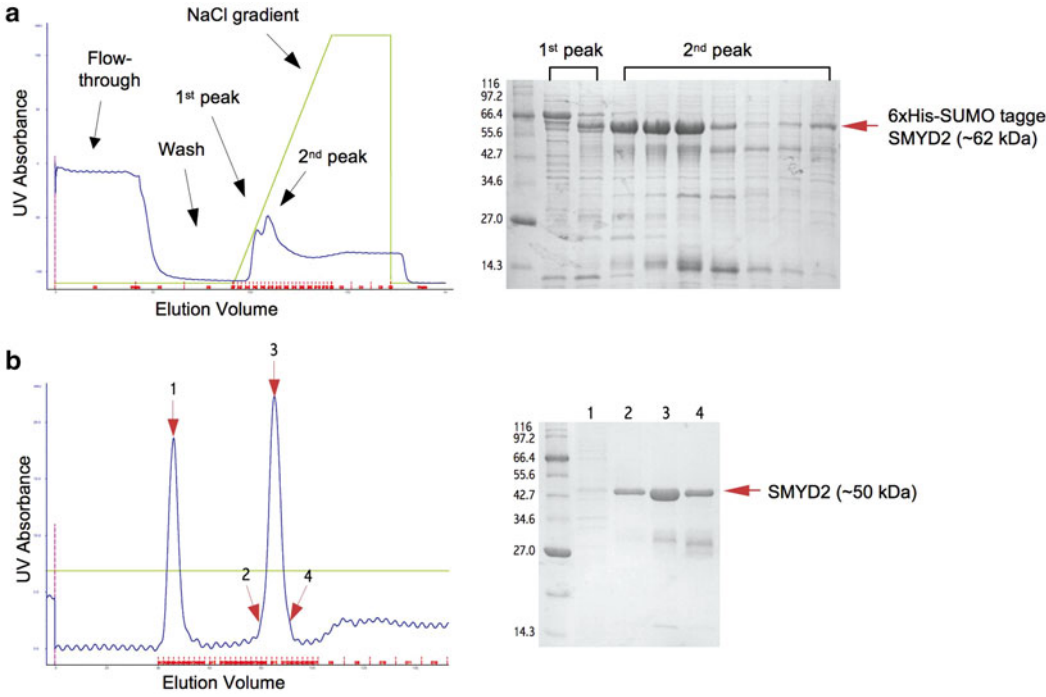

Fig. 3 Proteinpurification. (**a**) Elution profile of the first HisTrap column (*left*). Proteins are separated and eluted out using a linear gradient. SDS-PAGE analysis of elution fractions (*right*). (**b**) Elution profile of the gel filtration column (*left*). SDS-PAGE analysis of elution fractions (*right*). Lanes of SDS-PAGE correspond to the labels in the elution profile

8. Load the cleaved protein onto the desalting column, and elute with the HisTrap HP column-binding buffer. Collect elution fractions. The goal of this step is to exchange the buffers and prepare for a second Ni^{2+} affinity purification, which will separate SMYD2 from the cleaved 6×His-SUMO tag.
9. Pool the fractions containing SMYD2 according to chromatography.
10. Load the sample onto the second HisTrap HP column.
11. Wash the column with 10 column volumes of HisTrap-binding buffer.
12. Collect "flow-through" and "wash."
13. Pool the "flow-through" and "wash" from the previous step. Concentrate to ~1 mL using a 30K-MWCO (molecular weight cutoff) concentrator (*see* **Note 11**).
14. Transfer the concentrated protein to a clean pre-chilled microcentrifuge tube. Centrifuge at 13,000 × *g* for 10 min to remove any precipitate.
15. Load the supernatant onto the gel filtration column.
16. Elute with one column volume of gel filtration buffer. Proteins will be separated according to their size (Fig. 3b).
17. Analyze the peak fractions using SDS-PAGE (Fig. 3b).
18. Pool the SMYD2-containing fractions and concentrate to a final concentration of ~20 mg/mL using a 30K-MWCO concentrator.
19. Aliquot and store at −80 °C (*see* **Note 12**).

3.5 Crystallization

1. Exchange the buffers of the protein sample with Micro Bio-Spin Chromatography Columns. The new buffer is the protein sample buffer (*see* **Note 13**).
2. Measure protein concentration according to the absorbance at 280 nm.
3. Dilute the protein concentration to 10 mg/mL.
4. Incubate the protein sample with 2 mM AdoHcy at 4 °C for 2 h.
5. Centrifuge at 13,000 × *g* for 1 min to remove any precipitates; collect the supernatant.
6. Set up crystallization using the hanging drop vapor diffusion method at 20 °C. First, add 500 μL well solution *S1* (from **step 2**, Subheading 2.4) to the wells of a pre-greased 24-well plate (*see* **Notes 14** and **15**).
7. Pipette a 1 μL drop of well solution S1 onto a siliconized cover slide.
8. Add 1 μL of the protein sample (from **step 3**, Subheading 3.5) to the drop. Then, flip the cover slide, put it on top of the well, and press down gently until the grease seals the well.

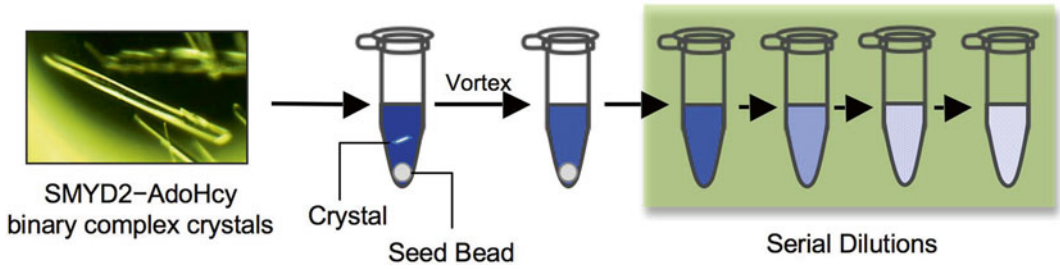

Fig. 4 Preparation of microseed solutions

9. Keep the plate in a 20 °C-crystallization incubator. Crystals typically appear overnight and achieve a full size in a week (Fig. 4).
10. Take a clean cat whisker or MicroGripper and dip into the drop to pick up seed crystals (*see* **Note 16**).
11. Transfer the seed crystals onto a clean cover slide with a fresh drop of 5 μL well solution. Soak for 30 s to wash off any amorphous precipitates.
12. Place the washed crystals in a microcentrifuge tube containing the Seed Bead and 50 μL of the well solution. Vortex for ~3 min or until the crystals are crushed into microcrystalline particles. This seed stock can be stored at 20 °C for later use (up to 2 days).
13. Perform serial dilutions of the seed stock (1:5, 1:10, and 1:100) using well solution S1. Check each dilution under the microscope. This step is useful for controlling the number of the seeds in the experiment (Fig. 4).
14. Prepare a protein sample for crystal seeding: Dilute the protein from **step 2**, Subheading 3.5 to a lower protein concentration of 3 mg/mL.
15. Incubate with 2 mM AdoHcy and 2 mM ERα peptide at 4 °C for 2 h.
16. Centrifuge at 13,000 × *g* for 1 min to remove any precipitates. Collect the supernatant.
17. Set up crystal seeding using the hanging-drop vapor diffusion method at 20 °C. First, add 500 μL well solution *S2* (from **step 2**, Subheading 2.4) to the wells of a pre-greased 24-well plate.
18. Pipette 0.7 μL of well solution S2 onto a siliconized cover slide. Add 1 μL protein sample (from **step 16**, Subheading 3.5) and 0.3 μL seed solution (from **step 13**, Subheading 3.5) to the drop.
19. Flip the cover slide, put it on top of the well, and seal.
20. Keep the plate in the 20 °C-crystallization incubator.
21. Crystals appear within a day and achieve a full size in a week. These crystals are suitable for X-ray diffraction data collection.

4 Notes

1. Prepare all buffers and solutions using deionized water and molecular biology-grade reagents.
2. Choose an appropriate solvent to dissolve peptides. The best solvent to use will depend on the solubility properties of the peptides. Acidic peptides can be dissolved in water or basic buffers, whereas basic peptides can be dissolved in water and acidic solutions. For very hydrophobic peptides, try dissolving the peptides in a small amount of organic solvent such as DMSO, methanol, or isopropanol and then dilute with water. Do not use DMSO for cysteine-containing peptides as it may oxidize the side chain of cysteine [7]. Also, peptides used for co-crystallization should have high purity (>95 %).
3. All buffers used in the ÄKTA FPLC protein purification system, including water (for washing) and 20 % ethanol (for storage), must be filtered through 0.22 μm filter and degassed for 2 h. Store buffers at 4 °C. β-Mercaptoethanol (BME) or DTT should be added to buffers before use.
4. The optimal imidazole concentration in the binding buffer is protein dependent. Typically, 20–40 mM is suitable for many proteins.
5. Small-scale expression test is recommended before proceeding to a large-scale protein production in order to optimize expression conditions. The conditions can be varied including inducer concentration and induction time and temperature.
6. Inducing protein expression at a lower temperature, between 15 and 20 °C, may help increase yields of properly folded protein [8]. Lowering the temperature gradually from 37 to 15 °C can reduce the induction of *E. coli* heat-shock protein 70 (HSP70), which is sensitive to rapid temperature change.
7. The choice of lysis buffer depends on protein properties and purification strategy. In this protocol, the lysis buffer is the HisTrap HP column-binding buffer, which is chosen because the first step of purification is Ni^{2+} affinity chromatography.
8. The pressure cell must be pre-chilled at 4 °C prior to use. If the French Press process takes more than 30 min, cool the pressure cell at 4 °C for 10–15 min and then continue.
9. Alternative method for cell lysis. Use sonication to lyse cells. Keep the cell suspension on ice during sonication. Sonicate the cells using a number of pulses (10 s) followed by pauses (20 s) for cooling. Sonication lyses cells by liquid shear and cavitation. It also shears DNA; thus DNase I is not needed.
10. Avoid introducing air into the FPLC system as it may affect resolution and cause a noisy baseline in the chromatograms.

Keep the pump running while connecting the column to the system. Use a "drop-to-drop" technique.

11. Select the concentrator based on the protein size. For a protein that has a molecular weight of 40 kDa, a 30K MWCO concentrator is appropriate to use.
12. Steps in Subheadings 3.3 (Lyse Cells) and 3.4 (Protein Purification) together can be completed in 5 days. It is recommended that after cell lysis purification should be started immediately to avoid protein degradation. Experiment timeline: Day 1, all steps in Subheading 3.3, **steps 1–4**, in Subheading 3.4; day 2, **steps 5–7** in Subheading 3.4; day 3, **steps 8–12** in Subheading 3.4; day 4, **steps 13–16** in Subheading 3.4; day 5, **steps 17–19** in Subheading 3.4.
13. In the sample buffer, the concentration of pH buffer should be 10–20 times lower than that in the crystallization condition or well solution. If a well solution contains 0.1 M Tris–HCl, pH 8.5, then no more than 10 mM pH buffer should be included in the sample buffer.
14. It is recommended that a crystallization trial should be performed to screen for optimal conditions for crystal growth. Promising conditions can be further optimized to obtain crystals with desired quality [9].
15. To make a greased plate, apply Dow Corning vacuum grease to the upper rim of each well.
16. Because a typical size of crystals is small (under 1 mm), crystal picking needs to be performed under a microscope.

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Chapter 17

Gold Nanoparticle-Based Förster Resonance Energy Transfer (FRET) Analysis of Estrogen Receptor: DNA Interaction

Xiaodi Su, Khin Moh Moh Aung, Steven Lukman, and Bin Liu

Abstract

Estrogen receptors play critical roles in regulating genes responsible for development and maintenance of reproductive tissues and other physiological function. The interaction of ERs with DNA sequences, known as estrogen response elements (EREs) (a palindromic repeat separated by three-base spacer, 5′GGTCAnnnTGACC-3′), is required for estrogen regulation of target gene expression. Here, we describe a simple "mix-and-measure"-based method for detecting ER:ERE interactions using ERE-immobilized metal nanoparticles and water-soluble conjugated polyelectrolytes (CPEs) as cooperative sensing elements. This method can differentiate the distinct DNA-binding affinity between ERα and ERβ, and determine ER:ERE-binding stoichiometry. This method can also accurately detect all 15 singly mutated EREs (i.e., three possible base substitutions at each of one to five positions from left to right of the 5′ end half site, GGTCA) for their binding energy to ER. This method is compatible with 96-well plate format for high-throughput analysis.

Key words Förster resonance energy transfer, Gold nanoparticles, Water-soluble conjugated polyelectrolytes, Transcription factor, Protein:DNA interactions, Estrogenreceptor

1 Introduction

Estrogenreceptors play critical roles in regulating genes responsible for development and maintenance of reproductive tissues and other physiological function. Two major ER subtypes (ERα and ERβ) are arranged into similar domains and the degree of homology varies widely among the regions. Despite the high degree of similarity in the DNA binding domain (96 % amino acid identity) and biochemical properties, ERα and ERβ differ substantially in tissue distribution [1]. The interaction of ERα and ERβ with DNA sequences, known as estrogen response elements (EREs) (a palindromic repeat separated by three-base spacer, 5′GGTCAnnnTGACC-3′), is required for estrogen regulation of target gene expression [2].

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Here we introduce a rapid and quantitative method for measuring ER:ERE interactions in a "mix-and-measure" manner [3]. In this method, double-stranded DNA (ERE)-conjugated gold nanoparticle (denoted as dsDNA-AuNPs or ERE-AuNPs) and water-soluble conjugated polyelectrolytes (CPEs) are used as collaborative sensing elements. The unique optical and charge properties of CPEs [4, 5] and the prominent property of 13 nm AuNPs as superquencher [6, 7] are exploited. The detection of ER binding to ERE is based on the Förster resonance energy transfer (FRET) and/or nanoparticle surface energy transfer (NSET) between the CPEs and ERE-AuNPs. The signal readout is the differential CPE emission determined by ER binding to ERE that changes the interaction between CPEs and ERE-AuNPs (Fig. 1).

Three water-soluble CPEs of different charge properties (cationic and anionic) and emission wavelengths (i.e., 410, 430, and 560 nm) have been chosen to demonstrate the concept. Through a proper selection of CPEs in terms of charge properties and emission wavelengths, ER binding can be detected by fluorescence recovery (e.g., light-on, Fig. 1a), fluorescence quenching (e.g., light-off, Fig. 1b), or both ways (e.g., two-way, Fig. 1c). With this method, we can differentiate the distinct DNA-binding affinity between ERα and ERβ, and determine ER:ERE-binding stoichiometry. We can also accurately detect singly mutated EREs for their binding strength to ER. The binding energy matrix measured for the total of 15 singly mutated EREs (each of them carrying one base variation in the 5′ end half-site with all possible base substitutions at each of the five possible sites) correlates to an in vivo energy model determined by thermodynamic modeling of ChIP-seq (TherMos) with a rank correlation coefficient of 0.98 [2]. This method has been validated for other transcription factors, for example, FoxA1 and Ap2γ and DNA-proteins of known and/ or unknown charge properties [8], which is not always feasible with FRET assays using other donor-acceptor pair [9].

2 Materials and Apparatus

1. Purified recombinant human estrogen receptors ERα (3770 nM) in 50 mM Tris–HCl, pH 8.0, 500 mM KCl, 1 mM EDTA, 1 mM Na_3VO_4, 2 mM DTT, and 10 % glycerol and ERβ (4500 nM) in 50 mM Bis-Tris-propane, pH 9.0, 400 mM KCl, 2 mM DTT, 1 mM EDTA, and 10 % glycerol (*see* **Note 1**).
2. Custom-synthesized ERE sequences (35 bp oligonucleotides: one wild-type ERE "wtERE," one scrambled ERE "scrERE," 15 singly mutated EREs—"mut1" to "mut15") are listed in Table 1. The wtERE contains perfect core sequence, 5′-GGTCAnnnTGACC-3′, and scrERE has both the 5′ and 3′

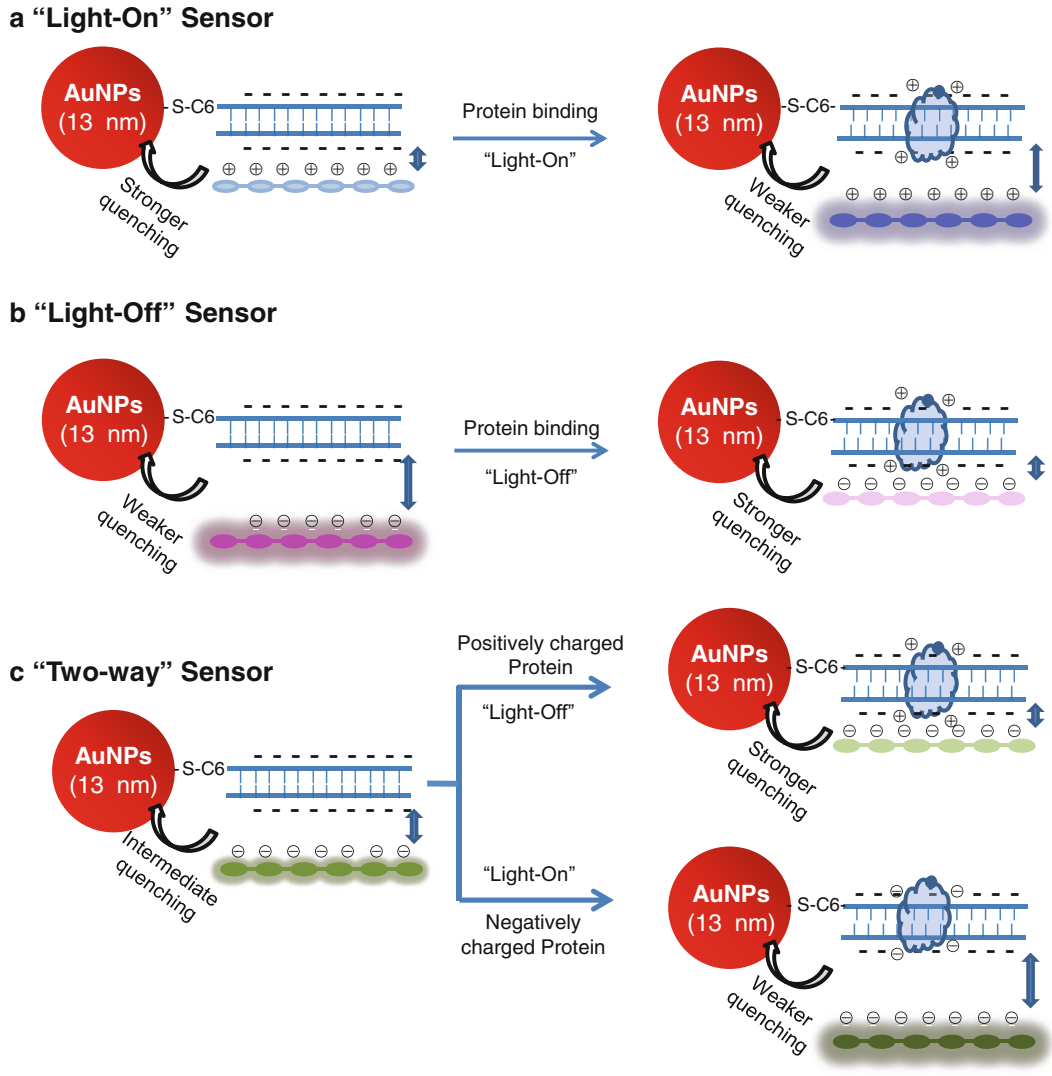

Fig. 1 Schematic drawings of (**a**) "light-on," (**b**) "light-off," and (**c**) "two-way" hybrid probe for detecting protein-DNA binding, according to the initial quenching, i.e., substantial quenching, minor quenching, or intermediate quenching, respectively

half-sites completely scrambled. The thiolated version of these sequences (used for conjugation on AuNPs) are denoted as thiol-wtERE and thiol-scrERE, respectively.

3. Water-soluble conjugated polyelectrolytes (CPEs) were utilized, including two anionic CPEs (ACPE) and one cationic CPE (CCPE). CPEs are designated by their charge and peak emission wavelengths as ACPE-430 (synthesized in our lab [9]), ACPE-560, and CCPE-410 (Sigma Aldrich) (Fig. 2a).

Table 1
ERE sequences

ID	Sequence
wtERE	5′-AGTAAGCT cca**GGTCA** TTA **TGACC**tgg AGCTTACT-3′
mut1	5′-AGTAAGCT ccaT **GTCA** TTA **TGACC**tgg AGCTTACT-3′
mut2	5′-AGTAAGCT ccaC **GTCA** TTA **TGACC**tgg AGCTTACT-3′
mut3	5′-AGTAAGCT ccaA **GTCA** TTA **TGACC**tgg AGCTTACT-3′
mut4	5′-AGTAAGCT cca**G** T **TCA** TTA **TGACC**tgg AGCTTACT-3′
mut5	5′-AGTAAGCT cca**G** C **TCA** TTA **TGACC**tgg AGCTTACT-3′
mut6	5′-AGTAAGCT cca**G** A **TCA** TTA **TGACC**tgg AGCTTACT-3′
mut7	5′-AGTAAGCT cca**GG** G **CA** TTA **TGACC**tgg AGCTTACT-3′
mut8	5′-AGTAAGCT cca**GG** C **CA** TTA **TGACC**tgg AGCTTACT-3′
mut9	5′-AGTAAGCT cca**GG** A **CA** TTA **TGACC**tgg AGCTTACT-3′
mut10	5′-AGTAAGCT cca**GGT** G **A** TTA **TGACC**tgg AGCTTACT-3′
mut11	5′-AGTAAGCT cca**GGT** T **A** TTA **TGACC**tgg AGCTTACT-3′
mut12	5′-AGTAAGCT cca**GGT** A **A** TTA **TGACC**tgg AGCTTACT-3′
mut13	5′-AGTAAGCT cca**GGTC** G TTA **TGACC**tgg AGCTTACT-3′
mut14	5′-AGTAAGCT cca**GGTC** T TTA **TGACC**tgg AGCTTACT-3′
mut15	5′-AGTAAGCT cca**GGTC** C TTA **TGACC**tgg AGCTTACT-3′
scrERE	5′-AGTAAGCT ccaTAGCG TTA CGCTAtgg AGCTTACT-3′

The degree of quenching of CPEs by dsDNA(ERE)-conjugated AuNPs (Fig. 2b) is 15 %, 50 %, and 85 %, respectively, depending on the spectral integral, charge property, and quantum yield [3].

4. 40 mM sodium citrate, 1 mM gold (III) chloride trihydrate ($HAuCl_4$), round-bottomed flask, reflux condenser, large stir bar, aqua regia.
5. Tris (2-carboxyethyl) phosphine, 3 kDa molecular weight cut-off (3 kDaMWCO) centrifugal filtration device (e.g., Microcon YM-3).
6. 0.1 M HCl, 1.71 M NaCl, 0.1 M NaOH, 5 mM HEPES buffer.
7. 100 μM thiazole orange, 1 mM potassium cyanide (KCN), 10 mM PBS buffer: 2.7 mM KCl, 137 mM NaCl, pH 7.4.
8. Nanodrop 3300 fluorescent spectrophotometer (Thermo Scientific).
9. Infinite M200 monochromator microplate reader (TECAN).

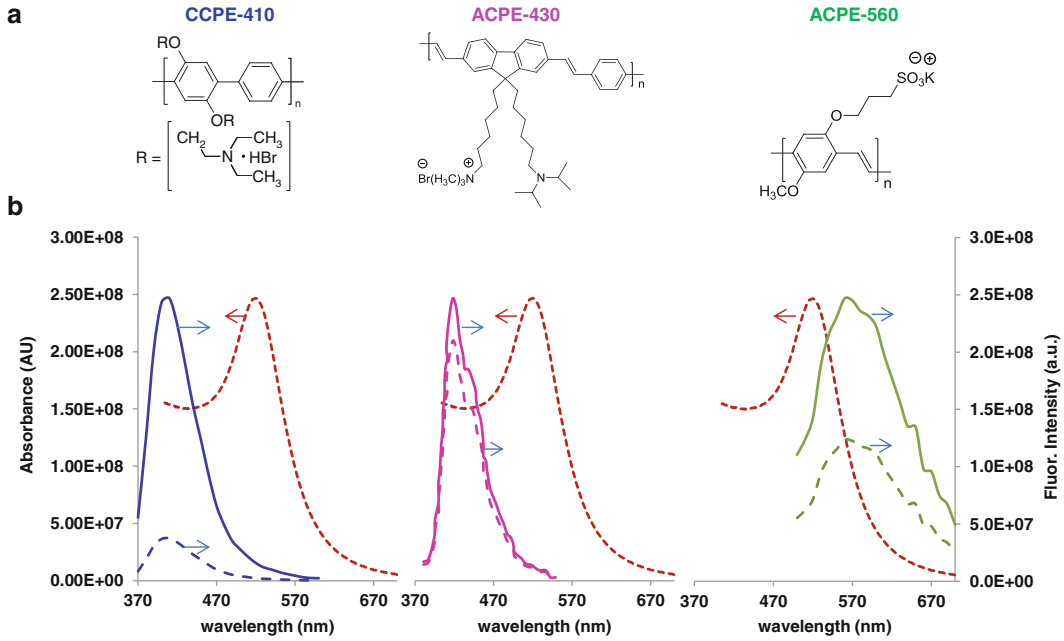

Fig. 2 (**a**) Chemical structures of three CPEs used in this study. (**b**) Absorbance spectra of AuNPs (*dashed red lines*). Fluorescence emission spectra of free CPEs (*solid lines*) and after initial quenching by dsDNA–AuNP conjugates (*dashed lines*)

3 Methods

3.1 Preparation of Gold Nanoparticles

Gold nanoparticles (AuNPs) of 13 nm in diameter are prepared by the citrate reduction of $HAuCl_4$.

1. Prepare 1 mM (50 mL) gold chloride $HAuCl_4$ solution and 40 mM (5 mL) sodium citrate solution.
2. Heat and stir $HAuCl_4$ solution at 100 °C under reflux until it boils and starts condensation.
3. Add 5 mL of sodium citrate solution rapidly. Within several minutes, the color of the solution will change from pale yellow to red.
4. Continue heating for 30 min to ensure complete reduction.
5. Continue stirring for an additional 15 min after heating is stopped and allow to cool to room temperature (*see* **Note 2**).

3.2 Preparation of dsDNA-Conjugated AuNPs

1. Prepare 50 μM thiolated single-stranded DNA (ssDNA) in 5 mM HEPES buffer.
2. Activate thiolated ssDNA with tris (2-carboxyethyl) phosphine (TCEP, 10 fold molar excess to the ssDNA) and stir for 10 min (*see* **Note 3**).

3. Centrifuge the final solution through a 3 kDa MWCO centrifugal filter device at 19,064 × *g* for 30–45 min to remove TCEP before conjugation to AuNPs. The final volume should be around 5–10 μL. Afterwards, top up the solution with 5 mM HEPES buffer and repeat the washes for another 2–3 times (*see* **Note 4**).
4. Conjugate activated thiolated DNA to AuNP [10]. To prepare 500 μL of DNA conjugated AuNP solution, first mix activated DNA with AuNPs at desired ratio (100:1, for example, 500 nM:5 nM) and incubate for 5 min (*see* **Note 5**). Then add 5 μL of 0.1 M HCl to lower the pH of the solution to pH 3 and wait for 5 min before adding 9 μL of 1.71 M NaCl to make the final salt concentration of 30 mM. Incubate the sample for another 5 min and bring the pH back to 7 by adding 10 μL of 0.1 M NaOH. Top up the final solution to 500 μL with distilled water.
5. Anneal the ssDNA-AuNPs conjugates (500 nM) with their complementary DNAs (500 nM) at 90 °C for 5 min before cooling down to room temperature (*see* **Note 6**).
6. Wash the obtained conjugation mixture through three rounds of centrifugation and rinsing with 5 mM HEPES buffer (pH 7.4) to remove the unbound DNA strands and stored at 4 °C (*see* **Note 7**).

3.3 Quantification of dsDNA on AuNPs

Quantification of dsDNA coverage on dsDNA-AuNP conjugates was done using thiazole orange (TO) intercalation method [10].

1. Dilute 25 μL of DNA-AuNP conjugate solution to 100 μL with HEPES buffer.
2. Add 5 μL of 1 mM KCN to dissolve the AuNPs completely and release attached dsDNA (*see* **Note 8**).
3. Mix 0.5 mL of 100 μM thiazole orange with the released dsDNA in the supernatant for 15 min, and record the emission of thiazole orange at 530 nm using a Nanodrop 3300 fluorescent spectrophotometer. The fluorescence intensity from thiazole orange will be proportional to the amount of dsDNA since thiazole orange dye only gives appreciable emission as it intercalates into the dsDNA. Use a series of known concentrations of dsDNA to generate a calibration curve, then measure, and quantify the samples accordingly.

3.4 ACPE-430-Based Detection of ERα and ERβ Binding to wtERE-AuNPs

Conduct the ER:ERE-binding assay in four main steps.

1. Incubate 10 μL of 500 nM ERE-AuNPs with 25 μL of ER ranging from 0 to 1 μM in a microplate (384-well flat clear-bottom black polystyrene TC-treated microplate, Corning) containing 55 μL PBS buffer, for 30 min at room temperature. (For determination of the binding constant (K_d) and the

stoichiometry (n), titrate the protein with increasing concentration from 0 to 250 nM.)

2. Add 10 μL of 1 μM ACPE-430 solution into above solution in **step 1** and incubate for another 10 min. In the solution the final concentrations of ERE-AuNPs, ER, and CP are 50 nM, 0–250 nM, and 100 nM, respectively.
3. Measure the fluorescence spectra of the final solution, using a microplate reader (Infinite M200, Tecan) from 300 to 700 nm at an excitation wavelength of 370 nm, and compare the spectra in the presence and absence of protein. (In control experiments of testing CPE emissions in the presence of DNA or protein without AuNPs, 50 nM dsDNA or 250 nM protein were added into 100 nM ACPE-430 in PBS buffer.) (*See* **Note 9**.)
4. With the [ER] dependent fluorescence intensity scales, calculate the protein-DNA binding affinity constant K_d quantitatively through $(F_0 - F)/(F - F_{sat}) = ([\text{protein}]/K_d)n$ (Fig. 3c).

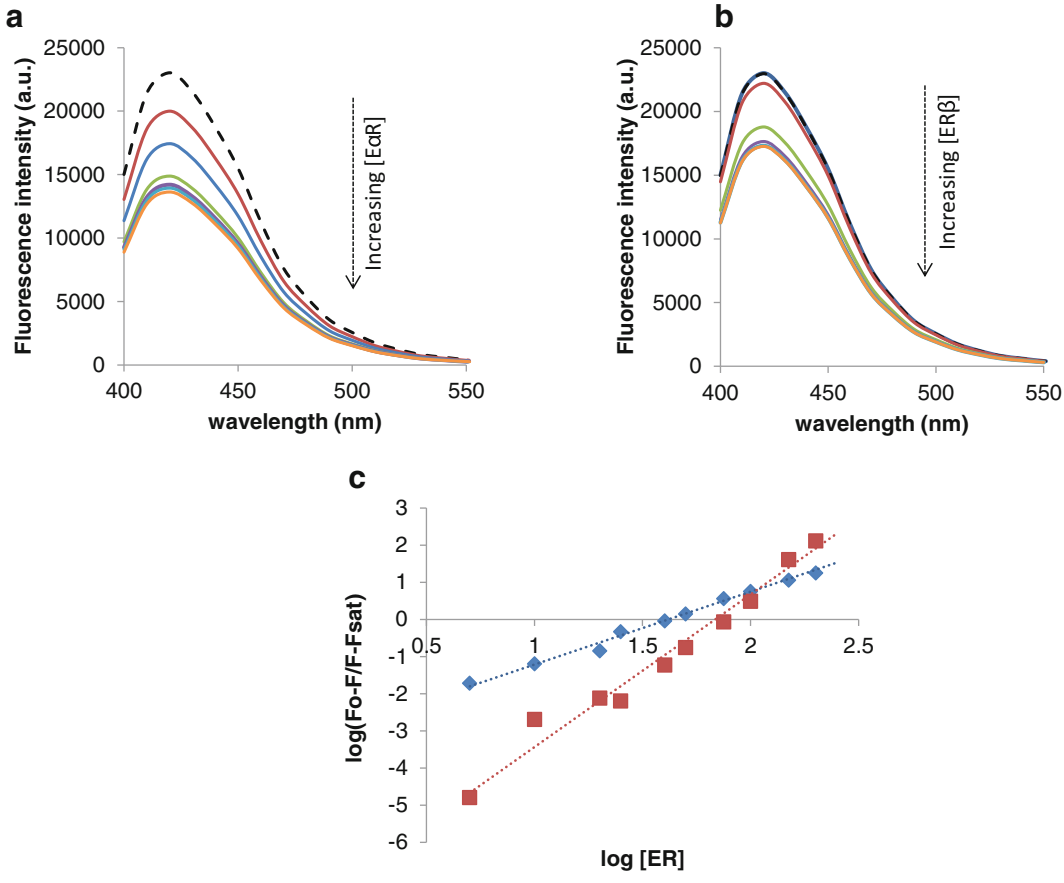

Fig. 3 Fluorescence emission spectra of ACPE-430 mixed with wtERE-AuNPs without ER (*black dashed line*) and with increasing molar concentration of (**a**) ERα and (**b**) ERβ relative to DNA (0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0fold). (**c**) Logarithmic plot of log $[(F_0 - F)/(F - F_{sat})]$ vs. log [ERα (*diamond*) or ERβ (*square*)] for deducing binding constant (K_d) and binding stoichiometry (*see* text for more detail)

Table 2
Binding constant (K_d) of ERα and ERβ with wtERE measured by three CPE/DNA-AuNP hybrid sensors[a,b]

	ERα		ERβ	
Hybrid sensors	K_d **(nM)**	***n***	K_d **(nM)**	***n***
ACPE-430/dsDNA-AuNPs	42.95 ± 1.85	1.93 ± 0.07	74.09 ±3.28	4.05 ± 0.13
ACPE-560/dsDNA-AuNPs	41.31 ± 1.54	1.96 ±0.10	72.90 ± 2.39	4.01 ± 0.23
CCPE-410/dsDNA-AuNPs	45.77 ± 4.89	1.97 ±0.09	76.04 ± 3.35	3.71 ± 0.19

[a] K_d for ERα-ERE from Sepharose chromatograph is 46 nM [11]

[b] ERβ is known to have a two times lower affinity than ERα [1]

Obtain the K_d by plotting log $[(F_0 - F)/(F - F_{sat})]$ versus log [protein], where F_0 and F_{sat} are the relative fluorescence intensities in the absence of protein and in protein saturation, respectively. The value of log [protein] at log $[(F_0 - F)/(F - F_{sat})] = 0$ equals to the logarithm of the K_d. The slope, n, is the binding stoichiometry of protein to DNA. The K_d values and stoichiometric (n) values (Table 2) confirm the following characteristics: ERα binds to wtERE as a dimer (n~2) and more strongly than ERβ ($K_{d,\,ER}\alpha < K_{d,\,ER}\beta$), and ERβ binds with a ratio of approximately 4 to 1 molar ratio with wtERE as determined previously using SPR spectroscopy [1]. The K_d obtained for ERα correlates well to the value of 46 nM obtained using Sepharose chromatography [11].

3.5 CCPE-410-Based Detection of ERα and ERβ Binding to wtERE-AuNPs

Relative to the "light-off" principle, a "light-on" assay can offer an additional benefit, that is, the solution glows upon protein binding. The human eye can quickly adapt to the background illumination and rapidly catch the change in intensity under UV light to distinguish objects from background. CCPE-410-based detection of ERα and ERβ binding to wtERE-AuNPs was conducted by the same steps as in ACPE-430-based detection, except that the polyelectrolyte used was CCPE-410.

CCPE-410, which exhibits opposite charge from DNA, initially is quenched by 85 % (Fig. 2). Since ERα and ERβ are positively charged at pH 7.4, ERs binding to ERE lower the charge of DNA-AuNPs conjugates and thus reduce the electrostatic attraction or the affinity of CCPE-410 to AuNP. Thereby fluorescence recovery is observed as the measure of ER-ERE interactions (Fig. 4a, b). This CCPE could be the polymer of choice if visual detection is desirable through the noticeable fluorescence recovery (Fig. 4c). Quantitative measurement of ER affinity with this CCPE-410/AuNPs hybrid sensor gives the same result as the previous ACPE-430 sensor; that is, ERα has a stronger affinity to wtERE than ERβ, and ERα binds to ERE as a dimer, whereas ERβ binds to ERE as a tetramer (Table 2).

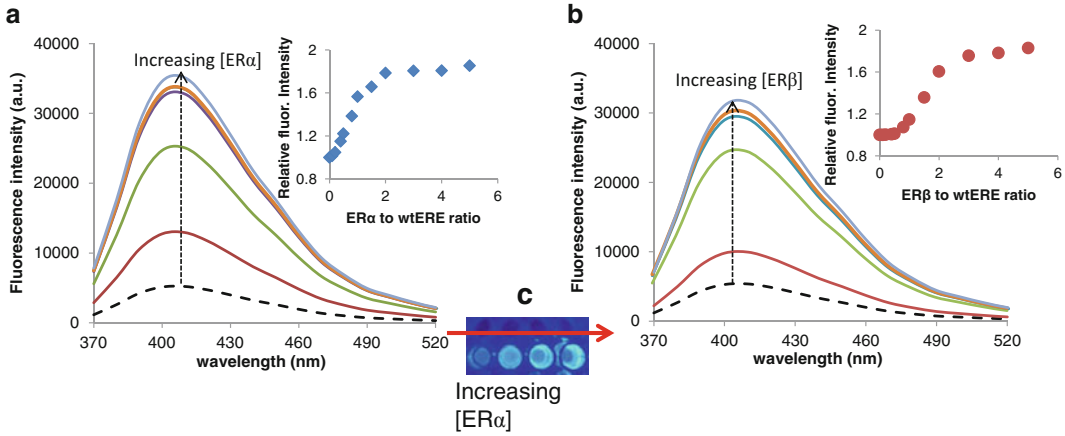

Fig. 4 Fluorescence emission spectra of CCPE-410 mixed with ERE-AuNPs without ER (*black dashed line*) and with increasing molar concentration of (**a**) ERα and (**b**) ERβ relative to DNA (0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 fold). The *insets* summarize change in fluorescence intensity at 410 nm as a function of [ER]. (**c**) Change in fluorescence emission of CCPE-410 as the ratio of ERα to ERE is varied from 0, 1, 2 to 5, respectively, under UV light

3.6 ACPE-560-Based Detection of ERα and ERβ Binding to wtERE-AuNPs

ACPE-560-based detection of ERα and ERβ binding to wtERE-AuNPs was conducted by the same steps as in ACPE-430-based detection, except that the polyelectrolyte used was ACPE-560.

The ACPE-560 has a weak hydrophobic interaction that is not favorable for distance dependent energy transfer. However, the higher overlap integral of its emission spectrum with the absorption spectrum of AuNP renders an enhanced quenching to ~50 % of the original fluorescence intensity (Fig. 2). The intermediate fluorescence quenching basically gives ACPE-560 its unique dual applicability, in which it can be used to detect protein binding either by fluorescence quenching or by recovery. This "two-way" fashion allows for detecting of proteins of positive or negative charge or proteins of unknown charge properties.

At pH 7.4, ERα is positively charged; this renders the fluorescence of the ACPE-560 to be further quenched with its binding to DNA (Fig. 5a). At pH 9.5, ERα is negatively charged. The binding to ERE-AuNPs at this pH manifests as the recovery of the anionic ACPE-560 fluorescence intensity (Fig. 5b) due to the repulsion between the CPE and the bound protein. Figure 5c shows a summary of ER concentration dependent ACPE-560 intensity change at pH 7.4 and 9.5 for ERα and ERβ. The K_d values measured by ACPE-560 (Table 2) are the average values from both methods. The results from ACPE-560 closely agree to those measured using the other two CPEs.

3.7 Binding Energy of ERα to Singly Mutated ERE: Using a Competition Approach

A perfect ERE contains a palindromic consensus sequence separated by a three-base pair spacer, 5′-GGTCAnnnTGACC-3′. Despite the consensus ERE delineated from conserved *cis-regulatory* elements, the majority of in vivo EREs deviate from the consensus,

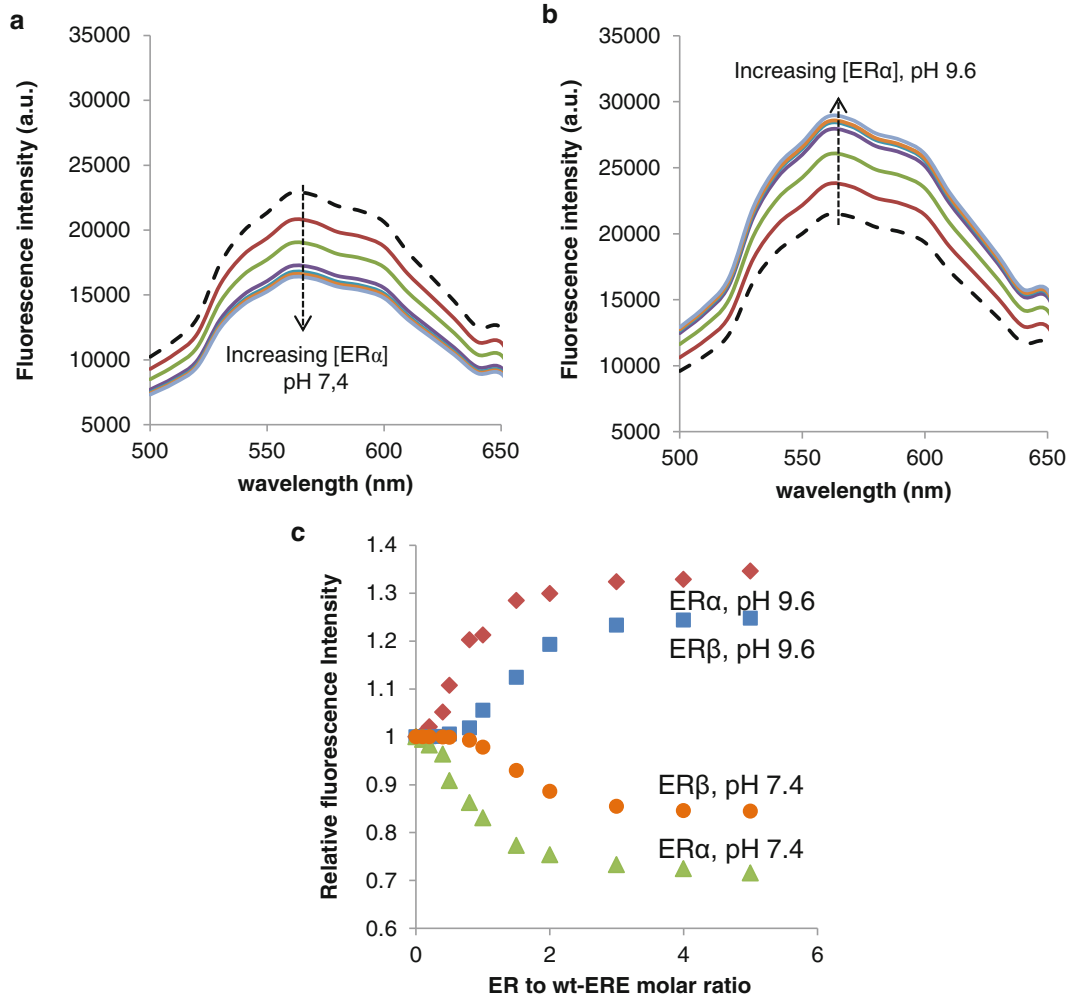

Fig. 5 (**a**, **b**) Fluorescence spectra of ACPE-560 mixed with ERE-AuNPs without ER (*black dashed line*) and with increasing molar concentration of ERα at (**a**) pH 7.4 and (**b**) pH 9.5. (**c**) The relative change in fluorescent intensity at 560 nm of ACPE-560 as the function of ER concentration for ERα at pH 7.4 (*triangle*), pH 9.5 (*diamond*) and ERβ at pH 7.4 (*circle*), pH 9.5 (*square*)

with one half-site identical to that in the consensus and the second half-site having nucleotide variant(s). To detect the binding energy of ERα to singly mutant EREs (one nucleotide variant in one of the half-sites), a competition approach is used with the CCPE-410 "light-on" probe.

1. Incubate 10 μL of 1 μM ERα solution with 5 μL of 1 μM DNA solution (competitor DNA, i.e., the mutEREs) in a microplate (384-well flat clear-bottom black polystyrene TC-treated microplate, Corning) containing 65 μL PBS buffer for 10 min.
2. Add 10 μL of 500 nM wtERE-AuNPs conjugate to above mixture and incubate for another 30 min at RT.

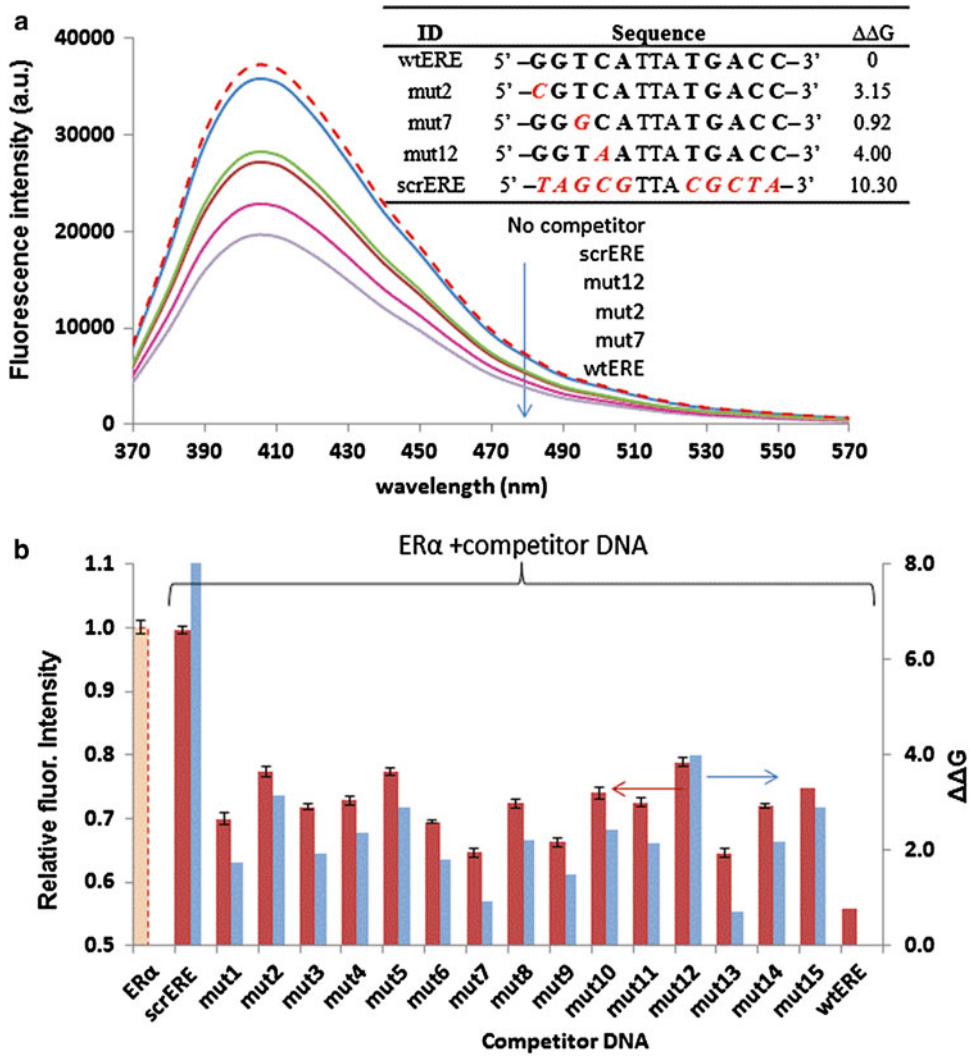

Fig. 6 (**a**) Fluorescence intensity plot of CCPE-410 responding to ERα binding to wtERE-AuNPs in the presence of various competitors DNA, i.e., wtERE, scrERE, smut2, smut7, and smut12. The *red dashed line* refers to emission intensity with ERα binding without any free competitor DNA in solution. (**b**) Relative fluorescence intensity bar plot for all single mutated EREs as competitor (*red bars* (bars with error bars)), with that without competitor DNA as reference (*leftmost bar*). The *blue bars* (the bars without error bars) are calculation free energy using TherMos thermodynamic prediction [2]

3. Add 10 μL of 1 μM CCPE-410 solution (100 nM CCPE-410) (e.g., 2:1 molar ratio to dsDNA-AuNPs) to the mixture of **step 2** and incubate for an additional 10 min to reach equilibrium.
4. Measure and compare the fluorescence spectra of the final solution (same as in **step 3**, ACPE-430-based detection) in the presence and absence of protein-mutERE mixture (Fig. 6).

When the competitor has a weaker affinity to ERα (e.g., the scrERE), more ERα (positively charged at pH 7.4) will be available to bind to wtERE-AuNPs, which leads to a stronger repulsion of the CCPE-410 and thus high emission intensity ("light-on"), and vice versa (Fig. 6a for scrERE, mut12, mut2, or mut7). The intensity trend for these competitors correlates well with the trend of their free energy value (Table inset in Fig. 6a, the larger the value, the lower the affinity), giving the following affinity order wtERE > mut7 > mut2/mut12 > scrERE. For all 15 singly mutant ERE variants, the position- and type-specific affinity trend measured by the DNA-AuNPs/CCPE-410 hybrid sensor correlates with the energy matrix derived from the in vivo model using TherMos (Fig. 6b) with a rank correlation efficient of 0.98.

4 Notes

1. At first use, dispense the proteins into 10 μL aliquots, as is, without additional storage buffer and store at −80 °C to avoid multiple freeze-thaw cycles. The proteins should be mixed gently and not vortexed. Before use, thaw the aliquots in a room temperature water bath and return to 4 °C to maintain activity.
2. Before setting up for AuNPs synthesis, a round-bottom flask, a reflux condenser, and a large stir bar should be washed with first aqua regia and then thoroughly with water. Aqua regia is noxious and extremely caustic. Handle with extreme care in a well-ventilated fume hood. Make sure that $HAuCl_4$ solution is boiling before adding the sodium citrate solution. Condensation droplets should be seen inside the flask. Sodium citrate solution should be added to the mixture as rapidly as possible. If the addition is done correctly, the solution should turn from yellow to purple and then to deep red.
3. Thiol-modified oligonucleotides are supplied in the protected form with the disulfide linkage intact to minimize the potential for oxidation, which results in oligo dimer formation. To use the free thiol (-SH) in the application the disulfide linkage must be reduced with supplier's recommended reducing agent.
4. Time and rpm of the centrifugation needs to be optimized accordingly but do not exceed 21,885 × *g*.
5. The incubation time can be lengthened if necessary.
6. The caps of the centrifuge tubes should be sealed with parafilm to prevent sample loss due to evaporation during heating. After heating to 90 °C, turn the heating block off and leave the tubes in the block until it cools down to achieve a gradual

cooling step. For sequences with significant hairpin potential, a gradual cooling/annealing step is beneficial.

7. The conjugates should be stable and no appearance of any precipitation over several weeks. The stability of AuNPs after DNA conjugation can be tested using high-salt solution [3, 8].
8. Care must be taken when using potassium cyanide since it is highly toxic. Work involving sodium or potassium cyanide should only be conducted in an operational fume hood. Adequate personal protective equipment must be worn and great care should be taken that cyanide salts do not come into contact with acid, which liberates highly toxic hydrogen cyanide gas. Cyanide waste solutions must be collected in a dedicated waste bottle and labeled as hazardous waste.
9. ERα and ERβ (isoelectric point, pI ~8.3 and 8.8, respectively) [12] are positively charged at pH 7.4. The anionic ACPE-430 was initially quenched merely by 15 % by wtERE-AuNPs (Fig. 2b), due to the limited interaction with the ERE. Binding of ERα and ERβ to wtERE (Fig. 3a, b) lowers the overall negative charge of DNA-AuNPs (more positive), leading to the increased magnitude of quenching with "light-off" principle.

Acknowledgements

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Chapter 18

Analysis of Interaction of Estradiol with Estrogen Receptor by NMR Spectroscopy

M.K. Thakur and V. Paramanik

Abstract

Following binding to estradiol, estrogen receptors (ER) α and ERβ recruit a number of interacting proteins and mediate a plethora of functions. The binding of estrogen with the receptors shows changes in the resonance structure and movement of protons. We cloned ERβ and its trans-activation domain (TAD) and ligand-binding domain (LBD), expressed them in prokaryotic expression vectors, purified them, and studied their interaction with estradiol. In this chapter, a detailed method of preparation of recombinant proteins, SDS-PAGE, silver staining, and NMR are described. Such methods are useful to check the biological activity of bacterially expressed proteins and are applicable to basic and applied research.

Key words Estrogen receptors, Cloning, SDS-polyacrylamide gel electrophoresis, Nuclear magnetic resonance spectroscopy, Estradiol

1 Introduction

Estradiol mediates its multiple functions through well-characterized ERα and ERβ receptors [1, 2]. The interaction of estradiol with ER can be studied in vitro by a number of methods including nuclear magnetic resonance (NMR) spectroscopy. Basically, NMR spectroscopy is useful to study the interaction of ligands with receptors and check the activity of newly synthesized molecules.

E. coli is widely used for the production of recombinant proteins. ERβ and its transactivating domain (TAD) and ligand-binding domain (LBD) were subcloned and expressed in *E. coli*. They were purified by Ni^{+2}-IDA-affinity chromatography. Thereafter activities of purified proteins were checked by NMR [3]. ^{1}H NMR studies of recombinant ERβ and its domains showed conformational change in the presence of estradiol (Fig. 1).

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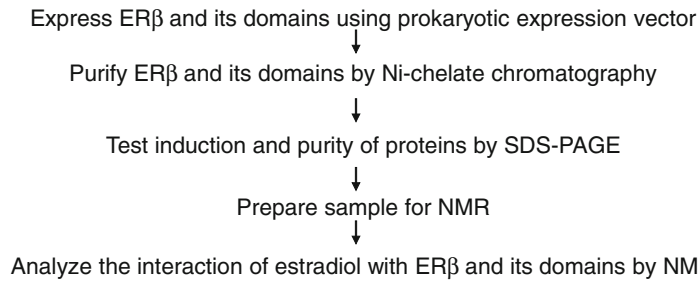

Fig. 1 Schematic procedure to study interaction of ERβ and its domains with estradiol using NMR

2 Materials

Prepare all solutions using autoclaved triple-distilled deionized water and analytical grade reagents. Store all reagents as mentioned either at room temperature, 4 °C or −20 °C.

2.1 Cloning Components

1. Plasmid containing ERβ.
2. Expression vectors pRSETA, pIVEX.
3. Restriction enzymes: *PvuII*, *EcoRI*, *XhoI*, *BamHI*.
4. DNA ligase.
5. ERβ and its domain constructs (TAD and LBD).
6. BL21 (DE3) competent *E. coli* cells.

2.2 Protein Expression Components

1. SOB medium: Add 0.5 g NaCl, 250 mM KCl, and 100 mM $MgCl_2$ in 1 L deionized water and adjust to pH 7.4. Autoclave and bring to room temperature before use.
2. *E. coli BL21* (*DE3*) containing recombinant plasmid (ERβ, TAD, and LBD).
3. Ampicillin (100 μg/mL).
4. IPTG (0.05 mM and 0.1 mM). Prepare fresh before use.
5. IDA sepharose beads. Use fresh beads.
6. Nickel sulfate (0.1 M). Prepare fresh before use.
7. Lysis buffer: 20 mM Tris–HCl pH 8.0, 0.5 M NaCl, 1 % Triton X-100, 10 % glycerol and 1 mM PMSF. Prepare fresh before use.
8. Binding buffer: 20 mM Tris–HCl, pH 8.0, 10 mM IPTG, 150 mM NaCl, 0.2 % NP-40, 2 mM β-mercaptoethanol. Prepare fresh and keep on ice during use.
9. Washing buffer: 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.2 % NP-40, 2 mM β-mercaptoethanol with varying concentrations of imidazole (50 mM, 100 mM, 150 mM,

200 mM, and 250 mM). Prepare fresh and keep on ice during use.

10. Elution buffer: Washing buffer containing 300 mM imidazole. Prepare just before use.

2.3 SDS-PAGE Components

1. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8. Weigh 181.7 g Tris and add 900 mL water. Mix and slowly adjust pH with 1 N HCl; avoid sudden drops in pH. Raise volume to 1 L with water. Store at 4 °C.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Weigh 60.6 g Tris and add 900 mL water. Mix and carefully adjust pH with 1 N HCl. Raise volume to 1 L with water. Store at 4 °C.
3. Thirty percent acrylamide/bis solution (29:1 acrylamide:bis): Weigh 29 g of acrylamide and 1 g bis-acrylamide. Dissolve in 50 mL of water in a 100 mL graduated cylinder. Mix solution for about 30 min. Raise volume to 100 mL with deionized water. Store in a dark bottle at 4 °C (*see* **Note 1**).
4. Ammonium persulfate: 10 % solution in deionized water.
5. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED). Store at 4 °C.
6. SDS-PAGE running buffer: 0.025 M Tris–HCl, pH 8.3, 0.192 M glycine, 0.1 % SDS.
7. SDS lysis buffer (5×): 0.3 M Tris–HCl, pH 6.8, 10 % SDS, 25 % β-mercaptoethanol, 0.1 % bromophenol blue, 45 % glycerol. Aliquot and store at 4 °C.
8. Loading buffer: 50 % glycerol, 0.05 % bromophenol blue, Tris–HCl, pH 6.8. Prepare aliquot and store at 4 °C.
9. Protein molecular weight markers.
10. 0.1 % sodium dodecyl sulfate.
11. Resolving gel: Mix 30 % acrylamide stock, 0.1 % SDS, and 375 mM Tris–HCl, pH 8.8 for a 10 % acrylamide gel. Add APS and TEMED to catalyze polymerization of the gel.
12. Stacking gel: Mix 30 % acrylamide stock with 0.1 % SDS and 125 mM Tris–HCl, pH 6.8.

Add APS and TEMED to catalyze polymerization of the 4.5 % acrylamide gel.

2.4 Silver Staining Components

1. Fixative solution: 50 mL methanol, 12 mL acetic acid, 1.35 mL 37 % formaldehyde, 37 mL H_2O.
2. Sensitizing solution: H_2O containing 200 μL 10 % $Na_2S_2O_3$.
3. Silver staining solution: 0.2 g $AgNO_3$, 200 μL 37% formaldehyde in 100 mL H_2O.

4. Developing solution: 6 g $Na_2S_2O_3$, 130 μL 37% formaldehyde, and 4μL $Na_2S_2O_3$ in 100 mL H_2O.
5. Stop solution: 50 mL methanol, 12 mL acetic acid, 38 mL H_2O.
6. Storage solution: 10 mL of 35 % ethanol in 195 mL H_2O.

2.5 NMR Components

1. Bovine serum albumin (BSA).
2. 200 μM purified ERβ, TAD, and LBD.
3. 10 nM 17β-estradiol.
4. 40 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 10 % glycerol, 1 μg/μL BSA.
5. JEOL AL300 FT NMR.

3 Methods

3.1 Cloning

1. Clone mouse ERβ cDNA and LBD in *PvuII/EcoRI* and *XhoI/EcoRI* sites, respectively, of the prokaryotic expression vector pRSETA (*see* **Note 2**).
2. Clone TAD in *XhoI/BamHI* sites of pIVEX (*see* **Note 2**).
3. Transform *BL21* (*DE3*) *E. coli* competent cells with recombinant constructs [4].

3.2 Overexpression of Recombinant Proteins

1. Grow overnight culture of transformed *E. coli BL21*(*DE3*) carrying the sequences for recombinant ERβ, TAD and LBD.
2. Dilute the culture (1:100) in fresh SOB medium containing ampicillin (100 μg/mL) and grow at 37 °C up to 0.6 A_{600} (*see* **Note 3**).
3. Induce ERβ with 0.05 mM IPTG; induce TAD and LBD with 0.1 mM IPTG [3] (*see* **Notes 4** and **5**).
4. Lyse the bacteria in lysis buffer and incubate at 30 °C for 30 min. Centrifuge at 10,000 × *g* for 5 min at 4 °C and collect the supernatant and pellet for use.

3.3 Purification of Recombinant Proteins

1. Prepare IDA sepharose beads with 2 volumes of 0.1 M nickel sulfate in a 2 mL microfuge tube.
2. Wash with water and equilibrate with binding buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole 0.2 % NP-40, and 2 mM ME) (*see* **Note 5**).
3. Load 500 μg of bacterial proteins in three tubes containing sepharose beads, one for each of the proteins to be purified (ERβ, TAD, and LBD) (*see* **Note 6**).
4. Incubate in ice for 1 h.

5. Centrifuge the microfuge tubes containing charged beads with protein containing ERβ, TAD, and LBD at 10,000 × *g* for 5 min at 4 °C.
6. Collect the pellets and wash with buffer (20 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.2 % NP-40, and 2 mM ME) containing varying concentrations of imidazole (50, 100, 150, 200, and 250 mM).
7. Elute ERβ, TAD, and LBD proteins with 300 mM imidazole [3].

3.4 SDS-PAGE

1. Resolve purified proteins (ERβ, TAD, and LBD) by 10 % SDS-PAGE (1 mm thick, 5 cm) [5].
2. Prepare a 10 % resolving gel and allow it to polymerize.
3. Prepare the stacking gel and allow to polymerize.
4. Mix a sample of each of the purified proteins with an equal volume of 5×SDS loading buffer and resolve along with a molecular weight marker.
5. Resolve proteins through the gel by electrophoresis.

3.5 Detection of Purified Proteins

1. For silver staining of the gel, place the gel in which purified ERβ, TAD, and LBD have been resolved in fixative solution overnight.
2. Wash the fixed gel (3 × 5 min each) in 35 % ethanol.
3. Soak the gel in sensitizing solution for 2 min.
4. Wash the gel quickly (3 × 5 min each) in water.
5. Incubate the gel in silver staining solution for 20 min.
6. Wash the gel quickly with water (2 × 1 min each).
7. Add developing solution to visualize the purified proteins in the gel.
8. Stop the reaction with stop solution for 20 min (*see* **Note 7**).
9. Take a photograph and match the molecular weights of the purified proteins with the molecular weight markers [6].

3.6 NMR Analysis

1. Calculate the concentration of the purified protein by Bradford method using BSA as a standard.
2. Incubate 200 μM of purified ERβ, TAD, and LBD with 10 nM 17β-estradiol in 40 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 10 % glycerol, and 1 μg/μL BSA at room temperature (*see* **Note 8**).
3. Record NMR spectra for purified proteins in the presence and absence of 17β-estradiol by NMR (e.g., JEOL AL300 FT).
4. Analyze NMR spectra on the basis shifting of ^{1}H and resonance structure (*see* **Note 9**).

4 Notes

1. You must wear a mask and gloves while preparing acrylamide-bis-acrylamide solution as it is toxic. Store it at 4 °C.
2. To clone and express recombinant ER and its domains, TAD, and LBD, in prokaryotic expression vectors, the sequences and the vectors must have matching restriction enzyme sites. Clone ERβ in pRSETA with XhoI/EcoRI sequence. Clone TAD in pIVEX with XhoI/BamHI sequence, and clone LBD in pRSETA with PvuII/EcoRI sequence.
3. Measure absorbance of bacterial culture using spectrophotometer at 600 nm. Use bacterial culture medium as a blank.
4. Use different concentrations of IPTG (0.025, 0.05, and 0.1 mM) in three different flasks containing bacterial culture to determine the optimal concentration of IPTG for protein induction. 0.05 mM IPTG was found to be the optimal concentration for the induction of ERβ. In the case of TAD and LBD, 0.1 mM IPTG was found to be the optimal concentration
5. Prepare binding buffer, washing buffers and elution buffer in separate tubes and keep on ice. Weigh imidazole as needed to prepare varying concentration of washing buffer.
6. Measure absorbance at 595 nm immediately after adding Bradford reagent to the samples. Use Bradford reagent as blank.
7. Prepare the stop solution before developing the bands in silver staining. When the bands have reached the desired intensity, immediately stop the reaction.
8. There is no need to purify protein from the gel. Recombinant proteins were purified using affinity chromatography. After elution, check the purity of a small sample of the purified proteins by SDS-PAGE. The remainder of the purified protein sample will be used for NMR spectroscopy.
9. Use purified protein for NMR. Estimate the purified protein concentration by Bradford method using BSA as a standard. Prepare estradiol solution fresh. Analyze NMR on the basis of changes in resonance structure and shifting in H^1 in the absence and presence of estradiol.

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Chapter 19

Application of Circular Dichroism Spectroscopy to the Analysis of the Interaction Between the Estrogen Receptor Alpha and Coactivators: The Case of Calmodulin

Emeric Miclet, Sandrine Bourgoin-Voillard, Cillian Byrne, and Yves Jacquot

Abstract

The estrogen receptor α ligand-binding domain (ERα-LBD) binds the natural hormone 17β-estradiol (E_2) to induce transcription and cell proliferation. This process occurs with the contribution of protein and peptide partners (also called coactivators) that can modulate the structure of ERα, and therefore its specificity of action. As with most transcription factors, ERα exhibits a high content of α helix, making it difficult to routinely run spectroscopic studies capable of deciphering the secondary structure of the different partners under binding conditions. Ca^{2+}-calmodulin, a protein also highly structured in α-helix, is a key coactivator for ERα activity. Here, we show how circular dichroism can be used to study the interaction of ERα with Ca^{2+}-calmodulin. Our approach allows the determination not only of the conformational changes induced upon complex formation but also the dissociation constant (K_d) of this interaction.

Key words Estrogenreceptor, Calmodulin, Circular dichroism, Peptide, Helix content, K_d

1 Introduction

The human estrogen receptor alpha (ERα) is a transcription factor that is composed of two principal regions: a DNA-binding domain (DBD) and a ligand-binding domain (LBD). In physiological conditions, ERα phosphorylation and dimerization, which occur principally in the nucleus, are followed by the binding of the resulting complex to short DNA regions called estrogen response elements (EREs), thereby inducing transcription (Fig. 1a) [1–4]. As shown in Fig. 1b, the LBD contains at least four binding regions: (1) a BF1 domain in charge of the recruitment of ligands such as 17β-estradiol (E_2) or tamoxifen [5–8], (2) a BF2 domain that is responsible for the recruitment of LxxLL-containing coactivators (where L corresponds to leucine and x corresponds to any amino acid) [5, 9–11], (3) a BF3 domain, whose function remains

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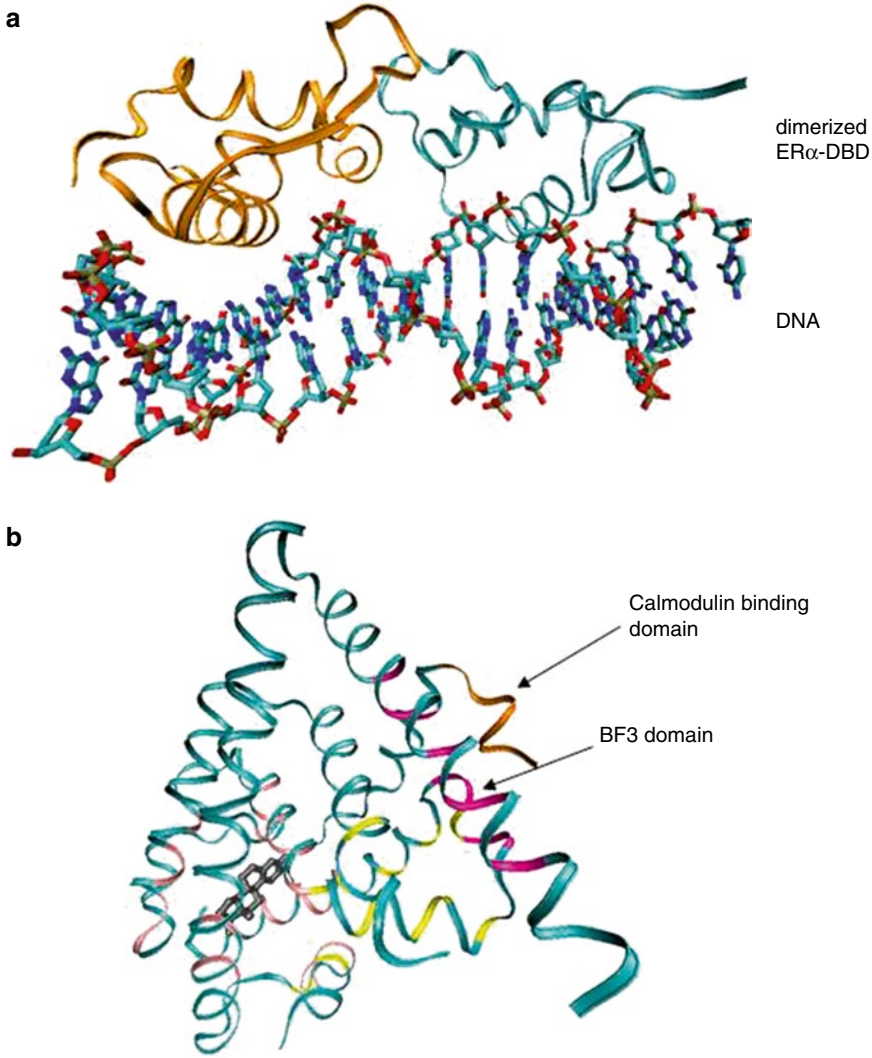

Fig. 1 (**a**) Interaction of the ERα-DBD with DNA. ERα-DBD homodimers (one monomer in *orange* and one monomer in *cyan*) are drawn in ribbon form (PDB code: 1HCQ [1]). (**b**) The ERα-LBD (drawn in ribbon form, PDB code: 1ERE [58]). Estradiol (E_2) is drawn in *grey* and the key residues delimiting the BF1 domain are drawn in *pink* [59–61]. The residues delimiting the LxxLL-binding domain (BF2 domain) are in *yellow* [9, 11, 62] and the calmodulin-binding domain [63], which is on the BF3 domain (in *pink* [64]) is in *orange*. The pictures have been created using the software VMD®

somewhat enigmatic but shows a propensity to interact with non-inflammatory drugs and the immunophilin FKBP52 [12–14], and, lastly, (4) a Ca^{2+}-calmodulin-binding site located at the N-terminus part of the LBD [15–18]. Thus, the ERα-LBD can be considered as a multiplatform protein. Even if principally located in the nucleus, the ERα is subjected to turnover and shuttling processes;

thus, the whole protein (or truncated forms) can be found in the plasma membrane, where it exerts indirect (rapid or non-genomic) mechanisms [19–25].

Protein-protein and protein-peptide interactions are complex processes that are classically studied by biochemical, biophysical, and analytical techniques such as ultracentrifugation, affinity chromatography, microdialysis-HPLC, mass spectrometry, electromigration methods, microcalorimetry, surface plasmon resonance, and protein cross-linking [26, 27]. Equally, spectroscopic methods (X-ray diffraction, nuclear magnetic resonance, circular dichroism) may also be used. Whereas biochemical, biophysical and analytical approaches are poorly informative in terms of folding, X-ray and NMR methods allow the elucidation of structural information at the atomic scale. However, these two techniques are costly in both time and material, complicating their routine use. Thus, circular dichroism (CD) spectroscopy is attractive as an alternative, although it provides global conformational information.

Briefly, CD spectroscopy is a light absorption method that consists of measuring the difference in absorbance of left- and right-circularly polarized light as a function of the wavelength. In the presence of chiral molecules containing a chromophore, a signal resulting from the absorption difference (ΔA) in the left and in the right directions can be recorded [28]. In the context of peptides and proteins, the most common chromophore is the peptide bond NH-C = O, which absorbs between 180 nm and 260 nm (far-UV region). The anisotropic character induced by the secondary structure of polypeptides and proteins is responsible for absorption differences, leading to spectra reflecting conformational states (typically helices, β-sheets, "random" forms, and so forth), as shown in Fig. 2 [28–34].

Thus, CD could be a useful technique for following the interaction between ERα and its protein and peptide partners when the binding process is accompanied by specific conformational changes of one of the two partners. Here, we illustrate the interaction between the ERα-LBD and its coactivatory partner Ca^{2+}-calmodulin (holocalmodulin, Fig. 3). As previously stated, the ERα-LBD, which is flexible, is largely structured in α-helix. The small protein Ca^{2+}-calmodulin, which contains ~142 amino acids, also displays a strong CD helix signature by itself and is subjected to a local (region Arg-74 to Glu-82) and limited destabilization upon ligand binding [35–40]. Accordingly, it would be difficult to carry out a quantitative study of the interaction of Ca^{2+}-calmodulin with the whole ERα or with the ERα-LBD by CD.

By limiting the ERα-LBD to its calmodulin-binding site (295–311 region of the ERα, peptide ERα17p, sequence: H_2N-PLMIKRSKKNSLALSLT-OH, determined by using the specific antibodies G-20 and AER-308 [17]), it is possible to study the

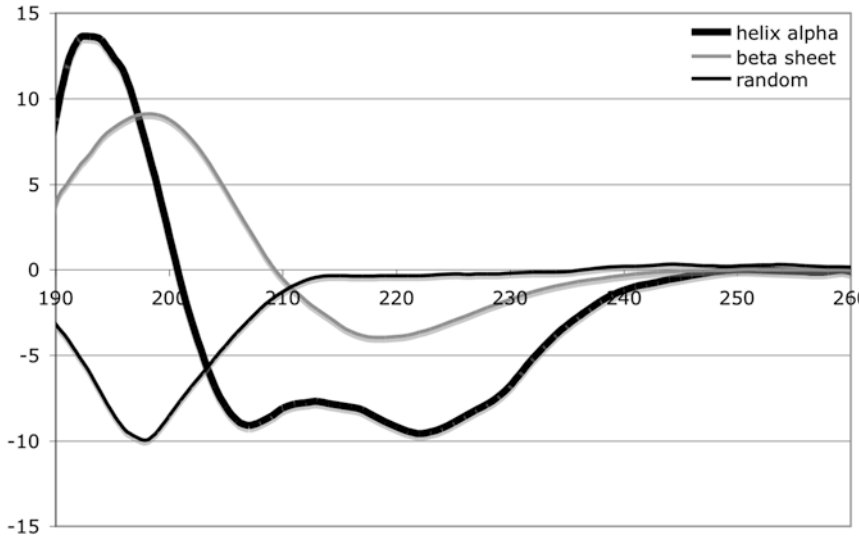

Fig. 2 Typical random, sheet, and helix CD signatures

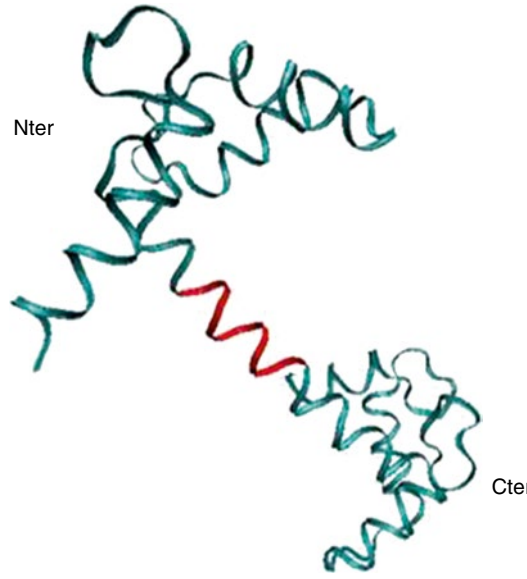

Fig. 3 Structure of free calmodulin (PDB code: 1CLL [65]). In red, the Arg-274 to Glu-82 region that is subject to modification under ligand binding. The picture has been created using the software VMD®

conformational changes accompanying its interaction with Ca^{2+}-calmodulin. It should be noted that the peptide ERα17p binds in C-terminus of Ca^{2+}-calmodulin, presumably with a stoechiometry 1:1 [52]. In addition, it shares a basic block (KRSKK motif, third nuclear localization signal of ERα) and a primary amphipathic character, two important characteristics that are required for Ca^{2+}-calmodulin association [41–50].

As observed for most calmodulin peptide ligands, the peptide ERα17p, which is random when alone in solution [51], adopts an α-helical structure when bound to Ca^{2+}-calmodulin [52]. Whether these interaction patterns would also be observed with the whole ERα is a question of interest that has not yet been resolved. During the titration step, we observed an increase of the helix content of the peptide ERα17p leading to a plateau (Fig. 4a, b). This feature reveals that this process is saturable and, therefore, specific. The percentage of α-helix for bound ERα17p ranges from 72 to 87 %, confirming the strong α-helix propensity of ERα17p when associated with Ca^{2+}-calmodulin. We were also able to estimate a K_d value of 30.7 ± 2.4 μM. This chapter describes the protocols for the application of circular dichroism to the qualitative and quantitative study of the interaction of ERα with Ca^{2+}-calmodulin.

2 Materials

2.1 Peptide Synthesis and Purification

1. Phenylacetamidomethyl (PAM) resin preloaded with a Boc-Thr(Bzl) amino acid (substitution: 0.25 mmol/g) for solid-phase peptide synthesis (SPPS) (Novabiochem Merck Biosciences). Store at 4 °C.
2. Boc-Ala-OH, Boc-Leu-OH, Boc-Ile-OH, Boc-Pro-OH, Boc-Met-OH, Boc-Lyz(2-ClZ)-OH, Boc-Arg(Tos)-OH, Boc-Ser(Bzl)-OH, and Boc-Asn(Trt)-OH amino acids (Novabiochem Merck Biosciences). Store at 4 °C.
3. Trifluoroacetic acid (TFA), diisopropylethylamine (DIPEA), dimethylsulfoxide (DMSO), methanol, acetic anhydride, fluorhydric acid (HF), calcium gluconate/chlorhexidine digluconate gel, acetonitrile, diethylether, and anisole (Sigma-Aldrich).
4. Dicyclohexylcarbodiimide (DCC) 1 M in NMP, 1-hydroxybenzotriazole (HOBt) 1 M in NMP, methanol, and 1-methyl-2-pyrrolidone (NMP) (Applied Biosystems).
5. Dichloromethane (DCM) (Carlo Erba).
6. Automatic Peptide Synthesizer (433A apparatus, Applied Biosystems, Foster City, CA), using tert-butyloxycarbonyl (Boc) strategy.
7. Dessicator with P_2O_5 (Sigma-Aldrich).

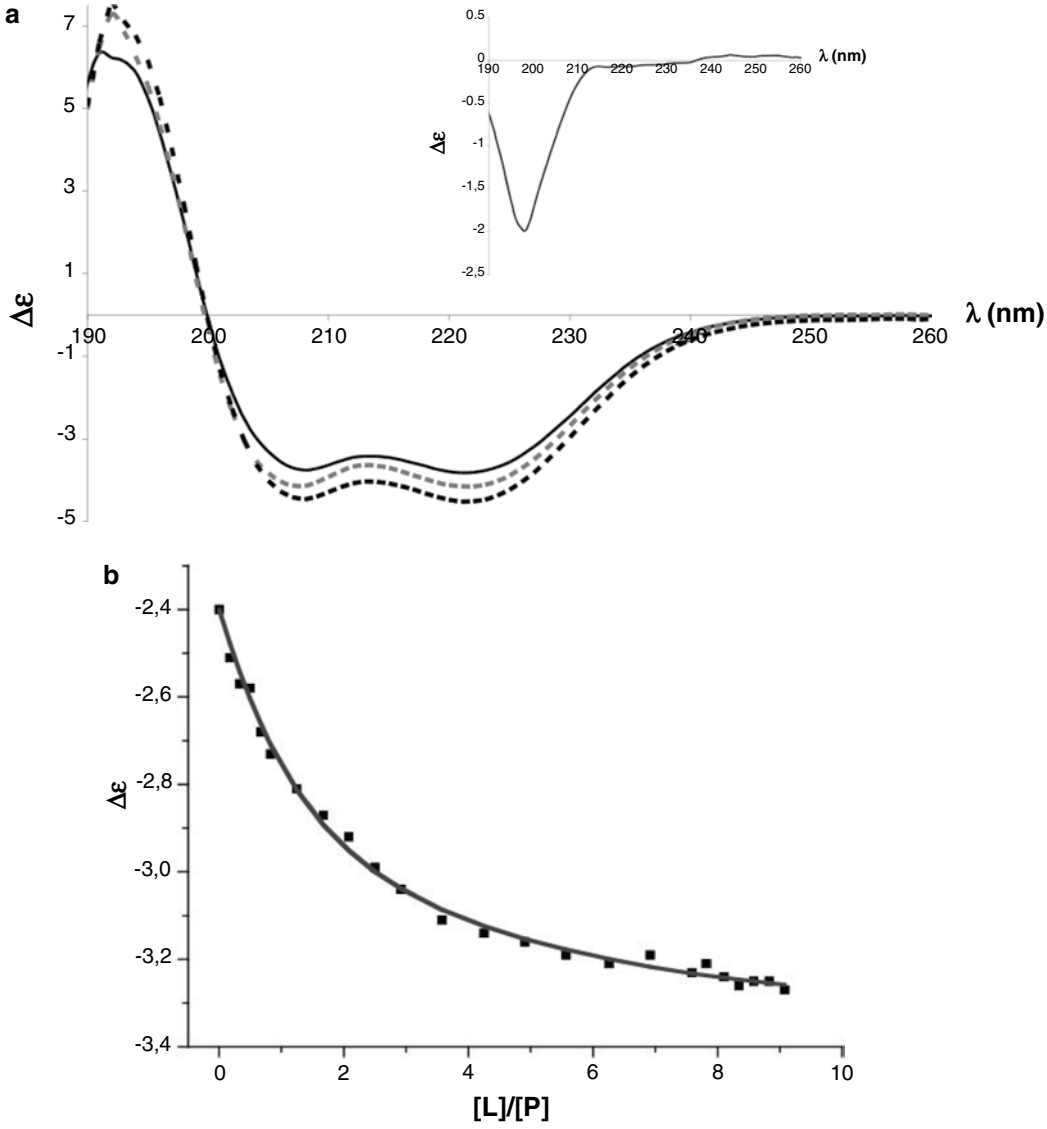

Fig. 4 (**a**) Far-UV CD spectra of 30 μM Ca^{2+}-calmodulin alone (*black solid line*) or in the presence of 1 equivalent (*grey dashed line*) and 2 equivalents (*black dashed line*) of ERα17p in 10 mM NH_4OAc buffer solution (*inset*: spectrum of ERα17p alone in buffer solution). $\Delta\varepsilon$ values are expressed in $M^{-1}.cm^{-1}$ per residue. (**b**) Titration of Ca^{2+}-calmodulin and ERα17p interaction by far-UV CD. CD titration was performed by the addition of aliquots of 300 or 500 μM ERα17p solution (2, 5, or 8 μL) in an initial solution containing 30 μM Ca^{2+}-calmodulin. Molar dichroism data (normalized for calmodulin) at 222 nm are reported for different ERα17p: Ca^{2+}-calmodulin ratio ($\Delta\varepsilon$ is in $M^{-1}.cm^{-1}$ per residue). The fit has been performed using the equation described in the text (See Note 22) and giving accurate values for the dissociation constant and the mean residue molar dichroism of the bound peptide

8. Fluorhydric acid (HF) apparatus (Peptide Institute, Inc.).
9. Dimethylsulfide (Acros Organics).
10. Filter flask, fritted glass, glass rod, round-bottomed flask, vacuum pump.
11. Centrifuge tubes, conical bottom, 15 mL, sterile, polypropylene.
12. Distilled water apparatus.
13. Liquid nitrogen.
14. Freeze-dryer.
15. Centrifuge (microfuge, Sigma 3-18K, Sigma Centrifuge).
16. 10 mL syringe equipped with a Minisart® hydrophilic syringe 0.2 μm filter (Sartorius).
17. Semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a Dionex HPLC system (Dionex P580 Pump, Dionex AD25 Absorbance Detector, Dionex 4400 Integrator) equipped with an ACE C_8 column (10 mm × 250 mm, 5 μm particle size, 300 Å pore size) and the Chromeleon® software (Thermo Fisher Scientific) or equivalent.
18. Analytical reverse-phase high-performance liquid chromatography on a Waters HPLC system (Waters 600 Pump and Controller, Waters 2487 Dual λ Detector, Waters 746 Data Module) using an ACE C_{18} RP-HPLC column (4.6 mm × 250 mm, 5 μm particle size, 300 Å pore size) and Breeze® software (Waters SAS).
19. Rotavapor R-210 equipped with a vacuum pump V-700 and a pump controller V-850 (Büchi).

2.2 Peptide Identification

1. 100 μL Hamilton syringe with a needle.
2. α-Cyano-4-hydroxycinnamic acid (HCCA).
3. V700666 100-well Voyager DE, DE PRO, DE STR sample plate (Applied Biosystems).
4. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (4700 Proteomic Analyzer, Applied Biosystems, or equivalent) for peptide identification.

2.3 Ca^{2+}-Calmodulin Stock Solution Components

1. Calmodulin from bovine testes: 99 % identity with human calmodulin, 146 amino acids, MW = 16,792 Da, acetylated at its N-terminus and with a trimethyllysine in position 115 [53]. Ref. P1431, lyophilized powder, purity ≥ 98 %, ≥40,000 units/mg of proteins (Sigma-Aldrich).
2. Ammonium acetate (NH_4OAc) and calcium chloride ($CaCl_2$) (Fluka).
3. RiOs-DI Water Purification Systems® (Millipore®).
4. NH_4OAc buffer: 10 mL of 10 mM NH_4OAc, prepared with 10 MΩ deionized water. Store at 25 °C.

5. Centrifugal ultrafiltration cartridges such as the Amicon Microcon® 10 kDa cartridges (Millipore).
6. Heidolph Top Mix 94323 (Fisher Bioblock Scientific), or equivalent, for mixing.
7. Double beam Uvikon Spectrophotometer 931 (Kontron Instruments), or equivalent, for calmodulin titration with 1 cm path length quartz cuvettes.
8. Nitrogen gas.
9. Ion trap electrospray ionization mass spectrometer (ESI, Esquire 3000, Bruker) equipped with a 3D quadripolar ion trap and with an orthogonal electrospray ionization source, operating in negative mode for the detection of non-covalent complexes and to verify the presence of four calcium ions/calmodulin molecule [54].
10. Laboratory pipettes (20–100 μL and 50–200 μL) with appropriate tips.
11. Microcentrifuge tubes (1.5 mL).

2.4 Far-UV Circular Dichroism Spectroscopy

1. Jobin Yvon CD6 spectropolarimeter (Jobin Yvon).
2. Peltier with temperature controller Haake F3 Fisons (Haake Manufacturing Company).
3. Quartz cuvette (1 mm cuvette quartz suprasil).
4. Far-UV CD data processed with the Dichrograph® software, or equivalent.
5. Origin® software for spectra analysis using the Chi-square minimization procedure.

3 Methods

3.1 Peptide Synthesis (*See* Note 1)

1. Place the bottles of reactants (DCC and HOBt 10 % in NMP, DIPEA, acetic anhydride) and solvents (NMP, methanol, DCM, DMSO) for Boc strategy on the synthesizer according to the manufacturer's instructions.
2. Carry out the requisite calibration/flow tests and program the automatic peptide synthesizer (Boc strategy) following the manufacturer's instructions.
3. Weigh 0.1 mmol of PAM resin preloaded with Boc-Thr(Bzl) amino acid (substitution: 0.25 mmol/g) appropriate for the synthesis of the peptide of interest (H_2N-PLMIKRSKKN SLALSLT-OH, ERα17p, residues 295–311 of the hinge region of the human estrogen receptor α) and place it in the reaction vessel equipped with a filter.
4. Weigh out 1 mmol of each of the Boc-protected amino acids in cartridges.

5. Seal the cartridges with pierceable caps.
6. Place the cartridges on the machine rack as specified by the manufacturer.
7. Fix the reaction vessel as specified by the manufacturer.
8. Run the synthesis as specified by the manufacturer.
9. At the end of the synthesis, make sure that the resin has been rinsed with DCM and methanol and that it has shrunk.
10. Place the resin in the dessicator (P_2O_5 is used as drying agent) for at least 4 h.

3.2 Peptide Cleavage

1. Weigh the resin and place it in the reaction vessel of the HF (fluorhydric acid) apparatus.
2. Add the scavengers, dimethylsulfide (0.25 mL/g), and anisole (1.25 mL/g) (*see* **Note 2**).
3. Screw the reaction vessel into the HF reaction apparatus following the instructions of the manufacturer and switch on the pump to create a vacuum.
4. Freeze the reaction vessel in liquid nitrogen (over 15 min at a minimum).
5. Transfer HF following the protocol of the manufacturer (*see* **Note 3**).
6. Replace the liquid nitrogen with a solution of ice water. Reaction time: 2 h at 0 °C with stirring (*see* **Note 4**).
7. Remove the HF under vacuum. Make sure that all HF is removed.
8. Unscrew the reaction vessel and manually add approximately 5 mL of ice-cold diethylether to the reaction vessel.
9. Triturate the mixture with a glass rod and filter through a fritted glass filter (*see* **Note 5**).
10. Remove the diethylether solution.
11. Add degassed distilled water:acetic acid (9:1) (*see* **Note 6**) through the fritted glass filter containing the peptide and the resin and mix thoroughly with a glass rod (*see* **Note 7**).
12. Collect the aqueous solution containing the peptide into a clean round-bottomed flask.
13. Lyophilize the filtered solution at −80 °C and with an internal pressure < 5 mBar.

3.3 Peptide Purification

1. Dissolve the lyophilized peptide in distilled water (100 mg of crude product in 25 mL of distilled water at 45 °C and a small amount of acetonitrile (~2 mL) to accelerate solubilization).
2. Centrifuge the solution (1500 × *g*) for 5 min.

3. Filter the supernatant with a 10 mL syringe equipped with a hydrophilic syringe filter of 0.2 µm.
4. HPLC conditions for purification: Eluent A: distilled water with 0.1 % of trifluoroacetic acid (TFA); eluent B: acetonitrile with 0.1 % TFA. UV detection: 220 nm, flow rate: 5 mL/min. Gradient conditions: from 20 % to 40 % of solvent B over 20 min (*see* **Notes 8** and **9**).
5. Conditions for analytical HPLC: Eluent A: distilled water with 0.1 % of TFA; eluent B: acetonitrile with 0.1 % TFA. UV detection: 220 nm, flow rate: 1 mL/min. Gradient conditions: from 5 % to 60 % of solvent B over 20 min (*see* **Note 10** and Fig. 5a).
6. Collect all the HPLC fractions obtained at Rt = 11.02 min.
7. Evaporate the acetonitrile at 40 °C and under vacuum (*see* **Notes 11** and **12**).
8. Freeze the peptide solution (composed of water, only, after the removal of acetonitrile) in liquid nitrogen.
9. Lyophilize at −80 °C and with an internal pressure < 5 mBar.

3.4 Peptide Identification

1. Mix 1 µL of the peptide fraction issued from the RP-HPLC with 1 µL of a saturated solution of HCCA in acetonitrile/water (50:50) with 0.1 % TFA and place 1 µL of this solution on the MALDI-TOF plate.
2. Dry.
3. Introduce the plate in the MALDI-TOF apparatus and record the spectra in positive mode according to the manufacturer's instructions (Fig. 5b, *see* **Note 13**).

3.5 Ca^{2+}-Calmodulin Stock Solution Preparation [54]

1. Dilute the lyophilized powder of commercial calmodulin in a volume of 10 mM NH_4OAc buffer solution (pH 6.5–7.5) made from 10 MΩ (at 25 °C) deionized water to reach a final concentration of 1 mg/mL (final stock solution of ~60 µM).
2. Put in a 1.5 mL centrifuge tube.
3. Vortex for a few seconds.
4. The peptide ERα17p is desalted and buffer exchanged against 10 mM NH_4OAc using a centrifugal ultrafiltration cartridge to afford a final concentration of 500 µM. Store at −30 °C (*see* **Note 14**).
5. Add 1 µL of 20 mM $CaCl_2$ (made with 10 MΩ deionized water at 25 °C) into the desalted solution of calmodulin (*see* **Note 15**) to obtain a final concentration of 50 µM $CaCl_2$.
6. Perform a titration by UV spectroscopy (λ = 180–400 nm) to precisely define the final concentration of Ca^{2+}-calmodulin. The final concentration of Ca^{2+}-calmodulin must be ~25 µM.

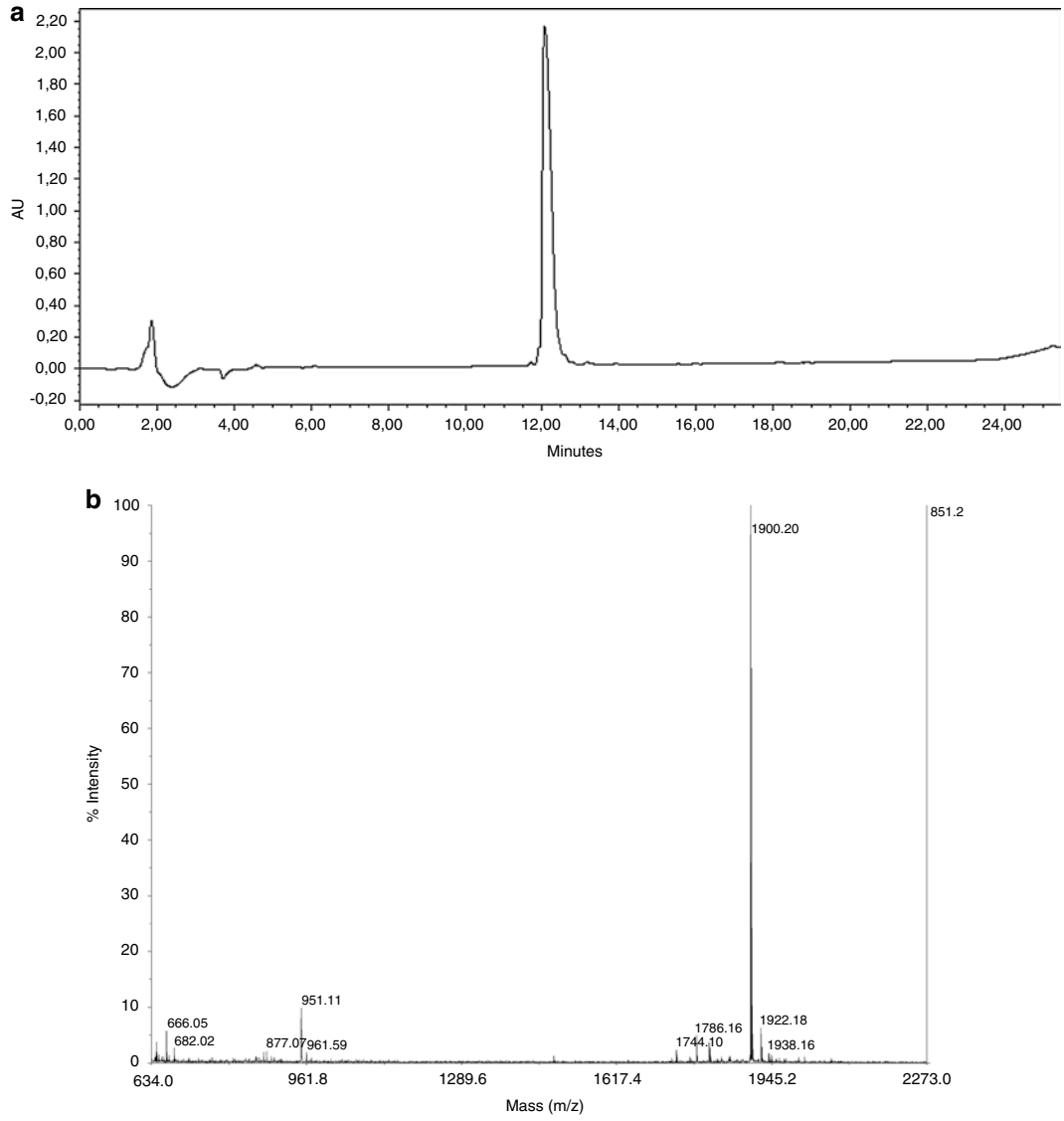

Fig. 5 (**a**) Analytical reverse HPLC chromatogram and (**b**) MALDI-TOF spectrum of the peptide ERα17p

3.6 Verification by Electrospray Ionization of the Presence of Ca^{2+}-Calmodulin [54]

1. Infuse samples into the ESI source at a flow rate of 200 μL/h using a syringe pump.
2. Accumulate the spectra for 2–5 min to achieve a good S/N ratio.
3. Use nitrogen as a nebulizing (6 psi) and drying gas (200 °C, 5 L/min).

4. Record the ESI mass spectra in negative mode by using a standard resonant analytical scan (13,000 Th/s, from m/z 50 to m/z 3000) and with resonant ejection at $\beta_z = 2/3$ ($q_z = 0.78$).
5. Skimmer and capillary exit voltage values of −30 V and −110 V, respectively, are appropriate for preserving in-solution preformed non-covalent complexes (*see* **Note 16**).
6. Automated ion charge control (ICC) must be set to 20,000 (*see* **Note 17**).

3.7 Circular Dichroism Experiments

1. Place 200 μL of a 25 μM buffer solution of Ca^{2+}-calmodulin in the quartz cell, at room temperature.
2. Record far-UV circular dichroism spectra (190–260 nm) at room temperature using a 250 μL (0.1 cm path length) quartz cell.
3. Make a titration by adding in the cuvette 2, 5, or 8 μL of the peptide stock solution at the concentrations of 300 or 500 μM and record the CD spectrum after each addition and a short time of mixing to homogenize the sample.
4. Average each spectrum over four scans.
5. Smooth each spectrum after buffer and background subtraction.
6. Transform molar circular dichroism data ($\Delta\varepsilon$, $M^{-1}.cm^{-1}$) to mean residue molar ellipticity [θ] (deg.cm^2/dmol) (*see* **Note 18**).
7. Process Far-UV CD data with the Dichrograph® software (*see* **Note 19**).
8. Analyze spectra using the Chi-square minimization procedure implemented in the Origin® software (Levenberg-Marquardt algorithm).

3.8 K_d Calculation

1. To determine the dissociation constant (K_d), perform circular dichroism titration by the addition of small amounts of a 300 or 500 μM ERα17p stock solution (2, 5, or 8 μL) (*see* **Note 20**).
2. Titration must be continued until a plateau is reached (*see* **Note 21**).
3. Determine the $\Delta\varepsilon_{PF}$ (PF: protein free) and $\Delta\varepsilon_{LF}$ (LF: ligand free) values at 222 nm from the circular dichroism spectra of isolated Ca^{2+}-calmodulin and ERα17p, respectively.
4. The K_d and $\Delta\varepsilon_{LB}$ (LB: ligand bound) values are determined by fitting the function $\Delta\varepsilon = f([L],[P])$ (for the quantitative analysis, *see* **Note 22**).
5. Determine the percentage of helix of ERα17p using the fitted $\Delta\varepsilon_{LB}$ value (*see* **Note 23**).

4 Notes

1. This protocol describes peptide synthesis using Boc strategy on an Applied Biosystems 433A automated peptide synthesiser; however, any automated synthesiser could be used. Equally, the synthesis of this peptide has also been successful using the Fmoc strategy [55].
2. The scavengers are used to trap carbocations issued from protecting groups during the cleavage step.
3. *Warning*—Use acid-proof gauntlet gloves. A calcium gluconate/chlorhexidine digluconate gel must be kept nearby in case of skin contact with HF.
4. One hour is usually sufficient for the cleavage of the peptide and for the deprotection of the amino acids. However, when arginines are protected with a tosyl protecting group, two hours are required.
5. This stage is used to remove the scavengers from the reaction mixture and to precipitate the peptide (after this stage, the fritted glass will contain the peptide and the resin).
6. The use of degased distilled water will prevent the oxidation of the methionine in the position 3 of the peptide ERα17p.
7. This stage is used to dissolve the peptide (the resin will stay on the fritted glass).
8. The yield of the purification is limited (~40 %) since the peptide ERα17p forms fibers and hydrogels, which are removed in the filtration step [56].
9. Retention time: Rt = 11.02 min.
10. Retention time: Rt = 12.32 min (Fig. 5a).
11. Maintain the bath temperature of 40 °C to avoid peptide degradation.
12. The removal of acetonitrile is highly recommended since it may damage the freeze-dryer.
13. m/z $(M + H^+) = 1900.20$; m/z $(M + Na^+) = 1922.18$; m/z $(M + K^+) = 1938.16$.
14. For this step, insert the ultrafiltration concentrator device containing a 10 kDa membrane filter into a 1.5 mL tube. Wash the membrane filter by introducing 400 µL of 10 mM NH_4OAc buffer solution (pH 6.5–7.5) into the tube and centrifuge at 14,000 × *g* for 20 min. Repeat this washing step once. Introduce 400 µL of calmodulin at 1 mg/mL on the concentrator filter, and then centrifuge at 11,000 × *g* for 20 min. The calmodulin (MW = 16,792 Da) is retained on the membrane filter while salts are eliminated. Introduce 500 µL of 10 mM NH_4OAc

buffer solution (pH 6.5–7.5) and centrifuge at 14,000 × *g* for 20 min. Repeat three times. Separate the tube from the membrane device. Place a clean tube over the top of the membrane device. Invert the membrane device and centrifuge for 3 min at 1000 × *g* to transfer desalted calmodulin into the clean tube. Add approximately 370 μL of 10 mM NH_4OAc buffer solution (pH 6.5–7.5) to the tube to achieve a final volume of 400 μL. After this step, the loss of calmodulin is not negligible. The new concentration of calmodulin will be measured in **step 6** after adding $CaCl_2$.

15. Commercial calmodulin is already in its "holo" (i.e., calmodulin complexed with four calcium ions) form but $CaCl_2$ in excess is added to the solution to compensate for Ca^{2+} losses occurring during the desalting step.
16. In such soft conditions, in-source dissociation processes are avoided.
17. This value avoids space charge effects that can lead to m/z shifts and resolution loss.
18. Molar circular dichroism data ($\Delta\varepsilon$, $M^{-1}.cm^{-1}$) is converted to mean residue molar ellipticity $[\theta]$ (deg.cm^2/dmol) by using the relation $[\theta] \approx 3298\Delta\varepsilon$. During calculations, consider the dilution effects induced by the addition of the peptide (5 μL in time).
19. α-Helix far-UV circular dichroism spectrum consists of one intense positive maximum at 192 nm (perpendicular $\pi \rightarrow \pi^*$ transition) and two negative minima at 208 nm (parallel $\pi \rightarrow \pi^*$ transition) and 222 nm ($n \rightarrow \pi^*$ transition).
20. The increase in the concentration of ERα17p is associated with an increase of the intensity of the signal at $\lambda = 222$ nm (Fig. 4). The increase of the signal at $\lambda = 222$ nm is proportional to the increase in the α-helix content (as illustrated in Fig. 4a).
21. The fact that we reached a plateau shows that this process is saturable and therefore characteristic of a specific interaction between ERα17p and Ca^{2+}-calmodulin.
22. Collect a large number of circular dichroism spectra in the presence of increasing amounts of ERα17p (we have recorded 24 spectra, as shown in the Fig. 4b). Importantly, do not subtract free calmodulin from the spectra. Since 10 % of the sequence is flexible, it would introduce a bias in calculations. The following equations take account of the complex (bound calmodulin and bound peptide), free calmodulin and free peptide. Raising the [L]/[P] ratio (where [L] = [ERα17p] and [P] = [Ca^{2+}-calmodulin]) allows precision fitting of the K_d and molar circular dichroism $\Delta\varepsilon_{LB}$ (LB: ligand bound) parameters

of the bound peptide. At 222 nm, both the peptide and Ca^{2+}-calmodulin contribute to the CD signal, either in their free or bound states. Thus, the total differential absorption of left- and right-handed circularly polarized light ΔA displayed by the sample at a defined wavelength can be presented as follows:

$$\Delta A = \Delta A_{PF} + \Delta A_{LF} + \Delta A_{PB} + \Delta A_{LB} \quad (1)$$

where ΔA_{PF}, ΔA_{LF}, ΔA_{PB} and ΔA_{LB} are the differential absorptions of the free Ca^{2+}-calmodulin, the free ligand, the bound Ca^{2+}-calmodulin and the bound ligand, respectively. Considering $\Delta\varepsilon$, [X], and N_X (that correspond to the molar circular dichroism, the concentration and the number of residues for the Ca^{2+}-calmodulin (X = P) or the peptide ligand (X = L), respectively), the Eq. 1 can be transformed as

$$\begin{aligned}\Delta A = {} & (\Delta\varepsilon_{PF} \times [P]_{PF} \times N_P) + (\Delta\varepsilon_{LF} \times [L]_{LF} \times N_L) \\ & + (\Delta\varepsilon_{PB} \times [P]_{PB} \times N_P) + (\Delta\varepsilon_{LB} \times [L]_{LB} \times N_L)\end{aligned} \quad (2)$$

Referring to Ca^{2+}-calmodulin total concentration [P] and the number of residues that comprise Ca^{2+}-calmodulin (N_P), CD signal depicted by Eq. 2 can be expressed in circular dichroism unit:

$$\begin{aligned}\Delta\varepsilon = {} & (\Delta\varepsilon_{PF} \times [P]_{PF} / [P]) + (\Delta\varepsilon_{LF} \times N_L / N_P \times [L]_{LF} / [P]) \\ & + (\Delta\varepsilon_{PB} \times [P]_{PB} / [P]) + (\Delta\varepsilon_{LB} \times N_L / N_P \times [L]_{LB} / [P])\end{aligned} \quad (3)$$

In addition:

$$[P]_{PF} = [P] - [P]_{PB}, [L]_{LF} = [L] - [L]_{LB}, [L]_{LB} = [P]_{PB} = [PL]$$

Hence,

$$\begin{aligned}\Delta\varepsilon = {} & (\Delta\varepsilon_{PF} \times ([P] - [PL]) / [P]) + (\Delta\varepsilon_{LF} \times N_L / N_P \times ([L] - [PL]) / [P]) \\ & + (\Delta\varepsilon_{PB} \times [PL] / [P]) + (\Delta\varepsilon_{LB} \times N_L / N_P \times [PL] / [P]),\end{aligned}$$

or

$$\begin{aligned}\Delta\varepsilon = {} & \Delta\varepsilon_{PF} + (\Delta\varepsilon_{LF} \times N_L / N_P \times [L] / [P]) \\ & + (\Delta\varepsilon_{PB} - \Delta\varepsilon_{PF} + (\Delta\varepsilon_{LB} - \Delta\varepsilon_{LF}) \times N_L / N_P)[PL] / [P]\end{aligned}$$

Introducing

$$K_d = [L]_{LF} \times [P]_{PF} / [PL],$$

$$K_d = ([L] - [PL]) \times ([P] - [PL]) / [PL] \text{ or } [PL]^2 - ([P] + [L] + K_d) \times [PL] + [P] \times [L] = 0$$

Thus,

$$[PL] = \frac{([P]+[L]+K_d) - \sqrt{([P]+[L]+K_d)^2 - 4[P][L]}}{2}$$

giving the following equation which is used to fit the values of K_d and $\Delta\varepsilon_{LB}$:

$$\Delta\varepsilon = \Delta\varepsilon_{PF} + (\Delta\varepsilon_{LF}\frac{[N_L]}{[N_P]}\frac{[L]}{[P]}) + [\Delta\varepsilon_{PB} - \Delta\varepsilon_{PF} + (\Delta\varepsilon_{LB} - \Delta\varepsilon_{LF})\frac{[N_L]}{[N_P]}]$$
$$\frac{([P]+[L]+Kd) - \sqrt{([P]+[L]+Kd)^2 - 4[P][L]}}{2[P]}$$

where $\Delta\varepsilon_{PF}$ (PF: protein free) and $\Delta\varepsilon_{LF}$ (LF: ligand free) are accurately determined from the circular dichroism spectra of isolated Ca^{2+}-calmodulin and ERα17p, respectively. At 222 nm, $\Delta\varepsilon_{PF} = -2.40$ $M^{-1}.cm^{-1}$ per residue and $\Delta\varepsilon_{LF} = -0.07$ $M^{-1}.cm^{-1}$ per residue. $\Delta\varepsilon_{PB}$ (PB: protein bound) was also estimated as only 9 out of 96 residues (~10 %) lose the α-helix conformation upon ligand binding [37–40]. Thus, $\Delta\varepsilon_{PF} < \Delta\varepsilon_{PB} < 0.9\Delta\varepsilon_{PF}$. Two different fits have then been performed using this equation: Fit 1, where $\Delta\varepsilon_{PB} = \Delta\varepsilon_{PF} = -2.40$ $M^{-1}.cm^{-1}$ per residue and Fit 2, where $\Delta\varepsilon_{PB} = 0.9\Delta\varepsilon_{PF} = -2.17$ $M^{-1}.cm^{-1}$ per residue. Accordingly, two mean residue molar dichroism values associated to the ligand-bound species ($\Delta\varepsilon_{LB}$) were obtained, i.e., $\Delta\varepsilon_{LB} = -8.21 \pm 0.17$ $M^{-1}.cm^{-1}$ per residue (Fit 1) and $\Delta\varepsilon_{LB} = -9.94 \pm 0.17$ $M^{-1}.cm^{-1}$ per residue (Fit 2). In contrast, K_d values were not affected by far-UV CD signal differences observed between the free and bound states of Ca^{2+}-calmodulin. Indeed, the same K_d values (30.7 ± 2.4 μM, convergence $\chi^2 = 0.00036$) were found for both fits.

23. According to the equation established to relate the average fractional helicity (FH) of peptides to the molar ellipticity, i.e., $FH = [\theta]_{222}/([\theta_{H\infty}]_{222}\ (1–2.5/N))$ [57] (where $[\theta_{H\infty}]_{222}$ is the mean residue molar ellipticity of a completely helical peptide of infinite length and N is the number of residues). In our case ($N = 17$), $FH = -8.21/(-44{,}000 \times (1–2.5/17) = 72 \pm 2$ % for the bounded peptide (Fit 1) or $FH = -9.94/(-44{,}000 \times (1–2.5/17) = 87 \pm 2$ % for the bounded peptide (Fit 2).

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Chapter 20

Surface Plasmon Resonance Study of Cooperative Interactions of Estrogen Receptor α and Specificity Protein 1 with Composite DNA Elements

Xiaodi Su and Hong Yan Song

Abstract

Estrogen receptor α (ERα) and Specificity protein 1 (Sp1) are transcription factors (TF) that are involved in regulating progesterone receptor (PR) gene expression through cooperative interactions with DNA. The natural composite DNA +571 ERE/Sp1 site in promoter A of the progesterone receptor contains a half-site of estrogen response elements (½ERE) upstream of two Sp1 binding sites (the proximal Sp1 (Sp1/P) and distal Sp1 (Sp1/D)) with a 4 bp spacer. Here, we have developed a protocol for studying the cooperative interaction of Sp1 and ERα with the composite DNA of +571 ERE/Sp1 site using Biacore T200, a high sensitivity surface plasmon resonance spectroscopy. With this protocol, we have concluded that Sp1 binding enhances the overall ERα binding to the composite DNA. We have also determined the optimal spacer distance between the ½ERE and Sp1/D for the best cooperative protein binding. This study is pivotal in guiding the bioinformatics simulation to yield an exact model of the spacer dependency of the transcription factor/cofactor–DNA interactions, which is important for understanding the nuclear receptor regulating activity through other coactivators.

Key words Transcription factor, Estrogenreceptor, Sp1, Cooperative protein–DNA interaction, Surface plasmon resonance, Biacore T200

1 Introduction

Estrogen is a hormone of critical importance in the development and maintenance of reproductive tissues and in cardiovascular and bone physiology. Many of the effects of estrogen are mediated through its interaction with the intracellular estrogen receptors, ERα and ERβ. Classical models of estrogen action have proposed that the binding of ERs to specific DNA sequences, estrogen response elements (EREs) (a palindromic repeat separated by a three-base spacer, 5′-GGTCAnnnTGACC-3′), is essential to initiate the cellular response to estrogen [1]. In the context of complex regulatory regions, in which imperfect EREs are encased among motifs for other transcription factors, estrogen regulation and gene

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transcription mechanisms are much more complicated, involving complex interactions among multiple proteins and DNA. Human progesterone receptor (PR) [2–4], heat shock protein 27 (Hsp 27) [5], cathepsin D [6], retinoic acid receptor [7], rat creatine kinase B (*CKB*) [8], and c-fos protooncogene [9], for example, are all estrogen-inducible genes. However, the estrogen responsive regions of these genes contain no classic palindromic EREs, but do contain an ERE half-site (½ERE) adjacent to Sp1/GC-rich binding site(s). How ERs and Sp1 activate gene expression through these composite DNA elements and how ERs mediate estrogen responsiveness are issues requiring extensive studies of protein–DNA (e.g., ER–DNA and Sp1–DNA) and protein–protein (e.g., ER–Sp1) interactions.

Promoter A of the human PR gene contains the +571 ERE/Sp1 composite site which contains a ½ERE upstream of two Sp1 binding sites, the distal Sp1 (Sp1/D) and proximal Sp1 (Sp1/P) sites. It has been speculated that Sp1 binding to the Sp1/D will promote the binding of ERα to the DNA via protein–protein interactions [2, 3]. To examine this hypothesis and to determine the optimal distance between the Sp1 sites and the ½ERE for cooperative binding, we developed a surface plasmon resonance protocol to quantify cooperative protein binding to a series of composite DNA sequences designed from the +571 ERE/Sp1 composite site. The spacer distance between the ½ERE and the Sp1/D was extended from 4 bp to 18 bp. Since surface plasmon resonance only measures total protein bound on the surface plasmon resonance chips, but does not provide identification, anti-ERα IgG was added following protein binding to quantify the amount of ERα bound to DNA, especially for those from ERα/Sp1 protein mixture. With this analytical protocol, we affirm that Sp1 protein is important to enhance ERα binding, and there exist the optimal spacers between the ERα and Sp1/D that allow the best ERα/Sp1 cooperative binding. This chapter describes the surface plasmon resonance protocol for studying multiple proteins binding to DNA.

2 Materials

1. Purified recombinant human estrogen receptor α (ERα), usually 2050 nM. Aliquots of a stock solution of ERα were stored in HEPES buffer containing 10 % glycerol at −80 °C.
2. Aliquots of a stock of Sp1 protein (usually 4938 nM) were stored in AM1 buffer (20 mM Tris–HCl, pH 8, 20 % glycerol, 100 mM KCl, 1 mM DTT, and 0.2 mM EDTA) at −80 °C (*see* **Note 1**).
3. Rabbit anti-ERα (antibody directed against the C-terminus of human ERα, HC-20, Santa Cruz). Aliquots of a stock solution were stored at −20 °C.

4. HEPES buffer: 40 mM HEPES, 10 mM $MgCl_2$, 200 mM KCl.
5. HEPES-T buffer: HEPES with 0.1 % Triton X-100.
6. Biotinylated oligonucleotides (38–52 bp, depending on spacers between ½ERE and Sp1/D sites) were custom synthesized. Table 1 lists all of the biotin labeled sense strands. Each contains a half site of the palindromic ERE (½ERE) and two CG-rich

Table 1
ERa-Sp1 composite site—spacer effect

Name	Sequence
Scrambled	5′-/Biotin/TAG GAG C**CG TTA** AGCG **TT GTA C**TC CC**T TGT AC**C CGA CC-3′
PRA_4spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGCG **CC GCC C**TC CC**C C**GC **CC**C CGA CC-3′
PRA_5spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_6spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGAGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_7spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_8spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATAGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_9spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_10spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATAGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_11spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATATGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_12spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATATAGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_13spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATATATGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_14spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATATATAGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_15spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATATATATGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_16spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATATATATAGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_17spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATATATATATGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′
PRA_18spacer_Fw	5′-/Biotin/TAG GAG C**TG ACC** AGATATATATATATAGCG **CC GCC C**TC CC**C CGC CC**C CGA CC-3′

regions (CCGCCC) termed distal Sp1 (Sp1/D) and proximal Sp1 (Sp1/P). The ½ERE and Sp1/D were separated by 4–18 bp (*see* **Note 2**). The scrambled sequence carries a false ½ERE (CGTTA) and two false Sp1 binding sites (TTGTAC), which are separated by 4 bp (between ½ERE and Sp1/D) and used as a control. The flanking sequence remains the same for all 16 sequences. The biotinylated sense strands and the non-labeled antisense strands (sequences not shown) were annealed in PBS buffer (10 mM PBS, pH 7.4, 100 mM NaCl, 10 mM EDTA) at 95 °C for 5 min.

7. The surface plasmon resonance experiments were conducted using the four-channel Biacore T200. Streptavidin sensor chips (Series S SA chip) (GE Healthcare) were used, onto which biotinylated pre-annealed DNA was immobilized. HEPES buffer was used as running fluid for biotinylated DNA immobilization. HEPES-T buffer was used for protein binding (*see* **Note 3**).
8. Preconditioning buffer: 1 M NaCl, 50 mM NaOH.
9. Regeneration reagent: 0.1 % SDS.

3 Methods

3.1 Immobilization of Composite DNA onto Streptavidin Sensor Chip

1. Precondition the streptavidin sensor chip with preconditioning buffer and HEPES buffer (*see* **Note 4**).
2. Immobilize biotinylated dsDNA (from a 100 nM solution) onto a streptavidin sensor chip. Set the DNA immobilization level at a lower density of 20 fmol/mm^2 (*see* **Note 5**).
3. Use the four flow cells (Fc1–Fc4) in a parallel manner so that four different DNAs are injected into different sensor cells (Fig. 1a) (*see* **Note 6**). In every experiment, immobilize the

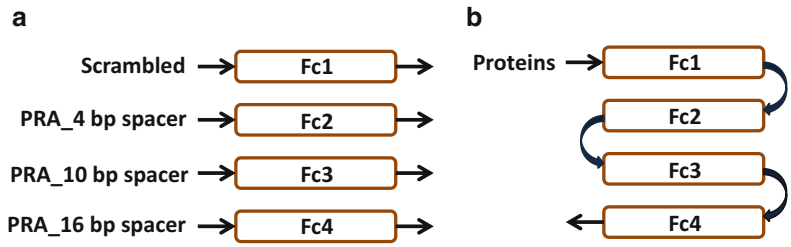

Fig. 1 Biacore T200 fluidic cells (Fc), defined by Biacore Integrated micro Fluidics Cartridges. (**a**) For DNA immobilization, the different composite DNA promoter sequences flow through respective flow cells in a parallel manner; for example, composite DNAs with 4 bp, 10 bp, and 16 bp spacer are immobilized in Fc2, Fc3, and Fc4, respectively. The scrambled DNA is always immobilized in Fc1 as a control. (**b**) For protein binding analysis, protein solution passes through Fc1–Fc4 sequentially

Table 2
DNA surface coverage

½ERE-Sp1	MW[a]	Set[b] (RU)	Measured[c] (RU)	Density[d]	
				ng/mm²	fmol/mm²
Scrambled	23,763	450	454 ± 29	0.454 ± 0.029	19.3 ± 1.1
PRA_4spacer_Fw	23,772	450	462	0.462	19.4
PRA_5spacer_Fw	24,390	462	513	0.513	21.0
PRA_6spacer_Fw	25,007	474	547	0.547	19.0
PRA_8spacer_Fw	26,242	497	478	0.478	18.2
PRA_10spacer_Fw	27,477	520	559	0.559	20.3
PRA_11spacer_Fw	28,094	532	506	0.506	18.0
PRA_12spacer_Fw	28,711	544	530	0.53	18.5
PRA_14spacer_Fw	29,947	567	597	0.597	19.9
PRA_15spacer_Fw	30,564	579	571	0.571	18.7
PRA_16spacer_Fw	31,181	590	608	0.608	19.5
PRA_18spacer_Fw	32,416	614	599	0.599	18.5
Average				0.543 ± 0.05	19.2 ± 0.94

[a]Molecular weight
[b]The set biotin-DNA immobilization level in RU that is calculated according to the ~20 fmol/mm² density we set and the molecular weight of respective DNA
[c]The measured immobilized biotin-DNA on sensor surface in response units (RU)
[d]Calculated based on Biacore SPR sensitivity factor of 1000 RU = 1 ng/mm²

scrambled DNA in the first flow cell (Fc1) as a control, and immobilize three composite DNA sequences of different spacer length to the remaining flow cells, Fc2, Fc3, and Fc4, respectively. Table 2 shows the DNA immobilization characteristics (*see* **Note 7**).

3.2 Determination of Protein Binding and Cooperative Effect

1. Inject ERα (100 nM) or a mixture of ERα and Sp1 (both at 100 nM) through the dsDNA immobilized channels for 3 min at a flow rate of 30 μL/min (*see* **Note 8**). The protein will flow through the four flow cells (Fc1–Fc4) sequentially (Fig. 1b), thus the protein solution passes through all sensor cells (each containing different dsDNA) under the same conditions.
2. At the end of protein binding run, rinse the surface of the flow cells with binding buffer for 1 min to remove unbound proteins.
3. Record the resonance units (RU value) in the binding buffer at the stages before and after protein binding by the software

from the real-time SPR sensorgram, which is a plot of SPR angle change in RU caused by analyte adsorption over time (1000 RU is equal to 1 ng/mm^2 of protein) (*see* **Note 9**).

4. To determine the amount of bound ERα, inject 130 nM anti-ERα antibody for 90 s at a flow rate of 10 μL/min (*see* **Note 10**). In our previous studies [10, 11], we have shown that anti-ERα binding signal is proportional to the bound ERα. Here the anti-ERα sensorgram (Fig. 3b for DNA of 6, 11, 16; Fig. 3c for 8, 14, 18 bp spacer length) shows clearly that in the presence of Sp1, ERα binding is largely increased for all DNA sequences tested. This result supports the hypothesis that Sp1 enhances ERα binding. Figure 3a, d, are the schematic description of the protocol and the summary of the antibody signal for all spacers, respectively.
5. To obtain average protein and antibody binding signals, repeat each measurement three to four times on the DNA immobilized sensor channels. After ERα, Sp1, and anti-ERα binding, apply regeneration reagent (0.1 % SDS) to the sensor surface for 30 s at a flow rate of 30 μL/min to remove the surface bound ERα, Sp1 and anti-ERα (*see* **Note 11**).
6. To determine the optimal spacer for the best cooperative effect, repeat **steps 1–5** to test DNA of all spacer lengths (4–18 bp) (*see* **Note 12**).
7. This protocol can be modified for detecting cooperative protein–DNA binding using nuclear extracts, in which the only modification is to include another step of secondary antibody binding following anti-ERα binding. The use of secondary antibody is to boost the binding signal so that the minor amount of targeted proteins in the nuclear extract can be detected upon this amplification step [12].

4 Notes

1. ERα activity is highly sensitive to temperature fluctuation. It is recommended to aliquot it into small volume according to the amount needed in one experiment or one injection to minimize the freeze and thaw cycles. Before using, allow the frozen protein to equilibrate to room temperature.
2. A series of composite DNAs with spacers were designed based on the +571 ERE/Sp1 composite site in promoter A of the human PR gene. This distance range (between ½ERE and Sp1/D from 4 bp to 18 bp) was chosen because it covers the natural shortest spacer (4 bp) to the longest spacer (17 bp) that was predicted in the literature to have a cooperative effect. The base pairs were chosen to avoid intrachain hairpins.

3. Prepare HEPES buffer from HEPES hemisodium salt in purified water (resistivity 18.2 MΩ). Filter the buffer through a 0.45 μm bottle top filter and degas before use.
4. Inject the 1 M NaCl/50 mM NaOH for 2 min at a flow rate of 50 μL/min three times to remove loosely bound streptavidin. Wash the cells with the HEPES buffer between each injection.
5. Considering the large protein size relative to DNA, the DNA coverage should be relatively low. Our previous ERα–DNA study suggested that it is difficult to saturate a closely packed DNA layer of 50 fmol/mm^2 (3 DNA molecules per 100 nm^2) even with a very higher concentration of ERα (125 nM) [11]. However, a lower density DNA surface of 15 fmol/mm^2 (0.9 DNA molecule per 100 nm^2) provides enough space for ERα binding to the surface DNA at reasonable protein concentrations [13].
6. According to the set DNA density, for 100 nM DNA the instrument automatically computes the volume of DNA sample needed, which is 128 μL for this case. Avoid injection bubbles by preparing an excess volume of 20 μL extra for the injection.
7. For example, set resonance units (RU), experimentally obtained immobilization value in RU, and the calculated DNA density (in ng/mm^2 and fmol/mm^2). The purpose of scrambled DNA in Fc1 as a control is to test the nonspecific protein and anti-ERα binding and to ensure that the four channels have similar physical and physiological environments. The control scrambled DNA is immobilized into Fc1 multiple times in repeated experiments to complete for all 4–18 bp DNA spacers. The obtained immobilization value is averaged at 19.3 ± 1.1 fmol/mm^2 or 0.45 ± 0.03 ng/mm^2 (with the relative standard deviation (RSD) of ~6 %). This reproducibility is reasonably good. For the other composite DNAs immobilized in the Fc2, Fc3, or Fc4 cells, the measured RU are very close to the set RU (<6 % variation). The resulting DNA surface coverage is 19.2 ± 0.94 fmol/mm^2 which is very close to the set value.
8. The protein solution required for this injection is 118 μL, as automatically calculated by the software according to the set flow rate (30 μL/min) and interaction time (3 min), as well as a dead volume of 28 μL (as suggested by the Biacore T200 technical support engineer). In order to avoid injection bubbles, we prepared an additional 20 μL extra, so the total volume is 138 μL.
9. The surface plasmon resonance sensogram exemplified by that for DNA of 4, 10, 16 bp spacer and scrambled DNA (Fig. 2) shows that the binding of ERα alone is below 300 RU, whereas

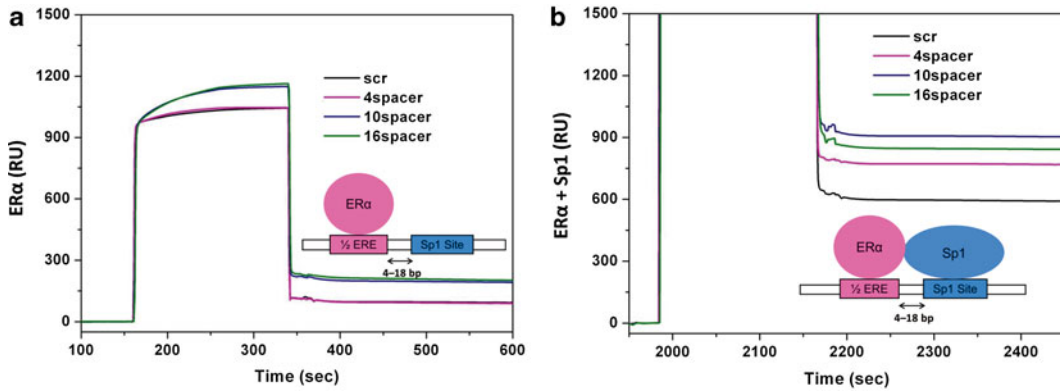

Fig. 2 Time resolved surface plasmon resonance sensorgram of protein binding from (**a**) ERα (100 nM) alone, and (**b**) ERα/Sp1 mixture (100 nM/100 nM). In (**b**) the ***y***-axis is scaled down to the same scale as in (**a**) so that increased total protein binding (the difference before and after protein binding) can be seen clearly

from the mixture of ERα and Sp1, the total protein bound is up to 900 RU.

10. Anti-ERα solution required in this injection is 43 μL as automatically calculated by the software, according to the flow rate (10 μL/min) and the flow time (90 s), and the dead volume of 28 μL (*see* **Note 8**). We prepared 20 μL extra to avoid injection bubbles, so the total volume is 71 μL
11. If the regeneration solution has removed all the bound proteins and antibody, the baseline in binding buffer should return to the level before protein and antibody binding. If the baseline is not totally returned, inject the regeneration solution one more time.
12. The increased anti-ERα signal following cooperative ERα/Sp1 binding relative to ERα alone (Fig. 3d) shows that Sp1 enhances the binding of ERα for all tested DNA promoters and the most significant binding enhancement is observed when ½ERE and Sp1/D were separated by 11–14 bp. We speculate that this distance range is optimal to promote protein–protein interactions, therefore the combined interactions between protein–DNA (ERα–½ERE, Sp1–Sp1 site) and protein–protein (ERα–Sp1) lead to the best cooperative effect. However, when the spacer is shorter (i.e., PRA_4 spacer, PRA_5 spacer, and PRA_6 spacer) or longer (>14 bp), although the cooperative effect is still remarkably detected, the magnitude of increment is smaller, due presumably to unfavorable protein–protein interactions associated with either the steric hindrance (shorter spacer) and far exceeding the distance for a perfect protein–protein contact (longer spacer).

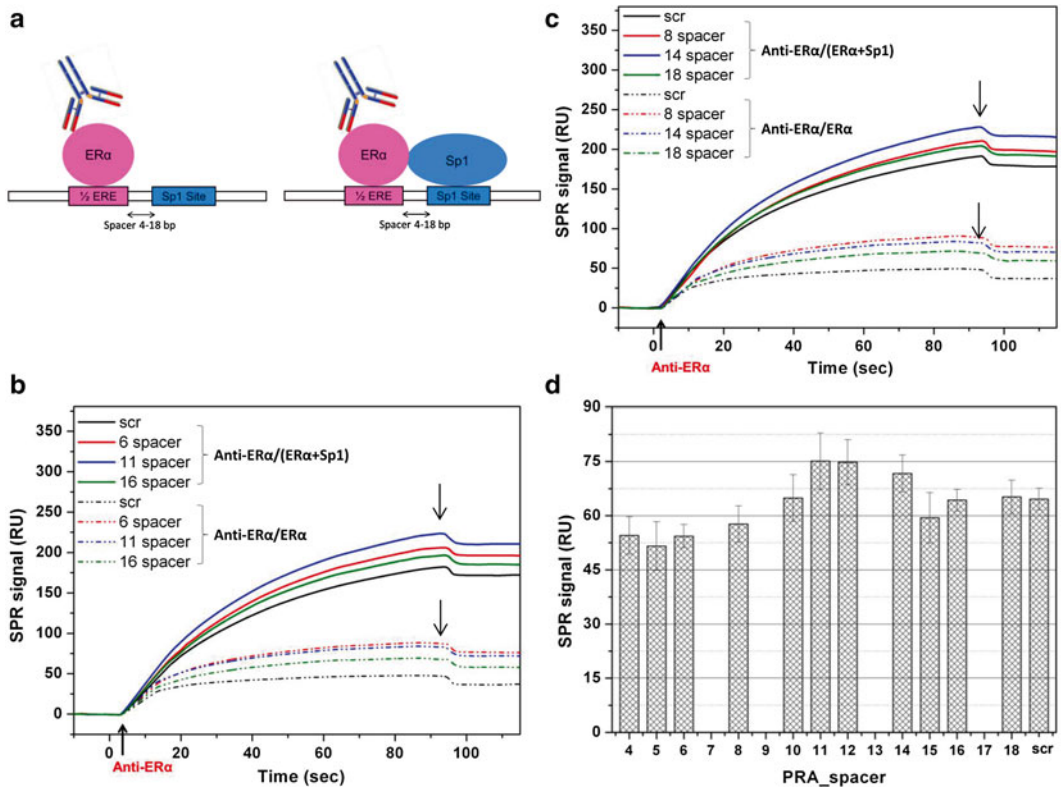

Fig. 3 (**a**) Schematic description of the analytical protocol for experiment with (1) only ERα and (2) ERα/Sp1 mixture. (**b**, **c**) Surface plasmon resonance sensorgram of anti-ERα binding following ERα binding (*dotted lines*) and ERα/Sp1 mixture (*solid lines*) for (**a**) spacers 6, 11, 16 and (**b**) 8, 14, 18. (**d**) A summary of RU of anti-ERα for all spacers tested. At the end of protein binding, the cells are rinsed using binding buffer as shown by the *down arrows* in **b** and **c**

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Chapter 21

Tips and Tricks for Successful Application of Statistical Methods to Biological Data

Evelyn Schlenker

Abstract

This chapter discusses experimental design and use of statistics to describe characteristics of data (descriptive statistics) and inferential statistics that test the hypothesis posed by the investigator. Inferential statistics, based on probability distributions, depend upon the type and distribution of the data. For data that are continuous, randomly and independently selected, as well as normally distributed more powerful parametric tests such as Student's *t* test and analysis of variance (ANOVA) can be used. For non-normally distributed or skewed data, transformation of the data (using logarithms) may normalize the data allowing use of parametric tests. Alternatively, with skewed data nonparametric tests can be utilized, some of which rely on data that are ranked prior to statistical analysis.

Experimental designs and analyses need to balance between committing type 1 errors (false positives) and type 2 errors (false negatives). For a variety of clinical studies that determine risk or benefit, relative risk ratios (random clinical trials and cohort studies) or odds ratios (case–control studies) are utilized. Although both use 2×2 tables, their premise and calculations differ. Finally, special statistical methods are applied to microarray and proteomics data, since the large number of genes or proteins evaluated increase the likelihood of false discoveries. Additional studies in separate samples are used to verify microarray and proteomic data. Examples in this chapter and references are available to help continued investigation of experimental designs and appropriate data analysis.

Key words Descriptive statistics, Parametric tests, Nonparametric tests, Type 1 and type 2 errors, Microarray studies

1 Introduction

Thirty or forty years ago a scientist could collect and publish a complete set of data with very little statistical analysis. The advantage of this approach to current readers is the ability to reanalyze these data and sometimes come to different or additional conclusions. The disadvantage of lack of statistical analysis in the older papers is that the original conclusions were not always accurate, but they have been perpetuated in review articles. Times have changed and now journals and reviewers require authors to report the experimental designs and statistical methods that they used to

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analyze their data and why they chose particular approaches. Since different computer programs use a variety of statistical equations to analyze data, authors need to also report the program they employed. Importantly, articles may be held up for publication because there are questions about the statistics utilized. The purpose of this chapter is to empower you to determine which statistical tests to use for your specific experimental design and why. Further readings are cited to will allow readers to investigate experimental designs and statistical analysis methods beyond the scope of this chapter.

Before computer programs became readily available, statistics were performed by hand or with the aid of a simple calculator. Before undertaking such a task, the researcher was forced to make sure that the test selected to evaluate the data was the correct one. Consequently, they also had more insight into why and how they used a given test. With the advent of powerful statistical computer programs to analyze and graph results, data can now be easily evaluated and presented, but the researcher still needs to choose the tests and interpret the results of the statistical analysis. Thus, another purpose of this chapter is to allow you to understand the differences among types of data and statistical tests, as well as how to interpret the outcomes you obtain.

2 Statistics and Probability: Conventions and Assumptions

Statistical tests depend on probability distributions. That is, a comparison is made between the calculated statistic determined by the outcome of a study and a critical value obtained from a distribution specific for that statistical test. There is a "*P*" or probability value assigned to the relationship. The convention is to accept a *P* value of less than 0.05 (also known as the alpha value) as the "cut-off" of what we consider to be statistically "significant". This convention suggests that the researcher is willing to accept a 1 in 20 chance of being wrong when asserting that a true difference exists between groups. Or put another way, there is a 5 % chance that there is no difference between the effects of two treatments even though we report that the differences are significantly different ($P < 0.05$). This cut-off was not determined by scientific study! It was an arbitrary choice that was made, based on the level of chance of being wrong versus being right that investigators were willing to accept. Of course, most investigators are more confident if the *P* value for their data is 0.001 because that suggests that you are now accepting a 1 in a 1000 chance of being wrong when asserting that a true difference exists between two groups.

Thus, conclusions obtained using statistics depend upon the alpha value selected (usually 0.05) remembering that the conclusions are associated with probabilities, not absolute conclusions. Furthermore conclusions from large and complicated studies may

be "boiled down" to a conclusion that an effect is statistically significant based only on a *P* value. What happens if the *P* value obtained is 0.055? Another approach to deal with this dilemma is to determine effect sizes or the magnitude of the differences between groups or effects of perturbations. Effect sizes are more closely related to "biological" significance and not just "statistical" significance. Paul Ellis published an excellent book that discusses what effect sizes are and how they are determined in a variety of experimental designs and appropriate statistical tests [1].

Another statistical interpretation pitfall is making an assertion that differences in the magnitude of *P* values denote differences between groups that are not directly compared. For example, a *P* value of 0.001 suggests that there is a 1 in 1000 chance no difference exists between the effects of two treatments. However, if treatment "A" shows a *P* value of 0.01 and treatment "B" a *P* value of 0.001 relative to respective controls, that does not mean that treatment "A" has a more significant effect than treatment "B"! Only by conducting appropriate statistics that directly compare the two treatments can one make any conclusion regarding the differences between two treatments.

3 Signal to Noise Ratios and Statistics

Statistical tests are really used to evaluate the "signal" to "noise" ratio. The "signal" is the difference of mean values between two groups. The "noise" refers to the variability due to underlying biological factors, and/or the level of precision and accuracy of the experimenter and methods utilized. One way that "noise" can be decreased is by increasing the number of samples used. However, there are restraints to increasing sample sizes, including ethical issues. Other approaches to decrease "noise" include improving the accuracy and precision of the methods employed and being more selective of subjects designated for a study. For example, in studies of reproductive age females, the "noise" in the data will be greater and may cloud the outcomes of the study if the stage of the menstrual cycle/estrus cycle is not taken into consideration. Another historical way to increase "noise" is mix results by gender, instead of designing and evaluating experiments in male and female subjects separately. With current mandates from the NIH, hopefully this latter approach will be more prevalent.

4 Types of Statistics: Descriptive and Inferential

4.1 Descriptive Statistics

There are two broad categories of statistics: descriptive and inferential. Descriptive statistics, as their name implies, describe the characteristics of the data. Descriptors of data include measures of central tendencies such as the mean that is calculated by summing values

and dividing them by the number of values (N). Another measure is the median, which refers to 50 % of a data distribution. The mode refers to the most frequent value. In normally distributed data, the mean, mode and median are very similar.

Aside from central tendencies of the data, the variability of the data needs to be considered. Variability measures of data include the variance which is determined as the sum of the mean minus the individual values (xi) divided by the sample size (N) minus one or: sum $(\text{mean} - \text{xi})^2 / N - 1$. A related measure is the standard deviation (SD) which is calculated as the square root of the variance. Another frequently used measure of variability is the standard error of the mean (SEM) which is determined by dividing the SD by the square root of the number of samples. Since the SEM is smaller than the SD, the SEM is commonly presented in graphs and tables to describe variability. However, the SEM actually quantifies the uncertainty of the estimate of the population mean when several samples are taken. It is not really appropriate unless the sample size is given so that the standard deviation can be determined Additional measures of descriptive statistics include the range of values covered by the data from the smallest to the largest, as well as quintals that define 25 % and 75 % of the data.

Two other types of descriptive statistics are confidence intervals and the coefficient of variation. Confidence intervals are used as descriptive statistics and also to determine the range of 95 % or 99 % of the values of a sample. To determine a 95 % confidence interval, first determine the standard error of the mean ($\text{SEM} = \text{SD} / [N^{1/2}]$) and then multiply it by 1.96. The result is added to and also subtracted from the mean ($\text{mean} \pm 1.96\ \text{SEM}$). For example, if the mean of a sample of 100 subjects is 30 and the SD is 6, then the CI is calculated as follows: $6/(10) \times 1.96 = 1.18$, $30 + 1.18 = 31.18$ and $30 - 1.18 = 28.82$. The mean and confidence interval are reported as follows: 30 (31.18 – 28.82). This confidence interval is relatively small because of the large mean and small standard deviation. Confidence intervals can be used in place of standard errors in graphs (and should be designated as such in the figure legend or in the statistical methods section) and can also be used to determine a "normal" range of data such as the levels of growth hormone in a population.

The coefficient of variation percent is calculated by dividing the standard deviation by the mean and multiplying the result by 100. The coefficient of variation is used to report variability results in many types of experiments, including hormone assays and microarray studies. It helps define the accuracy and precision of the methods used. It is also influenced by the sample size and may be decreased by increasing the number of biological replicates. Interestingly enough, the coefficient of variation may itself be used as a variable when determining if a treatment "normalizes" variability [2].

4.2 Inferential Statistics

4.2.1 Student's t Test: Comparing Two Groups

The second type of statistical tests is inferential statistics used to determine if the results really support or refute the null hypothesis. The null hypothesis states that the effect of two treatments is comparable. However, what we actually want to determine is the alternative hypothesis, which is that the effects of the two treatments are different. For example, we want to compare the effects of a control treatment and estrogen on the expression of gene 1 in a cell culture study. The appropriate statistical test for this experimental design is a Student's *t* test. The Student's *t* test is calculated by dividing the difference between the means of the outcomes of the two treatments (levels of gene expression in this case) by the sum of the two variances each divided by their sample size. Thus, the mean differences represent the "signal" and the variances the "noise".

We designate that significance is achieved if the calculated statistics using the Student's *t* test is greater than an alpha value of 0.05 for a critical value. Thus, if the calculated value is greater than the critical value, the null hypothesis is rejected, and we conclude that there is a significant difference, in our example, between the control and estrogen treatment on the expression level of gene 1. A graphic representation of individual data points and means and standard deviations for this experiment is shown in Fig. 1. Significant differences in gene 1 expression between the two treatments are represented with an asterisk.

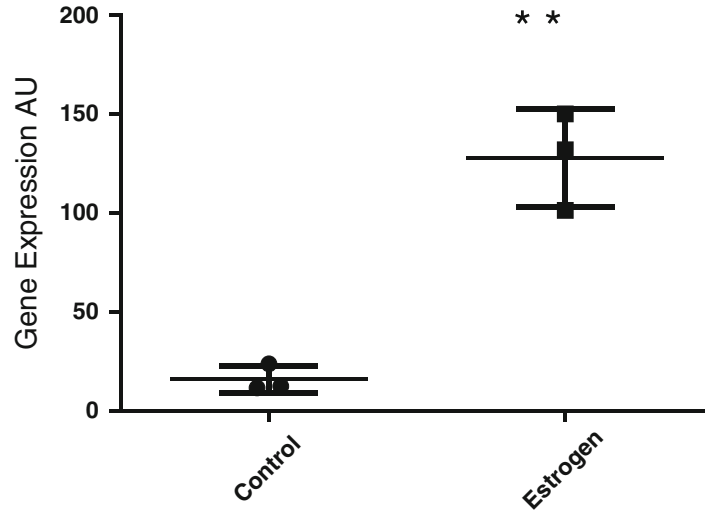

Fig. 1 Individual values of three biological replicates investigating the influence of a control treatment and estrogen on gene 1 expression in arbitrary units (AU). Means and standard deviations for each treatment group are shown. The *asterisks* indicate a significant effect of estrogen treatment ($P < 0.0001$)

4.2.2 Analysis of Variance (ANOVA): Evaluating Significance with Two or more Groups

If we were to now include additional treatment groups (e.g., tamoxifen and raloxifene) to evaluate their effect on the level of gene 1 expression, one obvious approach to determine differences among treatments would be just to do repeated Student's *t* tests comparing the control to each treatment group. The problem with repeating Student's *t* tests is that the probability of the outcomes being significant becomes greater as the number of comparisons conducted increases. We are at risk of committing a Type 1 error, of accepting a result as true when in fact it is not. Another term for a Type 1 error is a false positive. If we reject a finding as significant because the *P* value is 0.057, for example, we could be committing another type of error, a Type 2 error or rejecting a finding as being nonsignificant when, in fact, it is. This occurs frequently when sample sizes are very small and variability is large.

Getting back to the problem at hand: the descriptive statistics (mean and standard deviation) for the effects of control and three treatments with estrogenic compounds on the expression level of gene 1 are shown in Fig. 2. To prevent the possibility of making a Type 1 error we would first conduct a one way analysis of variance (ANOVA). This test would tell us if there were any differences in gene 1 expression level as a result of any of the four treatments. This test evaluates the relative size of the "signal" or the effects of the treatments relative to the "noise" or variability related to the experimental procedures also known as the *F* ratio. In addition to

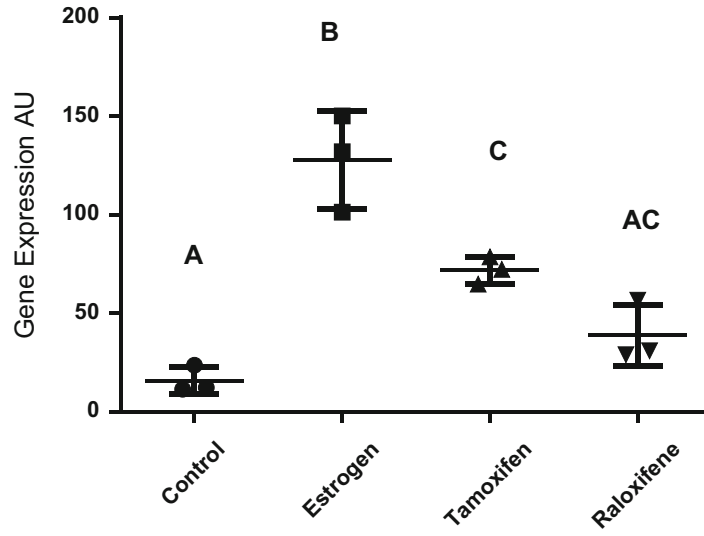

Fig. 2 Individual values of three biological replicates investigating the influence of a control treatment, tamoxifen, raloxifene, and estrogen on gene 1 expression in arbitrary units (AU). The *different letters* indicated that estrogen and tamoxifen are significantly different from control and that estrogen differs from tamoxifen as well as raloxifene. Means and standard deviations for each treatment group are shown

the P value, knowledge of the degrees of freedom is needed to determine the critical value that will be compared to the calculated statistic or F value. Degrees of freedom are obtained by knowing the number of treatment groups ($N-1$) and the number of subjects (N). If this test is significant, which it is in this example, results are reported as follows: $F_{3,8}=30$, $P=0.0001$. The 3 and 8 are known as degrees of freedom for treatments (4-minus-1) and for number of subjects ($[2\times4]=8$). This test only tells us that there are differences among the groups in their ability to change expression levels of gene 1, but not which treatment has different effects compared a second treatment.

4.2.3 Post hoc Tests: Comparing Treatment Effects to the Control

Since the ANOVA was found to be significant, we can conduct post hoc tests to determine where the significance lies; that is, which groups are significantly different. If the goal is to determine whether each individual treatment is different from control, the post hoc tests that make this comparison include the Bonferroni test, Dunnett's test, and Holms test (in order of most conservative to least conservative test). Both Bonferroni and Holms tests typically use the alpha value of 0.05 and divide 0.05 by the number of comparisons required. For the Bonferroni test 0.05 is divided by 3 (number of treatment comparisons to the control) which yields a P value of 0.016. Thus, for each of the treatments, the calculated statistic relative to the critical statistic needs to reach a level of significance of 0.016 to be considered significant. The Holms test is less conservative. For this test a comparison of the greatest difference between the control and a treatment (in this case estrogen) is required to reach the 0.016 benchmark. However, for the subsequent two comparisons, they only need to reach benchmarks of 0.025 (next largest mean difference relative to control) and 0.05 for the last comparison. Results of our experiment indicate that both estrogen and tamoxifen are significantly different from control.

4.2.4 Post hoc Tests: Comparing All the Treatment Group Responses

If our hypothesis is that all four treatments are different from each other, a variety of post hoc tests can be conducted to investigate this possibility. The most common "work horse" test utilized is a *Student–Newman–Keuls test* or SNK test. Other tests to accomplish the same task are the *Tukey test*, the Bonferroni test (alpha is now divided by 6 if there are to be 6 comparisons requiring the P value to be less than 0.0008), the *Holms test* (correcting for number of comparisons, but not as conservative as the Bonferroni test). The least conservative of all post hoc tests is called the *Least Square Means test*. Thus, if our goal is to determine whether each individual treatment is different from every other treatment, the post hoc tests include Tukey and Bonferroni (both very conservative), Student–Newman–Keuls test and Holms (less conservative than Tukey and Bonferroni but similar to each other in level of conservation), and least square means test (least conservative).

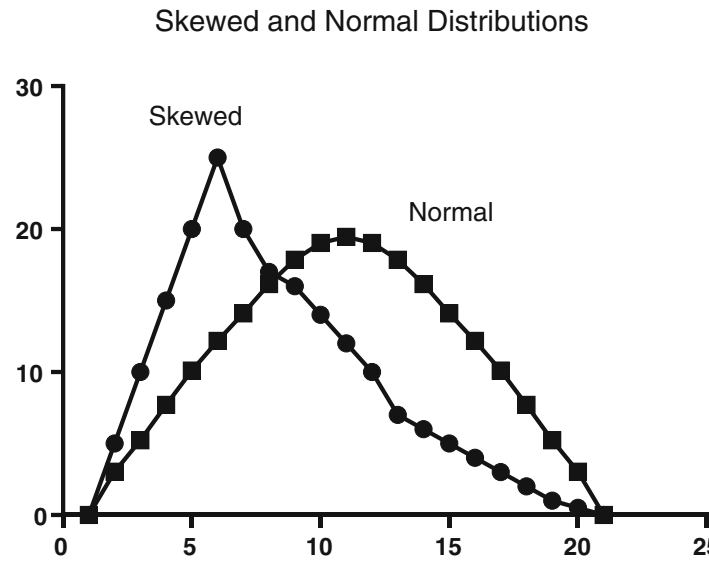

Fig. 3 Schematics illustrating normal and skewed distributions. In a normal distribution, the mean, mode, and median are the same

After conducting an ANOVA and SNK tests the results of our experiment to determine effects of the four treatments on expression levels of gene 1 are shown in Fig. 2. Significant differences among treatments on gene 1 expression levels are indicated by different letters above the data. Thus, we would conclude that both estrogen and tamoxifen affect gene 1 levels significantly different from control and estrogen differs in its effects on gene 1 expression levels from tamoxifen as well as from raloxifene.

4.2.5 Separate Groups and Repeated Experimental Designs

The experimental designs and analyses in the examples discussed above were conducted on separate groups, 2 for the Student's *t* test example and 4 for the ANOVA. However, what if we wanted to determine the effects of treatments on estrus cycles in the same female rats? In this example, each rat would receive all treatments. If we were only using a vehicle control and one treatment, we would conduct a *paired* Student's *t* test. For this test we would determine the difference in the response of each female to vehicle versus her subsequent response to the treatment. Calculation of the Student's t statistic consists of dividing the mean difference by the standard error of the means of the differences. In this case if there are ten rats in the study, we would use the critical *t* statistic with a *P* value of 0.05 for 10-minus-1 or 9 degrees of freedom. If we employ more than two treatment groups in the study, then we would use an ANOVA but with repeated measures. Now the *F* ratio, a measure of the "signal" to "noise" ratio is determined by the within-subjects treatment effect divided by the residual effect as shown in Table 1.

Table 1
Descriptive statistics (Table 1a) and an example of a repeated ANOVA table with the *F* ratio of the within subjects: treatment/residual effects and the corresponding *P* value are shown in Table 1b. In this example eight rats were given vehicle (control) in week 1. Over each of the next 3 weeks, the rats were given a different dose of a drug that affected serum estradiol levels

(a)					
Doses	**# Animals**	**Estradiol (pmol/L)**	**SD**		
Vehicle	8	68.5	8.28		
Dose 1	8	39.25	5.42		
Dose 2	8	23.38	4.75		
Dose 3	8	9.88	1.55		
(b)					
Source of variability	**Degrees of freedom**	**Sum of squares**	**Mean square**	**F ratio**	**P value**
Between subjects	7	267	38.14		
Within subjects	24	15,842			
Treatment	3	15,252	5084		
Residual	21	593.2	28.25	180	<0.000001
Total	31	16,112			

Some caveats are needed to use a repeated design. These include that none of the treatments can compromise the function of the cells, animals, or people studied (for example, a toxin causing cell death), the treatments must have no residual effects (e.g., as a result of a drug administration that has a very long half-life), and effects must be reversible. However there are several advantages of this type of experimental design. These include using a decreased number of subjects (in our example we would need only eight rats instead of 32 rats if each rat only received one treatment), there is less variability within subjects, and one can better assess the response of an individual to an intervention.

5 Parametric Versus Nonparametric Statistics

The types of inferential tests described above fall into the category of *parametric statistics*. Parametric statistical tests make certain assumptions about the distribution of the data; that is, the data must be normally distributed rather than skewed (Fig. 3). Another assumption is that variables must be continuous (e.g., temperature, blood pressure, or protein expression), the samples must be independently selected as well as randomly selected, and the variances of groups must be similar. It is a tall order to meet all of these

requirements, especially when sample sizes are small as in the example of estrogenic agents on gene expression (Fig. 2) and most molecular biology studies.

Parametric tests include not only the *Student's t-tests and ANOVA* (one way or much more complex two or three way ANOVAs depending upon the experimental design), but also *regression analysis and Pearson's correlation* analyses. Regression analysis is used to evaluate cause and effect. For example, regression analysis could be used if the goal of an experiment is to determine the effects of different doses of an estrogenic agent on the level of gene expression. In contrast, correlation analysis determines the effect of two dependent variables (say body mass index or BMI) and blood glucose levels. The results allow us to determine if there is an association (or not) between BMI and glucose levels. Both regression and correlation relationships (expressed as r values) range from −1 to +1. A negative r value denotes that an increase of one factor causes the other to decrease. A positive r value indicates an increase in one factor results in an increase in the other factor. The closer the r value is to 1 (or −1), the higher the likelihood is that the treatment has a major effect on the outcome. The effect size for both regression analysis and correlation analyses is determined by squaring the r value (r^2). Again, the higher the r^2, the greater the effect of the treatment on the outcome. More complicated types of regression and correlation analyses determine the effects of a number of variables on a particular outcome and are used especially in clinical studies to control for potential confounders. An example of a multivalent regression analysis is to determine what factors affect glucose levels in middle aged women. Potential confounders in the analysis could include BMI, caloric intake, activity levels, and insulin levels.

5.1 Nonparametric Tests

After careful examination of raw values and conducting some descriptive statistical tests (means and SD, for example), what happens when your data do not satisfy criteria listed for parametric tests? What happens if you have categorical data or proportions, the data are not normally distributed, or the variances among groups are different? One solution is to transform the data using log and inverse manipulation of the data and then use parametric statistics. Another solution is to employ nonparametric tests. Such "skewed" data is plotted using whisker box plots (Fig. 4).

Commonly used nonparametric tests include Chi-Square tests, z tests, relative risk ratios, odds ratios, logistic regression, Kruskal–Wallis, Mann–Whitney, Wilcoxon ranked sign tests, and Spearman correlation tests. In the last four tests cited, the raw data are ranked and statistics are conducted on the ranked data. Table 2 matches some commonly used experimental designs with comparable parametric and nonparametric tests. The bottom line is, if the data are skewed, nonparametric tests should be utilized.

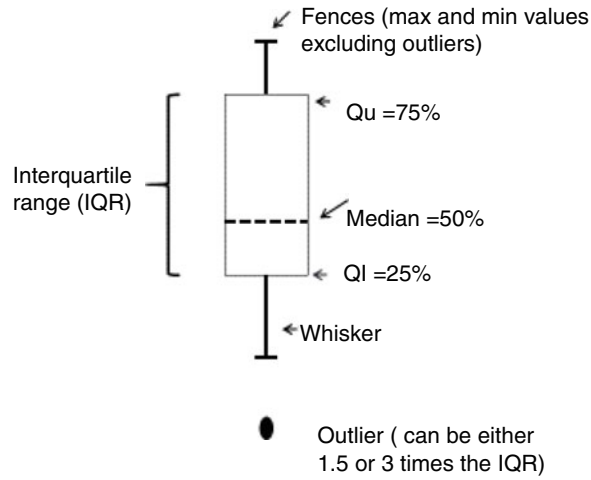

Fig. 4 Characteristics of the box and whisker plot which is used to display skewed data. Shown are the median, 25 % and 75 % quintiles (Qu), whiskers as the range of the data and the outlier is designated as a dot

Table 2
Experimental designs and corresponding examples of parametric and nonparametric tests

Experimental design	Parametric tests	Nonparametric (ranked) tests
2 groups	Unpaired *t* test	Mann–Whitney rank-sum
2 groups repeated	Paired *t* test	Wilcoxon's signed-rank
3 or more groups[a]	ANOVA	Kruskal–Wallis statistic
3 or more groups repeated[a]	Repeated ANOVA	Friedman statistic
Correlation	Pearson	Spearman

[a] Post hoc tests need to be conducted if significance (usually $P<0.05$) is determined in studies that include 3 or more groups. Repeated experimental designs denote that each subject receives all treatments

6 Relative Risk and Odds Ratios

In clinical experiments, both parametric and nonparametric tests are used to analyze data depending upon the experimental design, type of data collected, and distribution (normal versus skewed). Two types of proportional tests used in cohort and random clinical tests (prospective) and case–control studies (usually retrospective) are relative risk and odds ratios which evaluate the strength of exposures and disorders.

6.1 Types of Clinical Studies

A randomized clinical trial is an interventional study in which patients may receive various treatments. An example is the Women's Health Initiative in which postmenopausal women were treated

with placebo, estrogen alone, or estrogen plus progesterone [3]. Outcomes such as heart disease, bone fractures, and breast cancer incidence were evaluated.

A cohort study is a longitudinal investigation in which individuals are followed over time to determine what risk factors experienced by the participants may be responsible for development of disease. An example of a cohort study is the Framingham study which followed several thousand men and women over decades to evaluate risk factors (such as hypertension, obesity, and hyperlipidemia) associated with the development of heart disease.

Case–control studies are designed to determine if exposure to an agent in the individuals who develop an illness (the cases) is different from lack of a particular exposure of the controls who do not develop the disease. Matching subjects for factors such as age, gender, body mass index, and environmental behaviors helps to minimize bias, although recall bias may still exist. This type of design is useful for studying whether exposures affect the development of relatively rare disorders. Unlike random clinical trials and cohort studies, case–control studies prove association, but not causation. Further investigations need to be conducted to prove biological causation. However, this study design can have powerful effects on policy; for example, calling for recall of tainted foods and drugs that cause considerable morbidity and may lead to death [4].

6.2 The Relative Risk Ratio

The relative risk ratio ascertains the probability of an event in a treatment (or exposure group) divided by the probability of an event occurring in the control or non-exposed group. Table 3 illustrates an example of data in a 2×2 table used to calculate the relative risk ratio. To determine the relative risk ratio in this example we calculate the ratio of $312/327$ to $20/420 = 0.954/0.0476 = 20.003$. The interpretation of this relative risk ratio is that individuals who are exposed or treated have a 20 times greater probability of developing the disease compared to individuals not exposed or treated. Another component to this determination is the confidence interval whose calculation we will not go into here, but whose calculation is delineated in "Primer of Biostatistics" [6].

If the relative risk ratio and confidence intervals are greater than 1, for example 2.0 (1.5–2.5), then the exposure is considered

Table 3
Data used to calculate the relative risk ratio

	Disease	No disease	Total
Treated or exposed	312	15	327
Not treated or exposed	20	400	420
Total	332	415	747

Table 4
A 2 × 2 table used to calculate odds ratios for case–control studies

	Cases	Controls
Exposed to risk factor	312	15
Not exposed to risk factor	20	400
Total	332	415

harmful. If the relative risk ratio and confidence interval are less than 1, for example 0.6 (0.2–0.8), then the treatment or exposure is considered beneficial and significant. Finally if the relative risk ratio and the confidence span 1, for example 2.0 (0.8–5), the effect of treatment and exposure are not considered significant.

6.3 The Odds Ratio

The odds ratio evaluates the relationship between being a "case" due exposure to a risk factor that results in a disease, to being a "control" and exposed to a risk factor but not developing a disease. This calculation also uses a 2×2 table similar to that shown for relative risk ratio determination, but it is different since we are now comparing responses of cases and controls. For example in Table 4, cases and controls are placed in the cells for disease and no disease, respectively, whereas exposures are in the same cells as in Table 3.

In this example the calculation of odds ratio is $312/332$ divided by $20/332 = 0.94/0.06 = 15.6$. The interpretation of this result indicates that the cases exposed to a risk factor have a risk of developing the disease that is 15.6 times greater than the controls. Like relative risk ratios, confidence intervals are used to determine the significance of the outcome. For example, if the odds ratio is 15.6 and the confidence interval is 5–25, then the exposure is significantly harmful and may contribute to a disease. Importantly, the odds ratio does not prove that the exposure causes a disease, only suggests that it *may be* contributory. Further studies are needed to prove cause and effect.

7 Use of Statistics to Evaluate Large Data Sets

We have discussed that the likelihood of making a Type 1 error, of accepting a result as true when it is not, increases when repeated comparisons are made using inappropriate statistical tests. This problem is compounded when evaluating large data sets such as those that arise from the application of genomics and proteomics techniques. Not only is the likelihood of *false positives* increased, but so is the probability of *false negatives*, that is, rejecting a difference when it actually is true. Another term for "false" findings is *false discovery rates.*

A unique experimental design analysis is necessary to evaluate large biological data sets, such as microarray data, since so many comparisons are made within a given experiment. Prior to data analysis, the threshold levels of gene expression need to be validated. The "valid" data may be transformed or analyzed as raw signals. Statistical analyses called "multiple testing correction tests" assess the false discovery rate and are applied to overcome the statistical issue posed by the large number of comparisons made in genomics and proteomics studies. Multiple testing correction tests that are then utilized include the Bonferroni test, the Holms test, the Westfall and Young Permutation, and the Benjamini and Hochberg False Discovery Test. The first two have been described previously under inferential post hoc tests. These are the more conservative tests and decrease the likelihood of committing a type 1 error (or false positives), but may increase the likelihood of committing a type 2 error (false negative). The two other tests are less conservative and their application is more likely to result in committing a type 1 error. The following URL (http://www.chem.agilent.com/cag/bsp/sig/downloads/pdf/mtc.pdf) and references further detail the applications of these tests for microarray analysis [5–8].

As in other studies, the experimental design (two or more groups, case–control studies, regression or correlation), helps dictate the type of analyses utilized with microarray data. Moreover, confirmatory studies to verify that the changes in gene expression are "real" are critical. For this purpose, evaluation of RNA or protein levels for a particular gene are carried out in a set of separate samples using real time RT-PCR, Western Blot, ELISA, or immunohistochemistry.

Issues that introduce variability in other types of scientific experiments are also present in microarray experiments. In addition the small amounts of variability that they introduce are greatly compounded in microarray experiments by the large number of comparisons that are made in these experiments. Thus, biological variability may be introduced by obtaining samples at different times of day, the diet of the subjects, their gender, or in cancer studies, which tissues are selected. Other experimentally based factors contributing to variability include the method used to extraction of RNA, reagents used, the level of technical expertise, or the type of microarray platform utilized.

Another important factor to consider is the number of replicates and number of genes or proteins to be evaluated. Sample replicates are an important means of reducing biological variability. As in all scientific experiments, using replicates from the *same* sample is considered a *technical replicate* and actually represents an experimental number of one. Of greater use and validity are *biological replicates* or the number of individual subjects sampled in a study. The number of biological replicates dictates the sample size for that group unlike the technical replicates on one sample.

8 Conclusions

In conclusion, two types of statistical tests exist, one type that describe the characteristics of data (descriptive statistics) and the other, inferential statistics, that tests the hypothesis posed by the investigator. Inferential statistics, based on probability distributions, depend upon the type and distribution of the data acquired as well how the data were obtained. For data that are continuous, randomly and independently selected, as well as normally distributed, more powerful parametric tests can be used. For skewed data, transformations may normalize the data, or else nonparametric tests can be used. Experimental designs and analyses balance the possibly between committing Type 1 and Type 2 errors. For a variety of clinical studies that determine risk or benefit, relative risk ratios (RCT and cohort studies) or odds ratios (case–control) are utilized. Although both use 2×2 tables, their premises and calculations differ. Finally, special statistical methods are applied to large biological data sets, since the large number of genes or proteins evaluated increase the likelihood of false discoveries.

Thus, understanding the underpinnings of experimental design and analysis, covered in this chapter, will improve formulation of experiments and the analysis and interpretations of results. If in doubt, statisticians are good resources for sorting out the tangled web of vast numbers we collect in today's research.

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Chapter 22

The Synonymous Ala87 Mutation of Estrogen Receptor Alpha Modifies Transcriptional Activation Through Both ERE and AP1 Sites

Tamara Fernández-Calero, Gilles Flouriot, and Mónica Marín

Abstract

Estrogen receptor α (ERα) exerts regulatory actions through genomic mechanisms. In the classical pathway, ligand-activated ERα binds directly to DNA through estrogen response elements (ERE) located in the promoter of target genes. ERα can also exert indirect regulation of transcription via protein-protein interaction with other transcription factors such as AP-1.

Several ERα synonymous polymorphisms have been identified and efforts to understand their implications have been made. Nevertheless effects of synonymous polymorphisms are still neglected. This chapter focuses on the experimental procedure employed in order to characterize the transcriptional activity of a synonymous polymorphism of the ERα (rs746432) called Alanine 87 (Ala87). Activity of both WT and Ala87 ERα isoforms on transcriptional pathways can be analyzed in transiently transfected cells using different reporter constructs. ERα efficiency on the classical genomic pathway can be analyzed by determining its transactivation activity on an ERE-driven thymidine kinase (TK) promoter controlling the expression of the luciferase reporter gene. Transcriptional activity through the indirect genomic pathway can be analyzed by employing an AP-1 DNA response element-driven promoter also controlling the expression of luciferase reporter gene.

Key words Estrogenreceptoralpha (ERα), Estrogen transcriptional regulation, Estrogen-responsive element (ERE), Nonclassical pathway, AP-1pathway, Estrogenreceptor alanine 87 polymorphism

1 Introduction

The estrogen receptors ERα and ERβ mediate the pleiotropic and tissue-specific effects of estrogens. These transcription factors belong to the nuclear receptor superfamily and share a multidomain structure (Fig. 1). Two domains harbor activation functions (AF): the A/B domain contains AF-1 and the E domain contains AF-2 [1]. The respective contributions that AF1 and AF2 make toward the activity of the full-length ER are both promoter and cell specific, and also depend on the differentiation stage of the cell [2]. The classical mechanism of activation of ERs depends on ligand

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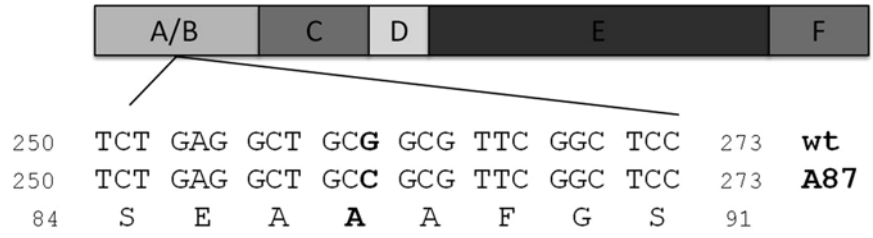

Fig. 1 Representation of ER functional domains. *A/B*: N-terminal domain (contains AF-1), *C*: DNA-binding domain, *D*: hinge, *E*: Ligand-binding domain (contains AF-2), *F*: C-terminal domain. ER wild-type (WT) and synonymous ERAla87 mutant coding sequences, and translated amino acid residues are shown (*below*). The codon variant Ala87 is indicated in *bold*. Reproduced from [14] with permission from Elsevier

binding to the receptors, after which the receptors dimerize and bind to estrogen response elements (EREs) located in the promoter of estrogen-responsive genes [3]. Ligand binding allows the interaction with specific cofactors and binding to DNA to activate or repress different sets of genes. In the nonclassical pathway of genomic activation, ERα binds indirectly to the DNA via tethering to other transcription factors such as specificity protein 1 (Sp1), activating protein 1 (AP-1), or nuclear factor-kappa B (NF-kB), and regulates estrogen transcriptional regulation in an ERE-independent manner [4]. For instance, ERα upregulates or downregulates transcription from genes that contain AP-1 sites, binding sites for the Jun/Fos complex, in a cell-type-specific manner [5].

Due to the central role of ERα in many physiological processes, the study of polymorphisms is of utmost importance for pharmacogenomics implications. In this work we investigated the functional properties of the ER variant assigned as BstUI [6]. It is a synonymous polymorphism, a single base mutation that changes the DNA sequence without changing the amino acid coded by that sequence, and the effect of this kind of variation has been much neglected until recently. This ERα polymorphism has been poorly characterized and no clear association of this SNP to human diseases has been reported yet [7]. It is located in exon 1 and corresponds to the alanine 87 (Ala87) synonymous mutation (GCG to GCC) (rs746432) (Fig. 1). The mean GCC allele frequency was estimated to be about 5 %, being almost 0 % for Asian populations to about 10 % for European populations [8].

Here we describe the experimental procedure employed to analyze the transcriptional activity of the Ala87 variant of ERα (ERAla87) comparatively to the ERα wildtype (WT), in two genomic pathways, the classical ERE pathway and the nonclassical genomic AP-1pathway. In order to compare the transcriptional activity of ERAla87 and WT ERα, cells lines that do not express a functional receptor are transfected with constructs that encode one or the other form of ER. At the same time, cells are transfected

with a second plasmid construct which includes a reporter gene (luciferase) under the control of a sensitive promoter containing ERE elements or the AP-1 element. A third DNA construct expressing beta galactosidase is included in the transfections for normalization of the experiments.

The ER transactivation efficiency is analyzed in HepG2 and HeLa cells, two epithelial-like cell lines which exhibit divergent differentiation phenotypes. HeLa cells, derived from a cervix carcinoma, present a poorly differentiated phenotype and exhibit a cell context strictly permissive to AF2 transactivation function of ERα. In contrast, the hepatocarcinoma HepG2 cell line appears more differentiated and in these cells AF1 is the dominant transactivation function involved in ERα transcriptional activity [2].

17β-Estradiol (E2) is a natural ligand of ERα. Other compounds that bind ERα have been synthesized such as the selective estrogen receptor degraders (SERDs) which lead to degradation of the receptor, and selective estrogen receptor modulators (SERMs) that exhibit mixed agonist and antagonist effects in a tissue-specific manner [9]. In this study, the transcriptional activity of ERα was studied in presence of E2, the SERD ICI 182,780 (ICI), and the SERM 4-hydroxytamoxifen (4-OHT), in order to characterize the transactivation properties of the variant ERAla87. The estrogenic activity of 4-OHT exclusively depends upon AF-1 of ERα, and is therefore observed only in cells sensitive to this activation function such as the HepG2 cell line [2, 10]. 4-OHT is an agonist on the transcriptional activity of the human complement C3 promoter (which contains ERE elements) [11], meanwhile 4-OHT and ICI were previously described as potent agonists on the ERE-independent AP-1 ERα pathway [5, 12, 13].

The procedures described in this chapter allowed the demonstration that the synonymous ERAla87 variant has a differential behavior compared to ERWT in transfected cells (Fig. 2). In comparison to the wild type receptor, results show that the Ala87 mutation reduces the transactivation efficiency of ERα on an ERE reporter gene (Fig. 2a) while its expression level remains similar. In contrast, this mutation enhances 4-OHT-induced transactivation of ERα on an AP1 reporter gene (Fig. 2c), suggesting the ability to establish other interactions as well. Finally, the mutation affects the subcellular localization of ERα in a cell type-specific manner [14]. We showed that the mutation does not affect the abundance of the ER mRNA as evaluated by real time RT-PCR, nor the expression level of the receptor in HepG2 or HeLa cells as determined by western blotting. The observed differences of the ERAla87 could be explained by subtle differences of conformation respect to the ERWT in the assayed transfected cells. The functional alteration of the ERAla87 reported in this work highlights the relevance of synonymous mutations for protein function and hence for biomedical and pharmacological points of view.

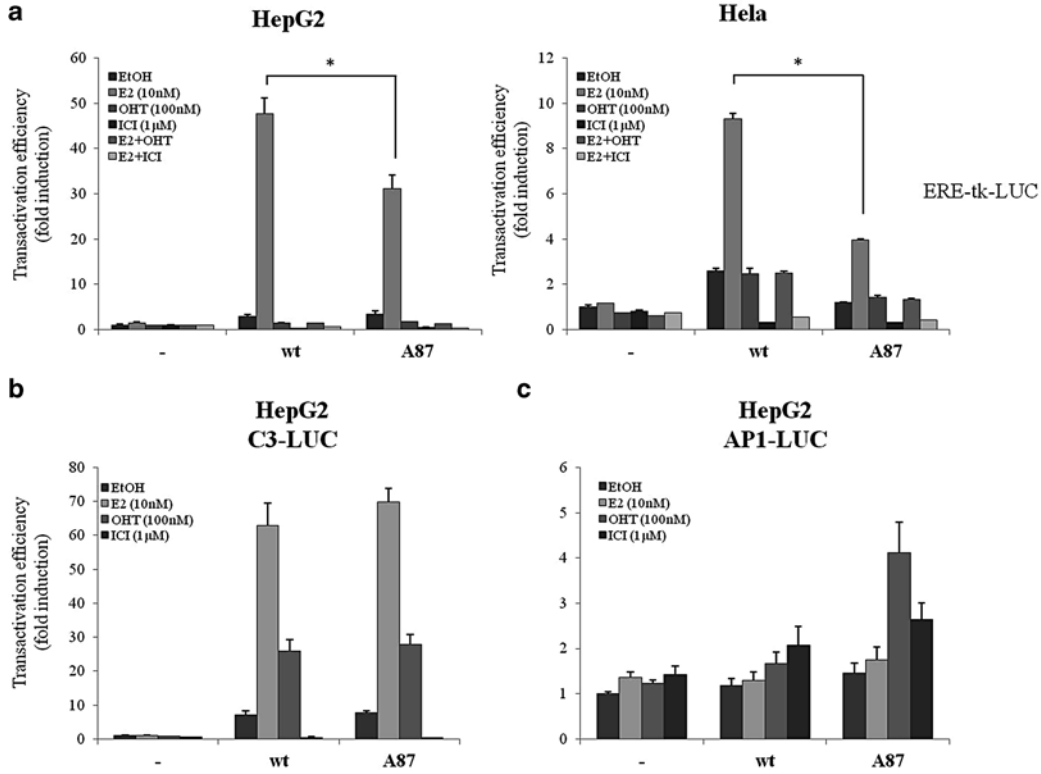

Fig. 2 Synonymous mutation Ala87 affects ER transactivation efficiency. ERα WT (wt) and ERAlaA87 (A87) transcriptional activity was determined in transfected HepG2 and HeLa cells on a ERE-luciferase reporter gene and in transfected HepG2 cells on the C3-luciferase and AP1-luciferase reporter genes. Cell lines were co-transfected with ERE-tk-LUC (100 ng) (**a**), C3-LUC (200 ng) (**b**) or AP1-LUC (200 ng) (**c**), together with pCMV SPORT-βgal (100 ng) and empty pSG5 (50 ng), pSG5-ERα (50 ng) or pSG5-ERαA87 (A87, 50 ng). Cells were treated for 24 h with EtOH as a control, 10 nM E2, 100 nM 4-hydroxytamoxifen (OHT), 100 nM ICI 182,780 (ICI), E2 + OHT or E2 + ICI. Luciferase activity was normalized against β-galactosidase activity and was expressed as fold increase above values measured with empty pSG5 treated with EtOH. Data corresponds to the mean ± SE of at least three independent experiments. $*p < 0.001$ two-way ANOVA with Bonferroni posttest. Reproduced from [14] with permission from Elsevier. Notice that the range of transactivation efficiency values (expressed as fold induction) varies among cell lines for the same reporter gene. Cell context permissiveness to different transactivation functions of ERα is one of the underlying causes for this. HeLa cells exhibit a cell context strictly permissive to AF2 transactivation function of ERα while in HepG2 cells AF1 is the dominant transactivation function involved in ERα transcriptional activity

2 Materials

HPLC-grade water or water with a similar purity grade (resistivity 18.2 MΩ) is recommended to prepare all solutions and suspensions.

2.1 Components for Cell Culture and Transfection

1. HeLa and HepG2 cell lines (American Type Culture Collection).
2. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL).

3. Transfection medium: Phenol red-free DMEM supplemented with 2.5 % charcoal-stripped calf serum (*see* **Note 1**).
4. Phosphate-buffered saline (PBS).
5. Transfection reagent such as jetPEI reagent (Polyplus).
6. Plasmids: pSG5, pSG5-ERwt, pSG5-ERAla87, ERE-tk-LUC, C3-LUC, AP1-LUC, and pCMV SPORT-βgal (*see* **Note 2**).
7. 24-Well cell culture plates, 5 % CO_2 incubator, sterile polypropylene tubes, microfuge, pipets, sterile pipette tips.
8. 150 mM NaCl.
9. Stock solution of ligands (E2, 4-OHT and ICI). Prepare stock solutions in ethanol following the manufacturer's instructions.
10. Reporter Lysis 5× Buffer (Promega).

2.2 Components for β-Galactosidase Assay

1. 0.1 M Phosphate buffer: pH 7.3. Mix 5.82 g of Na_2HPO_4 and 1.24 g of NaH_2PO_4 and transfer to a 500 mL graduated cylinder. Add water to a volume of 400 mL. Mix and check that the correct pH for the buffer is reached. Adjust slightly as necessary to pH 7.3, using phosphoric acid or sodium hydroxide. Raise volume to 500 mL with water. Store at 4 °C.
2. ONPG 4 mg/mL: Add 250 mg of *o*-nitrophenyl-beta-D-galactopyranoside to a 500 mL graduated cylinder. Add 0.1 M phosphate buffer, pH 7.3, to a volume of 200 mL. Gently warm with stirring in order to completely dissolve the product. Raise volume to 250 mL with 0.1 M phosphate buffer, pH 7.3. Make 10 mL aliquots and store at –20 °C.
3. 100× β-mercaptoethanol-$MgCl_2$: 0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol. To make, weigh 0.2 g $MgCl_2$ and add 3.19 mL β-mercaptoethanol. Raise volume to 10 mL with water. Store at 4 °C.
4. 96-Well transparent plates.
5. Microplate reader.
6. Mix for β-galactosidase assay: For 1 point mix 142 µL of 0.1 M phosphate buffer pH 7.3, 2.5 µL of β-mercaptoethanol-$MgCl_2$ 100× and 55 µL of ONPG 4 mg/mL.

2.3 Components for Luciferase Assay

1. Luciferase Assay System kit (Promega).
2. 96-Well opaque plates (*see* **Note 3**).
3. Luminometer.

3 Methods

In order to compare the ERAla87 to the ERWT transcriptional activity, HepG2 and HeLa cells, which do not express a functional receptor, are transfected with pSG5 vector encoding one of the

two ERα proteins. The pSG5-ERWT vector encodes the ERWT protein while the pSG5-ERAla87 vector encodes the ERAla87 protein. At the same time, cells are transfected with a second construct which includes luciferase as a reporter gene, under the control of a sensitive promoter. When analyzing the ERα transcriptional activity through the classical ERE pathway the vector used is the ERE-tk-LUC or the C3-LUC whose promoters contain ERE elements. Meanwhile when analyzing the ERα transcriptional activity through the nonclassical genomic AP-1pathway, the vector used is AP1-LUC whose promoter contains the AP-1-responsive element. For normalization of luciferase activity through the different experiments, the pCMV SPORT-gal plasmid encoding galactosidase is included in transfection. After 24 h of transfection, cells are treated for another 24 h with one of the ligands: E2, ICI, or 4-OHT. Cell lysates are prepared and luciferase and galactosidase activity are measured by light detection or color development following enzymatic reaction with their corresponding substrates. A general outline of the protocol is given in Fig. 3. All steps should be carried out at room temperature unless otherwise specified.

Notice that estrogenic activity of 4-OHT exclusively depends upon AF1 of ERα, and is therefore observed only in cells sensitive to this activation function such as the HepG2 cell line. As the 4-OHT is a potent agonist on the transcriptional activity of the human complement C3 promoter and of the AP-1 promoter, HepG2 cells are used for determining transcriptional activity of both promoters rather than HeLa cells.

Performing transfection experiments at least in triplicate is recommended to allow statistical analysis of the results.

3.1 Cell Culture and Transient Transfection

Keeping cells free from contamination is a critical factor for cell culture success. Use sterile materials and always follow an aseptic technique to reduce the probability of contamination.

1. The day before transfection trypsinize and count cells. Plate cells in cell culture medium at a density of 1×10^5 cells per well of a 24-well plate (*see* **Note 4**).
2. Before transfection wash cells with 0.5 mL PBS to remove all traces of cell culture medium. Add 0.5 mL of transfection medium on each well.
3. In a polypropylene tube dilute 500 ng of DNA/well in 150 mM NaCl, to a final volume of 50 μL. As a starting point use 200 ng of reporter gene (ERE-tk-LUC, AP1-LUC, or C3-LUC), 100 ng of pCMV SPORT-gal internal control, 50 ng of expression vectors (pSG5, pSG5-ERα or pSG5-ERAla87), and 150 ng of empty vector (pSG5) to make up to 500 ng total DNA per well (*see* **Note 5**).
4. Dilute 2 μL of jetPEI reagent/well into 48 μL in a second polypropylene tube and mix. Incubate for 5 min at room temperature (*see* **Note 6**).

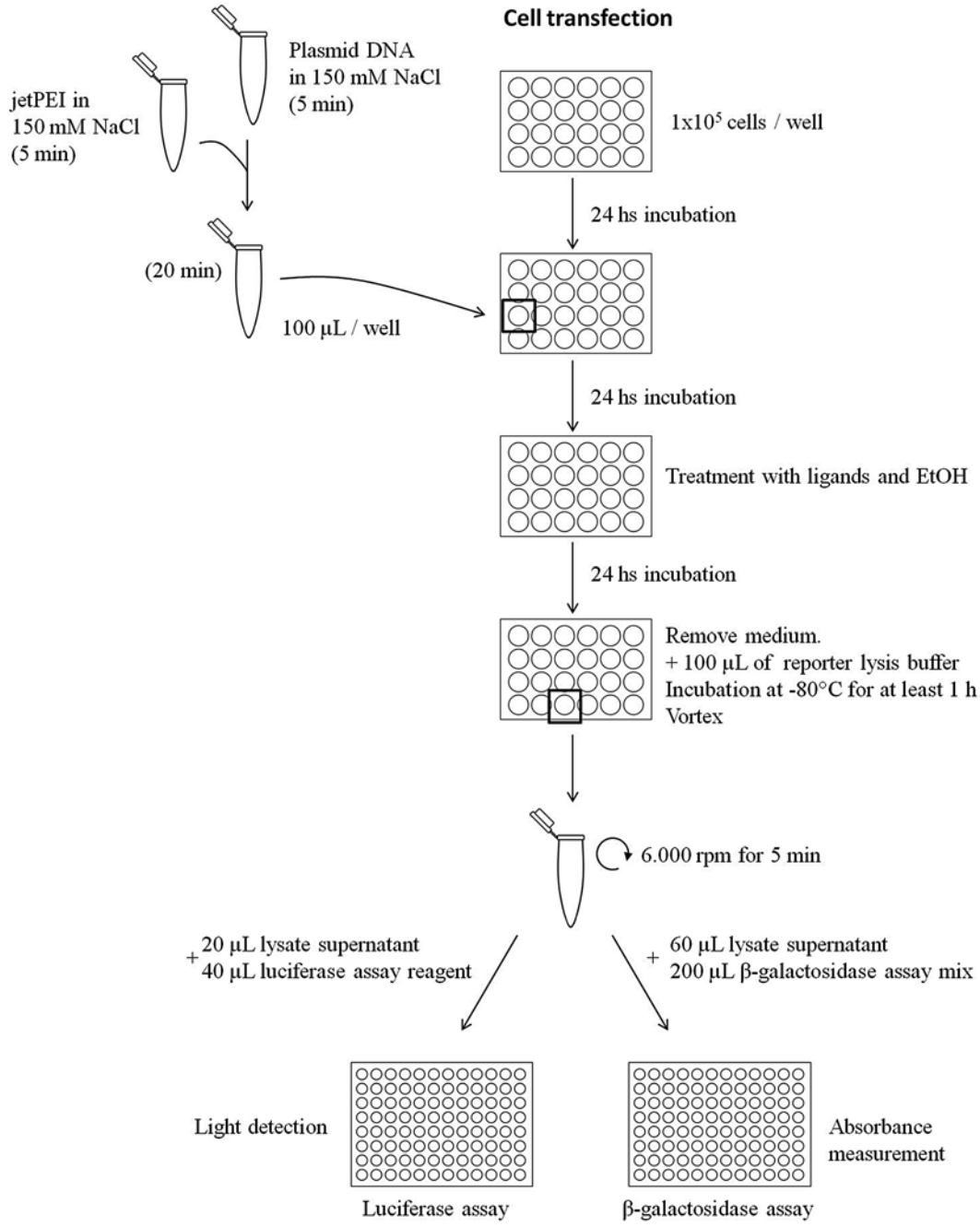

Fig. 3 Schematic representation of main protocol steps

5. Add diluted DNA into diluted jetPEI reagent, vortex, and spin down briefly. Incubate for 20 min at room temperature.
6. Add 100 µL of the jetPEI/DNA mix per well. Mix by gently swirling the plate. Incubate 24 h at 37 °C in 5 % CO_2 incubator.

7. Treat cells for 24 h with ligands or ethanol (vehicle control). Use as final concentration of ligands: 10 nM E2, 1 μM 4-OHT and 1 μM ICI diluted in transfection medium. Maintain 0.5 mL as final volume for each well.
8. Remove medium from the wells and add 100 μL of reporter lysis buffer. Store at −80 °C for at least 1 h (*see* **Note 7**).
9. Vortex the plate carefully. Transfer the cell lysate to a polypropylene tube and centrifuge at 6000 × *g* for 5 min. Transfer cell lysate supernatant to a new polypropylene tube and save it (*see* **Note 8**).

3.2 β-Galactosidase Assay

1. Add 60 μL of lysate supernatant into a well of a transparent 96-well plate. Add 200 μL of substrate mix for β-galactosidase assay to each well. Add 200 μL of substrate mix for β-galactosidase assay to three empty wells to use as blank samples.
2. Incubate until a faint yellow color has developed (*see* **Note 9**).
3. Take the plate to the microplate reader and measure absorbance at 405 nm.

3.3 Luciferase Assay

1. Add 10–20 μL of HeLa/HepG2 lysed cells into a well of an opaque 96-well plate.
2. Add 40 μL of Luciferase Assay Reagent per well (*see* **Note 10**). Immediately measure the light produced with the luminometer (*see* **Note 11**).

3.4 Activity

1. Compute galactosidase activity by subtracting the blank samples mean to each of the other samples.
2. Normalize luciferase activity against galactosidase activity within each sample.
3. Express normalized luciferase activity as a fold increase above the mean value measured with empty pSG5 treated with EtOH (*see* **Note 12**).
4. Compute average activity and the standard error within each condition.

4 Notes

1. In the charcoal stripped serum, lipid-like components including hormones, retinoids and fatty acid ligands of nuclear receptor transcription factors are depleted. Using charcoal-stripped calf serum in transfection medium is imperative so that these compounds will not interfere with the experiment.
2. DNA quality is a critical factor in transfection experiments. Plasmid quality and concentration must be thoroughly checked

before starting. A260/A280 ratio above 1.6 is recommended. DNA integrity can be verified on an agarose gel. At least 90 % of the DNA should be in the supercoiled conformation and no degradation products should be visible.

3. Do not use transparent plates. In transparent plates luminescence signals from adjacent wells can interfere with the signal that is being measured.
4. One of the most important factors to obtain reproducibility in transfection efficiency is working with a consistent number of cells. Transfection efficiency is sensitive to culture confluency; thus starting culture density should be optimized for each cell line. For an optimal transfection, jETPEI manual recommends 50–70 % of cell confluence on the day of transfection which can be achieved in a 24-well culture plate seeding 50,000–100,000 cells the day before transfection.
5. Plasmid/transfection reagent relation should be previously optimized for each transfection.
6. To account for normal loss during pipetting, all reagents should be prepared in excess.
7. Cells can be stored at –80 °C up to 1 month.
8. The lysates may be assayed directly or stored at –80 °C for up to 2 months.
9. Time elapsed for yellow color development may vary among cell lines. Color development in HeLa lysates is almost immediate. However HepG2 lysates should be incubated at 37 °C for at least 2 h for a slight yellow color appearance.
10. Do not forget to thaw luciferase substrate before starting the assay. Substrate should be at room temperature when performing assay. Maintain substrate in the dark.
11. Read time and delay reading time between two consecutive wells should be optimized for each experiment in order to get optimal light intensity and no interference between well measures. The typical delay time is 2 s, and the typical read time is 10 s. As light intensity of the reaction decays slowly, reading luciferase activity for several samples at the same time is not encouraged. When processing many samples luciferase assay reagent should be added to a few samples to measure light intensity (one row of a 96-well plate for example). Then, after completing measures of the first set, another set of samples can be processed.
12. The range of transactivation efficiency values (expressed as fold induction) varies among cell lines for the same reporter gene. Cell context permissiveness to different transactivation functions of ERα is one of the underlying causes for this. HeLa cells exhibit a cell context strictly permissive to AF2 transactivation function of ERα while in HepG2 cell line AF1 is the dominant

transactivation function involved in ERα transcriptional activity. As an example, ranges of transactivation efficiency values expressed as fold induction for ERE-tk-LUC induction in HeLa cells are usually below 10 while in HepG2 cells are usually above 40.

Acknowledgments

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Chapter 23

Selective Estrogen Receptor Modulators and the Tissue-Selective Estrogen Complex: Analysis of Cell Type-Specific Effects Using In Vivo Imaging of a Reporter Mouse Model

Sara Della Torre and Paolo Ciana

Abstract

Selective estrogen receptor modulators (SERMs) are a class of compounds that act differentially on the estrogen receptor (ER) in various tissues with a mixed agonist/antagonistic activity (agonistic in some tissues while antagonist in others). This peculiarity represents a challenge for developing new hormone replacement therapies (HRTs) and highlights the need of new tools to evaluate the specific effects of a given SERM in different organs/tissues of an entire organism and with time.

Reporter mice represent invaluable tools in pharmacology to analyze specific signaling in physiological conditions and monitor the effects of drugs acting on these signals in a *spatio*-temporal dimension. Here, we describe an in vivo protocol to examine the effects of different SERMs on estrogen receptor activity by using the ERE-Luc reporter model, a mouse that reports ER transcriptional activity.

Key words Reporter mice, Estrogen receptors, Luciferase, In vivo imaging, Selective estrogen receptor modulators (SERMs), Longitudinal studies, Clustering analysis

1 Introduction

Selective estrogen receptor modulators (SERMs) are structurally different compounds with a mixed agonist/antagonistic activity that interact with intracellular estrogen receptors (ERα and/or ERβ) in target cells and exert tissue-specific estrogen- or antiestrogen-like actions. Like any estrogenic compound, SERMs directly bind to ERs (α and/or β) which undergo structural modifications, dissociate from heat-shock proteins (HSPs), and directly bind to specific DNA sequences (named estrogen-responsive element or ERE) in the regulatory promoter regions of target genes, and promote or inhibit the transcription of those genes through the interaction with co-activators or co-repressors, respectively [1].

The current view is that the mixed agonist/antagonistic activity of SERMs are ascribable in part to ER conformational changes induced by SERM binding, resulting in a variety of specific interactions

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with other proteins, including co-regulator, but possibly also other signalling proteins such as other transcription factors or second messengers. Finally, SERMs may also regulate the activity of membrane-bound ERs (G protein-coupled receptor, GPR30) [2].

By such a complex mechanism of action, a SERM may act on ER activity by modulating a large number of physiologically relevant processes with consequences for health. Over the past decade the peculiarity of SERM action to enhance estrogenic benefits in certain tissues (i.e., bone and serum lipids) and minimize estrogenic risks in other tissues (i.e., breast tissue and uterus) has suggested the use of these molecules in the postmenopausal women's health.

Yet, in spite of the complexity underlying the mechanism of action of SERMs and their potential for adverse effects, the search for the "ideal" SERM is still ongoing and emphasizes the need for new experimental tools to test the activity of these substances on ER signalling in different tissues. Although animals are routinely involved in testing new SERMs, the multifaceted effects of these compounds in each tissue with time and in relation to the physiological/pathological context requires a *spatio*-temporal analysis which requires heavy labor and intensive use of animals.

To overcome these limitations, this protocol demonstrates the suitability of the ERE-Luc reporter mice as tools for the evaluation of the *spatio*-temporal action of SERMs. The ERE-Luc mice are genetically modified to respond to a specific stimulus with the production of a protein "reporter" (the luciferase), whose activity is easily detected and quantified by in vivo and ex vivo imaging. The ERE-Luc mouse was engineered with the random integration of a construct containing two ERE sequences driving the gene encoding the firefly luciferase, flanked by insulator sequences that protect the construct from the influences of the surrounding chromatin and ensure the ubiquitous expression of the transgene in all cells [3]. This bioluminescent reporter model was shown to be well suited for the *spatio*-temporal study of ER transcriptional activity in vivo and the screening and identification of compounds acting on ERs, including new SERMs.

Thus the reporter mouse, by enabling the in vivo study of estrogenic compounds, besides providing rapid and very reliable results, responds very well to the *3R* philosophy (*r*eduction, *r*eplacement, *r*efinement), enabling a significant reduction of the number of animals to be used.

In this chapter we describe a standard protocol used to study in acute (6 h) and in chronic (21 days) experiments the effects of raloxifene (RAL), bazedoxifene (BZA), and tissue-selective estrogen conjugate (TSEC), a new concept of SERMS based on the combination of bazedoxifene *plus* conjugated estrogen (CE) [4]. To unravel the estrogenic or anti-estrogenic activity of these compounds in the different tissue types, we compared the activity

exerted by these SERMs on ER signaling in the different tissues with those of female mice in physiological conditions (fertile females with a regular estrous cycle, CYC), in pathological conditions mimicking postmenopause (surgical ovariectomy, OVX), and in conditions mimicking HRT-treated postmenopausal women (OVX + CE).

2 Materials

Prepare all solutions using ultrapure water (resistivity 18.2 MΩ) and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise.

2.1 Transgenic Reporter Mice and Treatments

1. Heterozygous C57BL/6 ERE-Luc [3] female mice.
2. Test compounds: Conjugated estrogen (CE, 3 mg/kg), bazedoxifene (BZA, 2 or 10 mg/kg), tissue selective estrogen complex (TSEC = CE 3 mg/kg + BZA 2 or 10 mg/kg), raloxifene (RAL, 2 or 10 mg/kg). Dissolve all test compounds in dimethyl sulfoxide and subsequently dilute in the vehicle (2 % Tween 80 and 0.5 % carboxymethyl cellulose water solution).

2.2 In Vivo and Ex Vivo Bioluminescence Imaging

1. Syringes and micro-dissection tools, including forceps, scissors, and blades.
2. Anesthetic solution for mice: Isoflurane and Xenogen XGI-8 Gas Anesthesia System (Caliper Life Sciences) with the following setting: vaporizer value 2.5 %, oxygen flow 1.5 L/min in the induction chamber and 0.25 L/min to the mice during the in vivo imaging in the CCD-Camera.
3. Luciferin: D-Luciferin powder (beetle luciferin potassium salt, Promega) resuspended 25 mg/mL in water for injection.
4. Bioluminescence imaging unit: IVIS Lumina System (Caliper, PerkinElmer) consisting of a scientific grade thermoelectrically cooled CCD camera mounted on a light-tight imaging chamber.

2.3 Luciferase Enzymatic Assay

1. Tissue homogenizer (TissueLyser, Qiagen), including 5 mm inox beads and bead dispenser (Qiagen).
2. Refrigerated centrifuge with rotor for 96-well plates.
3. Microplate luminometer (Glomax, Promega).
4. Microplate reader with filter at 595 nm.
5. Single and multichannel pipettors.
6. Racked 1.2 mL deepwell strips (Starlab).
7. Transparent 96-well microplates.
8. Opaque white (not transparent) 96-well microplates (Corning).

9. Phosphate lysis buffer: add 25 mL 0.2 M Phosphate buffer, 50 µL 1 M DTT, 1 mL 0.2 M EGTA, 0.4 mL 0.5 M sterile EDTA, and double-distilled water to 50 mL. Store at 4 °C.
10. Recombinant firefly luciferase (QuantiLum, Promega).
11. Luciferase Assay System (Promega): (1) trycine buffer to dilute recombinant luciferase and (2) luciferin buffer, containing a patented mix of luciferase substrates and coenzymes including D-luciferin, ATP, Mg^{2+}, CoA.
12. Bradford Protein Assay Kit (Pierce), including protein standards.
13. Bovine serum albumin (BSA), lyophilized powder.

2.4 Analysis of Imaging Data

1. Imaging workstations and luminometers include proprietary software to operate the devices and allow the direct export of raw data to Microsoft Excel.
2. Perform statistical analyses with GraphPad Prism Software or equivalent.

3 Methods

3.1 Transgenic Reporter Mice and Treatments

1. House heterozygous C57BL/6 ERE-Luc [3] females individually in ventilated plastic cages with hardwood chip bedding. Feed ad libitum with a standard diet and provide with filtered water.
2. Maintain the animal room within a temperature range of 22–25 °C, relative humidity of 50 ± 10 %, and under an automatic cycle of 12-h light, 12-h dark (lights on at 0700 h). Ovariectomize the mice 3 weeks before the beginning of the study. Carry out the study in the mice at 2–3 months of age.
3. Three weeks after ovariectomy, assign ERE-Luc females to a specific experimental group (*see* **Note 1**).
4. Dissolve all test compounds in dimethyl sulfoxide and subsequently dilute in vehicle (2 % Tween 80 and 0.5 % carboxymethyl cellulose water solution).
5. Treat control animals (fertile cycling females and ovariectomized females) with vehicle.
6. Treat the animals by gavage for 6 h (acute treatment) or daily for 21 days (chronic treatment) with: vehicle (2 % Tween 80 and 0.5 % carboxymethyl cellulose water solution), conjugated estrogen (CE, 3 mg/kg), bazedoxifen (BZA, 2 or 10 mg/kg), TSEC (TSEC = CE 3 mg/kg + BZA 2 or 10 mg/kg), or raloxifene (RAL, 2 or 10 mg/kg).
7. Administer compounds at 9000 h and carry out the in vivo imaging sessions at 1500 h. It is advisable to measure bioluminescence

in the early afternoon (1500 h) to avoid confounding effects due to food consumption that occur mostly during the night and that are known to induce ER activity, particularly in liver [5] (*see* **Note 2**).

3.2 Bioluminescence Imaging

1. Anesthetize the reporter mice by gas anesthesia (expose the mouse to isoflurane for 2 min) prior to injecting them intraperitoneally (i.p.) with 80 mg/kg D-luciferin (*see* **Note 3**).
2. Take a photo of the mouse in dimmed light.
3. Turn on the imaging machine 10–20 min prior to beginning the imaging session to pre-cool the camera to −80 °C.
4. Measure photon emission 15 min after the i.p. injection (generally, photon emission is maximal between 10 and 25 min after the i.p. injection and then gradually decreases) (*see* **Note 4**).
5. The analysis of luciferase activity will be performed by measuring the photons emitted in the head, thymus, hepatic area, abdominal area, breast, limb, genital area, and tail, areas defined in Fig. 1a. Express data as photon counts/time/surface unit to assess the relative effect of the treatments in the different body areas.
6. Plotting the photon emission from the specific body areas as means ± SEM (Fig. 1b) and as plotted lines (Fig. 1c) will allow identification of the signal, the noise, and the signal/noise ratio.

3.3 Longitudinal Analysis of Fertile Cycling Reporter Mice

The profile of photon emission in cycling females changes during time in the various body areas and is ascribable to the sum of the liganded and unliganded factors that act on ER by modulating its activity during the normal ovarian cycle production of endogenous estrogen of the mouse and its downstream signaling. The physiological oscillation of ER activity in a tissue is associated with the modulation of specific target genes and pathways (Fig. 1b); the lack of estrogen signaling with ovariectomy impairs ER activity (Fig. 1c).

1. Treat control animals (fertile cycling females and ovariectomized females) daily with vehicle by gavage.
2. Carry out the treatment sessions at 9000 h and the in vivo imaging sessions at 1500 h on a daily basis.
3. Image bioluminescence as in Subheading 3.2 daily to obtain profiles of tissue-specific estrogen activity that correlate with the endogenous cyclic production of estrogen (*see* **Note 5**).

3.4 Screening of Pharmacological Compounds: A Dose-Response Analysis

Before beginning a long-term study with different estrogenic substances or SERMs, a study should first be performed in a small number of animals to evaluate the specificity of action of the given compound in each tissue and its ability to regulate the luciferase reporter when administered orally. The dose-response analysis is

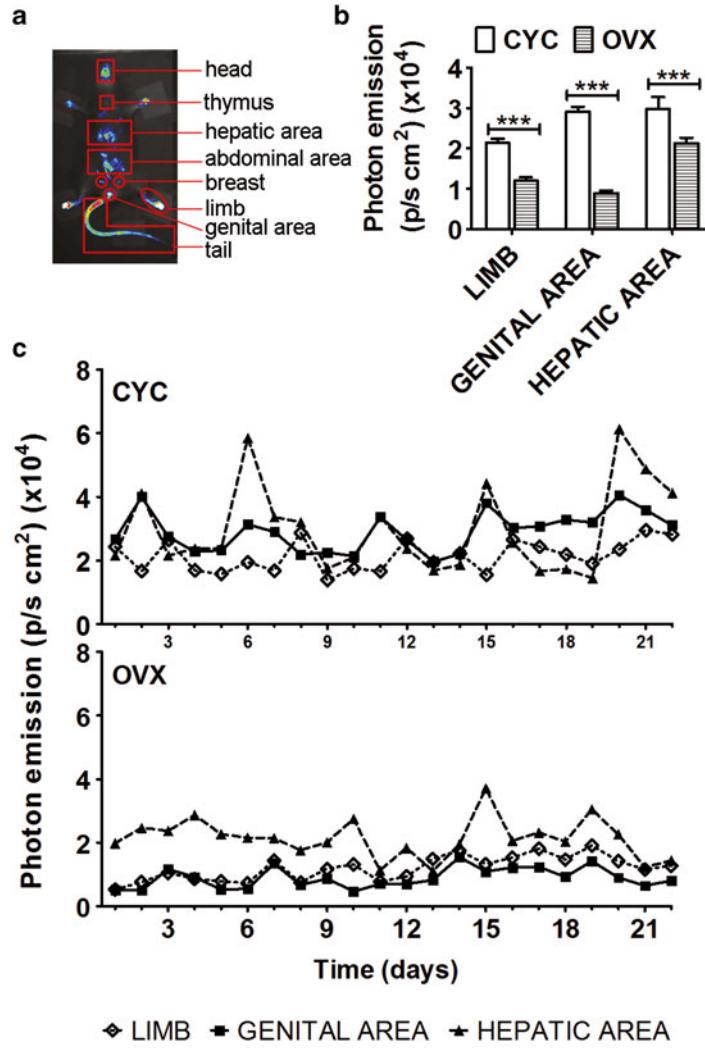

Fig. 1 In vivo measurement of ER activity in the ERE-Luc mouse. (**a**) After acquisition of total photon emission and reconstruction in pseudocolors of the regions where such emission occurs, the analysis can be improved by the definition of more selected body areas in which photon emission is measured. The figure represents the selections used for the quantification of photons emitted in head, thymus, hepatic, abdominal and genital area, breast, limb and tail. (**b**) Photon emission (photons/s/cm^2) of head, genital, and hepatic areas of CYC and OVX ERE-Luc females expressed as mean ± SEM. (**c**) Profile of photon emission in time in CYC and OVX ERE-Luc mice. The figure represents daily photon emission (plotted as photons/s/cm^2) in head, genital, and hepatic areas of a single mouse representative of the pattern of ER activity in intact (*upper*) and ovx (*bottom*) female mice. Reproduced from [13] with permission from Endocrinology

useful to identify the concentration necessary to provide the best signal-to-noise ratio in the desired target tissue. The preliminary study can also be designed to evaluate the time course and dose-response of the test substances. As an example, a dose-response

study was carried out to test a series of SERMs. Conjugated estrogen (CE, 3 mg/kg) was used as the reference ligand of known activity. The selection of the dosages to be administered with regard to BZA, and RAL (2 and 10 mg/kg), as well as CE, was based on previous reports and related to dosages previously found to mimic hormone therapy in humans [6–9]. For treatment with TSEC, two concentrations of BZA (2 and 10 mg/kg BZA + 3 mg/kg CE) similar to those of the other SERMs were chosen to identify the concentration necessary and sufficient to block CE effects when used in combined therapy (*see* **Note 6**).

1. For testing dose-responses, treat groups of five to eight mice by gavage with vehicle or one of the test substances (CE, BZA, RAL, or BZA + CE) at each of the proposed doses.
2. Measure whole body photon emission in each animal 6 h after treatment.
3. For the time course analysis, analyze whole body photon emission between 1 and 24 h after treatment with the estrogenic substance.
4. For a comparative analysis of the maximal effect of each compound in target tissues, measure photon emission in selected body areas as shown in Fig. 1a.
5. Express the data as photon counts/time/surface unit to assess the relative effect of the treatments in the different body areas as illustrated in Fig. 2.
6. This experiment defines the relative potency for each compound on the targets in different organs and defines their action after a single administration.
7. The results from dose-response and time course studies provide the foundation for long-term studies (e.g., 21 days) of the effects of estrogenic agents and SERMs in the reporter mice.

3.5 Ex Vivo Quantitative Assessment of Luciferase Activity in Tissue Extracts

At the end of the in vivo imaging experiment, tissues can be collected for the quantitative assessment of luciferase activity for a more precise analysis of the effect of the compounds in the study using the following protocol.

1. Euthanize the animals.
2. Manually dissect out the tissues of interest.
3. Immediately freeze the tissues on dry ice and store at −80 °C.
4. Homogenize the tissues by TissueLyser using 300 µL of the phosphate lysis buffer and putting a stainless steel bead in each 1.2 mL polyethylene microtube.
5. Centrifuge the homogenates at 5840 × *g* for 30 min 4 °C.
6. Transfer 20 µL of the supernatant to an opaque white 96-well plate for luminescence quantification by luminometer, with an

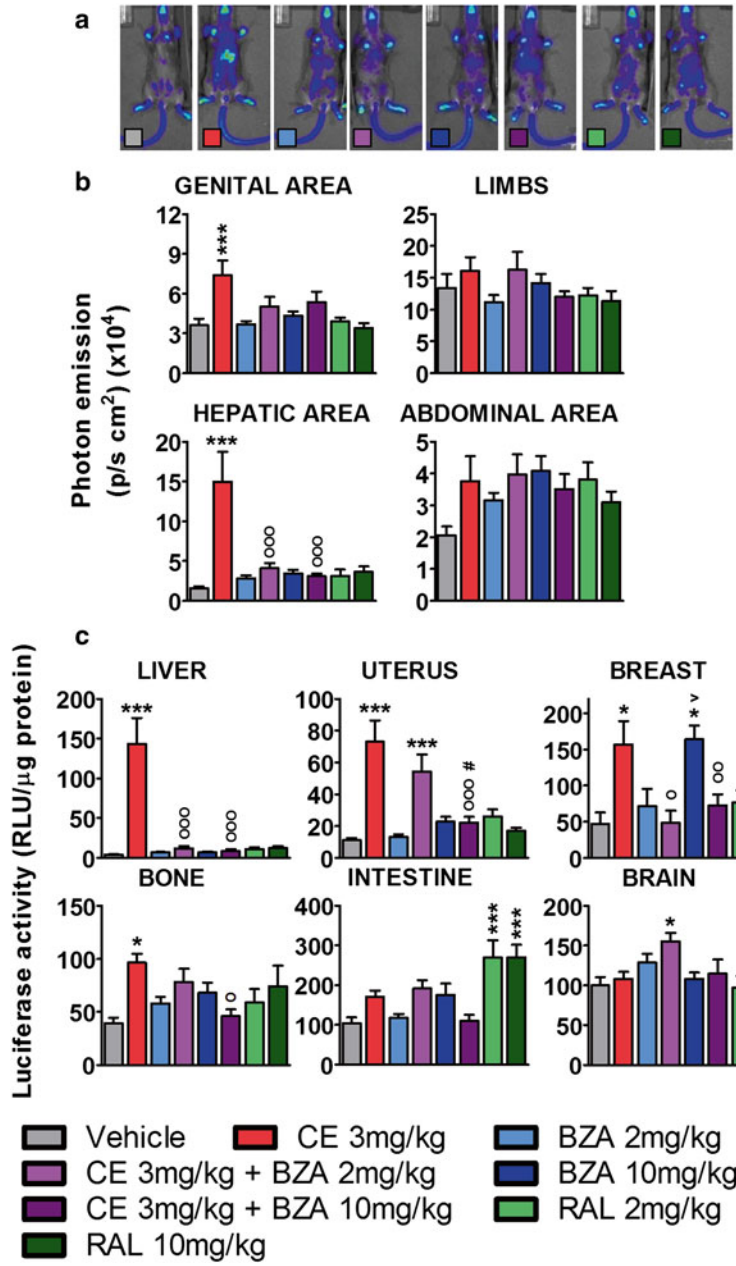

Fig. 2 A dose-response analysis of compounds with unknown pharmacological activity. (**a**) Representative pseudocolor images of photon emission from a representative animal/experimental group of OVX ERE-Luc mice 6 h after treatment with selected HRT by gavage. (**b**) Quantitative analysis of photon emission as measured by a 5 min exposure time to a CCD camera (see methodological section) in mice at 6 h after treatment with conjugated estrogen (CE, 3 mg/kg), bazedoxifene (BZA, 2 and 10 mg/kg) or BZA in association with 3 mg/kg CE (TSEC) and raloxifene (RAL, 2 and 10 mg/kg). *Columns* represent the mean ± SEM ($n = 8$). (**c**) After the imaging session, mice were euthanized and tissues rapidly dissected. Luciferase enzymatic activity was measured in tissue lysates and

integration time of 10 s, after machine-driven injection of 100 μL of luciferase assay reagent.

7. Record light measurements by the luminometer software GloMax.
8. Measure protein concentrations in the supernatants using a Bradford Protein Assay Kit, following the manufacturer's protocols and analyzing the data with a microplate reader.
9. Normalize the luminescence data to the protein content of each sample, and express the data as relative light units (RLU) per microgram of protein (Fig. 2c).

3.6 Cluster Analysis of the Potency of a SERM

As previously mentioned, reporter animals represent a unique tool for the study of the effects of novel pharmacological treatments and for the identification of undesired effects that could be additive and appear with time. Furthermore, some substances, by their chemical structures, could accumulate in fat tissue and, once mobilized, could exert their action on the whole organism by interfering with the physiological signaling of interest. The special feature of reporter mice allows one to monitor the activity of the reporter protein daily in a longitudinal study, and consequently, to evaluate the different dynamics of a specific compound in a tissue with time (Fig. 3). The bioluminescent data acquired during such an experiment can be analyzed by plotting the photon emissions measured daily in head, limbs, and genital, hepatic, and abdominal areas as a profile (Fig. 5). The profile of a specific compound should be compared with one of cycling and ovariectomized females to evaluate to what extent a given compound is able to reinstate, in that particular tissue of the ovariectomized and treated females, the ER activity typical of the physiological condition, and at the same time avoiding undesirable activation in some tissues (such as reproductive tissues).

1. Groups of a minimum of eight animals are caged separately and treated with the test substance daily by the selected route of administration (e.g., gavage).
2. Mice are subjected to bioluminescence imaging by CCD camera daily.
3. Calculate photon emissions for each tissue area of interest at 0, 3, 7, 14, and 21 days of treatment and plot the data as means ± SEM as described in Subheading 3.2 above.

Fig. 2 (continued) normalized on total protein content as RLU/μg proteins. *Columns* represent mean ± SEM ($n = 8$). *$p < 0.05$ and ***$p < 0.001$ vs. vehicle-treated animals; °$p < 0.05$, °°$p < 0.01$ and °°°$p < 0.001$ vs. CE-treated animals; >$p < 0.05$ vs. BZA 2 mg/kg; #$p < 0.05$ vs. CE 3 mg/kg + BZA 2 mg/kg. p values were calculated with one-way ANOVA followed by Bonferroni post hoc test. Reproduced from [13] with permission from Endocrinology

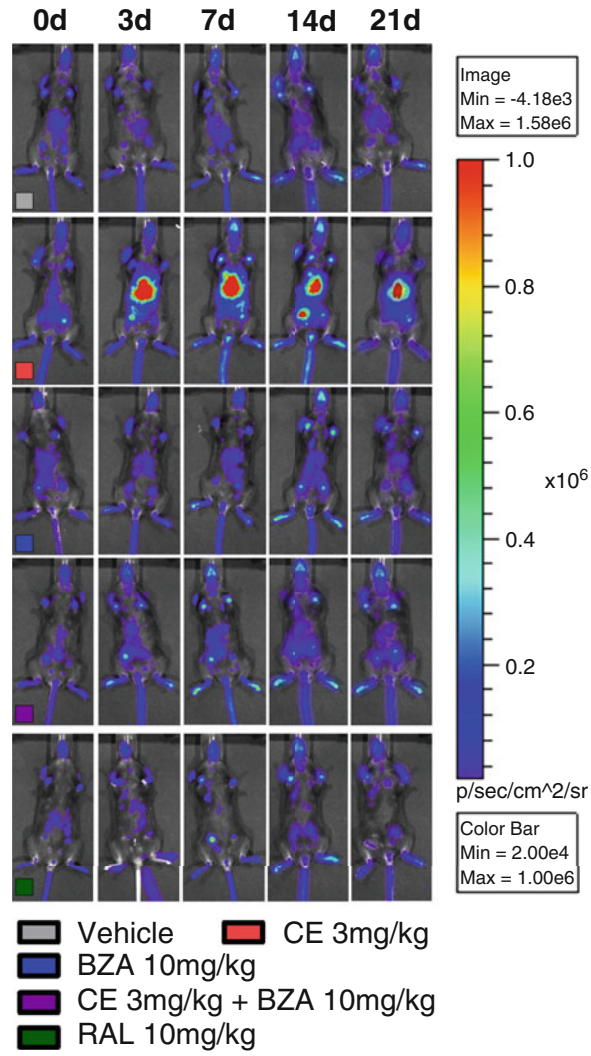

Fig. 3 Pseudocolor image of photon emission of ERE-Luc mice undergoing HRT. Pseudocolor image of photon emission from one representative animal/group at 0, 3, 7, and 21 days of chronic oral treatment with vehicle, CE (3 mg/kg), BZA (10 mg/kg), TSEC (CE3 mg/kg + BZA 10 mg/kg), and RAL (10 mg/kg). Reproduced from [13] with permission from Endocrinology

4. At the end of the 21-day-long experiment, euthanize the mice and collect and process tissues for quantitative analysis of luciferase in tissue extracts following the protocol described in Subheading 3.5.
5. Plot the data as means ± SEM (illustrated in Fig. 4).
6. Evaluate the potency of a given compound during a chronic experiment by analyzing the bioluminescent data acquired during the longitudinal study by calculating the area under

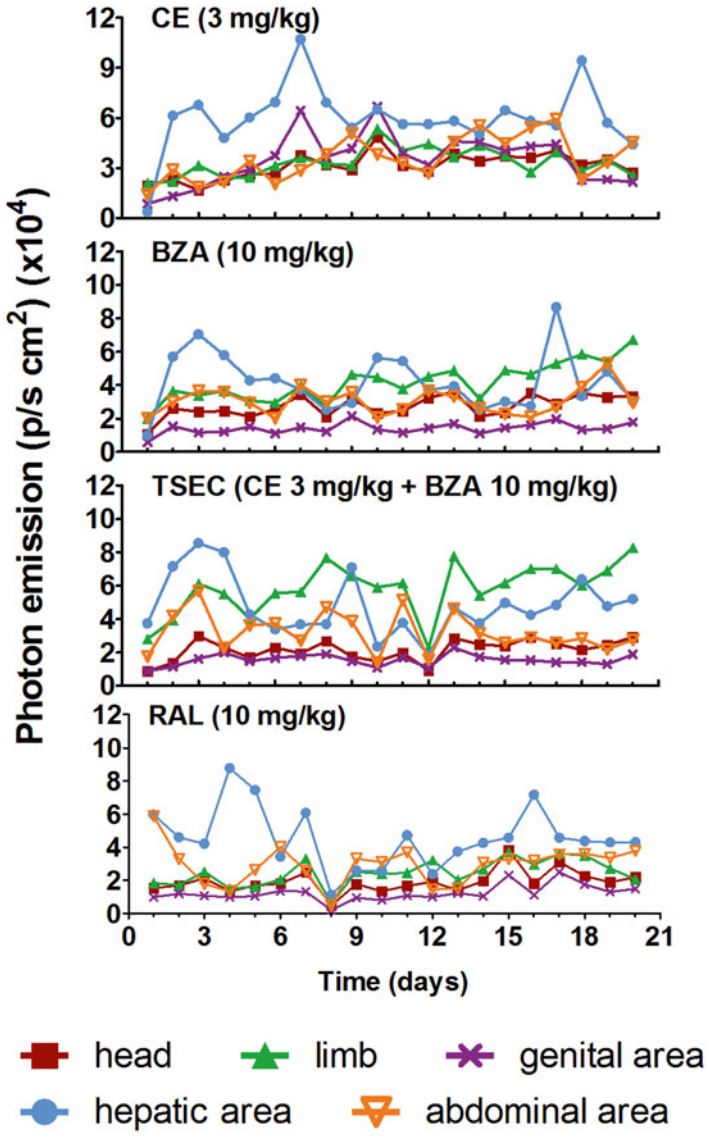

Fig. 4 Profile of photon emission in time in ERE-Luc mice undergoing HRT. Photon emission was measured daily in head, limbs, and genital, hepatic, and abdominal areas at 1500 h (6 h after the treatment). Graphs reproduce data obtained from a single, representative mouse/group. Reproduced from [13] with permission from Endocrinology

the curve (AUC) (Fig. 6a) and the coefficient of variation (%) (Fig. 6b) using statistical software such as GraphPad.

7. Perform Fourier transform analysis of the profile of ER activity in time [10] to determine the average amplitude of cycles, estimated by measuring the degree of displacement from the resting

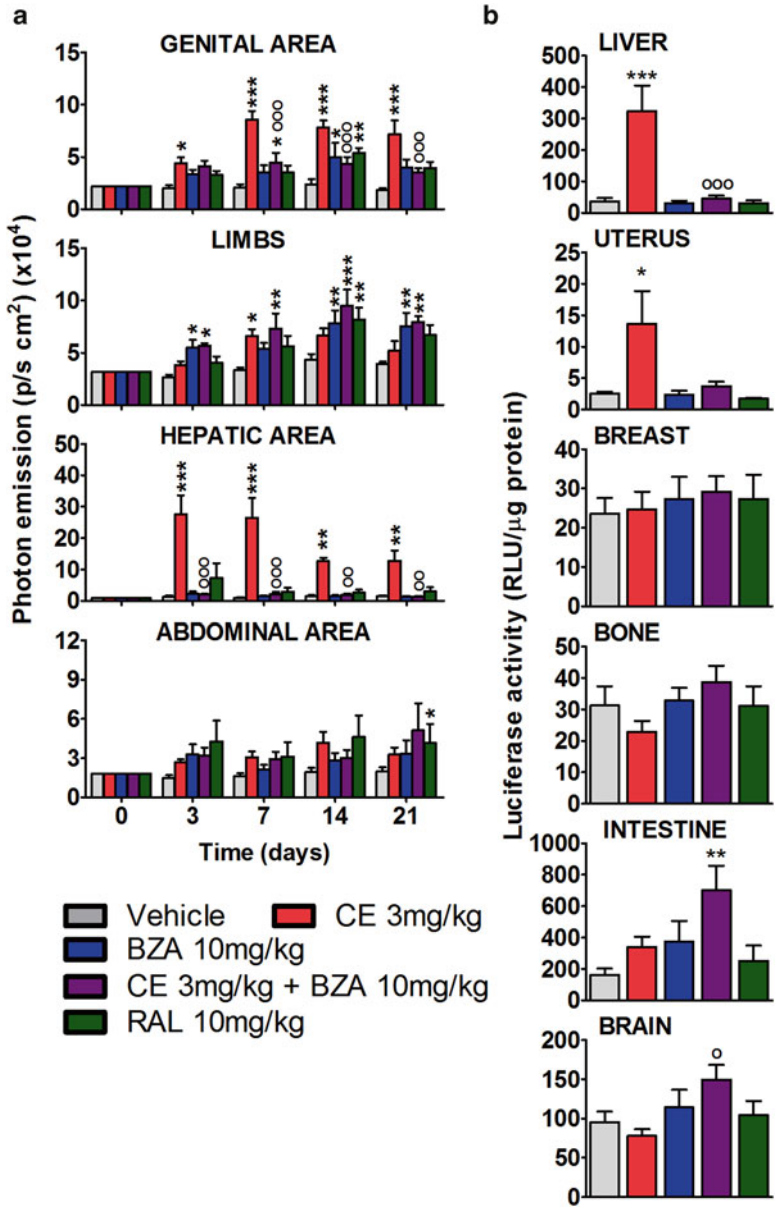

Fig. 5 Photon emission and luciferase activity of ERE-Luc mice after long-term HRT. (**a**) Photon emission measured as photons/s/cm^2 from specific body areas. Data represent mean ± SEM ($n = 8$). (**b**) After the imaging session at 21 days of treatment, mice were euthanized and tissues rapidly dissected. Luciferase enzymatic activity was measured in tissue lysates and normalized on total protein content (RLU/μg proteins; RLU = relative light units). *Columns* represent mean ± SEM ($n = 8$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. vehicle; °°$p < 0.01$ and °°°$p < 0.001$ vs. CE. p values were calculated with two-way ANOVA followed by Bonferroni post hoc test. Reproduced from [13] with permission from Endocrinology

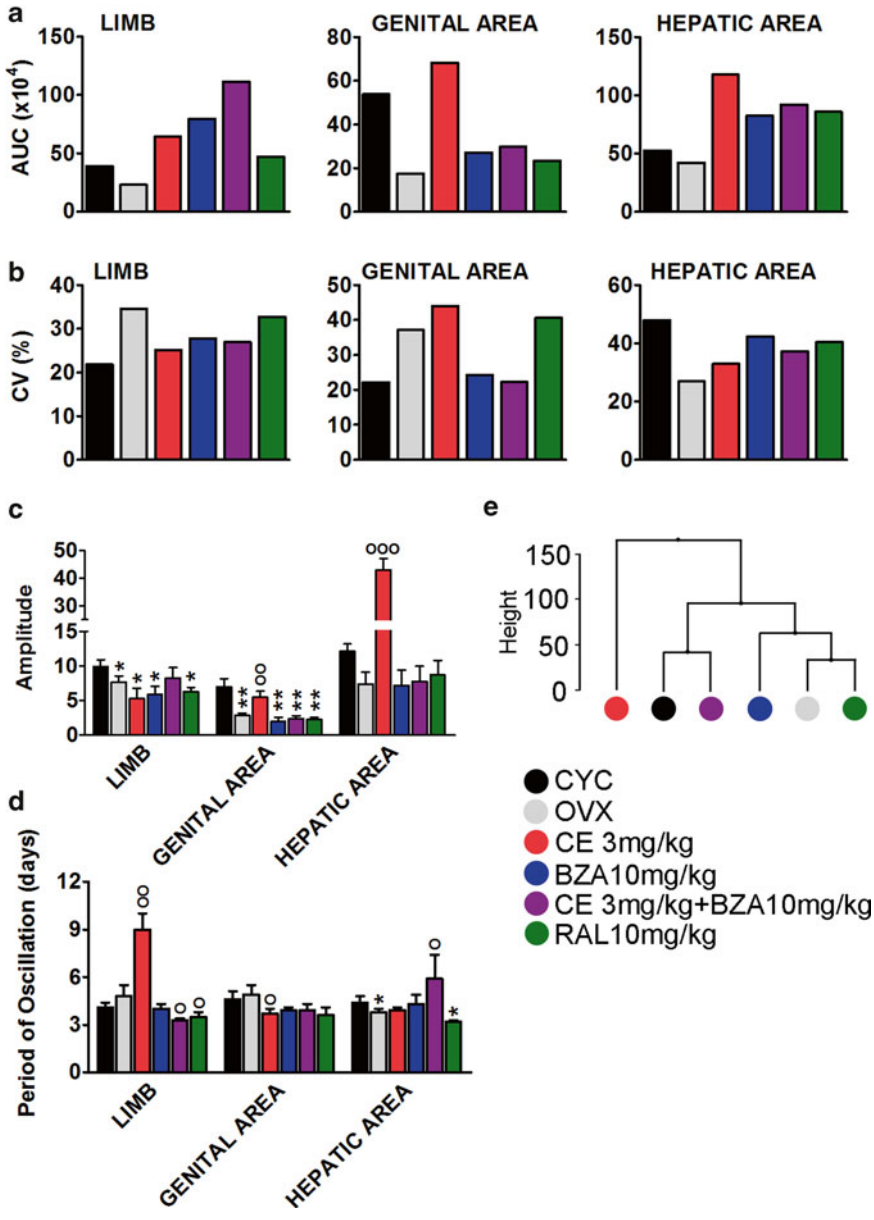

Fig. 6 Clustering analysis of photon emission of ERE-Luc mice undergoing HRT. Clustering analysis is applied to the data described in Figs. 1c and 5. (**a**) Area under the curve (AUC) and (**b**) the coefficient of variation (CV%) have been calculated with the GraphPad software. (**c** and **d**) After Fourier transform (FT), the average amplitude of cycles (**c**) in each group of mice is estimated by measuring the degree of displacement from the resting state (calculated as the square root of the 95th percentile of the power spectra), while the period of oscillation (**d**) is estimated by the inverse of the frequencies under the amplitude previously calculated. Bars represent mean ± SEM ($n = 8$). (**e**) Dendrogram analysis of the efficacy of selected HRT. The distances between branch lengths represent the distances between the physiology model (CYC) and the surgical menopause model (OVX). The efficacy of HRT is measured by its ability to mimic ER activity in the cycling mice. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. CYC; $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$ vs. OVX. p values were calculated with one-way ANOVA followed by Bonferroni *post hoc* test. Reproduced from [13] with permission from Endocrinology

state (calculated as the square root of the 95th percentile of the power spectra) (Fig. 6c) and the period of oscillation, estimated by the inverse of the frequencies under the amplitude previously calculated (Fig. 6d).

8. The combinatorial analysis of all these measurements (BLI, AUC, CV%, amplitude, and period of oscillation) will return a better evaluation of the overall effects exerted by a given SERM on ER activity and may provide independent support (or contradiction) for various hypotheses about similarity and difference.
9. The clustering analysis can be performed with a free software such as that available at the web page http://www.wessa.net/rwasp_hierarchicalclustering.wasp that will return a dendrogram showing the efficacy of selected estrogenic compound (Fig. 6e). The distances between branch lengths represent the distances between the physiological model (cycling, CYC) and the surgical ovariectomy model (OVX). The efficacy of each estrogenic compound is measured by its ability to mimic ER activity in the cycling mice: in particular, in this analysis TSEC reproduces an ER activation pattern more similar to CYC than CE or other SERMs.
10. The *spatio*-temporal analysis allowed by BLI of ER activity in the ERE-Luc reporter mouse represents a unique tool for understanding the potency of a treatment and the effects exerted by a given SERM in various tissues with time. This evaluation takes advantage of the clustering analysis performed on different measurements (such as BLI raw data, AUC, CV (%), amplitude, and period of oscillations evaluation) that can give a better measure of the overall effect of a given compound on the entire organism and could help to prevent undesirable changes on ER signaling, in particular in organs/tissues that could promote pathological complications (such as breast or uterine cancer) (*see* **Note 7**).

4 Notes

1. A study should first be first carried out in a small number of animals [3–5] to establish the sample size by power analysis; the use of too many animals wastes animals, money, time, and effort, and it is unethical. Conversely, if too few animals are used the experiment may lack power and miss a scientifically important response to the treatment. This also wastes resources and could have serious consequences, particularly in safety assessment. For further specifications, consult the web page http://www.3rs-reduction.co.uk. In the preliminary study, identification of the signal, the noise and the signal/noise ratio

will establish the power analysis and then the number of mice per experimental group required to achieve the parameters described at the website http://www.3rs-reduction.co.uk/html/6__power_and_sample_size.html.

2. Animal physiology may influence in vivo imaging, thereby increasing variability. For example, in the ERE-Luc mouse diet has been shown to strongly influence the signal due to the metabolic modulation of estrogen receptors and by the presence of phytoestrogens present in soy based diets [5, 11]. Thus mice must be kept under standard alimentary condition with phytoestrogen-free diets and care must be taken to avoid differential metabolic states among the experimental groups. It is usually not necessary to shave the mice or otherwise prepare them for imaging; however, shaving may improve the image if the signal is very low. On the other hand, we prefer to not shave the mice in a chronic experiment because the effect on health of the mice could be significant and the inflammatory process induced by shaving could affect the signal itself.
3. Influence of anesthetic on the luciferase reaction. Several anesthetics have been shown to influence luciferase activity by competing for the ATP binding pocket of the enzyme. To avoid experimental artifacts, it is therefore compulsory to carry out preliminary experiments demonstrating that the effects of the chosen anesthesia on photon emission is limited.
4. Luciferase substrate distribution: To ensure reproducible results in the semiquantitative in vivo bioluminescence imaging analysis, it is important to assess whether the enzyme substrate, D-luciferin, reaches all organs at a concentration sufficient to saturate the enzymatic activity of the reporter [12] in the experimental conditions adopted. Indeed, the diffusion of D-luciferin may change in different mice strains and in relation to the specific formulation and route of administration used. Thus, a time-course and a dose-response study of the ability of the substrate to diffuse should be carried out following the protocols which are outlined here below.
   Time-course study:
   (a) Anesthetize two to three untreated reporter mice by gas anesthesia (2 min isoflurane) prior to intraperitoneal (i.p.) injection with 80 mg/kg D-luciferin.
   (b) Take a photo of the mouse in dim light, and then measure photon emission with a series of 5 min imaging sessions. Generally, photon emission is maximal between 10 and 25 min after the i.p. injection and then gradually decreases.
   (c) The photon emission measurement in the different body areas will enable experimental establishment of the window of time necessary to guarantee the optimal distribution of

the substrate (typically 20–25 min) for the dose-response study to be carried out as follows:

(d) Anesthetize two to three reporter mice/experimental group as above.

(e) Inject the mice i.p. with increasing concentrations of D-luciferin (10, 25, and 100 mg/kg).

(f) At the time point previously selected as suitable for the optimal distribution of the substrate (~20 min), subject the animals to a 5-min imaging session.

(g) Plot the photon emission from the required specific body area to establish the concentration of luciferin sufficient to carry out future studies.

5. The search for an ideal SERM should take into consideration as a reference the oscillating ER activity showed by cycling mice in some tissues (such as bone and hepatic area) and avoid producing an increased or altered ER activation in other tissues (such as breast or uterus).
6. In order to finally elucidate its potential antagonist effect a given compound should be administered together with a known agonist.
7. Studies of various estrogenic substances and SERMs show the extent to which a given chemical entity is active in each target tissue, and on the other hand may provide unexpected results by showing that a compound may act in tissues where it was not predicted to exert any effect, and that they may exert an agonist activity in some tissues and an antagonist activity in others.

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Chapter 24

Use of Reporter Genes to Analyze Estrogen Response: The Transgenic Zebrafish Model

Daniel A. Gorelick, Caroline Lucia Pinto, Ruixin Hao, and Maria Bondesson

Abstract

In vivo models to detect estrogenic compounds are very valuable for screening for endocrine disruptors. Here we describe the use of transgenic estrogen reporter zebrafish as an in vivo model for identification of estrogenic properties of compounds. Live imaging of these transgenic fish provides knowledge of estrogen receptor specificity of different ligands as well as dynamics of estrogen signaling. Coupled to image analysis, the model can provide quantitative dose-response information on estrogenic activity of chemical compounds.

Key words Transgenic zebrafish, Estrogen, Xenoestrogens, Fluorescence microscopy, Image analysis

1 Introduction

Estrogens are synthesized in all vertebrates, indicative of a common origin and involvement in important endocrine functions [1, 2]. During development, estrogens promote the formation of reproductive organs and are required for the proper function of nonreproductive organs such as brain, liver, heart, breast, skin and bone (reviewed in [3]). The importance of estrogen signaling for normal development is manifested by perturbed development of brain and gonads as well as aberrant behavior in both aromatase and ER knockout mice (reviewed in [3]). Reflecting the strict regulation of estrogen signaling, excess or deficiency of estrogen during development can lead to pathological conditions.

In addition to endogenous estrogens, a number of xenoestrogens have been identified to bind to and activate the estrogen receptors (ERs). These compounds include phytoestrogens, such as the isoflavones genistein and daidzein, and synthetic estrogens, such as bisphenol A. From high-throughput screening of chemical libraries with diverse *in vitro* models, several more estrogenic

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compounds have been identified [4]. To confirm whether these xenoestrogens are active in vivo, rodent models and assays, such as the mouse uterotrophic assay, have been used. However, these models are expensive and of low throughput.

Zebrafish (*Danio rerio*) have emerged as an economical model for studying in vivo effects of larger numbers of estrogenic compounds. Zebrafish combine the biological relevance of an in vivo model, in which systemic effects and chemical metabolism are taken into account, with high fecundity and small size of embryos, facilitating high-throughput screening. The zebrafish genome codes for three nuclear ERs; *esr1*, *esr2a*, and *esr2b* (also denoted ERα, ERβ2, and ERβ1, respectively). These receptors mediate the genomic responses to estrogen signaling through their function as transcription factors binding to DNA in the promoters of estrogen target genes. Classically, the expression of estrogen target genes, such as the liver-produced yolk vitellogenins 1 and 3 (encoded by *vtg1* and *vtg3*), and the brain-specific aromatase B (AroB, encoded by *cyp19a1b*) genes, have been measured to detect estrogenic activity of compounds both in laboratory raised zebrafish and in field monitoring of other fish species. These assays require sacrificing animals and performing histological staining or PCR on harvested tissues. The ability to assay estrogen-dependent gene expression (i.e., estrogen receptor activity) in live fish allows for the detection of dynamic changes in receptor activity. Additionally, detecting fluorescence from a reporter gene is more rapid than histology or PCR and may be quantitative. Several transgenic zebrafish lines have been developed, in which fluorescent protein expression reports estrogen receptor activity [5–8]. The transgenic line Tg(5xERE:GFP)$^{c262/c262}$ contains five tandem estrogen response element DNA sequences (ERE) upstream of green fluorescent protein (GFP) and is a powerful model to visualize estrogen receptor subtype-specific ligands and the developmental dynamics of estrogen-responsive tissues [6, 9, 10]. In this chapter, we provide a detailed description of how to screen and identify compounds with estrogenic activity using the transgenic estrogen reporter fish line. We also describe how information on ER specificity of different ligands can be detected with the transgenic fish.

2 Materials

The zebrafish line Tg(5xERE:GFP)$^{c262/c262}$ was constructed as described previously [6]. The transgenic zebrafish line contains five tandem estrogen response element DNA sequences (EREs) upstream of green fluorescent protein (GFP). Treatment with estrogenic compounds confers a conformational change of the estrogen receptors, allowing receptors to migrate into the cell nucleus, bind ERE DNA as well as co-activator complexes, and activate GFP expression.

2.1 Solutions for Zebrafish Embryo Work

1. Embryo medium (E3): 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$.
2. Pronase A: 200 mg/mL stock solution.
3. 1-Phenyl 2-thiourea (PTU): 10 mM stock solution.
4. Tricaine methanesulfonate (MS 222): 4 mg/mL stock solution. A solution of MS222 in water is acidic and should therefore be buffered to neutral pH using sodium bicarbonate.

2.2 Chemicals

The following chemicals were used at 1 mM stock solutions in 100 % dimethylsulfoxide (DMSO). The stock solutions were stored at −20 °C.

1. 17β-Estradiol (E2).
2. 17α-Ethinylestradiol (17αEE2, EE2).
3. 16α-Lactone estradiol (16αLE2).
4. Diarylpropionitrile (DPN), 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT).
5. ICI 182,780 (ICI).
6. 4-Hydroxytamoxifen (4-OHT).

3 Methods

3.1 Zebrafish Maintenance

1. Work with vertebrate models requires an Institutional Animal Care and Use Committee (IACUC)-approved protocol. It should be conducted according to relevant national and international guidelines. The animal work presented here was approved by the IACUCs of the University of Houston and the University of Alabama at Birmingham.
2. Maintain adult fish in a commercial zebrafish tank system supplied continuously with circulating filtered water at 28 °C under 14 h of light and 10 h of dark cycle (14:10 LDC; lights on 8 AM; lights off 10 PM). A detailed description of zebrafish maintenance has been published by Avdesh and colleagues [11]. The aquaculture system water is buffered with sodium carbonate to pH 7, and salt (Instant Ocean) to around 500 μS. Dissolved oxygen should be kept above 6 mg/L. Feed fish two to three times/day with commercial flake food and baby brine shrimp. Other fish feeding schemes can also be used [12].

3.2 Breeding of Fish and Collection of Embryos

1. On the afternoon prior to the experiment, set up fish for breeding. Often three female fish are paired with two males in a small breeding tank filled half-way with aquaculture system water. To produce large numbers of embryos, several male and female fish can be placed in a mass breeding unit.

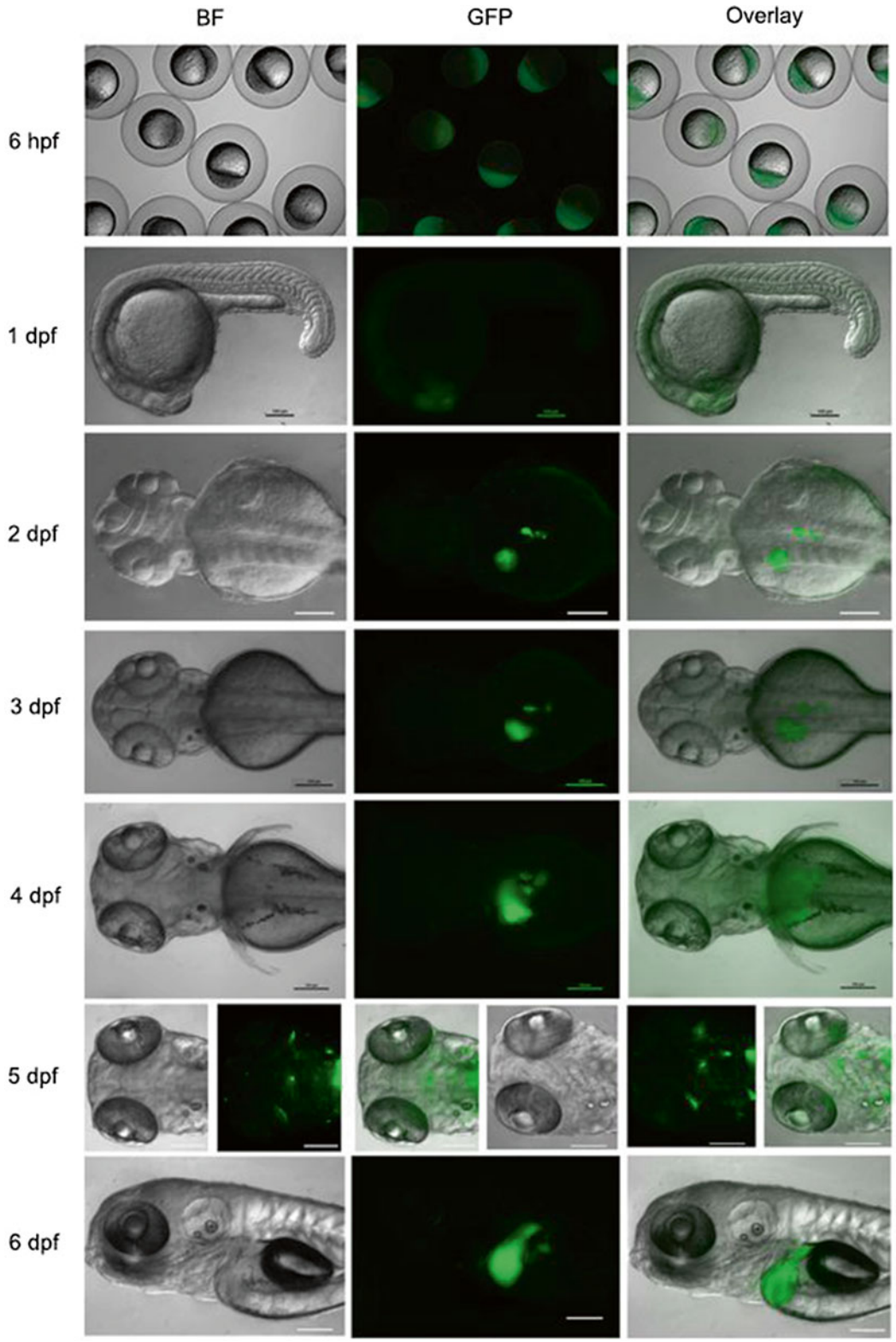
When the lights turn on in the morning of the day of the experiment, the fish breed. To time embryo fertilization, when setting up the fish in breeding tanks place dividers in the breeding tanks separating male and female fish, and pull the dividers in the morning to allow the fish to breed. After breeding, return the adult fish to permanent tanks.

2. Collect the embryos by pouring the water off the breeding tank through a plastic tea strainer. Thoroughly rinse the embryos with E3 and transfer embryos to a plastic 10 cm Petri dish by gently flushing with E3. Add 10–15 mL E3 per dish. Using a dissection microscope, remove dead or malformed embryos. Not more than 100 embryos should be put in one 10 cm dish. Place the Petri dish an incubator at 28.5 °C with 14/10 LDC (*see* **Note 1**).

3.3 Embryo Treatments

1. Initiate embryo treatments at different time points depending on the window of interest of the study. At different developmental stages the E2-treated Tg(5xERE:GFP)$^{c262/c262}$ embryos will fluoresce in different organs (Fig. 1). At 6 h post-fertilization (hpf), the early embryos fluoresce in the absence of estrogen because of maternal load of GFP in the egg (Fig. 1 and [10]). This expression has faded by 1 day post-fertilization (dpf), and instead embryo driven GFP expression mainly in the head is seen. At later time points, GFP expression in the heart valves, liver, and pancreas is observed (Fig. 1) (*see* **Note 2**).
2. Before treatment, dechorionate embryos. This can be performed manually using forceps, or chemically using pronase. For pronase treatment, place embryos on a glass Petri dish in 10 mL E3 and add 150 μL Pronase A (200 mg/mL stock concentration). Incubate for 1 min in room temperature, followed by several rinses with E3. For large-scale chorion removal, automated instruments have been developed [13].
3. At 24 hpf, replace E3 with E3 containing 200 μM 1-phenyl 2-thiourea (PTU) to prevent pigmentation of the embryos. By PTU treatment, the embryos and larvae will remain transparent for improved image clarity. If embryos are to be analyzed at 1 dpf, PTU treatment is not necessary.

Fig. 1 GFP expression visualizing estrogen activity in zebrafish from 6 hpf to 6 dpf. *First row*: Maternal load of GFP in Tg(5xERE:GFP)$^{c262/c262}$ embryos at 6 hpf in the absence of E2. *Second* to *seventh rows*: E2-induced GFP expression in Tg(5xERE:GFP)$^{c262/c262}$ fish during development. Zebrafish larvae were treated with 1 μM E2 (in 0.1% DMSO) or vehicle alone (control, 0.1 % DMSO) from 3 hpf and imaged at 1–6 dpf. Treatment induced fluorescence in the head (1 dpf), and liver and pancreas (from 2 dpf). *First column*, bright-field images; *second column*, corresponding GFP fluorescence images; *third column*, overlay of bright-field and GFP images. 1 dpf, lateral view; 2–4 dpf, dorsal view; 5 dpf, *left panels*, dorsal, and *right panels*, ventral view; anterior to the left. Scale bars, 100 μm. Reproduced from [3, 10] with permission from Elsevier


BF
GFP
Overlay
6 hpf
1 dpf
2 dpf
3 dpf
4 dpf
5 dpf
6 dpf

4. Place embryos in 96-well plates (one to three embryos/well) in 100 μL E3 + PTU. Cut a micropipette tip with a pair of scissors to increase the diameter of the hole of the tip allowing for transfer of the embryos. Use a micropipette set to 100 μL and transfer embryos into wells. Embryos younger than 1 dpf should be transferred using a glass pipette with soft edges, because they stick to plastic and may break. To prevent evaporation during prolonged treatment, omit embryos from outer rows and columns of the plate. Fill outer wells with 200 μL of E3 + PTU. Alternatively, seal the plates with a plastic film. Embryos can also be treated in clutches. Transfer 20–30 embryos into one well of a 6-well plates, remove E3 and then add 4 mL E3 + PTU.
5. Add chemicals to the desired concentration either directly to the wells with embryos or make an intermediate dilution in E3. The final concentration of DMSO should not exceed 1%. For positive controls, use either 1 μM E2 or 3 nM EE2, the latter compound being more stable in zebrafish (*see* **Note 3**).
6. Incubate the embryos with the chemicals for desired time period at 28.5 °C.

3.4 Imaging of Tissue-Specific Fluorescence

1. After treatment, the embryos can be imaged for fluorescence. Add 5 μL of tricaine methanesulfonate to each well of a 96-well plate to anesthetize the embryos/larvae. This will position the fish on their lateral side.
2. Use a fluorescent microscope with a GFP filter to image the fish. An automated stage will allow for automated imaging of several wells. We have used either an Olympus IX51 inverted fluorescence microscope using a 4× objective, and images captured using an Olympus XM10 camera with CellSens Dimension v1.9 software (Olympus), or a Zeiss Axio Observer Z1 microscope with a Zeiss HRm camera and Zen Blue 2011 software for imaging.
3. The fluorescence in images of the transgenic fish can be quantified. For quantitative analysis, use imaging software that includes the ability to mark regions of interest and measure fluorescence from multiple regions in an image. Most images can be analyzed using the freely available program Image J by the National Institute of Health (http://imagej.nih.gov/ij/index.html). By quantitative analysis of the fluorescence intensity, one can generate dose-response curves for any estrogenic compound of interest. Note that the dose represents the nominal dose of chemical in the water, and not the actual dose that enters the fish.

GFP is expressed in different tissues depending on the ER-specificity of the ligand. We have previously shown by in situ hybridization that esr1 mRNA is mainly expressed in the heart valves, while esr2b mRNA is expressed in the liver (Fig. 2 and [9]). Consequently, treatments with estrogen receptor ligands that preferentially activate one estrogen receptor compared with another (such as 16αLE2 or PPT) induce tissue-specific GFP expression (Fig. 2) (*see* **Note 4**).

3.5 Fluorescence Measurements

1. In addition to image analysis, fluorescence can be quantified in a fluorescence plate reader. In this case, transfer the embryos (one embryo/well in 100 μL E3 + PTU) to black U-shaped 96-well plates before chemical treatment, and perform the treatments in the plates. At the day of analysis, anesthetize the embryos with tricaine, briefly centrifuge the fish at low speed and place the plate in a fluorescence plate reader. Read fluorescence at excitation 395 nm and emission at 509 nm. Allow for several technical replicates for the plate reading, as the fluorescence intensity may vary somewhat between different embryos. We exposed the embryos from 1 dpf and measured the fluorescence at 4 dpf in a Perkin Elmer 2030 plate reader set to measure the fluorescence close to the bottom of the well (3 mm from bottom) (Fig. 3), but other exposure schemes can also be used (*see* **Notes 5** and **6**).

3.6 Measuring Estrogen Receptor Antagonists

1. The assay can also be used to detect and visualize estrogen receptor antagonists. To screen chemicals for antagonist activity, incubate transgenic embryos in E3 containing 1 μM E2 together with chemicals of interest. Measure fluorescence and compare localization and intensity between positive controls (E2 alone) and experimental samples (E2 plus chemicals of interest). Chemical antagonists will cause a reduction in fluorescence compared to positive controls (Fig. 4 and [6]) (*see* **Note 7**).

4 Notes

1. Some investigators incubate embryos in the dark, using incubators that lack a light. While there is data suggesting that altered light/dark cycles influence gene expression in embryos [14], we know of no studies demonstrating that light/dark cycles influence estrogen receptor activity in embryos.
2. Both the developmental stage and the orientation of the embryo are critical for visualizing tissue-specific GFP expression. Some orientations may mask the expression in different tissues. Fluorescence in the pancreas is easiest seen when the

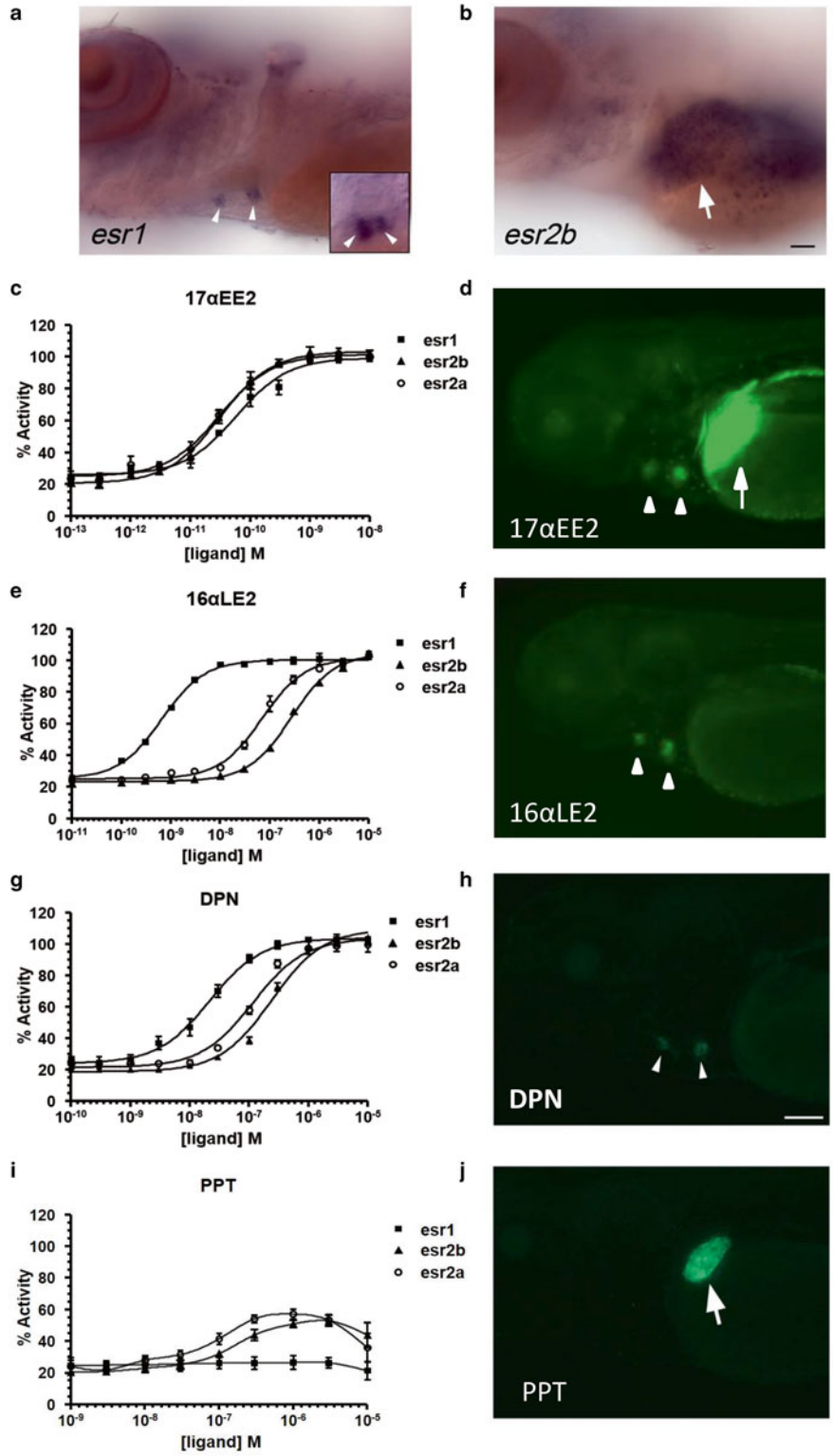


Fig. 2 Comparison of ligand-induced luciferase expression in zebrafish ER reporter cells for Esr1, Esr2a, and Esr2b with GFP expression in ERE reporter fish. Zebrafish were treated at 3 dpf with 1 nM 17αEE2, 100 nM 16αLE2, 1 μM DPN or 100 μM PPT, and images were captured at 4 dpf. In vitro selectivity of chemicals for Esr1 and Esr2a and b, assayed in zebrafish ER reporter cell lines (**c**, **e**, **g**, and **i**), correlates with GFP expression in

embryo is positioned with the dorsal side closest to the microscope objective [10]. Mounting of embryos in methyl cellulose (3%) or low melt agarose (0.3–1%) might be needed for imaging of embryos in different positions. In older embryos (5–6 dpf), the fluorescence in the liver is so strong that it may mask the fluorescence in other tissues. The image capture time needs to be optimized for detection of both liver and heart fluorescence.

3. DMSO is a preferred solvent because many steroids and endocrine disrupting compounds have high solubility in DMSO. Additionally, DMSO is relatively nontoxic to zebrafish compared to solvents such as acetone. There are no reports of DMSO affecting behavior at the concentrations used (0.1–1 % in embryo media). It is possible to use alcohols such as ethanol as solvents, but care should be taken not to expose zebrafish embryos to more than 0.1% final concentration. Always include control embryos exposed to solvent only.

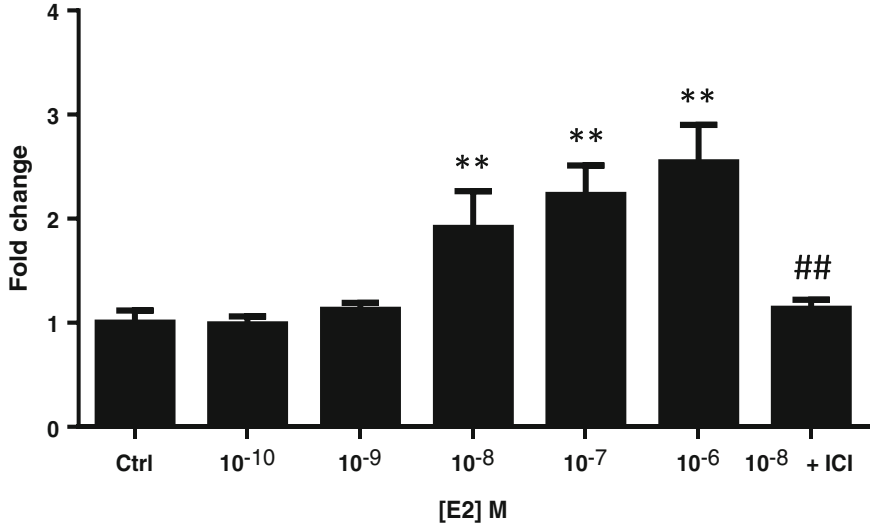


Fig. 3 Quantification of fluorescence in Tg(5xERE:GFP)$^{c262/c262}$ embryos. Embryos were placed in 96-well black plates and treated from 1 dpf with either E2 alone or E2 (10^{-8} M) together with the antagonist ICI (1 μM). After 3 days of treatment, the fluorescence was quantified in a plate reader. $N = 12$; $^{**}P < 0.01$ treatments compared to control; $\#\#P < 0.01$ E2 + ICI compared to E2 10^{-8} M

Fig. 2 (continued) the heart valves and liver of zebrafish larvae, respectively. It also correlates to the mRNA expression of Esr1 and Esr2b as determined by in situ hybridization (**a** and **b**). The pan-agonist 17αEE2 activates GFP expression in both the liver and heart valves of the ERE-GFP fish (**d**). The esr1 selective chemicals 16α LE2 and DPN induce GFP expression in the heart valves (**f** and **h**), whereas the Esr2 selective agonist PPT activates GFP expression in the liver (**j**). Zebrafish embryo with anterior to *left*, dorsal *top*; *arrow* = liver, *arrowheads* = heart. (**c**, **e**, **g**, and **i**) are adapted from [15] with permission from Elsevier. (**a**, **b**, **h**, and **j**) are reproduced from [9] in Environmental Health Perspectives

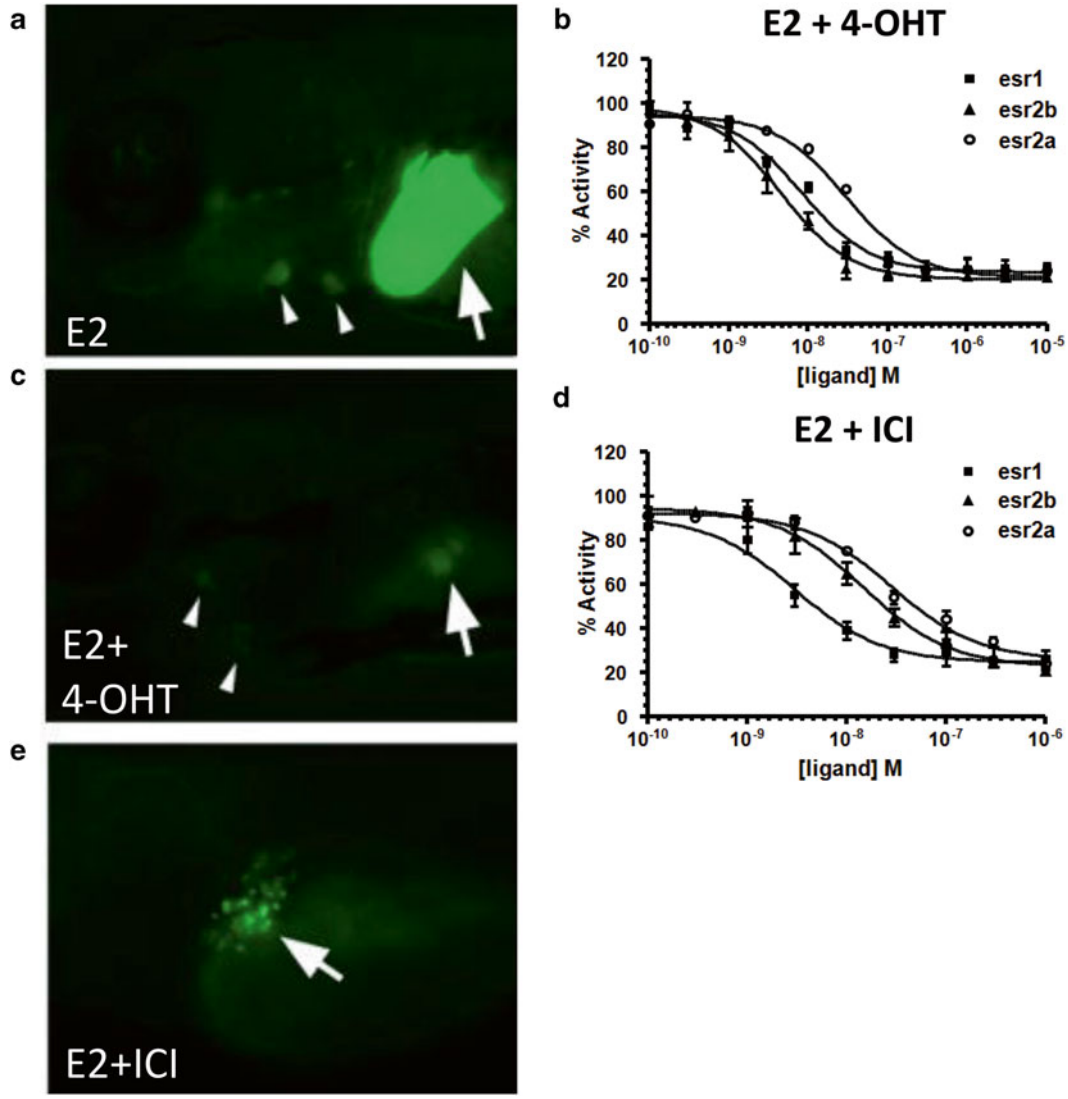


Fig. 4 Visualization of effects of estrogen receptor antagonists in reporter cell lines and Tg(5xERE:GFP)$^{c262/c262}$ embryos. Embryos were exposed to 1 μM E2 (**a**) only, or with 4-OHT (**c**) or ICI (**e**), and imaged at 4 dpf. Comparison is shown to zebrafish ER reporter cells, which were treated with E2 in the presence of 4-OHT (**b**) or ICI (**d**). Zebrafish embryo with anterior to *left*, dorsal *top*; *arrow* = liver, *arrowheads* = heart. Adapted from [6, 15] with permissions from the Endocrine Society and Elsevier

4. ERα vs. ERβ selectivity of ligands is different between zebrafish and human ERs (Fig. 2 and [15]). For example, the human ERβ selective ligand DPN is somewhat ERα (ESR1) selective in zebrafish. PPT, which is ERα selective in humans, only activates ERβ (ESR2) in zebrafish.
5. Remove dead embryos, as these will fluoresce strongly.

6. For high throughput screening of chemical libraries, note that some chemicals have intrinsic fluorescence. Measure the fluorescence in a plate with chemicals only to uncover the background fluorescence.
7. Some estrogenic compounds may have both agonistic and antagonistic activity on the zebrafish ERs. Treating the embryos with the compound both in the presence and absence of E2 will reveal agonism/antagonism effects.

Acknowledgements

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Chapter 25

Comparison of the Effects of the Selective Estrogen Receptor Modulators Ospemifene, Raloxifene, and Tamoxifen on Breast Tissue in Ex Vivo Culture

Natalija Eigeliene, Risto Erkkola, and Pirkko Härkönen

Abstract

Explant tissue culture provides a model for studying the direct effects of steroid hormones, their analogs, and novel hormonally active compounds on normal freshly isolated human breast tissues (HBTs). For this purpose, pre- and postmenopausal HBTs can be maintained in this culture system. The results demonstrate that the morphological integrity of HBT explants can be maintained in tissue culture up to 2 weeks and expression of differentiation markers, steroid hormone receptors, proliferation and apoptosis ratios can be evaluated as a response to hormonal stimulation. This chapter describes an ex vivo culture model that we have applied to study the effects of various hormonally active substances, including 17β-estradiol and selective estrogen receptor modulators (SERMs), on normal human breast tissues.

Key words Human breast tissue, Explant tissue culture, Estradiol, Estrogenreceptoralpha, SERMs

1 Introduction

The optimal method for evaluation of the effects of steroid hormones or any hormonally active compound on normal breast tissues is a large-scale clinical study in a normal female population [1]. On the other hand, these studies would take many years and need large numbers of healthy participants. Experimental studies using cell lines and experimental animals (e.g., rodents), although helpful, have their limitations due to lack of epithelial–stromal interactions in cell cultures [2] or the biological differences between the human and rodent mammary gland [3]. There are also studies on the non-human primate mammary gland, which have well-documented similarities to the anatomy of the human mammary gland, reproductive physiology, and peripheral steroid hormone metabolism [4–6]. These studies are of great value, but they are expensive, long, and require animals to be sacrificed.

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DOI 10.1007/978-1-4939-3127-9_25,

Many experimental models have shown that epithelial–stromal interactions are essential for proliferation and differentiation of epithelial cells both in vivo and in vitro [7–9]. The advantage of ex vivo tissue culture over cell lines lies in the maintenance of the three-dimensional structure of the tissues and in the maintenance of epithelial–stromal interactions. Previous studies have demonstrated that tissue-specific hormonal effects are maintained more properly in tissue culture than in primary cell culture models [7–9].

A major problem in studying hormonal effects in vivo is that the direct effects of the hormones (or any added factors) are difficult to verify, the hormonal interactions cannot be controlled, and the actual hormone concentrations at the level of target organ are not known. Ex vivo tissue culture possesses some evident advantages in studies of this complicated system, such as lack of systemic effects, easy manipulation of the environment, and the ability to study the direct effects of active compounds [10]. In the case of explant tissue culture, the epithelial–stromal interactions are maintained, which is important for a glandular tissue such as mammary gland [7].

This chapter describes an ex vivo culture model that we have modified and applied [1, 11, 12] to study the effects of various hormonally active substances, including 17β-estradiol and selective estrogen receptor modulators (SERMs) such as ospemifene, raloxifene, and tamoxifen, on normal human breast tissues (HBTs). This method allows the maintenance of tissue architecture, hormone responsiveness, and cell-to-cell signaling of tissue microenvironment in a dynamic state, which can be manipulated in the laboratory setting. Compared with current cell line- and animal-based models, ex vivo cultured HBTs enable robust quantitative evaluation of clinically relevant end points including drug efficacy, investigation of therapy resistance, and validation of possible biomarkers.

HBTs can be obtained from three different types of operations: reductive breast surgeries (plastic surgeries), surgeries performed for tumors (benign and malignant tissues), and breast tissue surrounding the tumor (peritumoral tissue or adjacent to tumor tissue). The main source of normal breast tissue for culturing ex vivo is reductive mammoplasties, which are performed on women at pre- and postmenopausal age.

2 Materials

Make sure that there is approval by the local Human Subjects/Ethics Committee to perform the planned study, and obtain written consent of every patient whose HBTs are going to be studied. Take into consideration that HBTs should only be obtained from patients who have not received any hormonal treatment during at least 6 months before the surgery.

Prepare all reagents and stocks to be ready for tissue culture ex vivo in advance. Carefully follow all waste disposal regulations when discarding biological waste materials.

1. 10× Phosphate buffered saline (PBS): 10.9 g Na_2HPO_4, 3.2 g NaH_2PO_4, 90 g NaCl. Mix to dissolve and adjust pH to 7.3. Raise volume to 1000 mL. 10× stock of PBS can be stored for several months at room temperature.
2. 1× PBS: working dilution of PBS is 1:10 (10× PBS:distilled water). Dilute just before use and adjust pH if necessary.
3. 10 mM sodium citrate buffer: 2.94 g tri-sodium citrate per 1000 mL distilled water. Mix to dissolve. Adjust pH to 6.0 with 5 M HCl and then add 0.5 mL of Tween®20 and mix well.
4. Peroxidase blocking solution (0.3 % H_2O_2): 10 mL of 30 % H_2O_2, 90 mL methanol. Prepare just before use and mix well.
5. Neutral formalin (10 %): 100 mL of 35–40 % formaldehyde, 3.5 g NaH_2PO_4, 6.5 g Na_2HPO_4, 900 mL distilled water. Filter the formaldehyde before adding to the solution. Mix well to dissolve. pH should be 6.5–7.0.
6. Mayer's Hemalaun solution: 1 g hematoxylin, 50 g aluminum potassium sulfate, 0.2 g sodium iodate, 1000 mL distilled water. Dissolve the hematoxylin in 500 mL distilled water. Add the other reagents and mix, then raise the volume to 1000 mL with distilled water. Some particles may remain undissolved so filter before use.
7. Basal culture medium: phenol red-free DMEM/F12 medium, 10 % dextran-charcoal stripped fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 1 % insulin-transferrin-sodium selenite (ITS) supplement, 100 mM hydrocortisone, and 10 ng/mL epidermal growth factor (EGF).
8. 17β-estradiol and SERM stocks. Prepare 17β-estradiol (E_2) at 10 nM and ospemifene, raloxifene and tamoxifen at 1–10 nM stocks. Dissolve all four compounds in dimethylsulfoxide (DMSO). The final concentration of DMSO in the culture medium is optimally 1:1000. Culture control HBTs in basal culture medium supplemented with DMSO (*see* **Note 1**).
9. Transportation liquid: sterile saline solution or phenol red-free DMEM/F12 medium supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL), kept at 4 °C.
10. Stainless steel grids to support the tissue explants. We use homemade grids (squares or circles) of stainless steel prepared by handy colleagues (material from which these grids were made is acid-resistant steel AISI 316, wire thickness 0.3 mm, size of holes 0.75 mm, purchased from www.spinea.fi). The size and height can vary depending on the size of tissue culture plates and could be decided differently in different labs (Fig. 1).

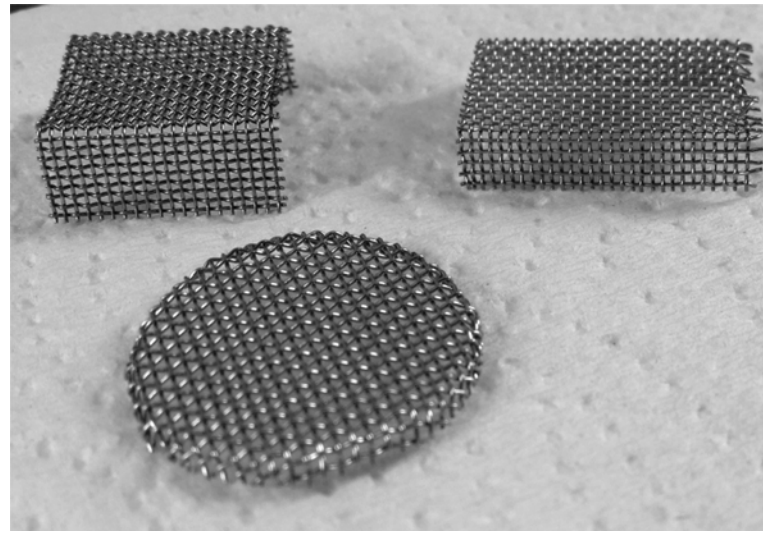

Fig. 1 Stainless steel grids used for ex vivo cultures. These grids were home-made and serve as support and tissue holder at the same time. The grids can vary in size, height and shape, depending on the size of the tissue culture plate and the skills of the person who makes them. The easiest to make is a rectangle; there is need only to fold two edges of the square. (**a**–**c**) Square and round grids suitable for 6-well plate; (**d**) for 24-well plate

11. Lens paper, which should be cut in small pieces according to the shape and size of the grids, then sterilized in autoclave (e.g., Whatman lens cleaning tissue).
12. Mesh biopsy cassettes, paraffin molds, microtome, glass slides for immunohistochemistry, permanent mounting medium, and coverslips.
13. 70 %, 85 %, 96 % and absolute ethanol, absolute ethanol:xylene (1:1), xylene, and paraffin.
14. Bovine serum albumin (BSA), 1 % in PBS (1 g BSA in 100 mL of PBS 1×).
15. Normal horse serum, mouse anti-human ERα antibody (Dako, Clone 1D5) (1:1000 in 1 % BSA/PBS), biotinylated horse anti-mouse IgG (5 μL in 1000 μL PBS).
16. Immunohistochemical staining enhancement kit such as the VECTASTAIN® ABC kit. For ABC reagent: add 10 μL reagent A into 1000 μL 1×PBS, mix gently by hand for 30 s, then add 10 μL reagent B and mix well. Prepare this reagent 30 min before use.
17. Eosin prestain solution: 3 mL of 1 % Eosin G solution (dissolve 1.0 g Eosin G powder in 100 mL distilled H_2O, mix well) in 100 mL of 96 % ethanol.
18. Chromagen substrate/DAB peroxidase substrate (Peroxidase substrate kit (DAB) SK-4100, Vector Laboratories, Inc.).

3 Methods

3.1 Preparation of HBTs

The culture method of Trowell [13] with modifications [11, 12] (Fig. 2) is used to culture HBT explants.

1. HBTs should be immediately immersed in cold transportation liquid and transported to the laboratory as soon as possible.
2. Prepare the HBT cultures within 1–2 h of the tissue removal during the surgery.
3. Place the tissue in DMEM/F12 medium. Using fine scissors and tweezers and a stereomicroscope, remove as much of the adipose tissue as possible, saving the soft collagenous tissue. The ducts and lobules (glandular compartment) are found mostly in this collagenous tissue (*see* **Note 2**).
4. Place the explants into a Petri dish containing DMEM/F12 medium for further processing.
5. Cut the tissues with fine scissors into pieces of approximately $2 \times 2 \times 2$ mm in DMEM/F12 medium supplemented with antibiotics.

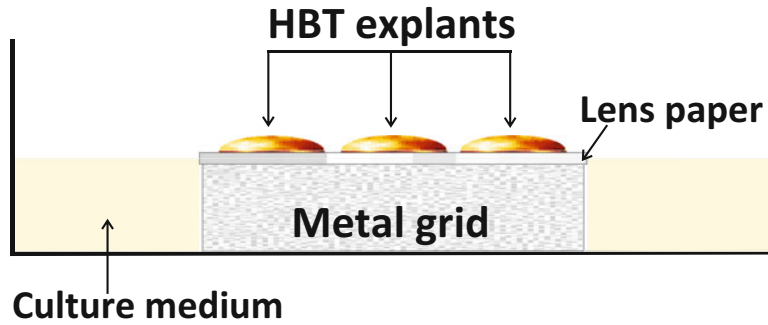

Fig. 2 Modified ex vivo culture method. Four to seven pieces of breast explants can be transferred onto lens papers lying on stainless steel grids in tissue culture plate (6–12–24 well plates can be used depending on the grid size) and cultured for different periods. The grids do not allow small tissue explants to be completely submerged, which is crucial step for tissue ex vivo cultures. In addition, it is very easy to change culture medium without sucking small tissue explants inside the pipette tips, because tissues are placed on the grids. Sterile lens paper should be cut according to the grid shape and sterilized and placed on the top of the grid. The one corner of this paper should be slightly immersed into culture medium, then the rest of it gets wet very quickly. The role of the lens paper is to moisture the explants and to prevent tissue overgrowth into the holes of the grids. The explants are kept in a humidified atmosphere with a mixture of 5 % CO_2 and 95 % air at 37 °C in culture medium. The culture medium should be changed every second day and fresh batches of steroids added. Two to three parallel dishes with samples obtained from each patient should be cultured for every treatment group and every time point

6. Rinse the small explants with fresh DMEM/F12 medium two to three times by transferring HBTs from one to another Petri dish.
7. Place stainless steel grids into culture dishes. Add the culture medium until it reaches the top of the grid. Place a sterile lens paper onto the grid.
8. Transfer and place three to seven small explants on grid, covered with wet lens paper.
9. Very carefully add 100–200 μL of culture medium on the top of HBTs, to make sure they are supplemented with medium.
10. Add treatments (E_2 or SERMs) or vehicle to the appropriate dishes; include duplicates or triplicates for each treatment.
11. Keep the explants in a humidified atmosphere in a mixture of 5 % CO_2 and 95 % air at 37 °C.
12. Replace the culture medium every second day. It is better to prepare a larger volume of culture medium with E_2 and SERMs, than to add a very small amount to each well or plate individually. Thus, calculate how much fresh culture medium you need with different compounds (E_2 and SERMs) for replacement, based on how many cultures you have. For example, if you need 10 mL fresh culture medium for E_2, ospemifene, raloxifene, and tamoxifen, take 10 mL per each treatment of basal culture medium in separate vials, then add compounds at desired concentration and mix well. Take out 50 % of the old CM and add 50 % of fresh culture medium with different hormones.
13. At least duplicate or triplicate parallel dishes with samples obtained from each patient should be cultured for every treatment group and every time point. Explants can be collected at any time point of interest (*see* **Note 3**).

3.2 HBT Fixation and Paraffin Embedding

1. Collect the cultured HBTs at time points of interest and immediately fix with 10 % formalin/PBS for 12 h at room temperature. Use a mesh biopsy cassette to handle the small explants (*see* **Note 4**).
2. After fixation, rinse fixed HBTs in 70 % ethanol for 1 h, then transfer them to fresh 70 % ethanol for 24 h at room temperature. After this time point proceed to dehydration and paraffin embedding (*see* **Note 5**).
3. First, HBTs need to be dehydrated through graded alcohols as follows:
   (a) 85 % ethanol: 8 h
   (b) 96 % ethanol (*see* **Note 6**): 12 h
   (c) Absolute ethanol: 1 h × 2

(d) Absolute ethanol:xylene: 15–30 min

(e) Xylene: 15–30 min × 2

(f) Paraffin (52 °C): 12–24 h

(g) Paraffin (56 °C): embedding paraffin

4. Embed HBTs in paraffin wax (56 °C) using molds. Keep them at room temperature until the wax solidifies (1–2 h), then keep at 4 °C overnight. On the following day, remove the paraffin blocks from the molds.
5. Section the paraffin blocks on a microtome to obtain 4–5 μm thick sections and place them on the special glass slides for immunohistochemistry. After sectioning, keep the slides at 37 °C overnight and then at 4 °C until immunohistochemical staining is carried out (*see* **Note 7**).

3.3 Immunohistochemistry of ERα

1. On Day 1: Clear paraffin sections in xylene for 3 × 10 min, then rehydrate them through 100 % → 96 % → 70 % ethanol for two times per each for 5 min each.
2. Briefly rinse the sections under tap water.
3. Peroxidase blocking: Incubate sections in a peroxidase blocking solution for 20 min at room temperature.
4. Epitope retrieval: Transfer slides to plastic coplin staining jars with sodium citrate buffer to microwave oven. Use the following settings: *low* 5 min → stop for 1 min → *medium high* 5 min → stop for 1 min → *high* 5 min.
5. Let the slides cool down in the same buffer for 20 min at room temperature.
6. Rinse the sections in PBS for 3 × 5 min.
7. Serum blocking: incubate sections with 10 % normal horse serum for 40 min at room temperature.
8. Carefully wipe away the excess serum blocking solution and apply the primary antibody.
9. Primary antibody: Incubate sections with mouse anti-human ERα (Dako, Clone 1D5) 1:1000 in 1 % BSA/PBS overnight at 4 °C. For the negative control incubate with only 1 % BSA/PBS.
10. On the second day, bring the slides to room temperature for 15–20 min.
11. Rinse the slides in PBS for 3 × 5 min.
12. Secondary antibody: Incubate the sections with biotinylated horse anti-mouse IgG diluted in PBS for 30 min at room temperature (5 μL into 1000 μL).
13. Rinse in PBS for 3 × 5 min.
14. *Note well*: Prepare the VECTASTAIN® ABC reagent at least 30 min before use.

15. Detection: Incubate sections with ABC reagent for 40 min at RT.
16. Rinse in PBS for 3 × 5 min.
17. Chromogen/Substrate: Incubate sections in DAB peroxidase substrate solution for 2–4 min.
18. Rinse briefly in distilled water.
19. Counterstain with Mayer's Hemalaun solution for 30 s.
20. Rinse under running tap water for 5 min.
21. Dehydrate through 70 % → 95 % → 100 % ethanol for two times per each, 5 min each.
22. Clear in xylene for 3 × 10 min.
23. Set a coverslip with permanent mounting medium on the sections and keep the slides in the laminar flow hood overnight.
24. Examine the immunostained sections under a microscope, photograph (Fig. 3) or scan the sections and quantify using appropriate methods.

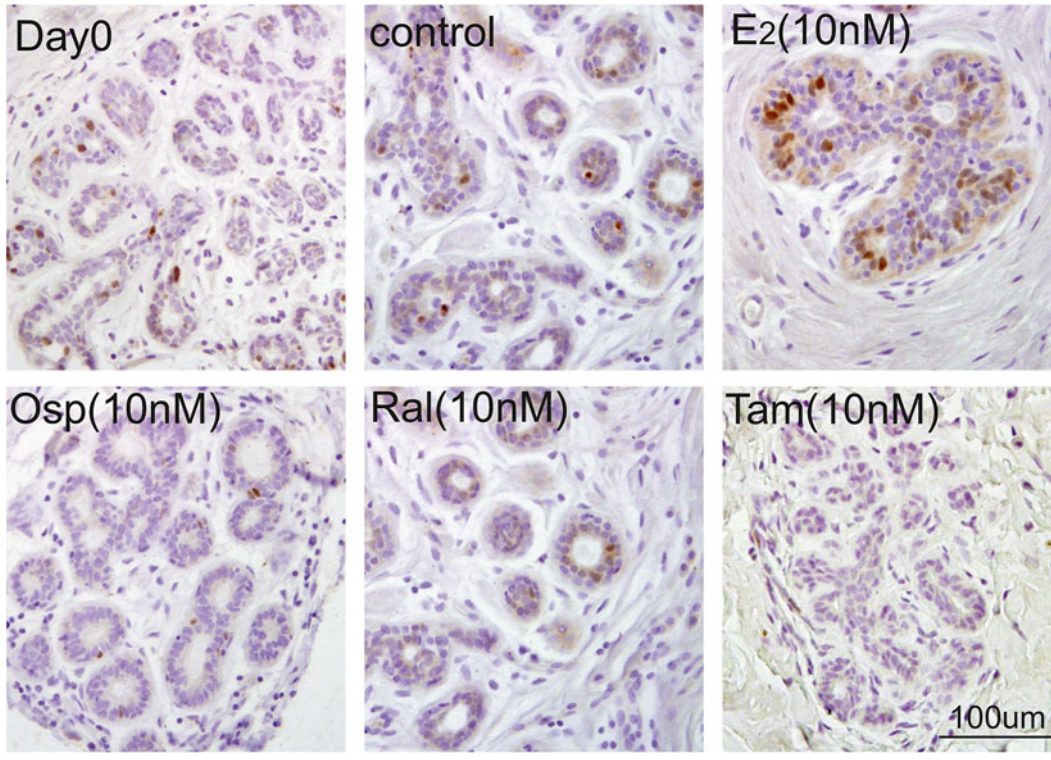

Fig. 3 Immunohistochemical staining of ERα in HBTs, obtained from one patient and cultured with 17β-estradiol, ospemifene, raloxifene, or tamoxifen for 7 days. Comparison between non- and cultured HBT explants. Control explants, cultured without additions. Bar 100 μm

4 Notes

1. Make 1–10 mM stock solutions of the hormonal compounds to be diluted and used at required final concentrations in culture medium upon culturing.
2. The fragile glandular compartment of HBTs is best seen and cut under stereo microscope. Use fine scissors and tweezers.
3. The level of ERα expression is maintained in the ex vivo cultures for the first 7 days. Thereafter, ERα staining is gradually decreasing.
4. The mesh biopsy cassettes are very useful for processing small HBTs for histology.
5. Formalin-fixed HBTs can be kept in 70 % ethanol for extended period of time, but optimally, they should be paraffin-embedded within 1–2 months after fixation.
6. Prestain very small HBT explants with eosin before paraffin embedding to make them clearly visible. Thus, use eosin prestain solution instead of of 96 % ethanol during HBT dehydration.
7. The HBTs contain a lot of fat tissue and often during antigen retrieval, especially in the microwave oven, samples detach from the glass slides very easily. Thus, on the day before beginning the immunochemistry procedure, put the specimens in the incubator at 55–58 °C for 2–3 h, then keep them at room temperature overnight. Proceed to immunohistochemistry staining the next day.

Acknowledgments

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Chapter 26

Estrogen Receptor Agonists and Antagonists in the Yeast Estrogen Bioassay

Si Wang and Toine F.H. Bovee

Abstract

Cell-based bioassays can be used to predict the eventual biological activity of a substance on a living organism. In vitro reporter gene bioassays are based on recombinant vertebrate cell lines or yeast strains and especially the latter are easy-to-handle, cheap, and fast. Moreover, yeast cells do not express estrogen, androgen, progesterone or glucocorticoid receptors, and are thus powerful tools in the development of specific reporter gene systems that are devoid of crosstalk from other hormone pathways. This chapter describes our experience with an in-house developed RIKILT yeast estrogen bioassay for testing estrogen receptor agonists and antagonists, focusing on the applicability of the latter.

Key words Yeast estrogen bioassay, In vitro, Estrogen receptor, Estrogen antagonist

1 Introduction

The estrogen receptor (ER) is a member of the nuclear receptor (NR) family, a class of gene transcription regulators which are activated upon binding of a low molecular weight ligand. The major endogenous agonist activating the ER is 17β-estradiol (E2), which regulates the expression of genes involved in the reproductive system and bone development, especially in females, but also in males [1]. Two types of ER can be found in mammals, ERα and ERβ. Transcription activation by the ER is dependent on binding of agonistic ligands to the binding site in the ligand-binding domain (LBD) of the ER polypeptide chain. The conformational change upon agonist binding results in uncovering of the DNA-binding domain and of the cofactor-binding motif within the LBD. Two functional classes of cofactors or coregulators have been found to interact with the ER and other nuclear receptors: coactivators and corepressors. These factors respectively stimulate or inhibit the basal transcription complex formed on promoter sequences.

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Coactivators and corepressors interact with nuclear receptors through their specific (iso)leucine-rich amino acid sequence motifs, LXXLL and LXXXIXXX(I/L), for activators and repressors, respectively [2]. Structural analysis of NR LBDs has established that agonist binding stabilizes the activation function-2 (AF-2) helix in an active conformation to form a charge clamp pocket, which is permissive for interactions with LXXLL motifs. In contrast, ER antagonists affect the positioning of the AF-2's mobile C-terminal helix (helix 12) to form a large binding pocket that interacts with the LXXXIXXXL motifs of corepressor proteins, thereby disrupting the LXXLL-binding site and preventing coactivator recruitment [3–5].

Modulation of ER activity is usually quantitatively analyzed by assaying ER binding, ER-controlled expression of a reporter gene or other downstream events such as estrogen receptor-mediated cell proliferation. Several reporter-gene assays have been developed and applied as screening tools to determine the estrogenic and anti-estrogenic activities of substances, as they are inexpensive, fast, robust, and have also been shown to produce relevant and reliable outcomes. By 1996, the first yeast estrogen screen assay (YES assay) was developed and applied to test for the presence of estrogenic substances in waste waters and river waters [6–8]. More recently, the RIKILT yeast estrogen bioassay (REA) was developed at RIKILT and this bioassay has been validated according to EU guidelines as a qualitative screening method for the determination of estrogenic activity in calf urine and animal feed and was subsequently accredited ISO17025 for both matrices [9–11]. Moreover, the REA bioassay was proven to be highly specific for testing pure estrogen receptor antagonists, selective estrogen receptor modulators (SERMs), and plant-derived substances [12, 13].

The REA is based on a clone of a Saccharomyces cerevisiae yeast cell that has been genetically modified to express the human estrogen receptor α (hERα) and contains a second construct that contains a sequence with estrogen-responsive elements (EREs) that drive the expression of yeast-enhanced green fluorescent protein (yEGFP) after the hERα is activated. yEGFP is thus used as the reporter gene to detect estrogenic activity. More specifically, upon ligand binding the receptor forms a homodimer and this ligand–receptor complex translocates to the nucleus where it binds specifically to the estrogen-responsive elements that subsequently induce the translation of yEGFP. The amount of fluorescent protein produced is directly proportional to the estrogenic activity of the test substance. In this chapter, we describe methods to detect substances with ER antagonistic properties using the RIKILT yeast estrogen bioassay.

2 Materials

1. Yeast cytosensor expressing the human estrogen receptor α (hERα) and yeast-enhanced green fluorescent protein (yEGFP) in response to estrogens. (A Petri dish with ER-α yeast colonies can be provided by RIKILT upon request. The ER-α yeast can be stored at 7 °C for maximum of 2 months.)
2. Minimum essential medium: 1.7 g yeast nitrogen base, 5.0 g ammonium sulfate, and 20.0 g dextrose, and transfer to a 1-L media storage bottle. Add purified water (resistivity 18.2 MΩ) to a volume of 1000 mL. Mix and autoclave the medium at 121 °C for 15 min. Store at room temperature (*see* **Note 1**).
3. L-leucine solution, 6 mg/mL: dissolve 0.3 g L-leucine in 50 mL purified water and filter through a 0.2 μm syringe filter, store at room temperature.
4. MM/L medium (Minimum essential medium supplemented with L-leucine): add 2 mL L-leucine solution (6 mg/mL) to 100 mL minimum essential medium, prepare freshly before each experiment.
5. Dimethylsulfoxide (DMSO).
6. 17β-estradiol (E2) from Sigma (E8875).
7. Prepare a 100 mM E2 stock solution in an amber glass vial. Make serial dilutions of E2 in DMSO using amber glass vials (*see* **Note 2**). Store the diluted E2 stock solutions at room temperature, protected from light.
8. Prepare serial dilutions of test substance in DMSO (*see* **Note 3**).
9. 50 mL sterile centrifuge tubes.
10. Flat-bottomed 96-well microplates.
11. Multi-detection microplate reader suitable for both fluorescence and optical density measurements.

3 Methods

3.1 Day 1

1. Add 10 mL MM/L medium into a 50 mL sterile centrifuge tube.
2. Take one colony of the ER-α yeast from the Petri dish and inoculate the 10 mL MM/L medium.
3. Grow the yeast culture overnight in a 30 °C incubator with vigorous orbital shaking at 225 rpm.

3.2 Day 2

1. Dilute the overnight yeast culture with fresh MM/L medium until an optical density (OD) at 630 nm is reached between 0.04 and 0.06 (*see* **Note 4**).

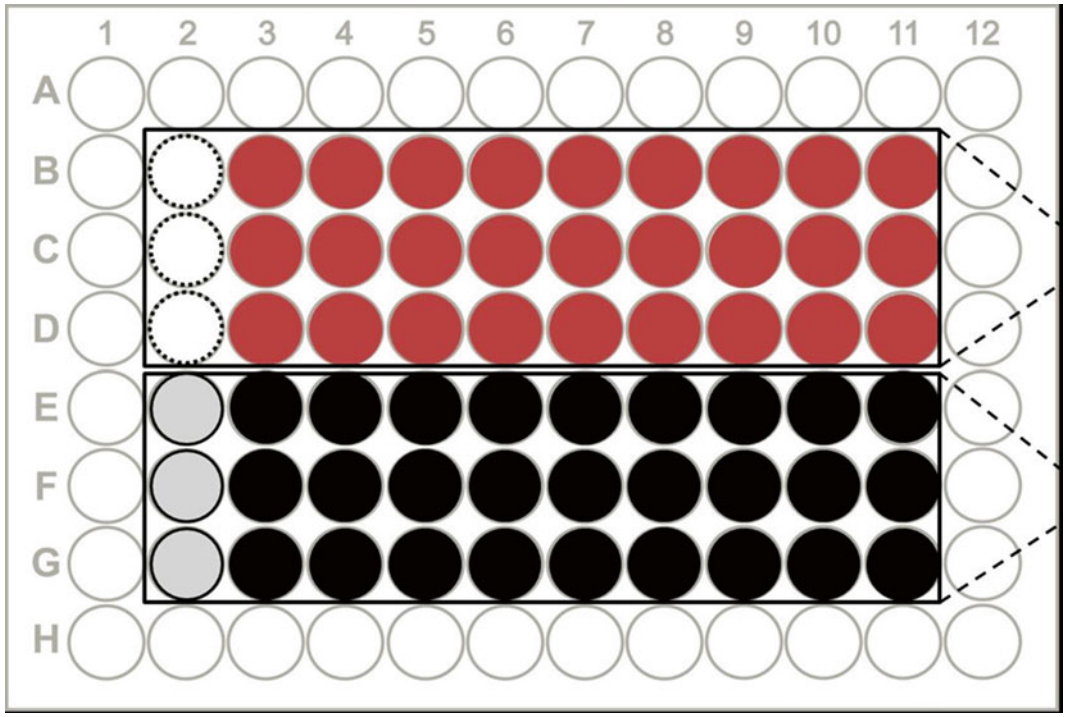

Fig. 1 Suggested plate layout for testing ER (ant)agonist in the yeast estrogen bioassay

2. Add 200 μL of the diluted yeast suspension to each well of a 96-well microplate (*see* **Note 5**).
3. For exposure, add 2 μL of E2 standard per well. Each E2 concentration is assayed in triplicate. DMSO is used as a solvent control (Fig. 1).
4. To test the antagonistic properties of the substance, first add 1 μL of 120 nM E2 to the appropriate wells (Fig. 1), this will result in a final concentration of 0.6 nM E2 in the well (*see* **Note 6**). Subsequently, add 1 μL of the test substance to the wells already containing 0.6 nM E2. Each test substance concentration is assayed in triplicate (*see* **Note 7**).
5. It is also possible to test the agonistic properties of the substance in the REA assay. This can be done in a similar way as described in Subheading 3.2, **step 3** for E2. Take another 96-well plate and add 2 μL of the substance standard to the wells already containing 200 μL diluted yeast suspension.
6. Measure the fluorescence at time zero (t_0) hour using a microplate reader with excitation wavelength at 485 nm and emission wavelength at 530 nm.
7. Measure the OD (630 nm) at t_0 hour using a microplate reader.

8. Put a lid on the plate and seal the plate by wrapping parafilm around the sides to prevent evaporation.
9. Incubate the plate for 24 h in a 30 °C incubator with orbital shaking at 125 rpm.

3.3 Day 3

1. Measure the fluorescence and OD at t_{24} hour (*see* **Note 8**).

3.4 Data Analysis

1. Subtract the t_0 fluorescence data from t_{24} for each well.
2. Correct the fluorescence signal with the signals obtained with the MM/L medium containing DMSO solvent only.
3. Calculate the EC_{50} and IC_{50} values for E2 and the test substance (antagonist), respectively, with a sigmoidal four-parameters Hill (logistic) model using a statistics software package such as Graphpad Prism software.

4 Notes

1. We find that it is best to store the medium under daylight (e.g., near the windows) for about a month to reduce the background color of the medium.
2. For a dose–response curve of E2, ideally threefold dilution in every step should be applied (e.g., 20 μM, 6 μM, 2 μM, 0.6 μM, 0.2 μM, 60 nM, 20 nM, 6 nM, 2 nM).
3. For a range finder experiment apply a tenfold dilution in every step.
4. In our hands, 1 mL overnight yeast culture in 30 mL fresh MM/L medium yields an OD of about 0.05 and is sufficient for fill in two 96-well microplates.
5. The outer 36 wells are not used for exposure and are filled with medium only, but can also be filled with 200 μL water.
6. The half-maximal response (EC_{50}) of E2 in the REA assay is approximately 0.6 nM.
7. This protocol provides procedures to study the antagonistic effects with a dose of an agonist (i.e., E2) that gives a half-maximal response. It is advisable to also study the antagonistic effects with a dose that gives a near maximal response (e.g., add 1 μL of 600 nM E2 in 200 μL yeast suspension, resulting in a final concentration of 3 nM E2 in the well).
8. Normally, the OD at 630 nm after exposure increases from 0.05 at 0 h to about 0.9 at 24 h. If the OD at 24 h is below 0.7, the concentration of the substance tested is considered to cause cytotoxicity and is excluded from further data analysis.

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Chapter 27

Silencing Estrogen Receptor-α with siRNA in the Intact Rodent Brain

Ana C. Ribeiro, Anders Ågmo, Sergei Musatov, and Donald W. Pfaff

Abstract

The ability to silence the expression of gene products in a chemically, spatially, and temporally specific manner in the brains of animals has enabled key breakthroughs in the field of behavioral neuroscience. Using this technique, estrogen receptor alpha (ERα) has been specifically implicated in a multitude of behaviors in mice, including sexual, aggressive, locomotor, and maternal behaviors. ERα has been identified in a variety of brain regions, including the medial preoptic area, ventromedial hypothalamus, and amygdala. In this chapter we describe the techniques involved in the generation of the small hairpin RNAs (shRNAs) specifically designed to silence ERα, the construction of the adeno-associated viral (AAV) vector for delivery of the shRNA, the procedures to confirm the silencing of ERα (in vitro and in vivo) and in vivo delivery of the shRNAs to the brains of animals.

Key words Adeno-associated virus, Estrogenreceptoralpha, RNA interference, shRNA, Behavior, Mice

1 Introduction

One of the prevailing trends in behavioral neuroscience is to elucidate the chemical and cellular substrates underlying specific behaviors. Traditionally, this was achieved using transgenic models that lacked the gene of interest (knockout animals). However, this approach, while informative at the time, had several major flaws, including the lack of specificity of the tissues in which the gene was being silenced and the developmental compensatory mechanisms that likely overcame the elimination of the gene.

Even when the effects of total silencing of the gene yielded fruitful behavioral results, such as the case with ERα knockout mice (ERKO) [1, 2], it still left many unanswered biological questions. For example, what are the specific neuronal substrates of these specific behaviors? The technique of viral vector delivery of shRNAs overcomes these limitations and offers critical insights into the specific chemical and cellular substrates of any behavior. Essentially, AAV delivery of a specific shRNA abolishes the expression of the

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gene of interest without destroying the infected neurons—in fact, they maintain normal morphology [3].

Major advantages of using such a technique include the ability to monitor the same animal before and after viral vector silencing (temporal specificity), to link the behavioral phenotype to a specific and quantifiable decrease in the number of neurons expressing the gene-product (neuronal specificity) and to compare the effects in silenced (correct shRNA) versus control shRNA-injected animals (chemical specificity). This technique and specific viral vector construct have been used extensively to elucidate estrogenic control of behavior in mice and rats [3–12]. Most studies conducted thus far have involved relatively large injection volumes (1 μL), to ensure suppression over entire brain nuclei, such as the ventromedial nucleus of the hypothalamus (VMN) and medial preoptic region (MPOA). These studies have revealed a role for ERα in the VMN in sexual behavior [3], social recognition [9], and the regulation of metabolic processes [12]. In the MPOA, ERα has been implicated in maternal behaviors [5] and sexual incentive behavior [6]. A new phase of investigations involves fine-tuning these effects by specifically targeting smaller populations of neurons, by using smaller injection volumes, to regionally limit the suppression of the gene product and monitor the specific effects on behavior [13]. This chapter describes a protocol for delivering shRNA for ERα to brain nuclei, as well as a control shRNA for the non-silenced control group (shRNA for luciferase) (both shRNAs express green fluorescent protein GFP). After behavioral studies are carried out in the silenced animals, immunohistochemistry of the brains is used to demonstrate the correct placement of the injections (GFP immunohistochemistry), as well as the loss of ERα from those nuclei in the silenced mice (ERα immunohistochemistry).

2 Materials

2.1 Viral Vectors Containing shRNA for ERα and Luciferase (Luc)

pAAV.H1.Luc and pAAV.H1.ER1 were manufactured by Dr. Sergei Musatov of Neurologix (Fort Lee, NJ). The sequences for the primers used to generate the shRNA are:

Control Group Luciferase (LUC) (5′-GATCCCCCCGCTGG AGAGCAACTGCA TCTTCCTGTCAATGCAGTT GCTCTC CAGCGGTTTTTGGAA-3′ and 5′-CTAGTTC CAAAAACCG C TGGAGAGCAACTGCATTGACAGGAAGATGCAGTTGCTCT CC AGCGGGGG-3′),

Experimental Group ERα (5′-GATCCCCGGCATGGAGC ATCTCT ACACTTCCTGTCAT GTAGAGATGCTCCATGCCT TTTTTGGAAT-3′ and 5′-CTAGATTCCAAAAAAGGC ATGGA GCATCTCTACATGACAGGAAGTGT AGAGATGCTCCATGC CGGG-3′) (for specific details on how these viral vectors were

generated, *see* ref. 3). Expression of these shRNAs is driven by the human H1 promoter. Both vectors express GFP as a marker for the injection site, while the pAAV.H1.ER1 contains a sequence complementary to ERα and pAAV.H1.Luc contains a sequence complementary to luciferase [3].

2.2 Stereotaxic and Microinfusion Materials

1. Small Animal Stereotaxic Instrument (Kopf).
2. Programmable single microinfusion pump (World Precision Instruments).
3. Five microliter Hamilton syringe (Hamilton Company).
4. Injection cannula 28G (length required depends on the brain region being targeted) with PE50 tubing (Plastics One).

2.3 Immunocyto chemical Verification of shRNA Suppression

1. Phosphate-buffered saline (PBS): 0.1 M PBS, pH 7.4. For 1 L of 1 M (10×) PBS add: 18 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, 0.24 g $KHPO_4$, adjust pH to 7.4 with 1 N HCl. Dilute to 1× for use.
2. Heparin 0.1 % (v/v) in 0.1 M PBS, pH 7.4.
3. 4 % (w/v) Formaldehyde in 0.1 M PBS, pH 7.4.
4. 30 % (w/v) Sucrose in 0.1 M PBS, pH 7.4.
5. Sliding freezing microtome to section brains at 40 μm thickness.
6. Cryoprotectant: for 1 L add 300 mL of ethylene glycol, 300 mL of glycerol, 100 mL 0.2 M PBS and 300 mL dH_2O.
7. Netwell inserts (Corning).

2.4 Immunocyto chemistry

1. TBS: For 2 L, add 100 mL of 1 M Tris–HCL, adjust to pH 7.5 with HCl, 18 g NaCl, bring up to 2 L.
2. TBS-T: For 2 L, add 100 mL of 1 M Tris–HCL, adjust to pH 7.5 with HCl, 18 g NaCl, 4 mL Triton X100, bring up to 2 L.
3. H_2O_2 Blocking solution: 100 μL 30 % H_2O_2 for each 3 mL TBS (3 mL/well of a 12-well plate).
4. Blocking solution: For 15 mL, add 0.42 g Bovine Serum Albumin (BSA), 300 μL preimmune goat serum, and 13.7 mL TBS.
5. Primary antibody solutions: Rabbit polyclonal ERα antibody (C1355, diluted 1:10,000 in blocking buffer; Millipore) and rabbit polyclonal enhanced green fluorescent protein (EGFP) antibody (ab290, diluted 1:10,000 in blocking buffer; Abcam).
6. Secondary antibody solutions: Goat biotinylated anti-rabbit antibody diluted 1:200 in blocking buffer.
7. Avidin-biotin enhancement kit such as the ABC Elite Vectastain Kit (Vector Laboratories).

8. DAB (Diaminobenzidine) solution: 50 mg DAB, 150 mg ammonium nickel sulfate, 100 mL TBS and 50 μL 30 % H_2O_2 mixed just before use.
9. Mounting solution: 0.1 % Gelatin, 40 % (v/v) ethanol and PBS.
10. Permount.

2.5 Quantitative Imaging

1. Nikon Eclipse E400 microscope coupled to a Nikon CoolPix 990 camera or equivalent.
2. Adobe Photoshop 11.0 (Adobe Systems) or similar software.
3. Paxinos Mouse Brain Atlas images [14].

3 Methods

3.1 Delivery of Viral Vector shRNA to Animals

1. Prepare animals for brain surgery. Deeply anesthetize the animals, and shave fur extending from neck region to the nose, and as wide as the ears.
2. Place the animal in a stereotaxic frame and secure the animal with the ear bars and nose clip until the head is immovable. Clean area with ethanol and betadine.
3. Perform a midsagittal cut through the skin.
4. Clean the skull area until bregma and lambda are visible.
5. Place the top of the stereotaxic instrument in place.
6. Place the injection needle in the bregma position, take the three coordinates—anterior/posterior, medio/lateral, and dorso/ventral—and add or subtract to these values depending on area to be targeted.
7. Using a pen or pencil mark the area where shRNAs will be delivered.
8. Drill hole(s) in the skull and clean the surface if any blood is visible (*see* **Note 1**).
9. After drilling, fill the injection cannula and make sure that the pump is operational and the syringe is loaded.
10. Fill the injection cannula and tubing with 3–5 μL of the viral vector containing 2×10^9 genomic particles per μL (shRNA Luc (pAAV.H1.Luc) for control animals or shRNA ERα (pAAV.H1.ER1) for experimental animals) and eject a small volume (1 μL) of liquid to make sure that the cannula is working properly.
11. Re-zero the cannula at the bregma and re-calculate the coordinates for the targeted brain area.
12. Very gently lower the cannula into position and start ejecting the liquid very slowly. Total delivery time for 1 μL should be 10 min.

13. Leave the cannula in place for an additional 5 min after the microinjection, and then slowly raise the injection cannula.
14. Following the procedure verify that the cannula is still functional by ejecting an additional 1 μL of liquid. If the cannula is blocked with tissue and no liquid can be ejected, then this information should be noted, as the procedure may have not been effective.
15. In the case of bilateral nuclei, repeat the procedure for the other side.
16. Following the microinjections, suture or staple the scalp closed and apply topical antibiotic to the closed incision.
17. Mark the animals (e.g., with ear markings) and then group house the animals during recovery.
18. Monitor the animals for pain and distress and seek veterinary care when appropriate.
19. Animals need to recover for 2 weeks to enable silencing of gene products before behavioral testing. Behavior testing should be performed and behaviors should be scored by individuals blind to the experimental conditions.
20. This technique has been successfully used to examine the role of ERα on: running wheel activity, sexual incentive motivation, anxiety and maternal behaviors by microinjecting this vector into the medial preoptic area [5, 6], sexual behavior and feeding behavior in the ventromedial nucleus of the hypothalamus [1, 3, 12], and social behavior and recognition, aggression, and anxiety in the medial amygdala [7].
21. All procedures were approved by the Rockefeller University Institutional Animal Care and Use Committee and followed the Public Health Services Policy on Humane Care and Use of Laboratory Animals.

3.2 Verification of Silencing Efficiency

The following steps use double-staining immunohistochemistry techniques to demonstrate the efficacy of the silencing of ERα by the presence (or lack thereof) of ERα (black staining) and its colocalization with GFP (brown staining).

1. After the behavioral testing, perfuse all animals transcardially with 50 mL of 0.1 M PBS containing 0.1 % heparin. This can be done with an osmotic pump or manually with a 50 mL syringe at a rate of approximately one drop per second.
2. Perfuse the animals with 50 mL of 4 % paraformaldehyde.
3. Remove the brains and postfix the brains for 4 h in 4 % paraformaldehyde.
4. Place the brains in 30 % sucrose/0.1 M PBS overnight.
5. Use a sliding freezing microtome to section the brains at 40 μm thickness. Collect the brain sections in four series for the entire

area of interest, as well as control brain regions. Collecting brain slices in four series will enable the analysis of several combinations of expression profiles (we used only one series with double immunohistochemical detection for ERα and GFP) and, importantly, prevents the double or triple counting of neurons between the series. Store the sections in cryoprotectant at –20 °C until immunocytochemical evaluation can be carried out.

6. Carefully pick up the brain slices with a wet brush (not too wet) and move them to 12-well plates containing Netwell inserts and filled with TBS-T.
7. Wash the sections for 10 min with TBS-T.
8. Incubate the tissue sections in H_2O_2 blocking solution to remove endogenous peroxidase activity for 30 min at room temperature on a platform shaker.
9. Wash the brain sections for 10 min with TBS-T three separate times on a platform shaker.
10. Incubate the sections in blocking solution for 1–2 h at room temperature on a platform shaker.
11. Add primary antibody directly to the pre-measured blocking solution in the well, or a new blocking solution can be made. For the GFP antibody a 1:10,000 dilution has worked well in our experience. All antibody concentrations should be tested on control animals prior to processing experimental animals.
12. Incubate brain sections in primary antibody for 48 h at 4 °C with shaking. Wash three times for 10 min per wash with TBS-T with shaking. Prepare secondary antibody at a dilution of 1:200 in blocking buffer.
13. Incubate for 2 h at room temperature with shaking.
14. Wash the sections three times for 10 min per wash with TBS-T with shaking.
15. Incubate brain sections for 1 h at room temperature with the avidin-biotin reagent, prepared according to the manufacturer's specifications.
16. Wash the sections four times for 10 min per wash with TBS-T with shaking.
17. Incubate the brain sections with DAB reagent at room temperature on a platform shaker, using a white background to observe the brown color develop—usually 2–5 min.
18. Stop the reaction by washing with distilled H_2O.
19. The next steps repeat the immunohistochemical staining, but this time for ERα. Repeat the removal of endogenous peroxidase activity by performing a peroxide block for 30 min at room temperature on a platform shaker.

20. Wash the brain sections for 10 min with TBS-T three separate times on a platform shaker.
21. Incubate the brain sections in blocking solution for 1–2 h at room temperature on a platform shaker.
22. Add primary antibody directly to the pre-measured blocking solution in the well with the brain sections, or make a new blocking solution. For the ERα antibody a 1:10,000 dilution has worked well in our experience. All antibody concentrations should be tested on control animals prior to processing experimental animals. Primary antibody incubations should be carried out for 48 h at 4 °C on a platform shaker.
23. Wash the sections three times for 10 min per wash with TBS-T with shaking.
24. Prepare secondary antibody dilution 1:200 in blocking buffer. Incubate the antibody with the brain sections for 2 h at room temperature with shaking.
25. Wash the sections three times for 10 min per wash with TBS-T with shaking.
26. Incubate the brain sections for 1 h at room temperature with avidin-biotin enhancement reagent, prepared according to manufacturer's specifications.
27. Wash the sections four times for 10 min per wash with TBS-T with shaking.
28. Incubate the sections with DAB reagent with nickel at room temperature on a platform shaker, on a white background to see the black color develop—usually 2–5 min.
29. Stop the reaction by washing with distilled H_2O.
30. Prepare pre-coated slides to mount the brains. Double-coat the slides with mounting solution: dip the slides in mounting solution and allow them to dry, then dip in mounting solution again, and allow them to dry the second time before use.
31. When ready to mount, place the brain sections in equal volumes of TBS and mounting solution, and then carefully raise the sections onto the pre-coated slides using a camel hair paintbrush.
32. Incubate slides in an oven at 40 °C for 1 h or overnight at room temperature.
33. Dehydrate the sections by consecutive incubations in distilled H_2O (twice) for 3 m, graded ethanol concentrations (70, 95 and 100 %) two times per solution for 3 min each, and finally twice in xylene for 10 min.
34. Coverslip the slides with Permount and allow slides to dry overnight.

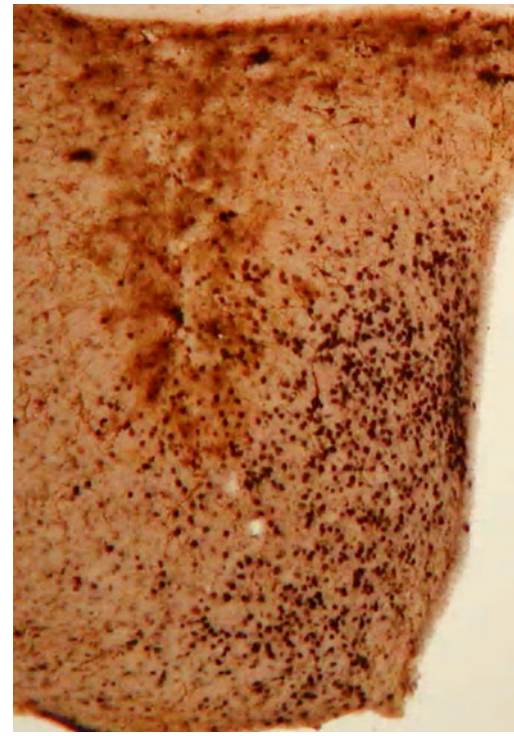

Fig. 1 Immunohistochemical detection of ERα and enhanced green fluorescent protein in brain tissue after viral vector delivery. DAB stained brain tissue: *brown* diffuse staining (DAB)—GFP; *black* nuclear staining (DAB with nickel)—ERα. *Solid black bar*: 200 μm. EGFP immunodetection should be performed first as the staining is lighter in color and will produce less background. EGFP staining is cytoplasmic and very diffuse and is found along the injection tracks. No quantitation of the staining for EGFP was done, only correct placement bilaterally was sought. Staining for ERα, which is found exclusively in the nuclei, should be performed last, and with DAB and nickel since the staining is much darker. The number of ERα immunoreactive cells was counted in the area of interest. It is particularly helpful to overlay a diagram from a mouse brain atlas over the staining image to mark the border of the region of interest and unambiguously count the number of immunoreactive cells. The number of ERα immunoreactive cells were counted in all control animals and averaged. For ERα-silenced mice, the number of immunoreactive cells was likewise counted, and only animals that had a greater than 80 % reduction in positive cells compared to control were included in the behavioral analysis

35. Take photomicrographs of brain areas of interest using a camera attachment for the bright-field microscope (Fig. 1). If the brain area is large, images can be joined using commercial software, such as ImageJ.
36. Overlay photomicrographs with panels from Paxinos Mouse Brain Atlas [14] using Adobe Photoshop 11.0 to determine whether the desired coordinates of injection were achieved.

37. ERα immunoreactive cells (black coloration) that are within the borders of the brain region of interest should be manually counted by an individual who is blinded to the experimental assignments.
38. EGFP immunoreactivity (brown coloration) should also be marked to evaluate microinjection coordinates of shRNA LUC animals (*see* **Note 2**).
39. Animals in the shRNAERα should have correct bilateral coordinates (based on GFP expression) as well as greater than 80 % suppression of ERα, compared to shRNA Luc animals (*see* **Notes 3** and **4**). Efficiencies for the surgery and bilateral suppression are usually in the 50 % range.
40. If animals in the control or experimental group do not have proper bilateral GFP expression in the targeted brain region they will not be included in the analysis.
41. Furthermore, in the experimental group (ERα-silenced animals), if the number of ERα-expressing neurons is greater than 20 % of the control (Luc) animals, then that animal will likewise not be included in the analysis. The number of neurons expressing ERα in the control groups are counted first, and the only animals in the experimental group that are included are those that contain less than 20 % of the control levels.

4 Notes

1. Special caution needs to be taken if coordinates are along the midline as extensive bleeding can occur. In such cases, stereotaxic delivery of the shRNA can be done at an angle to avoid the midline.
2. Injection tracts from the microinjections are not always visible, so all animals are immunostained for GFP (which is in both viral vector constructs) to determine injection sites.
3. Animal behavior should be scored only after immunocytochemical evaluation, as many animals will undoubtedly be eliminated. The efficiencies of correct bilateral injections and proper suppression of ERα are generally in the 50 % range.
4. In no case does this type of shRNA local manipulation result in a total loss of target protein, but significant behavioral results can, nonetheless, be demonstrated.

Acknowledgements

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Chapter 28

Silencing Estrogen Receptor-β with siRNA in Cultured Cells

Ren-Jun Hsu and Jui-Ming Liu

Abstract

Estrogen receptors α and β (ERα and ERβ) are the two genomic estrogen receptors. ERβ was the second of these receptors to be discovered; its structure is similar to that of ERα but they are different in histological distribution. However, the functions of ERα versus ERβ are still unclear. The ability of small interfering RNAs (siRNAs) to silence gene expression has proven to be invaluable for studying gene function in cultured mammalian cells. This chapter describes the use of siRNA to inhibit the expression of ERβ in renal cell carcinoma (RCC) and to further the understanding of ERβ function in RCC.

Key words Estrogen, Estrogen receptor β, Small interfering RNA, Transfection, Western blot

1 Introduction

Estrogen is a female hormone that is secreted mainly by the ovaries. It promotes the development of the female reproductive system and the proliferation of the endometrium as part of the menstrual cycle. Estrogen shows periodic changes with fluctuating secretion during the reproductive years. The functions of estrogen include mammary gland proliferation, water and sodium retention, bone maintenance, and promotion of subcutaneous fat accumulation [1–5]. Furthermore, estrogen reduces the risk of developing diseases such as coronary atherosclerosis [6–9], osteoporosis [10–12], and Alzheimer's disease [13].

Estrogen binds to genomic estrogen receptors, ERα and ERβ, and subsequently regulates the transcription of downstream genes. The correlation between ERα and breast cancer has been extensively studied while the actual molecular mechanism of ERβ is still unclear [14, 15]. Although the structures of ERα and ERβ

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are similar, their histological distributions and biological functions are different.

Previous studies have showed that the expression of ERβ in normal cells is higher than in cancer cells [16]. Other studies have demonstrated that ERβ can decrease proliferation and induce apoptosis [17]. Thus, we speculated that ERβ might play an important role as a tumor suppressor in carcinogenesis. We hypothesized that estrogen would inhibit carcinogenesis and progression in renal cell carcinoma (RCC) cells and that there might be a biological effect of estrogen on RCC. Our goal is to observe the biological and clinical effects of ERβ in RCC and we have observed that the effect of estrogen is strongest in the 786-O RCC cell line.

Small interfering RNAs (siRNA) are 21–23 nucleotide double-stranded RNA (dsRNA) molecules containing two unpaired bases at the 3′ end of each strand. Double-stranded RNA can be processed into single-stranded RNA (ssRNA) through a multi-protein complex. The siRNA and protein complex is called the RNA-induced silencing complex (RISC). RISC can perfectly match and bind to mRNA, then degrade the targeted mRNA to prevent its translation [18]. The significant silencing and strongly inhibitory effect of siRNA on its target genes has made siRNA an invaluable tool for studying gene function in cultured mammalian cells. The expectation is that siRNA will ultimately be useful for the prevention and treatment of diseases [19]. Indeed, siRNA has been shown to inhibit *Fas* gene expression in an animal model and to thereby protect mice from antibody or concanavalin A-induced hepatitis [20]. However, siRNA can only play a role in silencing genes if they enter target cells. Thus, the question how to transfect the exogenous siRNA into target cells becomes a key factor of gene silencing. Gene transfer can be carried out in many ways, such as calcium phosphate precipitation, electroporation, liposomes, microinjection, or retrovirus infection.

Retroviruses are the most efficient transfection vectors, but they are expensive and time consuming, so retroviruses are not usually the first option for transfecting genes. Liposomes are one of the most convenient ways to transfect genes in spite of its relatively low transfection efficient in primary suspension cells. This chapter describes the use of liposomes to transfect ERβsiRNA into RCC to inhibit ERβ expression.

2 Materials

2.1 Cell Culture and Chemicals

1. Human renal cell carcinoma (RCC) cell line 786-O (Bioresource Collection and Research Center).
2. Growth medium: RPMI media without phenol red (HyClone), supplemented with 5 % fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, and 1 % penicillin/streptomycin.
3. Transfection medium: DMEM or RPMI media (HyClone), supplemented with 2 mM glutamine, and 1 mM sodium pyruvate.
4. Dulbecco's Phosphate-Buffered Saline (DPBS) (HyClone).

2.2 siRNA Transfection

1. 20 μM/μL siERβ1 (HSS103378), siERβ2 (1HSS176622), siERβ3 (HSS103380) (Life Technologies).
2. Annealing buffer.
3. Lipofectamine® 2000 Reagent (Life Technologies). Store at 4 °C until use.
4. OPTI-MEM® I Reduced Serum Medium (GIBCO).
5. Silencer® Negative Control (Life Technologies).
6. 6 cm culture dishes.

3 Methods

1. Maintain 786-O cells in growth medium in a cell culture incubator at 37 °C, 5 % CO_2 (*see* **Note 1**).
2. Plate 1×10^6 cells in a 6 cm culture dish and culture for 8 h.
3. Remove the medium and wash the cells with DPBS.
4. Replace the medium with FBS-free medium overnight at 37 °C with 5 % CO_2 (*see* **Notes 2–4**).
5. Mix 20 μM nucleic acid (ERβsiRNA or controls in annealing buffer) with 200 μL OPTI-MEM. In a separate tube, mix 6 μL Lipofectamine 2000 reagent with 200 μL OPTI-MEM. Allow both mixtures to stand for 5 min at room temperature (*see* **Note 5**).
6. Add the contents of the Lipofectamine 2000 reaction to the nucleic acid-containing tube, and allow this mixture to stand for 20 min at room temperature (*see* **Note 6**).
7. Add the mixture to the 6 cm culture dish and incubate 37 °C and 5 % CO_2 for 8–12 h.

8. After the incubation, replace the transfection mixture with growth medium.
9. Culture the cells at 37 °C with 5 % CO_2 for another 48 h (*see* **Note 7**).
10. Collect the transfected cells and process for the extraction of RNA or protein assays (Fig. 1).

4 Notes

1. Check the cells for mycoplasma contamination before performing the transfection.
2. Transfect cells at 60–70 % confluence. For example, plate 1×10^6 of the 786-O cells in 2 mL of growth medium without FBS and antibiotics 1 day before transfection; cells should then be 60–70 % confluent at the time of transfection.
3. Use low-passage cells to make sure that cells are healthy and greater than 80 % viable before transfection.
4. Do not add antibiotics to the medium during transfection as it will reduce the transfection efficiency and cause cell death.
5. Usually, the volume of Lipofectamine® 2000 is two to three times that of siRNA.
6. Use Opti-MEM® I Reduced Serum Medium to dilute Lipofectamine® 2000 and siRNA prior to complex formation. Opti-MEM® I Reduced Serum Medium can enhance the efficiency of transfection, but is not necessary and it can be replaced by serum-free medium if it is convenient.
7. Culture the cells for 48 h after the transfection to allow time for ERβ protein turnover and to use ERβ siRNA to inhibit the translation of new ERβ mRNA.

Fig. 1 (continued) ERβ expression), or siERβ for 48 h. Panel **a** shows a representative western blot of ERβ protein in 786-O cells and panel **b** shows the corresponding densitometric quantitation of the western blot data from panel **a**. The cells were treated with vehicle control (Con), or 200 μM scrambled siRNA (Scr), siERβ1 (HSS103378), siERβ2 (1HSS176622), siERβ3 (HSS103380), or siER β123 mixture. In panel **c**, 786-O cells were transfected with siERβ1 and the ERβ mRNA expression was detected by real-time PCR. The western blotting data (panels **a** and **b**) show that the expression of ERβ was downregulated after transfection with siERβ, and that all three siERβ were equally effective. Panel **c** shows that ERβ mRNA expression was downregulated after transfection of 786-O with different concentrations of siERβ

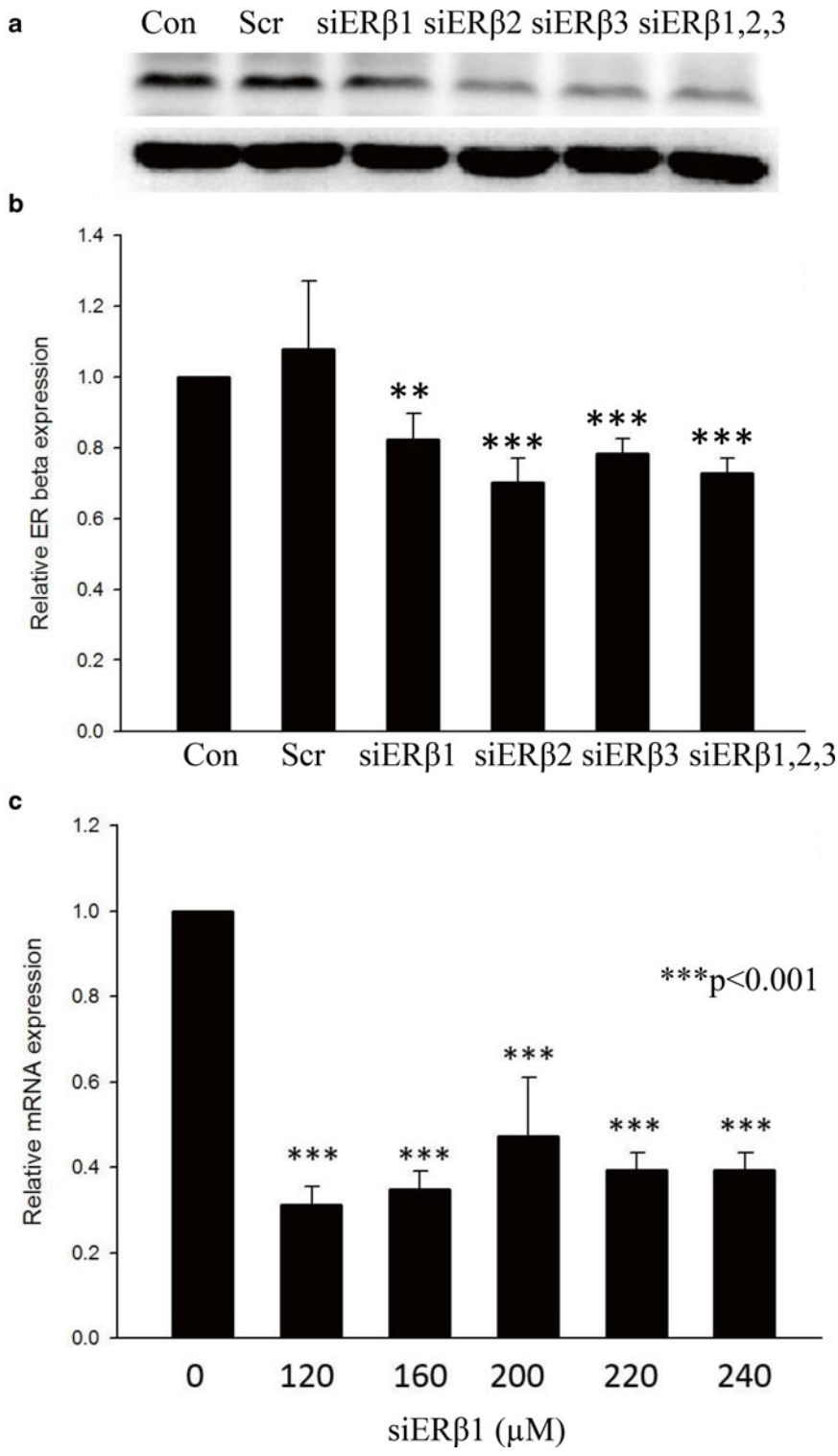

Fig. 1 siRNA for ERβ (siERβ) downregulates protein and mRNA expression of ERβ in 786-0 renal carcinoma cells. 786-0 cells were treated with vehicle, scrambled siRNA (nonsense siRNA sequence that would not affect

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Chapter 29

Experimental Procedures for Demonstration of MicroRNA Mediated Enhancement of Functional Neuroprotective Effects of Estrogen Receptor Agonists

Mrinmay Chakrabarti and Swapan K. Ray

Abstract

Protection of motoneurons is an important therapeutic goal in the treatment of neurological disorders. Recent reports have suggested that specific microRNAs (miRs) could modulate the expression of particular proteins for significant alterations in the pathogenesis of different neurological disorders. Thus, combination of overexpression of a specific neuroprotective miR and treatment with a neuroprotective agent could be a novel strategy for functional protection of motoneurons. The protocols described herein demonstrate that miR-7-1, a neuroprotective miR, can enhance the functional neuroprotective effects of estrogen receptor agonists such as 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT), Way 200070 (WAY), and estrogen (E2) in preventing apoptosis in A23187 calcium ionophore (CI) exposed VSC4.1 motoneurons. This article describes the protocols for the cell viability assay, transfection of VSC4.1 motoneurons with miRs, Annexin V/propidium iodide staining for apoptosis, Western blotting, patch-clamp recording of whole-cell membrane potential, and JC-1 staining for detection of mitochondrial membrane potential. Taken together, these protocols are used to demonstrate that miR-7-1 caused significant enhancement of the efficacy of estrogen receptor agonists for functional neuroprotection in VSC4.1 motoneurons.

Key words Apoptosis, miR-7-1, PPT, WAY, Estrogen, VSC4.1 motoneurons

1 Introduction

Spinal cord injury (SCI) and amyotrophic lateral sclerosis (ALS) are severe neurological disorders that manifest interruption in motor signals due to progressive death of motoneurons in the spinal cord. In SCI and ALS patients, current therapeutic agents cannot effectively prevent excessive neurodegeneration, especially of motoneurons. Thus, it is imperative to develop new therapeutic strategies that demonstrate the inhibition of signaling pathways responsible for apoptotic death of spinal cord neurons [1, 2].

Calcium ionophore (CI) is extensively used to mimic the effect of many pathophysiological stimuli that relate to Ca^{2+} overload [3, 4]. Some earlier reports indicated that CI caused over activation of

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glutamate receptors leading to induction of apoptosis [5, 6]. Therefore, we used CI in this study for demonstration of apoptotic death of motoneurons as a cell culture model of SCI.

MicroRNAs (miRs) play critical roles in modulation of the pathogenesis in neurological disorders including SCI and ALS [7]. Deregulation of many neuroprotective miRs have been observed in different pathophysiological processes such as inflammation, oxidative stress, apoptosis, glial scar formation, and axonal damage that ultimately lead to neurodegeneration [7–9]. Earlier studies indicated that miRs could modulate expression of many genes post-transcriptionally or through promoter interaction [10, 11]. In our investigation, we made an attempt to show ectopic overexpression of neuroprotective miR-7-1 for enhancing the functional neuroprotective effects of estrogen receptor (ER) agonists in a cell culture model of SCI.

The VSC4.1 motoneuron cell line was generated by fusion of dissociated embryonic rat ventral spinal cord neuron with mouse N18TG2 neuroblastoma cell [12]. Estrogen (E2) and other ER agonists provide neuroprotection in many neurodegenerative disorders, including SCI [2, 13]. ER agonists are well known to suppress intracellular free Ca^{2+}, activation of calpain, and apoptotic death through activation of ER alpha (ERα) and ER beta (ERβ) [14]. This article describes the protocols for the molecular biology methods and electrophysiological recordings that we have used to demonstrate that ectopic overexpression of miR-7-1 followed by treatment with an ER agonist can dramatically reverse CI toxicity in VSC4.1 motoneurons and maintain neuronal functionality. Thus, combination of overexpression of a neuroprotective miR and treatment with an appropriate ER agonist could be a novel therapeutic strategy for functional protection of motoneurons in SCI as well as in ALS. This article describes the protocols for the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell viability assay, transfection of VSC4.1 motoneurons with miRs, Annexin V/propidium iodide staining for apoptosis, Western blotting, patch-clamp recording of whole-cell membrane potential, and JC-1 staining for detection of mitochondrial membrane potential.

2 Materials

1. The hybrid VSC4.1 motoneuron cell line was generated by fusion of embryonic rat ventral spinal cord neuron with mouse N18TG2 neuroblastoma cell [12].
2. Growth medium: DMEM/F12 medium with 15 mM HEPES, 4 mg/L pyridoxine hydrochloride, and 3.7 g/L $NaHCO_3$, supplemented with 2 % Sato's components, 100 IU/mL of penicillin, and 100 IU/mL streptomycin, and 10 % heat-inactivated fetal bovine serum (FBS).

Sato's components: 200 mL PBS + 5.72 mL cell culture BSA (Sigma), 200 mL water + 322 mg putrescine dihydrochloride (Sigma), 20 mL ethanol + 8 mg L-thyroxine sodium salt pentahydrate (Sigma), 20 mL ethanol + 6.74 mg triiodo-l-thyronine sodium salt (Sigma), 1 mL progesterone stock solution (50 mL ethanol + 62.3 mg progesterone, Sigma), 1 mL selenium stock solution (50 mL water + 38.7 mg sodium selenide, Sigma).

3. Calcium ionophore A23187 (CI) (Sigma), 1,3,5-tris (4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT), Way 200070 (WAY), and estrogen (E2). CI stock solution (10 mg/mL) should be prepared in DMSO by gentle heating. It can be stored up to 3 months at –20 °C in dark. Stock solutions for PPT and WAY should be prepared in DMSO at a final concentration of 10 mg/mL and stored at –20 °C in dark. E2 is soluble in ethanol and a final concentration of 50 mg/mL can be made and stored at –20 °C. As E2 is air sensitive, it would be advisable to prepare fresh solutions rather than store solutions for future.
4. For transfection in VSC4.1 motoneurons, miR-7-1 mimics were purchased from Dharmacon. Briefly, centrifuge tubes containing miR mimics and then dissolved into a convenient stock solution using RNase-free water (for 20 nM miR mimics, add 200 μL water to get a final concentration of 100 μM). After brief vortexing, centrifuge the tubes containing miR mimics stock solution, aliquot into small volumes, and store at –20 °C. For best results, limit freeze–thaw events for each tube to no more than five times.
5. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide): 0.2 mg/mL in growth medium. Store at –20 °C (*see* **Note 1**).
6. Microplate reader (Biotek).
7. Isopropanol.
8. Lipofectamine 2000 transfection reagent and Opti-MEM medium (Invitrogen).
9. PBS (phosphate buffered saline), pH 7.4.
10. Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) (BD Biosciences).
11. Epics XL-MCL Flow Cytometer (Beckman Coulter).
12. Lysis buffer: 50 mM Tris–HCl, pH 7.4, 1 mM PMSF, 5 mM EGTA.
13. Coomassie-Plus protein reagent (Pierce Biotechnology).
14. 4–20 % sodium dodecyl sulfate (SDS) polyacrylamide gels.
15. Polyvinylidene fluoride (PVDF) membranes (Millipore).
16. Blocking buffer: 5 % nonfat milk.

17. Antibodies: primary IgG antibodies against β-actin, ERβ, ERα, Bax, and Bcl-2 (Santa Cruz Biotechnology). Secondary antibodies: horseradish peroxidase (HRP) conjugated IgG antibody.
18. X-OMAT AR films (Eastman Kodak).
19. HRP-Immunostar-chemiluminescence reagent (Bio-Rad Laboratories).
20. EPSON Scanner with Photoshop software (Adobe Systems) and Gel-Pro analyzer software (Media Cybernetics, Silver Spring, MD, USA).
21. Axopatch 200B amplifier (Molecular Devices) and AxographX software (Axograph).
22. Extracellular recording solution: 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 10 mM glucose, and 5 mM HEPES, pH 7.2, osmolarity adjusted to 325 mOsm/L with sucrose.
23. Internal electrode solution for patch electrodes (2.5–4.0 MΩ): 150 mM KCl, 2.5 mM NaCl, 4 mM Mg-ATP, 2 mM Na-ATP, 0.3 mM Na-GTP, 5 mM Na-phosphocreatine, 10 mM HEPES, pH 7.4, osmolarity adjusted to 310 mOsm/L with sucrose.
24. JC-1 fluorescent probe, 5 μg/mL (Life Technologies) in DMEM/F12 medium.
25. Fluorescent plate reader (Molecular Devices).
26. DNase-free RNase A, 2 mg/mL.
27. Statistical software such as Minitab 16 (Minitab, State College, PA, USA).

3 Methods

3.1 MTT Assay for Cell Viability

1. Use aseptic conditions in a laminar flow hood to carry out all cell culture work. Culture the VSC4.1 cells in monolayer to subconfluency in 75-cm^2 flasks containing 10 mL of DMEM/F12 medium.
2. Seed VSC4.1 motoneurons into 96-well micro-culture plates at 1×10^4 cells/well.
3. At 70–80 % confluence, insult the motoneurons with 25, 50, 100, 200, or 500 nM CI and incubate for 24 h in DMEM/F12 medium supplemented with 2 % FBS.
4. Replace with fresh medium containing 0.2 mg/mL MTT and incubate for 3 h.
5. Add 100 μL isopropanol to dissolve the MTT formazan crystals. Gentle stirring in a shaker and slow pipetting up and down helps completely dissolve the MTT formazan crystals (*see* **Note 2**).
6. Measure absorbance of the color at 570 nm with background subtraction at 630 nm in a microplate reader.

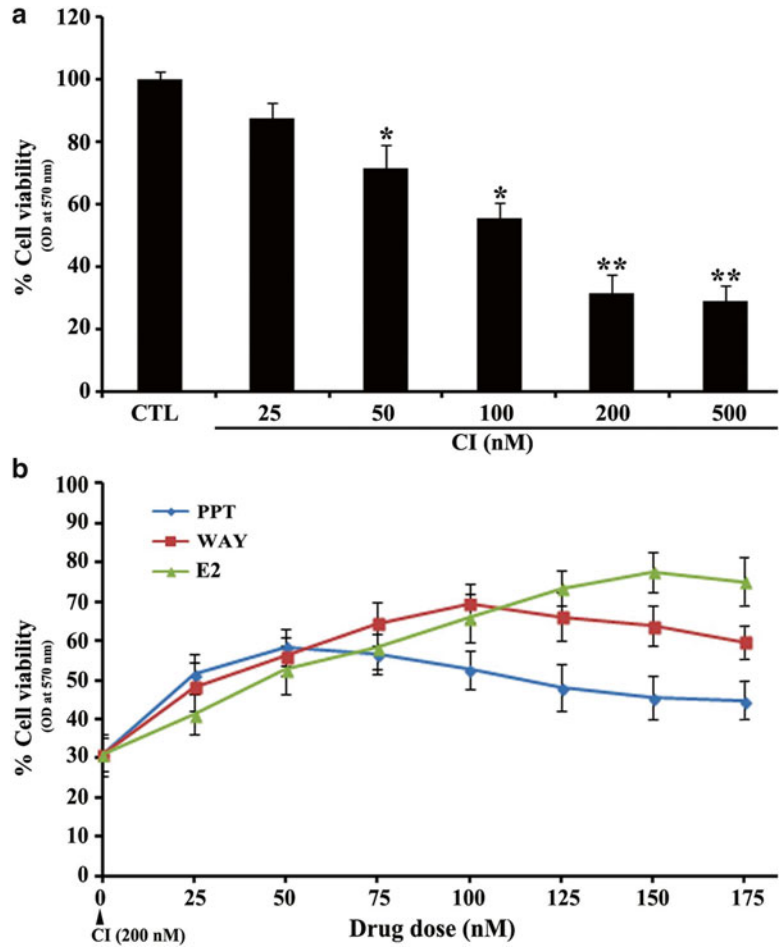

Fig. 1 The MTT assay was used to optimize the concentration of calcium ionophore (CI) needed for the induction of cell death in VSC4.1 motoneurons. Panel **a** illustrates residual cell viability in VSC4.1 motoneurons in untreated control (CTL) and in response to 25–500 nM CI for 24 h. Difference between CTL and a drug treatment was considered significant at $*p<0.05$ or $**p<0.01$. 200 nM CI was chosen as an optimum insult for induction of cytotoxicity in VSC4.1 cells. Panel **b** illustrates the effects of post-treatment of cells with ER agonists. VSC4.1 cells were treated with 200 nM CI for 24 h, then treated with increasing concentrations (0–175 nM) of E2, WAY, or PPT for 24 h, and the MTT assay was performed to determine residual cell viability. These data indicated that 150 nM E2, 50 nM PPT, and 100 nM WAY could most effectively protect motoneurons from the cytotoxic insult induced by CI. (Data are adapted from ref. [2] with permission)

7. Calculate cell viability from the MTT data as a percentage of viable cells in the total population (Fig. 1a).
8. To test the neuroprotective efficacy of ER agonists following CI insult, incubate VSC4.1 motoneurons with 200 nM CI for 24 h.

9. Post-treat the cells with 0–175 nM of PPT, WAY, or E2 and incubate for 24 h.
10. Perform the MTT assay as in **steps 3–6** above (Fig. 1b).

3.2 Transfection of VSC4.1 Motoneurons with a Specific miR

1. Seed VSC4.1 motoneurons at a concentration of 5×10^5 cells per well in 6-well plates and allow to grow overnight.
2. On the next day, transfect the cells with anti-miRs or pre-miRs of miR-7-1 oligomeric RNA at a final concentration of 50 nM using 20 μL Lipofectamine 2000 reagent and Opti-MEM medium following the manufacturer's protocol. Transfection of a negative control or anti-miR is necessary for all experiments to determine transfection efficiency and specificity of miR mimics under study. Results from the negative control should also be compared to results from untransfected cells (*see* **Note 3**).
3. After 12 h of transfection, replace the transfection medium with fresh growth medium containing 2 % FBS and nothing (control) or 200 nM CI and incubate the cells for 24 h.
4. At the end of the incubation, further treat the cells with 100 nM WAY or 150 nM E2 and grow for another 24 h.
5. Examine the neuroprotective effects of overexpression of miR alone or in combination with drugs by flow cytometry, Western blotting, and whole-cell membrane potential analysis (further protocols below).

3.3 Antibodies and Western Blotting

1. Following treatments as described in Subheading 3.2 above, homogenize the cells in lysis buffer.
2. Determine protein concentrations with Coomassie-Plus protein reagent.
3. Resolve equal quantity of proteins in each lane of 4–20 % polyacrylamide gels by SDS polyacrylamide gel electrophoresis (SDS-PAGE).
4. Transfer the resolved proteins from the SDS-PAGE gels to PVDF membranes electrophoretically.
5. Block the membranes in 5 % nonfat milk for 1 h.
6. Probe the PVDF membranes with specific primary IgG antibody (each antibody diluted at 1:1000) against β-actin (loading control), ERβ, ERα, Bax, or Bcl-2.
7. Detect the primary IgG antibody with horseradish peroxidase (HRP) conjugated secondary IgG antibody (1:3000) against the species-specific primary antibody.
8. After incubation with secondary antibody, incubate the membranes with HRP-Immunostar chemiluminescence reagent.
9. Expose the membranes to X-OMAT AR film.

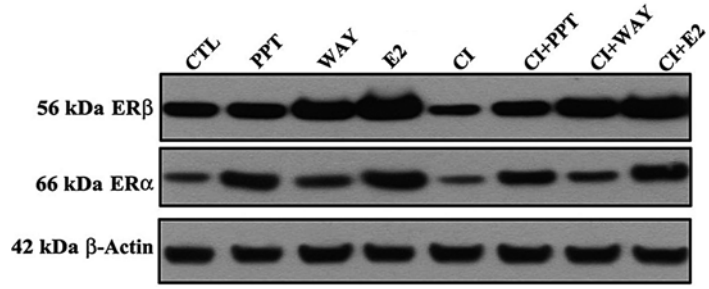

Fig. 2 Western blots demonstrated that treatment of VSC4.1 motoneurons for 24 h with 50 nM PPT, 100 nM WAY, or 150 nM E2 upregulated the expression of ERβ and ERα proteins when compared with control (CTL). The increases in ERβ and ERα protein persisted in cells that had been pretreated with 200 nM CI for 24 h before treatment with the estrogen agonists. β-Actin served as a loading standard. These data suggest that the ER agonists exert their neuroprotective efficacy via the genomic ER mediated mechanism. (Data are adapted from ref. [2] with permission)

10. Scan the autoradiograms on an EPSON Scanner using Photoshop software to identify alterations in expression of specific proteins (Fig. 2).
11. Quantify the density of the protein bands using Gel-Pro analyzer software (Fig. 3).
12. Perform all experiments in triplicate.

3.4 Annexin V-FITC/PI Staining Followed by Flow Cytometry

1. Harvest VSC4.1 cells that have been transfected with miR-7-1, insulted with CI, and post-treated with WAY or E2 as described in Subheading 3.2 above.
2. Wash the cells with PBS.
3. Fix the cells with 70 % ethanol for 15 min.
4. Pellet the cells by centrifugation at 800 × *g* for 10 min.
5. Aspirate residual ethanol from the cell pellets.
6. Treat the VSC4.1 motoneurons to treatment with 2 mg/mL DNase-free RNase A for 30 min at 37 °C. PI is a nucleic acid-specific, red-fluorescent dye that can bind both DNA and RNA. RNAse A treatment will digest all cellular RNA, so that the PI will specifically label the DNA.
7. Perform double staining of the cells with Annexin V-FITC/PI as per manufacturer's instructions (BD Bioscience). Briefly, wash cells thrice with cold 1× PBS and resuspend cells (10^6 cells/mL) in 1× Binding Buffer. Take 100 μL of cell suspension (~10^5 cells) in a 5 mL polystyrene tube, and add 5 μL of Annexin V-FITC and 5 μL PI. After vortexing gently, incubate cells for 15 min at room temperature (25 °C) in the dark, then add 400 μL of 1× Binding Buffer to each tube and perform flow cytometry analysis within 30 min (*see* **Note 4**).

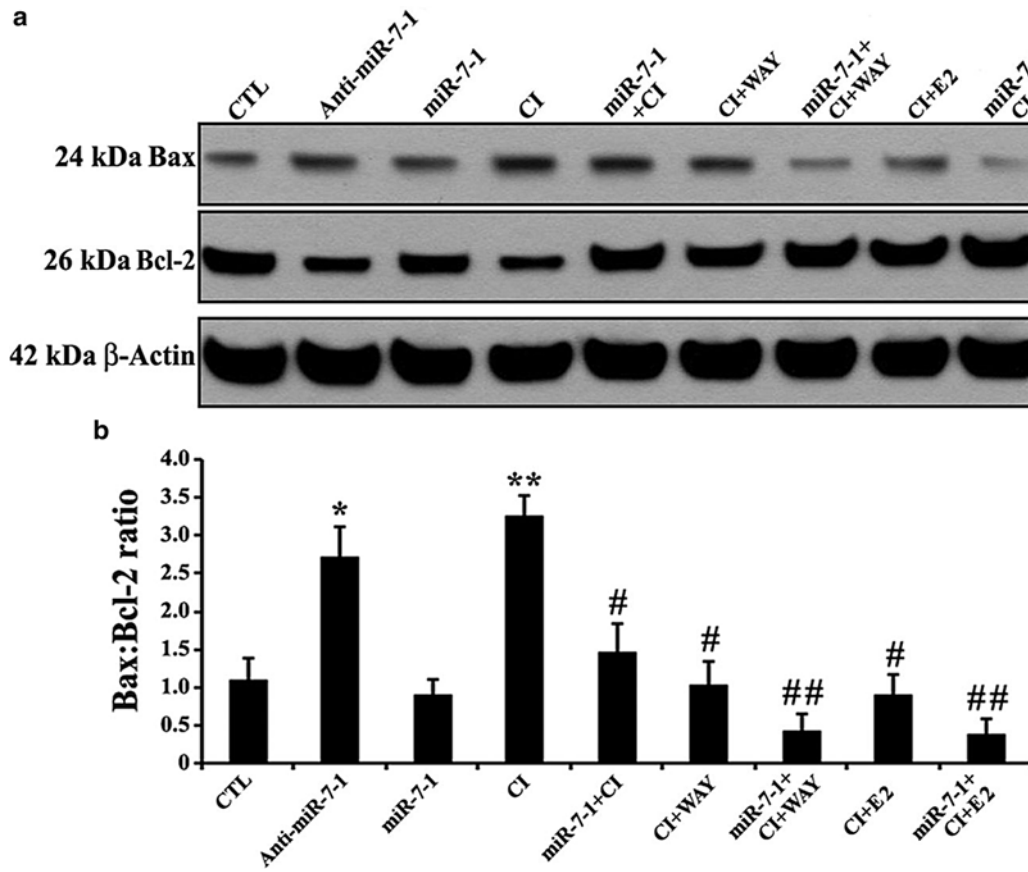

Fig. 3 The ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 was used as a marker of activation of the apoptotic signaling cascade. Treatments of the VSC4.1 motoneurons were: untreated control (CTL), anti-miR-7-1 (50 nM) for 12 h, miR-7-1 (50 nM) for 12 h, 200 nM CI for 24 h, miR-7-1 (50 nM) for 12 h + 200 nM CI for 24 h, 200 nM CI for 24 h + 100 nM WAY for 24 h, miR-7-1 (50 nM) for 12 h + 200 nM CI for 24 h + 100 nM WAY for 24 h, 200 nM CI for 24 h + 150 nM E2 for 24 h, and miR-7-1 (50 nM) for 12 h + 200 nM CI for 24 h + 150 nM E2 for 24 h. Panel **a** shows the effect of the various treatments on Bax, Bcl-2, and β-actin protein expression in a representative Western blot. Panel **b** shows the Bax:Bcl-2 ratio as derived from the densitometric analysis of the Western blot data. Differences were analyzed between CTL vs. treatment (designated as significant at $*p<0.05$ or $**p<0.01$) and CI vs. CI plus treatment (designated as significant at $\#p<0.05$ or $\#\#p<0.01$). The data indicate that monotherapy with miR-7-1, WAY, or E2 after CI insult can downregulate Bax:Bcl-2 ratio to some extent, but transfection with miR-7-1 followed by an ER agonist treatment suppresses the Bax:Bcl-2 ratio more effectively when compared with the CI treated VSC4.1 cells. Thus, these data confirm that sequential combination therapy with miR-7-1 and WAY or E2 can block the downstream apoptotic signaling cascade and provide neuroprotection in VSC4.1 motoneurons. (Data are adapted from ref. [2] with permission)

8. Analyze the Annexin V-FITC and PI stained cells by flow cytometry on an Epics XL-MCL Flow Cytometer with excitation wavelength of 488 nm and emission wavelength of 525 and 625 nm, respectively (Fig. 4). Approximately 20,000 cells per sample may be analyzed at a time. To detect the apoptotic population, dot-plots with PI−/Annexin V−, PI−/Annexin V+, PI+/Annexin V−, and PI+/Annexin V+ are drawn.

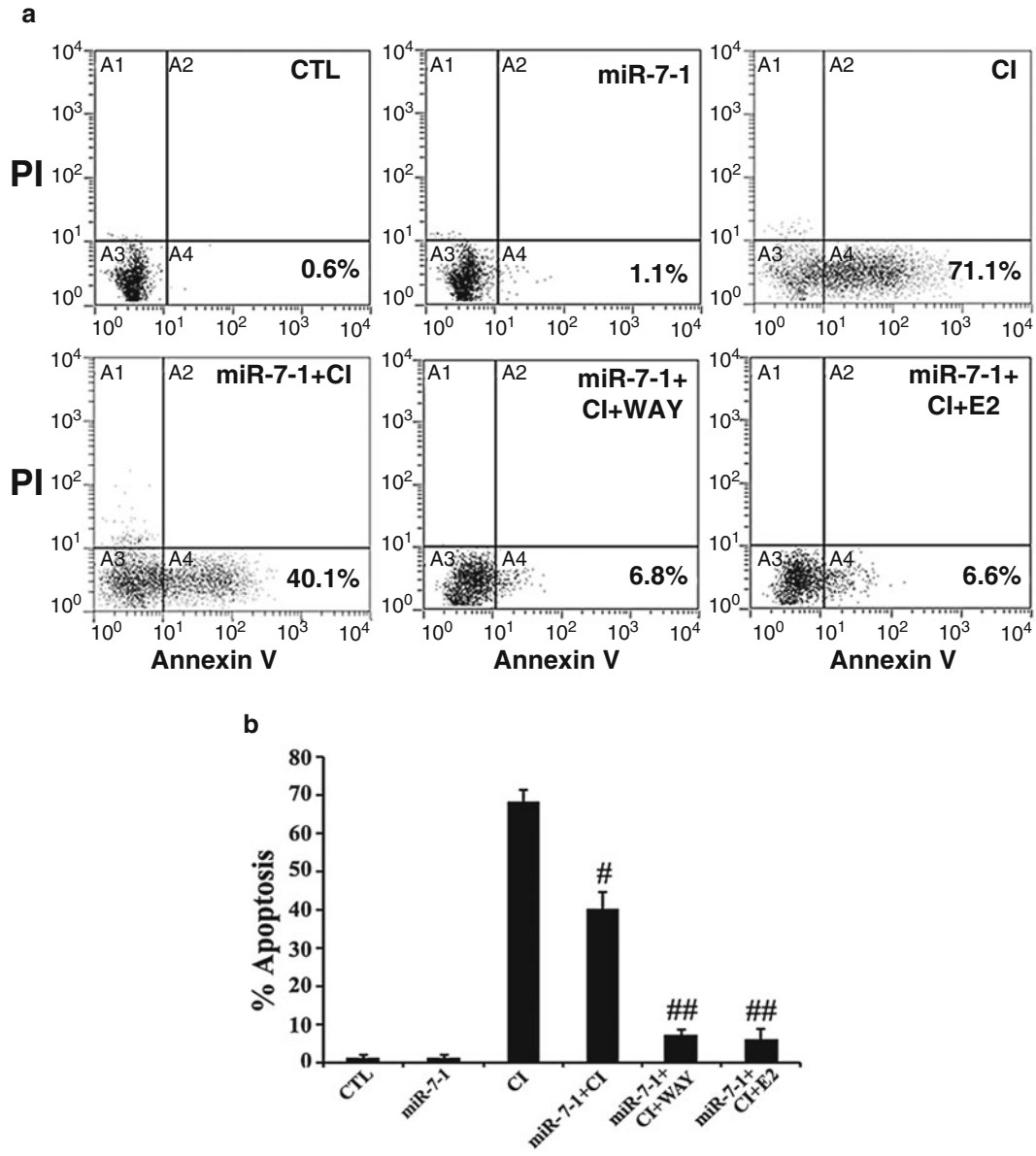

Fig. 4 Overexpression of miR-7-1 enhanced the neuroprotective efficacy of WAY and E2 in VSC4.1 motoneurons as measured by Annexin-V-FITC/PI staining followed by flow cytometry. Panel **a** shows the flow cytometry data for the following treatment groups: untreated control (CTL), miR mimics (50 nM) for 12 h, 200 nM CI alone for 24 h, miR mimics (50 nM) for 12 h + 200 nM CI for 24 h, miR mimics (50 nM) for 12 h + 200 nM CI for 24 h + 100 nM WAY for 24 h, and miR mimics (50 nM) for 12 h + 200 nM CI for 24 h + 150 nM E2 for 24 h. Panel **b** illustrates the percentage of apoptosis as identified by flow cytometry. Differences between CI insulted cells and other treatments of CI insulted cells were considered significant at $\#p < 0.05$ or $\#\#p < 0.01$. The data show that 200 nM CI induced approximately 70 % apoptosis in VSC4.1 motoneurons. Transfection with miR-7-1 decreased apoptotic cell death to approximately 40 % in CI insulted VSC4.1 cells. Sequential transfection with miR-7-1 followed by treatment with WAY or E2 significantly enhanced neuroprotection, decreasing the apoptotic cell population to less than 7 %. (Data are adapted from ref. [2] with permission)

Carry out the flow cytometric analysis within 30–45 min of completing the staining with Annexin V-FITC and PI as erroneous measurement of the amounts of apoptosis are obtained from cells that are kept on hold for a prolonged period before flow cytometry.

9. Interpretation of the results: cells that are negative for both Annexin V-FITC and PI are considered to be normal. Cells that stain positive for Annexin V-FITC and negative for PI are considered as being in early apoptosis. Cells that are positive for both Annexin V-FITC and PI are considered to be late necrotic. Cells that stain negative for Annexin V-FITC and positive for PI are considered to have been mechanically injured during the experiment (*see* **Note 5**).

3.5 Electrophysiological Recordings of Whole Cell Membrane Potential

1. After transfection or/and drug treatments as in Subheading 3.2 above, perfuse the cells at room temperature with extracellular recording solution.
2. Fill the Patch electrodes (2.5–4.0 MΩ) with internal solution.
3. Set the liquid junction potential at 4.1 mV and correct for this potential in all recordings. After seal formation and breakthrough in voltage-clamp (holding potential –60 mV), switch the amplifier to current-clamp mode with zero holding current and the record the resulting membrane potential.
4. After electrical compensation, determine access resistance in current-clamp mode and continuously monitor in voltage-clamp mode by measuring the size of the capacitance transient in response to a 10 mV hyperpolarizing step. The access resistance should be examined continuously in current-clamp mode and stopped if changes >20 % are encountered. All current-clamp recordings are performed in Axopatch 200B amplifier in conjunction with AxographX software and membrane potentials are corrected for the liquid junction potentials and to the null potential measured at the end of recording (Fig. 5a) (*see* **Note 6**).

3.6 Detection of Mitochondrial Membrane Potential

1. After transfection or/and drug treatments as in Subheading 3.2 above, incubate cells in DMEM/F12 medium containing 5 μg/mL of the fluorescent probe, JC-1, during treatment from 0 to 12 h to measure mitochondrial potential loss (*see* **Note 7**). Do not fix the cells.

Fig. 5 (continued) intact mitochondrial membrane potential. After exposure to CI, the mean fluorescence ratios of the mitochondria slowly decreased in a biphasic manner, indicating the loss of mitochondrial membrane potential. Transfection with miR-7-1 followed by treatment with WAY or E2 very effectively attenuated the loss of the mitochondrial membrane potential (Data are adapted from ref. [2] with permission)

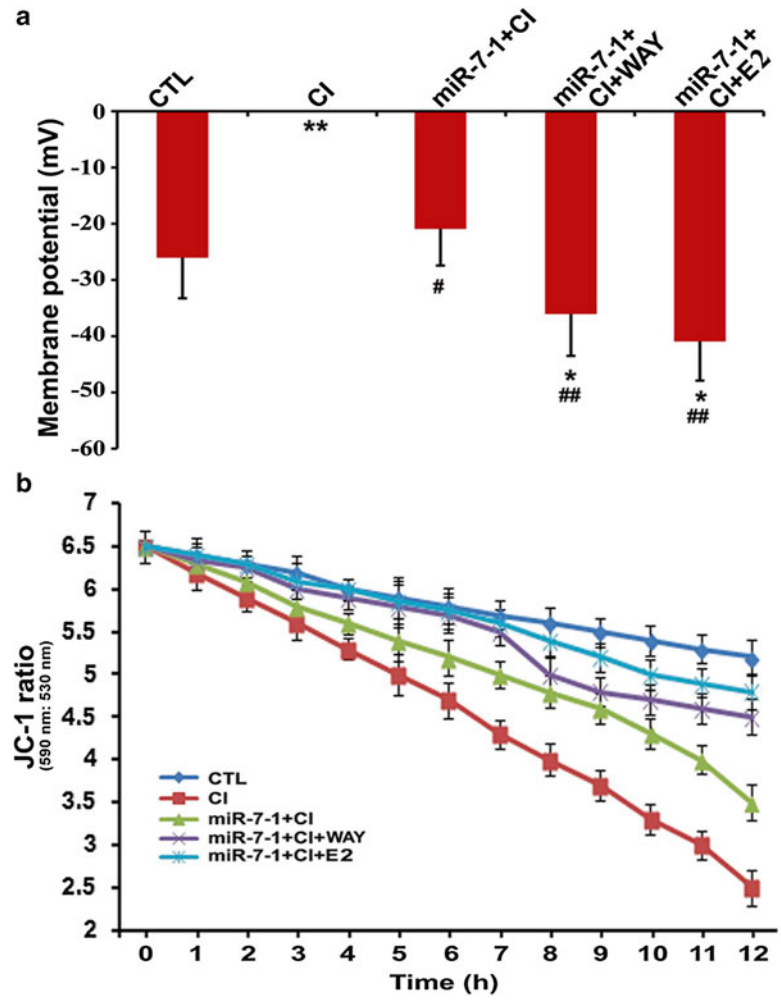

Fig. 5 The whole-cell membrane potential, measured by patch-clamp technique, and the mitochondrial membrane potential, measured by the ratio of JC-1 staining JC-1 staining, were used as indicators of the preservation of neuronal functionality. The treatment groups were untreated control (CTL), 200 nM CI for 24 h, miR-7-1 (50 nM) for 12 h + 200 nM CI for 24 h, miR-7-1 (50 nM) for 12 h + 200 nM CI for 24 h + 100 nM WAY for 24 h, and miR-7-1 (50 nM) for 12 h + 200 nM CI for 24 h + 150 nM E2 for 24 h. Differences were analyzed between CTL vs. treatment (designated as significant at $*p<0.05$ or $**p<0.01$) and CI vs. CI plus treatment (designated as significant at $\#p<0.05$ or $\#\#p<0.01$). Panel **a** illustrates the measurement of whole-cell membrane potential. It was difficult to measure membrane potentials in CI insulted cells as many of the cells were dead. Transfection with miR-7-1 followed by CI insult caused significant improvement in the whole cell membrane potentials when compared with the CI insulted cells. However, the most significant change in whole cell membrane potential was observed when cells were subjected to sequential therapy with miR-7-1 and WAY or E2, returning the electrophysiological function of these cells to a value similar to that of control cells. Panel **b** shows the JC-1 ratio (590 nm:530 nm) measured at 0–12 h after initiation of the treatments. A reduction in the JC-1 ratio indicates a loss of mitochondrial membrane potential and suggests mitochondrial swelling. These data show that control cells had a high JC-1 ratio indicating

2. After JC-1 staining, wash the cells twice with PBS (*see* **Note 8**).
3. Measure fluorescence in a fluorescent plate reader. Use an excitation wavelength of 488 nm, and read the fluorescence emission of JC-1 at wavelengths corresponding to its monomer (530 ± 15 nm) and J aggregate (>590 nm) forms (Fig. 5b) (*see* **Note 9**).

3.7 Statistical Analysis

Perform experiments in triplicate and analyze the results for statistical significance using statistical software such as Minitab 16. Express the data as mean ± standard error of mean (SEM) of separate experiments ($n \geq 3$) and compare by one-way analysis of variance (ANOVA) followed by the Fisher's post hoc test. Consider differences between control (CTL, the untreated group) and a treatment to be significant at $p < 0.05$.

4 Notes

1. MTT solution should be stored –20 °C. At 4 °C, MTT decomposes and thus yields erroneous results. Formation of dark color crystals is an indication of the product deterioration. Moreover, MTT may be cleaved by microbial contamination that can form MTT formazan yielding erroneous results.
2. MTT assay plates should be read within 30 min after adding MTT solution. High serum or albumin concentration in the culture medium may result in precipitation during addition of MTT solvent.
3. Cells should be transfected when they are 70–90 % confluent or in logarithmic growth phase. The optimal incubation time after transfection depends on the cell type, promoter strength, and expression product, all of which should be optimized experimentally. Similarly, the volume of transfection reagent used depends on the amount of miR-7-1 mimics and the cells to be transfected and should be optimized experimentally. For optimal results, transfection efficiency should be as high as possible. When performing start-up experiments or working with a new cell line, it is necessary to perform multiple transfections under different conditions to determine the optimal conditions for maximum transfection efficiency. Transfection conditions that induce maximum cell death in comparison to a negative control should be used for further studies. The transfection efficiency with Lipofectamine 2000 transfection reagent is very low in the presence of serum; thus transfection should be carried out in serum-free medium. The presence of cytotoxic agents in the growth medium may result in inefficient transfection and thus they should be added after the transfection and incubation period.

4. The intensity of staining with Annexin V-FITC depends on cell type, cell size, and the amount of phosphatidylserine exposure on the cell surfaces. Thus, the staining procedure should be optimized experimentally for different cell types.
5. Removal of adherent VSC4.1 cells from culture plates using trypsin digestion may cause cell membrane damage contributing to erroneous results. Therefore, cell exposure time in trypsin should be minimized for Annexin V-FITC staining assay and flow cytometric analysis. On the other hand, incorrect dilution of Annexin V-FITC reagent may not detect apoptosis that is present. To control for both types of errors, cells with induced apoptosis can be used as control to confirm a successful experiment.
6. The patch-clamp technique for whole-cell membrane potential is conducted by applying negative pressure and high voltage pulse to the recording pipette. Thus, there is high possibility of ruptures of cellular membrane inside the tip of the electrode. Therefore, components of the cytoplasmic location can diffuse out of the cell into the patch pipette, resulting inaccurate results.
7. Centrifugation of JC-1 staining solution should be avoided as this will precipitate the reagent. Concentrated JC-1 staining solution may provide erroneous results. In that case, JC-1 staining solution can be diluted before use.
8. Cells stained with JC-1 should not be exposed to strong light, which may yield erroneous results. Cells should be analyzed immediately after washing the JC-1 stained cells.
9. Mechanically injured or unhealthy control (untreated) cells exhibit a low ratio of red to green signal. Thus, only healthy cells should be used for JC-1 staining experiments.

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Chapter 30

Expression Profiles of Estrogen-Regulated MicroRNAs in Breast Cancer Cells

Anne Katchy and Cecilia Williams

Abstract

Molecular signaling through both estrogen and microRNAs are critical for breast cancer development and growth. The activity of estrogen is mediated by transcription factors, the estrogen receptors. Here we describe a method for robust characterization of estrogen-regulated microRNA profiles. The method details how to prepare cells for optimal estrogen response, directions for estrogen treatment, RNA extraction, microRNA large-scale profiling, and subsequent confirmations.

Key words Breast cancer, MicroRNA, Estrogen, Estrogenreceptor, Microarray, qPCR

1 Introduction

MicroRNAs (miRNAs) play critical roles in cells due to their major impact on posttranscriptional gene regulation. In cancer, miRNAs can function as oncogenes or tumor suppressors and are therefore promising biomarkers. miRNAs are endogenously expressed 19–24 nucleotide short, single-stranded, noncoding RNAs. They are transcribed by RNA polymerase II into primary miRNA transcripts (pri-miRNA) and then processed in the nucleus by Drosha into short-hairpin precursor-miRNAs (pre-miRNA). Pre-miRNA is exported to the cytoplasm and further processed by Dicer into mature miRNA, separated into single-stranded RNA that together with Argonaute proteins form RNA-induced silencing complexes (RISC). Within the RISC complex, the miRNAs can hybridize to the 3′-untranslated region (3′-UTR) of a target mRNA, leading to posttranscriptional regulation by blocking translation or degrading the target mRNA [1].

The hormone estrogen regulates numerous physiological and pathological processes, some of its most prominent functions being within the regulation of mammary gland development and breast cancer progression. Its activity is mediated through binding to the

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estrogen receptors ERα (ESR1) and ERβ (ESR2). The activated ERs can bind to DNA and function as transcription factors, regulating gene expression. ERα is upregulated in the majority of breast cancers and drives their proliferation [2]. These ERα-positive tumors depend on estrogen for growth, which has made ERα a main target for breast cancer treatment. About half of ERα-positive tumors, however, eventually develop resistance to this treatment, and understanding the underlying mechanism of ERα is important to overcome resistance, improve treatment outcomes, and understand how breast cancer develops. Whereas ERα's regulation of protein-coding genes is well established, its regulation of the non-coding miRNAs is less explored [3].

Due to the critical roles that both estrogen and miRNAs play in breast tumor development and progression, identifying miRNAs associated with normal or disrupted estrogen signaling is important. Initial studies aiming to elucidate ERα regulation of miRNA in breast cancers cell lines have yielded conflicting results, even when the same cell line was analyzed [3–6]. This may be a result of varying treatments, biological variation, the use of different techniques or the fact that the small sizes of miRNAs make them challenging to analyze. Here we describe a protocol that controls for variations and method artifacts. The main advantages of the miRNA assay approach described in this chapter is that it enables a fast screening of mature miRNAs in numerous samples, even with limited sample amounts. The lay-out, including the specific conditions for cell culture and estrogen treatment, biological and technical replicates, and large-scale screening followed by in-depth confirmations using separate techniques (Fig. 1), ensures a robust detection of miRNA regulations and reduces false positives and other artifacts. However, mutated or unknown miRNAs, or regulation at the primary and precursor transcript level, will not be detected by these methods.

In our study of estrogen-regulated miRNAs in breast cancer [7], we used two cells line models of the Luminal A (ERα-positive, progesterone hormone receptor (PR/PGR)-positive, Her2 (ERBB2)-negative) subtype: T47D and MCF7. Each cell line was analyzed in replicated cell cultures using different passages, each in technical replicates of treatment. This allowed for robust detection of reproducible ERα-regulated miRNAs. We compared the relative miRNA expression levels using both miRNA microarray and low-density TaqMan arrays (TLDA) and validated the results with specific quantitative real-time polymerase chain reaction (qPCR)

Fig. 1 (continued) followed by RNA extraction and quality control, before large-scale miRNA profiling is performed to identify possibly differentially expressed miRNAs. Finally, differentially expressed miRNAs are confirmed and validated using qPCR and time-series analysis

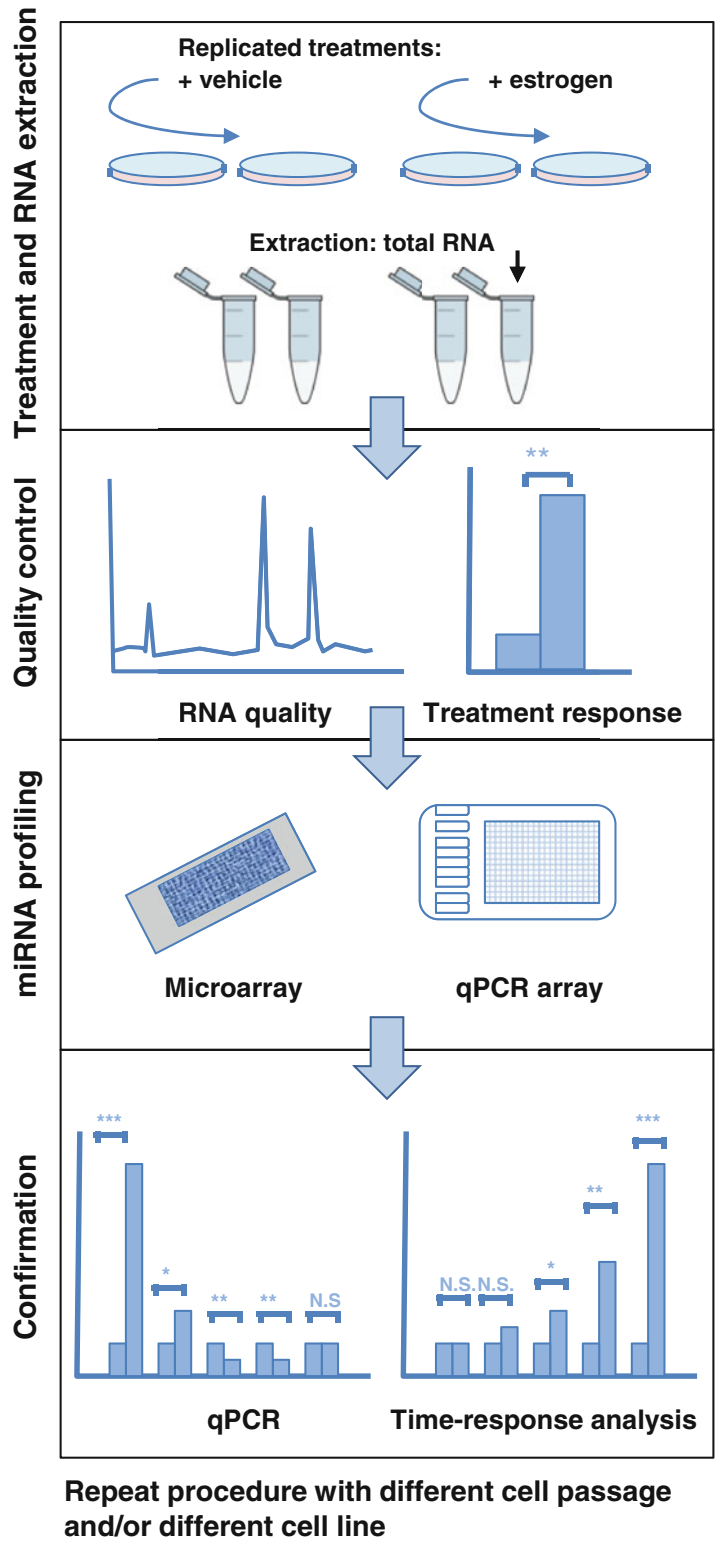

Fig. 1 An overview of the approach employed for detection of hormonal regulation of miRNA in breast cancer cells. The process begins with cell culture and treatment,

using both SYBR Green and TaqMan chemistries (schematically illustrated in Fig. 1). miRNA regulations were then further analyzed in time-series to define their exact regulation over time, which can help differentiate random or circadian variations from estrogen-impacted regulation, as well as differentiate primary effects from secondary effects. Bioinformatical comparisons with chromatin-binding studies of ERα can further aid in the establishment of direct transcriptional regulation. Our assay resulted in a reliable assessment of miRNA regulation in which we established that while protein-coding transcripts were readily regulated by 24-h E2 treatment, mature miRNAs were not [7]. However, it is possible that miRNAs are regulated by estrogen at other timepoints, as demonstrated in our time-series analysis of selected miRNAs [7], or in other cell types. The details need to be further explored and the protocol presented here provides a robust way to study hormonal regulation of mature miRNAs in breast cancer cell lines. A video demonstration of the protocol is available [8].

2 Materials

2.1 Select Cell Model

To identify which miRNAs are regulated by estrogen, selecting a well-defined model of ERα activity is a first important step. Several cell lines have been generated from human ERα-positive breast cancer tumors that are dependent on estrogen similar to the majority of clinical breast cancers. The Luminal A breast cancer subtype cell lines used in this study, T47D and MCF7 (American Type Culture Collection, ATCC), exhibit upregulated expression of nuclear receptors ERα and PR, and lack expression of membrane receptor Her2, along with expression of estrogen-responsive and luminal-epithelial differentiation genes [9–12]. Their ERα activities have been well characterized, both functionally and transcriptionally [13, 14]. Other cell lines can also be used (*see* **Note 1**).

2.2 Cell Culture Media and Treatment

1. T47D cell line culture media: Mix 500 mL Dulbecco's modified Eagle's low-glucose medium (DMEM) with 500 mL F12 [DMEM/F12 (1:1)] in an autoclaved 1 L bottle. Add 50 mL of fetal bovine serum (BSA), to yield 5 % FBS, and 10 mL of penicillin streptomycin for 1 % pen-strep. Mix contents completely. Store at 4 °C.
2. Reduced-serum culture media: replace DMEM with 500 mL phenol red-free DMEM, and replace FBS with 50 mL of dextran-coated charcoal-treated (DCC) FBS for 5 % DCC-FBS, or with 5 mL DCC-FBS for 0.5 % DCC-FBS. Mix contents completely. Store at 4 °C.
3. MCF7 cell line culture media: Mix 500 mL DMEM with 25 mL of the FBS (5 % FBS) and 5 mL of penicillin streptomycin (1 % pen-strep). Mix contents completely. Store at 4 °C.

4. For reduced-serum culture media: replace DMEM with 500 mL phenol red-free DMEM with 5 mL of 100× L-glutamine (200 mM final concentration). Replace FBS with 25 mL of DCC-FBS (Sigma-Aldrich) for 5 % DCC-FBS, or with 2.5 mL DCC-FBS for 0.5 % DCC-FBS. Mix contents completely. Store at 4 °C.
5. Phosphate-buffered saline (PBS) solution: Fill a 1 L autoclaved glass bottle with highly purified water (resistivity 18.2 MΩ). Add one PBS tablet (Medicago) and shake occasionally until tablet has dissolved. Autoclave PBS solution and store at room temperature.
6. 17β-estradiol (E2) stock: Prepare a 100 mM stock solution of E2 by dissolving 2.72 mg of E2 (1,3,5[10]-estratriene-3,17β-diol) in 100 μL vehicle (ethanol or DMSO) in a sterile 1.5 mL microcentrifuge tube, and mix contents gently. Centrifuge briefly and make serial dilutions (1:10) to yield 10, 1, and 0.1 mM stock concentrations using the vehicle. Store all stock solutions and solvents at −20 °C.
7. ICI stock: Prepare a 100 mM stock solution of ICI by dissolving 6.07 mg of ICI 182,780 in 100 μL vehicle (ethanol or DMSO) in a sterile 1.5 mL microcentrifuge tube, and mix contents gently. Centrifuge briefly and make serial dilutions (1:10) to yield 10, 1, and 0.1 mM stock concentrations using the vehicle. Store all stock solutions and solvents at −20 °C.
8. Trypsin–EDTA (0.05 %), phenol red (Life Technologies). Store stock at −20 °C. Aliquots can be prepared and stored at −20 °C. The aliquot in use should be stored at 4 °C.
9. Laminar flow hood and CO_2 incubator for cell culture, sterile serological and Pasteur pipets, hemocytometer or automated cell counter, rubber scrapers.

2.3 RNA Extraction, cDNA Synthesis and qPCR for mRNA Reagents

1. TRIzol.
2. Chloroform.
3. miRNeasy Mini Kit with DNase I for DNA degradation (Qiagen) or equivalent.
4. Agilent RNA 6000 Nano Assay (Agilent, Santa Clara, CA).
5. RNase-free water.
6. Random hexamer primers.
7. Superscript III cDNA synthesis kit (Life Technologies) or equivalent.
8. Gene-specific primers.
9. SYBR Green PCR master mix.
10. NanoDrop 1000 Spectrophotometer (Thermoscientific).
11. Heat blocks, water baths, microcentrifuge, real-time PCR machine and plates.

2.4 Low-Density Array Analysis

1. TaqMan miRNA RT kit (Life Technologies).
2. 10× Megaplex RT primers (Life Technologies).
3. TaqMan 2× Universal PCR Master Mix-No AmpErase UNG (Life Technologies).
4. TaqMan Low-Density Array (TLDA) plates (Life Technologies).

2.5 miRNA Confirmatory SYBR Green qPCR

1. NCode miRNA First-Strand cDNA Synthesis and qRT-PCR Kit (Life Technologies).
2. miRNA-specific primers.

2.6 miRNA Confirmatory TaqMan qPCR

1. TaqMan MicroRNA Reverse Transcriptase Kit (Life Technologies).
2. TaqMan 2× Universal PCR Master Mix-No AmpErase UNG (Life Technologies).
3. 20× TaqMan real-time assay primers.

3 Methods

This method details how to define estrogen-induced miRNA regulation, using estrogen-deprived culture conditions, treatment, the μParaflo-microfluidic microarray and/or the qPCR-based TLDA arrays (Fig. 1). The resulting profiles require validation and we include protocols for validation, using specific qPCR assays applying both SYBR Green-based and TaqMan-based chemistry, and time-series to identify regulation over time (*see* **Note 2**).

3.1 Cell Culture and Treatment

E2 is the dominant physiological form of estrogen in females, and the concentrations and time-points for optimal transcriptional activation of ERα have been characterized in multiple studies. In this protocol we use 10 nM E2 treatment for 24 h in T47D and MCF7 cells to study a near maximal ERα activity in breast cancer cells (*see* **Note 3**). In addition, time-dependent miRNA regulation can be specifically analyzed in a time interval of, for example, 1–72 h. Each ligand treatment should be performed in duplicate or triplicate plates for technical replicates. Repeat cell culture procedure and treatment using a different passage of the same cell line, and repeat the procedure with a different cell line to replicate your findings. All cell culture techniques should be performed under sterile conditions in a laminar flow hood.

1. Cell culture start-up: Warm the media to 37 °C in a sterile warm water bath and thaw a frozen vial of cells. Clean the outside of vial and media bottle with 70 % ethanol, and then place both vial and bottle in sterile laminar flow hood. Add 12–15 mL of appropriate media into a labeled sterile T-75 flask using a sterile serological pipet and ensure that the surface is completely covered

with media. Add the cells and mix gently. Place flask in a 37 °C incubator, supplied with 5 % CO_2 and incubate for 48 h. Change the media when needed, and allow cells to grow to 80–90 % confluence before preparing cells for the experiment.

2. Estrogen deprivation: Prepare cells before treatment by estrogen deprivation. In sterile hood remove media from flask using a sterile pasteur pipet connected to a vacuum. Gently wash attached cells twice with PBS. Add 1 mL of warm trypsin–EDTA to the flask, and place the flask in the incubator for 2 min for cells to detach. Add 3–4 mL of warm media to the flask and triturate the detached cells by taking up the cells in the 5 mL serological pipet and releasing the cells with the tip of the pipet placed against the bottom of the flask to increase pressure to break the cells apart into single cells. Count the cells using a hemocytometer or an automated cell counter such as the Countess Automated Cell Counter (Life Technologies), according to manufacturer's protocol. Label 100 mm plates (as needed) and add approximately 10 mL media to each plate. Plate approximately 2.0×10^6 cells per plate and distribute the cells by gently swirling the plates. Incubate cells for 24–48 h until approximately 80 % confluent (if cells are too confluent they will not respond well to E2). At 80 % confluency, wash cells twice with PBS, then add 5 % DCC-FBS media. Incubate cells for 24 h, wash again with PBS twice, add 0.5 % DCC-FBS media and incubate for additional 48 h. At this point, the cells are deprived of estrogen and the receptor largely inactive.
3. Cell treatment: Prepare one 15-mL conical tube for each plate and add 1 μL of the 0.1 mM ligand stock (E2 or ICI) or of the vehicle control to 10 mL of 0.5 % DCC-FBS media, for a final concentration of 10 nM. Mix contents in tube gently. Wash the prepared cells twice with PBS, remove as much of the PBS as possible and add the prepared ligand-media mix to the cells in the plate. Incubate cells for the chosen time point (0–72 h).

3.2 RNA Extraction and Quality Control

Extract both the mRNA and miRNA populations. Before analyzing for regulated miRNA profiles, you want to ensure that the estrogen treatment was efficient. This can be ascertained by measuring the regulation of well-characterized ERα-target genes with qRT-PCR. The target genes are cell type specific, but for MCF7 and T47D cells, pS2 (trefoil factor 1, TFF1) is an appropriate control.

1. Cell lysis: Wash cells twice with PBS, remove as much PBS as possible and add 1–2 mL TRIzol to each plate (*see* **Note 4**). Ensure that the volume of cells is no more than 10 % of the volume of TRIzol, and that the plate is covered with TRIzol. Allow to sit for 1 min, then scrape cells using a rubber scraper and transfer to a microcentrifuge tube. Cells can be stored in TRIzol at −80 °C.

2. RNA extraction: Extract and purify total RNA from each treatment using the miRNeasy Mini Kit (Qiagen) or equivalent kit including DNase I DNA degradation following the manufacturer's "Qiazol/TRIzol-spin column protocol for animal cells" instructions (*see* **Note 4**). CAUTION: TRIzol is toxic by contact with skin or eyes, by inhalation or if swallowed. Wear suitable protective clothing, gloves, and eye/face protection and use fume hood. After extraction, elute the RNA in 60 μL RNase-free water. Aliqout 1–2 μL RNA for quantification analysis and store remaining RNA at −80 °C. Measure the RNA concentrations using the NanoDrop 1000 Spectrophotometer according to manufacturer's protocol. Use approximately 100 ng RNA of each sample to analyze the RNA integrity using the Agilent RNA 6000 Nano Assay and ensure that the RNA integrity number (RIN) is at least 8.
3. cDNA synthesis: Transfer 500 ng or 1 μg of the total RNA to a new 1.5 mL microcentrifuge tube, and bring the volume to 10 μL with RNAse-free water. Add 2 μL of 50 μM random hexamer primers, heat to 70 °C for 10 min, and transfer the tubes to ice for 5 min. Make a master mix of 4 μL of 5× first strand buffer, 1 μL of 0.1 M DTT, 1 μL of 10 mM dNTPs, 0.5 μL of superscript III (from the Superscript III cDNA synthesis kit), and 1.5 μL RNase-free water, per sample, and add 8 μL of the master mix to each prepared RNA sample for a final volume of 20 μL. Place tubes at 25 °C for 10 min, followed by 46 °C for 1 h and 70 °C for 15 min (heat blocks, water bath or a PCR machine). Dilute the resulting cDNA to a 5 ng/μL stock (calculated using total RNA input) by adding water and store at −20 °C.
4. qPCR for mRNA analysis: Obtain qPCR primers for genes of interest (e.g., pS2) and reference/housekeeping genes (36B4, ARHGDIA, 18S, or other). Use triplicate reactions for each sample and each gene (technical replicates) in 96-well plates. For each qPCR reaction, add 10 ng cDNA (2 μL from stock concentration from Subheading 3.2, **step 3**), 1 pmol of relevant forward and reverse primers, and 5 μL of 2× SYBR green PCR master mix for a final reaction volume of 10 μL. Run the reaction in a real-time PCR machine. Make sure to perform melting-curve analysis to confirm the amplification of one specific fragment.
5. Analyze qPCR data using the $\Delta\Delta C_T$ formula: $\Delta C_T = C_T$ (target gene) $- C_T$ (reference gene). Calculate $\Delta\Delta C_T = \Delta C_{T(\text{treated/test sample})} - \Delta C_{T(\text{control/calibrator sample})}$. The calibrator sample is the vehicle-treated sample. Convert to fold-change (FC) values of each sample by: $FC = 2^{-\Delta\Delta C^T}$. The fold-change value of each sample gives the relative expression of the gene in the E2-treated cells compared to in the vehicle-treated cells. Then, calculate the total standard deviation (SD) for each sample (using the

STD DEV function on the C_T values in excel followed by Total $SD = (SD^2_{(target)} + SD^2_{(reference)})^{1/2}$). Student's *t*-test can be used for statistical analysis using two-tailed distribution and two-sample unequal variance parameters, and considered significant if $p < 0.05$. Confirm that the treatment resulted in significant regulation, at least twofold, of known targets before proceeding with miRNA profiling.

3.3 miRNA Profiling Analysis

1. miRNA microarray: Take 5 μg of isolated total RNA from cells treated with either vehicle or ligand, and place in a microcentrifuge tube. Adjust volume in tube to 20 μL. Each comparison should be performed in duplicates or triplicates. Send samples on dry ice to appropriate microarray facility if no in-house microarray facility is available. One method to measure miRNA microarray expression profiles is by μParaflo Microfluidic Biochip Technology [15]. This miRNA microarray contains 894 mature miRNAs and 50 control probes in quadruplets [7]. Perform hybridizations in duplicates in a dye-swap procedure. Results should show differentially expressed miRNAs, if any are changed (*see* **Note 5**).
2. qPCR-based TLDA: Each treatment should be analyzed in duplicates or triplicates. Place 700 ng of total RNA in a microcentrifuge tube for miRNA cDNA synthesis, and adjust volume in tube to 3 μL. Follow instructions from the Megaplex™ Pools protocol (Life Technologies) using the TaqMan miRNA RT kit with 10× Megaplex RT primers instead of the RT primers from the kit. Total volume for each Megaplex reaction should be 7.5 μL. Run the reaction in a thermocycler, according to the Megaplex Pools protocol. The resulting cDNA can be stored at −20 °C for at least 1 week. Place 6 μL of cDNA in a 1.5 mL microcentrifuge tube, add 450 μL of the TaqMan 2× Universal PCR Master Mix-No AmpErase UNG and 444 μL RNase-free water. Invert the tube about six times to mix contents, and centrifuge briefly. Load 100 μL into each of the eight "Fill ports" of a room-temperature 384-well TLDA plate in which primers and controls are pre-loaded (*see* **Note 6**). Centrifuge the array plate and run the reactions in a real time PCR system according to the TLDA protocol. Analyze the results using the $\Delta\Delta C_T$ formula (Subheading 3.2, **step 5**).

3.4 Confirmatory qPCR Analysis of Regulated miRNAs

After miRNA expression profiles are acquired as described above, it is crucial to confirm their regulation with independent methods using separate SYBR Green or TaqMan qPCR analysis (*see* **Note 7**).

1. For SYBR Green qPCR analysis: Perform miRNA-cDNA synthesis by placing 1 μg of total RNA in a 1.5 mL centrifuge tube, followed by polyA tailing and cDNA synthesis using the NCode miRNA First-Strand cDNA Synthesis and qRT-PCR Kit and protocol. Dilute the cDNA in a 1:10 ratio, and store at

–20 °C. For each miRNA qPCR reaction well, add 16 ng of poly-A cDNA, 2 pmol of the specific forward primer (equals the sequence of the mature miRNA as obtained from mirbase.org [16], with "U" converted to "T") and 2 pmol of the universal primer from the NCode miRNA qRT-PCR Kit, and 5 μL of 2× SYBR Green PCR master mix for a final reaction volume of 10 μL per sample. Ensure that there are triplicate wells for each sample for each miRNA (for technical replicates) and include analysis of one or two short RNA reference genes (for example U6 snRNA and a non-regulated miRNA). Run reactions and analyze as in Subheading 3.2, **step 5**.

2. TaqMan qPCR analysis: Place 10 ng of total RNA in a 0.2 mL microcentrifuge tube and adjust volume to 5 μL. Perform the reverse transcription (RT) using the TaqMan MicroRNA Reverse Transcriptase Kit (Life Technologies) and protocol [17]. Final reaction volume should be 15 μL. After the RT reaction, dilute the sample cDNA in a 1:5 ratio, and store all cDNA in the dark at –20 °C. For each miRNA qPCR reaction use 1.3 μL of the cDNA, add 10 μL of TaqMan 2× Universal PCR Master Mix-No AmpErase UNG, 7.7 μL of RNase-free water, 1 μL of 20× TaqMan real-time assay primer (specific for each miRNA) for a final volume of 20 μL. Mix contents gently, and centrifuge briefly. Use technical triplicates and include a short RNA reference gene (for example U6 snRNA). On a 7500 Fast Real-Time PCR System or equivalent qPCR machine, assign the reporter (FAM) and quencher (NFQ-MGB) for each miRNA (target) of interest, enter reaction volume, select the comparative threshold cycle ($\Delta\Delta C_t$) method, and define sample wells. Follow the instructions from the TaqMan microRNA assay protocol and analyze the run as in Subheading 3.2, **step 5**.

4 Notes

1. When analyzing other cell lines, ensure that an estrogen-responsive estrogen receptor of interest is expressed (ERα, ERβ or the membrane bound GPER1) at mRNA and protein level. If the experiment seeks to explore a receptor not expressed in the cells, it may be exogenously added (transiently or stably transfected). The receptor activity and function can be cell-type specific, but before proceeding to miRNA profiling, ensure that the receptor binds and responds to estrogen (e.g., ERE-luciferase transactivation assay or ligand-binding assay), and that characterized protein-coding target genes are regulated upon ligand treatment. In addition to E2, other endogenous, phytoestrogens or xenoestrogens, or various selective estrogen receptor modulators (SERMs) can be used.

2. Because of the small size of the miRNA, the fact that the mature miRNA sequence is found also in the pri-miRNA and the pre-miRNA, and because of heterogeneity in their GC content, miRNA expression profiling is more challenging than that for protein-coding RNAs [18]. Therefore, established techniques need special adaptation, which generates different risks of introducing artifacts. For genome-wide miRNA screening, there are three common miRNA profiling platforms to choose from at this time: miRNA-specific microarrays, low-density qPCR arrays, and next-generation sequencing. An overview of the microarray and qPCR approaches is presented in Fig. 1 and the nucleic acid modifications required for these techniques are visualized in Fig. 2. Next-generation sequencing, not described in this chapter, is the only technique that allows for the discovery of novel, mutated or edited miRNAs [19]. This technique, however, requires multiple steps to enrich small RNAs, add linkers and purify the fractions to produce a small RNA library, with subsequent enhanced risks of modulating their relative expression levels between samples. Given the various techniques for miRNA profiling, the most appropriate technique depends on the applications. Microarray gives the opportunity to observe the relative expression levels of a large number of genes simultaneously, is fast and suitable for screening of large number of samples, but can only analyze the sequences present on the microarray and will, therefore, not detect changes in unknown or mutated miRNAs. Microarrays are most suitable when material is relatively abundant and the interest is to define differential expression of already known miRNAs. Low-density qPCR arrays are beneficial when a limited amount of sample is available and a high sensitivity of low-expressed miRNAs is required. Sequencing has the main advantage of detecting unknown or mutated miRNAs. All technologies can provide false negatives. The normalization process presents a challenge when analyzing the relatively few miRNA genes, which are counted in thousands compared to the tens of thousands of mRNA transcripts. For the low-density qPCR, performed in 384-well plates with no in-plate replicates, multiple plates need to be analyzed for each sample and this makes the analysis costly. Also, in our hands, more false negative results were obtained using the TLDA analysis compared to the microarray. Given that the mature miRNA sequence is present in the pri-miRNA and the pre-miRNA sequences, it is of interest to further differentiate between these transcripts using specific PCR techniques [20]. TaqMan probes are available also for pre-miRNAs, and specific primers can be designed to exclude different mature or precursor variants using SYBR Green qPCR technology.
3. Culturing the cells to provide the optimal condition for hormone action is very important. The confluency of cells influences

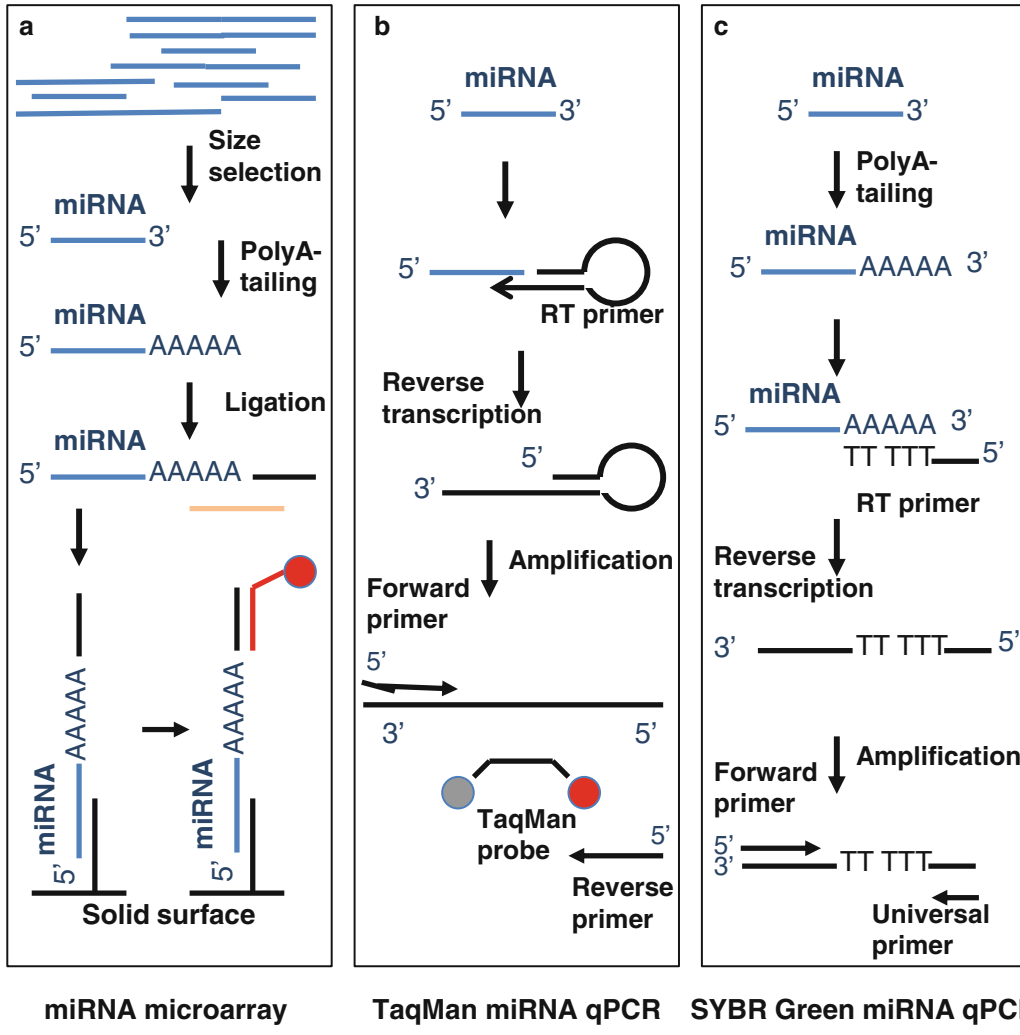

Fig. 2 Schematic illustration of the nucleic acid manipulations used for each miRNA detection technique. (**a**) A miRNA microarray has thousands of oligonucleotide probes designed to hybridize to particular miRNA sequences. The sample preparation for the μParaflo microarrays used in this study first enriches for short RNAs, followed by polyadenylation and linker ligations, before hybridization to the array probes and introducing fluorescent Cy5 and Cy3 capture-probe labeling onto the miRNAs. (**b**) TaqMan qPCR technology used in the TLDA technology, allows detection of transcript levels in relative amount. The miRNA template first requires conversion into cDNA, here by using a specific looped primer for each miRNA, which also ensures the analysis of only mature miRNA. This generates a cDNA template long enough to fit a miRNA-specific forward primer (complementary to the miRNA sequence), a universal reverse primer (complementary to the looped sequence), and a TaqMan probe. In this low-density setting, hundreds of miRNAs can be detected in parallel using one or two 384-well plates with individual primer pairs in each well [19]. This profiling requires less material (700 ng per sample and replicate), and allows for the detection and quantification of most known miRNAs. (**c**) SYBR Green technology, here used for validation of miRNA expression, includes polyadenylation of the miRNA sample, followed by cDNA synthesis with an oligo-dT primer that also harbors a universal primer sequence handle at its 5′ end, and finally, amplification including a universal primer (complementary to the primer sequence introduced through in the oligo-dT primer) and a miRNA-specific forward primer

cell-cell contact and behaviors such as cell-cell signaling and proliferation, and affects the response of cells to estrogen. Cells were therefore allowed to be 80 % confluent (Fig. 3a) and well attached to avoid cell contact inhibition and allow adherent growth and cell-cell signaling, but still generate abundant RNA output. Further, as phenol red media and serum can have estrogenic activity [21, 22], this protocol utilizes phenol red-free media and DCC-treated serum (that has been stripped of most of its hormone, cytokines, and growth factors) along with serum-starvation, to ensure that all hormonal and growth factor effects have been reduced to a minimum. The time of exposure of the cells and the concentration of the hormone is also of great importance. ERa in breast cancer cells are readily activated by 1–10 nM [23], whereas higher doses would be non-physiological. Some other ligands, such as phytoestrogens or bisphenol A (BPA), would require higher concentrations to generate equivalent ERα activation at around 1 μM [23]. For estrogen-associated regulation of genes, we have previously shown that 24-h exposure produces significant response of direct target genes in breast cancer cells [7, 14]. Since miRNAs are transcribed by RNA polymerase II, like mRNAs, it is conceivable that this is a suitable time point to detect direct regulation also of miRNAs, but other time points should be explored.

4. Extracting high-quality miRNAs requires specific considerations compared to that of longer RNAs. Because of their short sizes, miRNAs are not well retained in the total RNA preparation when regular TRIzol/chloroform/isopropanol RNA precipitations are performed, or when standard column purification kits are used. However, by increasing the volume of isopropanol, lowering the temperature before centrifugation (−80 °C) and omitting the washing in 70 % ethanol, the retaining of miRNAs can be enhanced during TRIzol/chloroform/isopropanol RNA precipitations. Or, as in this protocol, specific miRNA columns and buffers can be used. It is necessary to perform RNA extraction during RNase-free conditions and to check the quality of the RNA before proceeding with experiments. The number of cells, the extraction method, and the GC content of the miRNA can affect the yield and quality of both mRNAs and miRNAs [24]. After extraction, spectrophotometric analysis of the RNA provides information of the yield of the total RNA (containing both mRNAs and miRNAs), and provides a graphic representation of results as seen in Fig. 3b, and this allows for the observation of the purity of the sample. Poor yield of RNA would produce no specific absorption at 260 nm (Fig. 3b, bottom panel). Further, sizing quantitation analysis providing a RIN of between 8 and 10, with distinct peaks for 18S and 28S rRNA visible in the graphical representation of the RNA as exemplified in Fig. 4 (middle panel),

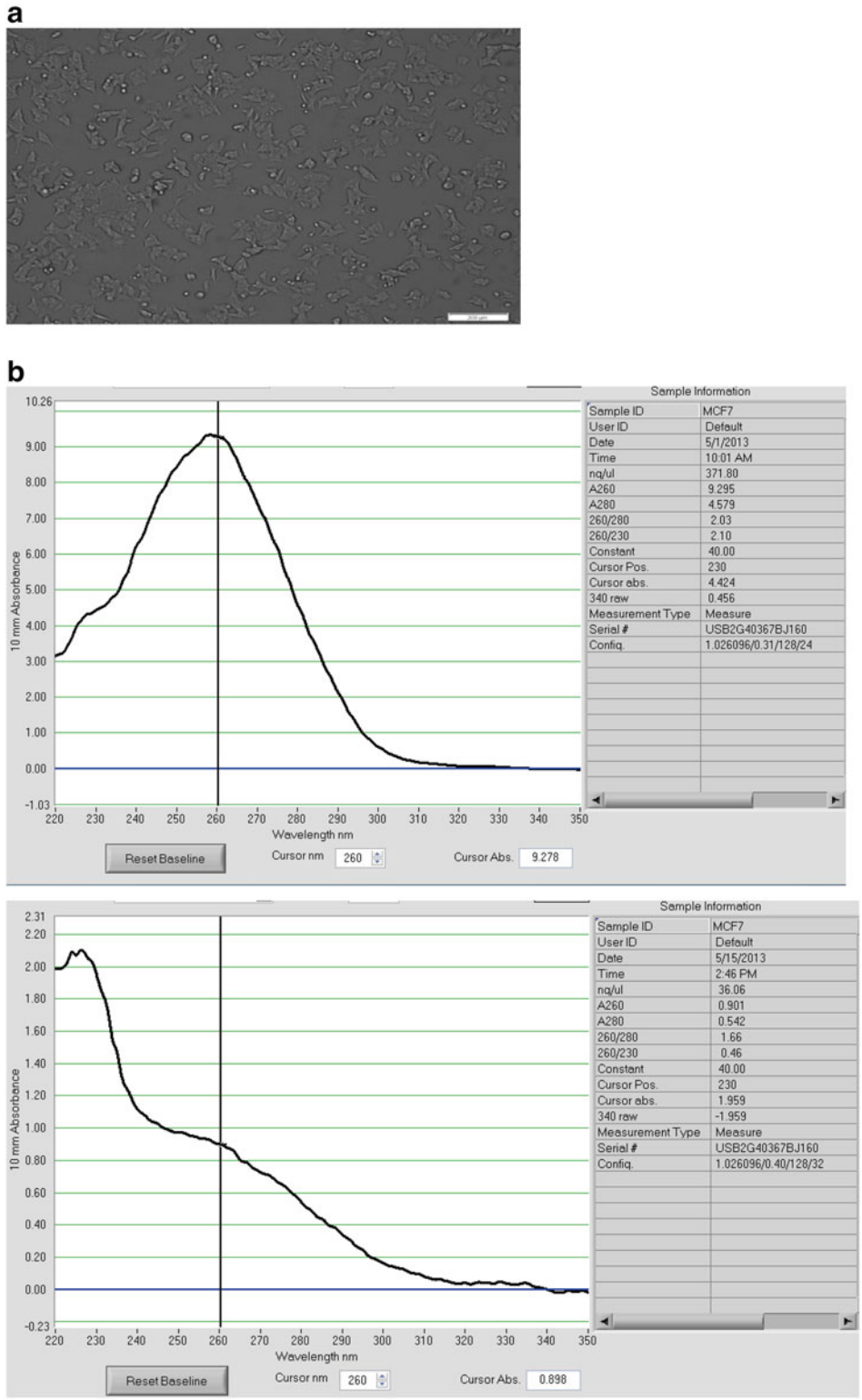

Fig. 3 Cell confluence and RNA extraction quality examples. (**a**) Cultured cells to illustrate the 80 % confluency recommended before treatment with ligand. (**b**) Graphical representation of RNA concentration and purity after extraction. Each graph represents a sample and a successful extraction process yields pure RNA (*top panel*). A poorly extracted RNA shows no clear absorption peak at 260 nm (*bottom panel*). Data are derived from NanoDrop 1000 Spectrophotometer. (Figure replicated from ref. [8] with permission)

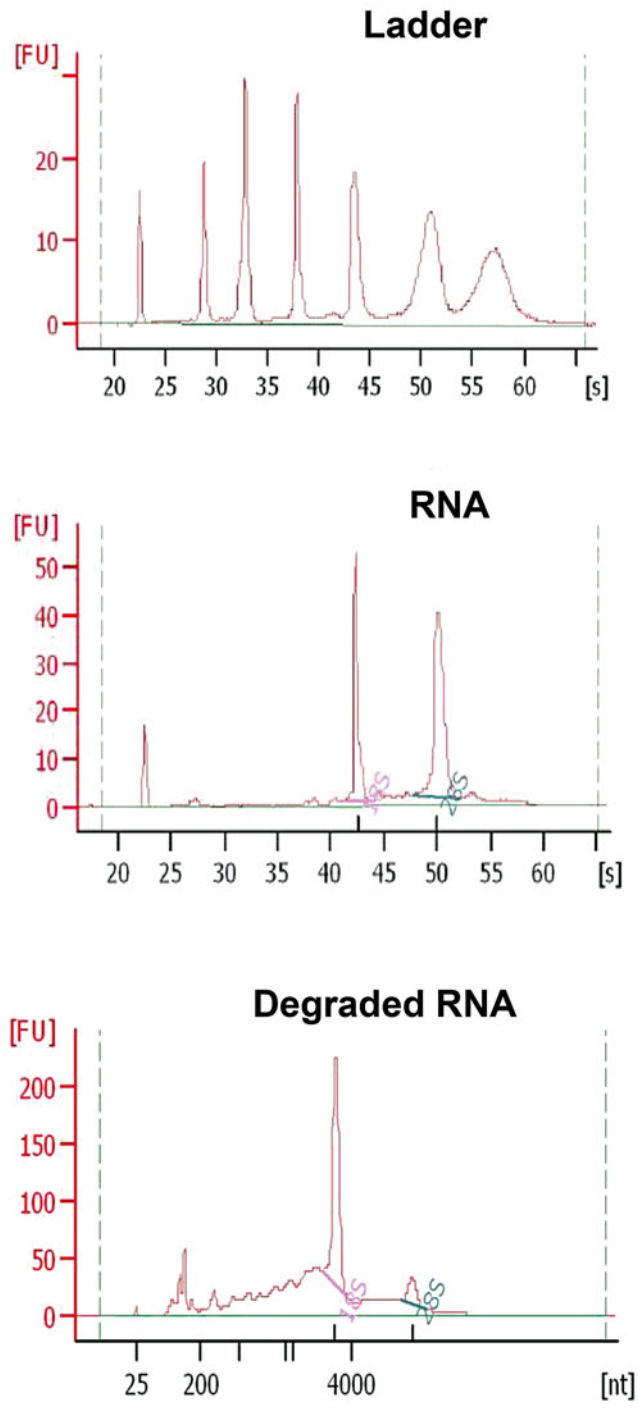

Fig. 4 RNA quality control. Results from RNA integrity analysis showing graphical representation for the ladder (*top*), a good quality RNA (*middle*) and a degraded RNA sample (*bottom*). Data are derived from Agilent RNA 6000 NanoAssay analysis. (Figure replicated from ref. [8] with permission)

ensures that the RNA is of sufficient quality. A degraded RNA would have a lower RIN, with less clear peaks and a reduced relative amount of the 18S and 28S rRNA (Fig. 4, bottom panel). The fraction of small RNAs can be further characterized using the Agilent Small RNA Kit. Once the quality of the RNA has been assessed, the experiment can proceed.

5. The miRNA microarray results are usually graphically represented by heat maps, as illustrated in Fig. 5a. The red indicates miRNAs upregulated in the E2-treated sample (higher expression), while the green indicates downregulated miRNA (lower expression). miRNA selected for confirmation should be significantly regulated, usually indicated by a *p*-value less than 0.05 although *p*-value up to 0.1 can also be considered for further confirmatory analysis using qPCR.
6. The TLDA array uses two 384-well plates (cards) in which pool A contains better characterized and more highly expressed miRNAs than pool B. Depending on the purpose of the study, either one or both cards can be chosen. The relative level of each miRNA is determined and one miRNA is analyzed per well (Fig. 5b). A higher C_t-value indicates lesser miRNA expression. Much like the miRNA microarray, results attained from the TLDA need to be further validated to ensure accuracy.
7. The miRNAs that the large-scale screening has indicated as being regulated need further validation in order to distinguish them from false positives. qPCR is a gold standard for validation of differential expression. The accuracy of the qPCR, however, depends on several parameters including RNA extraction, RNA integrity (quality), cDNA synthesis, primer design, detection method (chemistry), and the reference gene for data normalization [7, 18, 25]. The options for primer design for miRNA analysis are very limited. The short miRNA sequence length does not give room for much alternative primer design and only one primer can be harbored in this short sequence, hence, the need for alternative manipulations as illustrated in Fig. 2. The SYBR Green-based detection chemistry is different from the TaqMan chemistry used in the TLDA profiling and is suitable for confirmation purposes. It can be less specific than the TaqMan as it detects all amplified products, but performing a melting-curve analysis can assess the homogeneity of the amplicon. Figure 6a (left panel) shows the melting-curve analysis of an miRNA, in which a clearly defined uniform peak can be seen. Multiple peaks would be observed if the primer is nonspecific or if significant amounts of primer-dimer are formed (Fig. 6a, right panel). Amplification plots of each target miRNA can be observed, as exemplified in Fig. 6b. Although the TaqMan chemistry only detects the

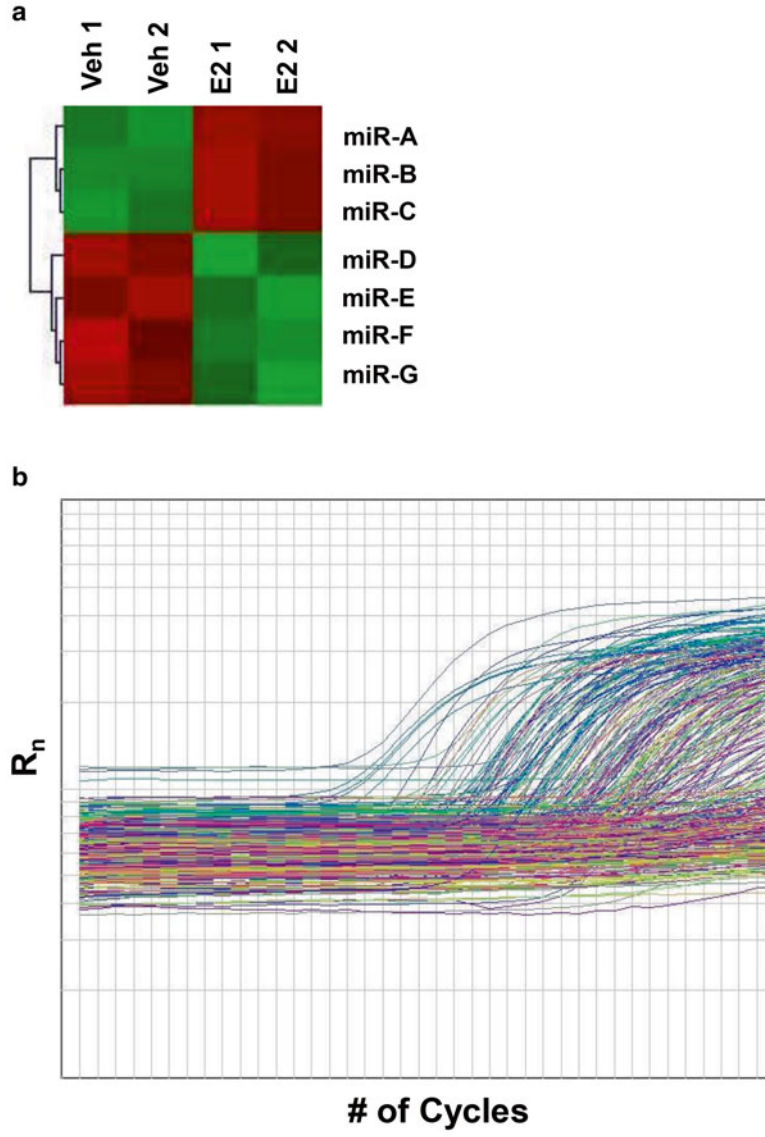

Fig. 5 An illustration of miRNA profiling results. (**a**) A heat map representation of miRNA microarray results for differentially expressed miRNAs. miR-A, B, C are upregulated in the E2 treated-sample as indicated in *red*, while miR-D, E, F, G are downregulated as indicated in *green*. (**b**) Amplification plots for each miRNA from the TLDA results. miRNA amplification can be noted by the increase in fluorescence as indicated by the *Rn* value. (Figure replicated from ref. [8] with permission)

amplification of the target miRNA, the option to control for multiple amplification products using the melting-curve analysis is not available with this chemistry, and while not detected it can still occur and influence the amplification efficacy of the target. We noted that the SYBR Green and TaqMan qPCR assays could generate slightly different results for some miRNAs in our study. Further, for both qPCR assays, normalization

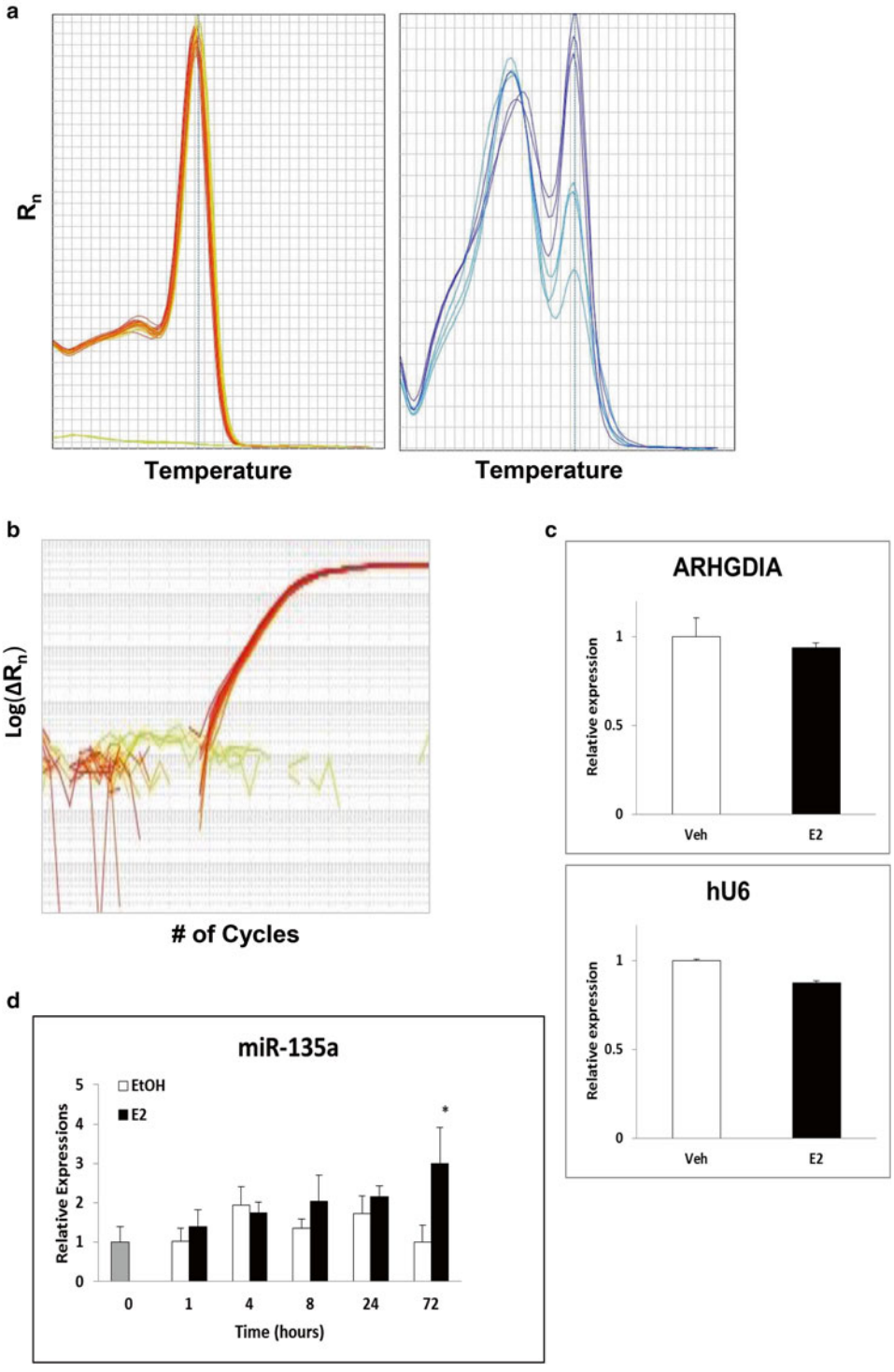

Fig. 6 qPCR analysis of miRNA regulations. (**a**) Melting-curve analysis for miRNA amplicon detection using SYBR Green, showing the emitted fluorescence (*Rn*) as a function of the temperature. The graph to the *left* shows only one amplified product, as indicated by complete homogeneity of the melting temperature of the samples. The graph to the *right* shows the amplification of multiple fragments as indicated by multiple melting

to a reference gene is required to determine the relative expression of genes between two samples. The reference gene, also referred to as housekeeping gene, should be a gene whose expression is not changed and which is expressed at similar levels as the gene of interest and uses the same procedure for its analysis. As there is no perfect reference gene, it is recommended to use at least two different, well-validated reference genes. For miRNA analysis, there is still a debate on a well-defined reference gene. So far, the U6 snRNA is widely used for small RNA analysis. U6 snRNA is a ~110-nucleotide-long, noncoding small nuclear RNA that functions in nuclear pre-mRNA splicing [26]. As observed in Fig. 6c (bottom), U6 expression levels are about the same across all samples shown, thus allowing for calculations of the variable levels of miRNA. A graphic representation of a miRNA qPCR result for a comparison between the vehicle and E2-treated cells that has been normalized to the reference U6 can be observed in Fig. 6d. This illustrates an miRNA that was detected as non-regulated after 24 h-E2 treatment, but which harbors ER chromatin-binding sites close to its genomic location, and time-series analysis identified significant regulation 72 h after treatment. Other miRNAs, which the large-scale screening indicated as unchanged, could also be evaluated for reference gene purposes. Technical replicates are important to validate the robustness of the amplification method and the process employed. Biological replicates are required for a representation of the general biological variation, including variable response to ligand treatment, and to show that effects seen in a cell are reproducible. Based on our experience, variations in miRNA expression between cell cultures of the same cell line can occur. Usually these differences are less than 1.3-fold, and the average of values and corresponding standard deviation identifies the natural and technological variation. This variation could result from various factors including varying cell density and the fact that cell functions could change from passage to passage. To identify statistically regulation resulting from ligand treatment, a widely accepted significance is a

Fig. 6 (continued) temperature peaks. (**b**) Amplification plots, indicated by ΔRn (*Rn* minus the baseline) for each amplification cycle for several samples for one miRNA. This can be either from SYBR Green or TaqMan detection methods. (**c**) Bar graph representation of suitable reference genes for mRNA analysis ARHGDIA (*top*), and for miRNA analysis snRNA U6 (*bottom*), illustrating no significant differences between samples when plotted against input cDNA. (**d**) miRNA qPCR results to illustrate comparison of relative expression levels of miR-135a over a time course of E2 treatment. Note that also vehicle-treated levels vary over time, making conclusion of (non-substantial) E2-dependent regulation challenging. Values are usually illustrated as the average of separate experiments (biological duplicates or triplicates) ± SD. Student's *t*-test is used to demonstrate significance: $*p < 0.05$. (Figure replicated from ref. [8] with permission)

p-value less than 0.05. Here, a student's *t*-test on the biological and technical replicates using the two-tailed distribution and two-sample unequal variance parameters has been used. Other *t*-test parameters exist, and the choice depends on the experimental design [27].

Acknowledgements

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Chapter 31

Estradiol-Induced Transcriptional Regulation of Long Non-Coding RNA, HOTAIR

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Abstract

HOTAIR (HOX antisense intergenic RNA) is a 2.2 kb long non-coding RNA (lncRNA), transcribed from the antisense strand of homeobox C (HOXC) gene locus in chromosome 12. HOTAIR acts as a scaffolding lncRNA. It interacts and guides various chromatin-modifying complexes such as PRC2 (polycomb-repressive complex 2) and LSD1 (lysine-specific demethylase 1) to the target gene promoters leading to their gene silencing. Various studies have demonstrated that HOTAIR overexpression is associated with breast cancer. Recent studies from our laboratory demonstrate that HOTAIR is required for viability of breast cancer cells and is transcriptionally regulated by estradiol (E2) in vitro and in vivo. This chapter describes protocols for analysis of the HOTAIR promoter, cloning, transfection and dual luciferase assays, knockdown of protein synthesis by antisense oligonucleotides, and chromatin immunoprecipitation (ChIP) assay. These protocols are useful for studying the estrogen-mediated transcriptional regulation of lncRNA HOTAIR, as well as other protein coding genes and non-coding RNAs.

Key words Long non-coding RNA, HOTAIR, Estradiol, Estrogen receptors, Gene expression, Epigenetics, Mixed lineage leukemia, Polycomb-repressive complex 2

1 Introduction

Non-coding RNAs (ncRNA) are class of transcripts that are coded by the genome but that are never translated into proteins [1]. NcRNAs are implicated in a variety of cellular and physiological functions and are emerging players in diverse human diseases, including cancers, cardiovascular diseases, and neurological disorders [1, 2]. Long non-coding RNAs (lncRNAs) are a class of ncRNAs that are more than 200 bases long. LncRNAs are critical players in numerous biological processes such as dosage compensation, genomic imprinting, chromatin organization, gene regulation, alternative splicing, and so forth. Mammalian genomes possess a large number of lncRNAs that exceed the fraction of protein coding genes. LncRNAs are transcribed by RNA polymerase II (RNAPII) [3–5]. Like protein coding mRNAs, lncRNAs

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are capped, spliced, and polyadenylated. Studies indicate that lncRNAs are dysregulated in various devastating human diseases including cancer. Although lncRNAs appear to be critical for various biological processes, their mechanism of action and transcriptional regulation remain elusive.

HOTAIR (HOX antisense intergenic RNA) is an example of 2.2 kb long lncRNA that is localized on chromosome 12 within the homeobox C (HOXC) gene cluster [6]. It is transcribed by RNAP II from the antisense strand and thus HOTAIR is an antisense transcript [6]. HOTAIR downregulates various genes in a genome-wide fashion, in trans [6, 7]. HOTAIR interacts with various chromatin-modifying enzymes that regulate gene expression. For example, HOTAIR, through its 5′-end, interacts with the histone H3 lysine 27 (K3K27)-specific methyl-transferase complex, PRC2 (polycomb-repressive complex 2) that is involved in gene silencing [6, 8, 9]. HOTAIR, through its 3′ end, interacts with LSD1 (histone demethylase-1)/CoREST/REST complex, leading to HOTAIR-mediated assembly of PRC2 and LSD1 complexes [8]. HOTAIR is highly expressed in primary breast tumors and many other carcinomas [2, 8, 10]. Recent studies showed that lncRNAs in the HOX loci become systematically dysregulated during breast cancer progression [10, 11]. HOTAIR expression is augmented in primary breast tumors and metastases, and its expression level in primary tumors is a powerful predictor of metastases and death [8]. Overexpression of HOTAIR in epithelial cancer cells induced genome-wide retargeting of PRC2 complex to an occupancy pattern more resembling embryonic fibroblasts [6]. Loss of HOTAIR expression inhibits cancer invasiveness, particularly in cells that possess excessive PRC2 activity [8].

We recently investigated the mechanism of gene expression of HOTAIR in the presence of estradiol (E2). These analyses demonstrated that along with estrogen receptors (ERs), mixed lineage leukemia (MLL)-histone methylases play crucial roles during E2-induced HOTAIR gene activation [11–14]. ERs, MLLs, and other ER coregulators such as CBP/p300 bind to the HOTAIR promoter in an E2-dependent manner, modify histone methylation and acetylation levels at the HOTAIR gene promoter, and ultimately induce transcriptional activation [11, 12]. Importantly, though these analyses are very focused on HOTAIR gene regulation analyses, the same techniques can be used for analyzing various other gene and non-coding RNA regulatory mechanisms [15], and they can be applied to both in vitro and in vivo studies. Many types of techniques can be applied to the analysis of E2 regulation of HOTAIR, including PCR and RT-PCR, immunoblot, and RNA-sequencing, among others. This chapter describes protocols for analysis of the HOTAIR promoter, cloning, transfection and dual luciferase assays, knockdown of protein synthesis by antisense oligonucleotides, and chromatin immunoprecipitation (ChIP) assay.

These protocols are useful for studying the estrogen-mediated transcriptional regulation of lncRNA HOTAIR, as well as other protein coding genes and non-coding RNAs.

2 Materials

2.1 Cell Lines

1. Estrogen receptor (ER)-positive cell lines: MCF7 (human breast adenocarcinoma cell line) and T47D (human ductal breast epithelial tumor cell line).
2. ER-negative cell lines: MDA-MB-231 (human breast adenocarcinoma) and MCF10 (mammary epithelial cells) [11, 12, 15–21].
3. Healthy control cell line: MCF10 (breast epithelial cell line).

2.2 Cell Culture Reagents

1. Growth media (for normal growth and maintenance of MCF7, T47D, MCF10, MDA-MB-231 cell lines): DMEM, 10 % heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 0.1 mg streptomycin/mL. After mixing store the media at 4 °C.
2. Treatment medium (medium for estrogen treatments): phenol red-free DMEM-F-12, 10 % charcoal-stripped FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 0.1 mg streptomycin/mL. After mixing store the media at 4 °C.
3. 1× PBS solution: Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 800 mL of deionized water. Adjust pH to 7.4. Adjust the volume to 1 L. Dispense in convenient volumes and sterilize by autoclaving. Store at room temperature.
4. Dextran-coated charcoal (Sigma).
5. 1× phosphate buffered saline (PBS).
6. Trypsin/EDTA solution.
7. 17β-estradiol (E2). E2 stock solution: 100 mM in ethanol. Store at −80 °C for 1 month or more. This stock solution is further serially diluted (using treatment medium) freshly to yield working concentrations as needed for treatment.

2.3 Cloning, Transfection, and Luciferase Reagents

1. Elongase (Invitrogen).
2. TA-cloning vector (pGEM-T).
3. pGL3-promoter vector.
4. ERE-pGL3 clones.
5. Restriction endonucleases.
6. iFect transfection reagent (K.D. Medicals).
7. Dual-Glo Luciferase assay kit (Promega).

8. DMEM-F-12 medium without serum, antibiotics or supplements.
9. Solution A: For each luciferase assay transfection, dilute 1.5 μg of ERE-pGL3 and 0.15 μg of reporter plasmid containing renilla luciferase (PRLTK, as an internal transfection control) into 100 μL of FBS-free DMEM-F-12 without any antibiotics or supplements.
10. Solution B: For each transfection dilute 6–8 μL of iFect transfection reagent into 100 μL of FBS-free DMEM-F-12 without antibiotics and supplements.
11. Solution A: For each antisense oligonucleotide transfection, dilute 1–9 μg of antisense oligonucleotides complementary to ERα, ERβ, or MLL1-4 mRNA into 100 μL of DMEM-F-12 without any antibiotics or supplements.
12. Luciferase assay reagent [10]: add 10 mL luciferase assay buffer to a vial of lyophilized luciferase assay substrate (from the Dual-Glo Luciferase assay kit). Store working aliquots of the LAR at −80 °C. Before each use allow LAR to equilibrate to room temperature.
13. Stop & Glo® Reagent: add 2.1 mL of 50× Stop & Glo® Substrate to 105 mL of Stop & Glo® reagent. Vortex 10 s and store at −20 °C.
14. DMEM media with 2× supplements: 20 % FBS, 2 % L-glutamine, 2 % penicillin and streptomycin.
15. ERα- and ERβ-specific antisense oligonucleotides (custom synthesized by IDT technologies).
16. DNA antisense design software (IDT).
17. Whole cell extraction buffer: 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.05 % NP-40, 0.2 mM PMSF, 0.5 mM DTT and 1× protease inhibitors mixture.

2.4 Chromatin Immunoprecipitation Assay

1. Antibodies against native protein of interest.
2. Formaldehyde (37 %).
3. Glycine, 1.375 M.
4. Protease inhibitors (Roche).
5. Protein A-agarose DNA beads (Millipore).
6. Proteinase K (20 μg/μL).
7. SDS Lysis Buffer: 1 % SDS, 10 mM EDTA, 50 mM Tris, pH 8.1.
8. ChIP Dilution Buffer: 0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl.
9. High Salt Immune Complex Wash Buffer: 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl.

10. Low Salt Immune Complex Wash Buffer: 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl.
11. LiCl Immune Complex Wash Buffer: 0.25 M LiCl, 1 % IGEPAL-CA630, 1 % deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1.
12. TE Buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
13. Elution buffer: 10 μL 20 % SDS, 20 μL 1 M $NaHCO_3$ and 170 μL dH_2O.
14. Ultrasonic Processor/Sonicator, 50-W model equipped with a 2 mm tip or equivalent.
15. 0.5 M EDTA, 8 μL 1 M Tris–HCl, 1 μL Proteinase K, pH 8.0.
16. 3 M sodium acetate, pH 5.2.
17. Ethanol, 70 and 95 %.

3 Methods

3.1 Charcoal Stripping of Fetal Bovine Serum (*See* Note 1)

1. Add 1 g dextran-coated activated charcoal per 50 mL heat-inactivated FBS, into sterile 50 mL tube.
2. Place on a shaker in a 4 °C cold room. Mix overnight (12 h). (Although it is difficult to maintain sterile conditions during charcoal stripping, care should be taken to avoid contamination and to keep the serum at 4 °C.)
3. Centrifuge for 30 min at 18,000 × *g* at 4 °C.
4. Gently pour off serum into new sterile centrifuge tube and centrifuge for another 30 min at 18,000 × *g* at 4 °C. Avoid taking any charcoal particles.
5. Repeat **steps 1–4**.
6. Gently pour off serum into new 50 mL sterile tubes and centrifuge for 30 min at 18,000 × *g* at 4 °C to remove any remaining charcoal particles.
7. Gently pour off serum into new sterile centrifuge tube, divide into 10 mL aliquots in sterile 15 mL tubes and store at −80 °C.
8. Once the final media is made with charcoal-stripped serum, filter-sterilize the medium using a 0.22 μm filter and collect in a sterile bottle. Store at 4 °C.

3.2 Treatment with Estradiol (E2)

For analyzing the impacts of E2 on HOTAIR gene regulation, cells are cultured specifically in phenol red-free media containing charcoal-stripped FBS for at least three generations prior to the treatment with E2.

1. Grow MCF7 cells in 100 × 20 mm tissue culture dishes under normal tissue culture conditions (37 °C with 5 % CO_2, humidified

cell culture incubator) for at least two generations (post thaw) using regular growth media. It is important to have healthy growing cells for the E2 treatment experiments.

2. After two generations of growth in regular media, split cells into treatment medium.
3. Grow and maintain for three generations in phenol red-free DMEM-F-12 treatment medium prior to use for E2 treatment.
4. Split the cells again in treatment medium into 100 × 20 mm cell culture plates and incubate until confluency reaches approximately 60 % (~ overnight).
5. Replace the old medium with new fresh medium and treat with E2 (add 10 μL E2 stock solution into 10 mL medium containing culture plate, 1000-fold dilution).
6. Treat control cells with equally diluted ethanol (vehicle).
7. After adding the E2 or vehicle treatment, incubate the cells for 4 h (or varying time periods as needed).
8. To harvest cells for various analyses, remove the growth medium.
9. Wash cells twice with 10 mL of cold 1× PBS.
10. Add 1 mL of 1× PBS and scrape the cells and transfer to 1.5 mL microcentrifuge tube.
11. Pellet the cells by centrifugation at 2000 × *g* for 10 min at 4 °C.
12. Remove the supernatant by pipetting and store the pellet at −80 °C until analysis, or the cells can be used directly for RNA (Fig. 1) or protein extraction.

3.3 Analysis of the HOTAIR Promoter

To understand the mechanism of HOTAIR gene regulation, we identified the HOTAIR promoter sequence and analyzed the promoter for the presence of various promoter elements, including EREs (Fig. 2). Identification and analysis of the HOTAIR promoter was performed using tools at the NCBI website (http://www.ncbi.nlm.nih.gov/gene?term=(hotair[gene])%20AND%20(Homo%20sapiens[orgn])%20AND%20alive[prop]%20NOT%20newentry[gene]&sort=weight).

1. Obtain the RNA sequence of HOTAIR from the gene records at NCBI (NR_047517.1).
2. Use the Blast tool to compare the RNA sequence to the whole human genome sequence.
3. Select the hit that has 100 % similarity to the HOTAIR sequence and obtain the gene map or the Gene Bank flat files.
4. Trace the Blast sequence in the obtained gene map and identify the start sequence of the HOTAIR lncRNA.
5. Take the 2500 bp region upstream of the start site as the promoter region.

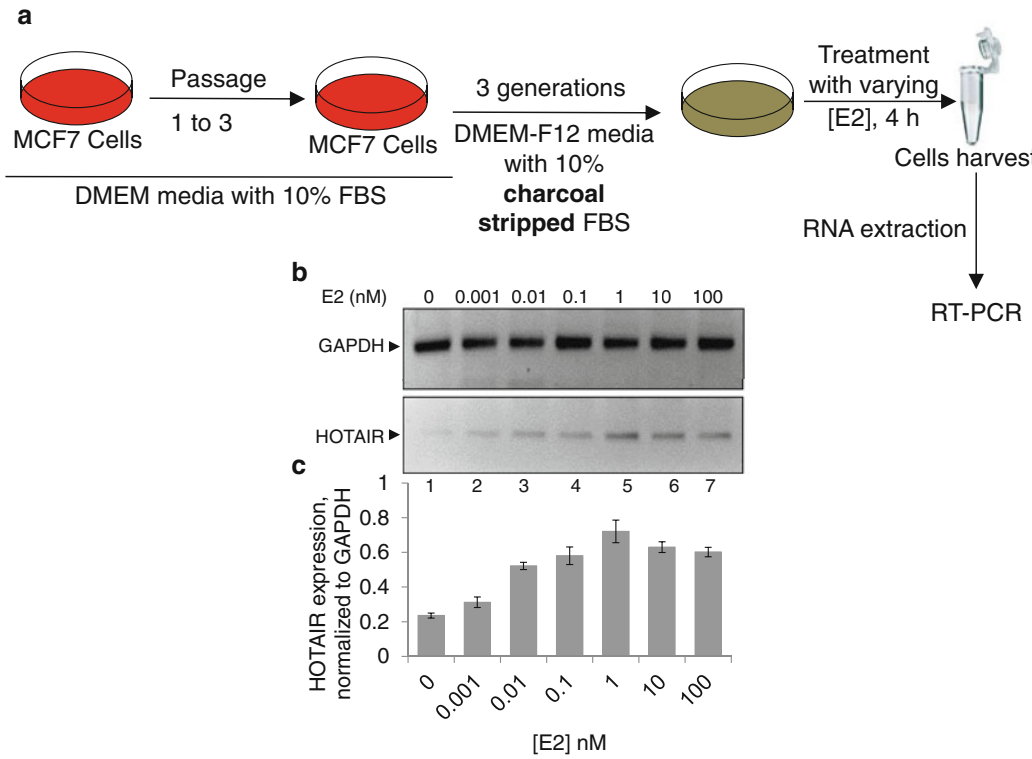

Fig. 1 Panel **a** shows the steps involved in the estrogen treatment of cells such as MCF7 breast cancer cells. Panels **b** and **c** illustrate the RT-PCR (**b**) and qPCR (**c**) analysis of HOTAIR expression in the MCF7 cells treated with increasing concentrations of E2 for 4 h. GAPDH was used as the loading and normalization control. These data demonstrate the E2 responsiveness of HOTAIR in MCF7 cells. (The experiment was repeated at least three times with three replicate treatments. Bars indicate standard errors.)

3.4 ERE Sequence Identification and Cloning

The consensus sequence of the full ERE consists of two palindromic half sites that are separated by a three nucleotide spacer: GGTCAnnnTGACC. We identified four putative EREs at −845 bp, −1486 bp, −1721 bp, and −1758 bp located upstream of the transcription start site in the HOTAIR promoter (Fig. 2). EREs located at the −845 bp and −1758 bp are putative $ERE_{1/2}$ sites (GGTCA). The EREs located at −1486 and −1721 were considered to be imperfect EREs since they had a two-base mismatch as compared to the consensus full EREs (Fig. 2). In order to characterize the functionality of HOTAIR promoter EREs, we cloned the respective EREs into the pGL3 vector, using the TA cloning procedure, followed by sub-cloning into the pGL3 vector (luciferase construct). The cloned ERE-pGL3 constructs were subjected to luciferase reporter assay (Fig. 2). Typically we clone approximately 300 bp region of the sequence from the genomic DNA with the ERE

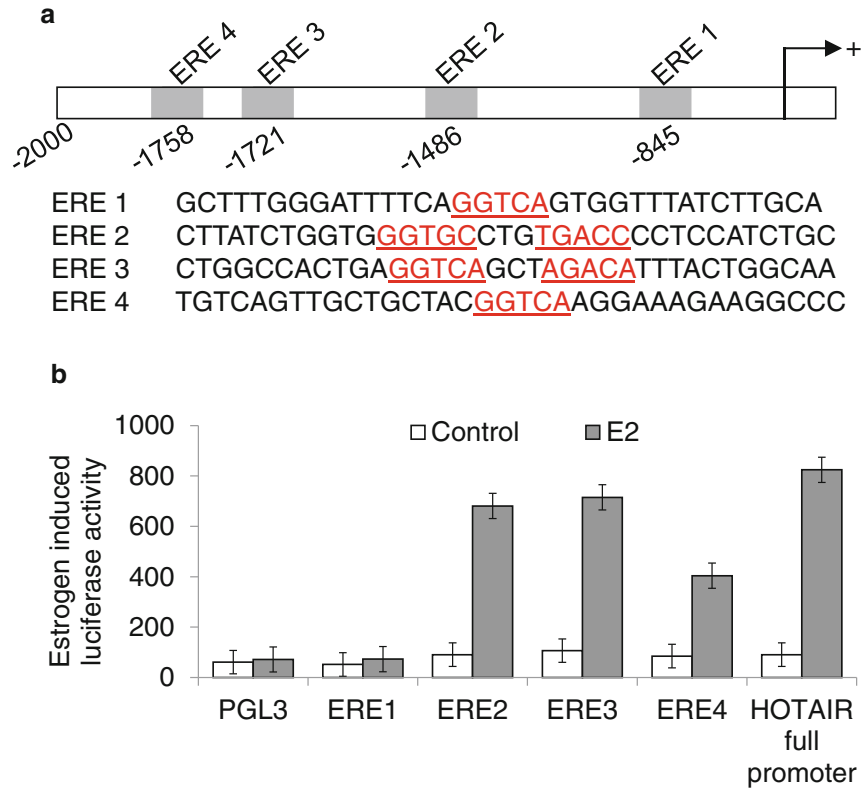

Fig. 2 Analysis of the HOTAIR promoter and luciferase assay: (**a**) The HOTAIR promoter carries four putative ERE sites (called ERE1, ERE2, ERE3, and ERE4). Each of the EREs was cloned individually into the pGL3 vector which carries the Firefly luciferase gene. Each of the four ERE-pGL3 vectors or empty-pGL3 (control vector) constructs was co-transfected along with the renilla luciferase construct (PRLTK) into MCF7 cells. The cells were then treated with 0.1 nM E2 for 4 h and subjected to luciferase assay. The Firefly luciferase activities, normalized to Renilla luciferase activity, were plotted. Panel **a** illustrates the locations and the neighboring sequences of EREs in the HOTAIR gene promoter. Panel **b** shows the luciferase-based reporter assay analysis. Bars indicate standard errors ($n=3$; $p \leq 0.05$). Induction of luciferase activity, upon treatment with E2 and in the presence of a specific ERE-pGL3 construct, indicates the potential involvement of that ERE in E2-induced gene activation. Based on the luciferase assay, ERE2 and ERE3 of the HOTAIR promoter appear to be involved in E2-mediated HOTAIR gene activation

placed in the middle of the insert sequence. The outline for cloning protocol is as follows:

1. PCR-amplify each ERE region (approximately 300 bp) or the full-length promoter (−2050 to +5 nt) from human genomic DNA with elongase.
2. Clone the insert into TA-cloning vector (pGEM-T).
3. Sequence the inserts to confirm the correct sequence and placement.
4. Sub-clone the inserts containing the ERE sequences into the pGL3 vector.

5. Confirm the correct sequence and placement by restriction nuclease digestion and sequencing.
6. Use the final ERE-pGL3 constructs (Fig. 2) for luciferase analysis.

3.5 Transfection with ERE-pGL3 and Luciferase Assay

1. Grow MCF7 cells for three generations in treatment medium.
2. Seed 2×10^5 MCF7 cells in 60 mm cell culture plates with 5 mL treatment medium. Incubate the plates in 5 % CO_2 incubator at 37 °C until approximately 60 % confluent (usually 12–14 h).
3. On the day of transfection, prepare transfection Solution A and Solution B.
4. Add Solution A to Solution B directly and mix gently by pipetting.
5. Incubate the mixture for 35–45 min at room temperature.
6. Wash the MCF7 cell thrice with DMEM-F-12 medium without additives.
7. For each transfection, add 0.8 mL DMEM-F-12 medium without additives to each tube containing the ERE-pGL2/iFect transfection reagent complex. Add the transfection mixtures into the respective plates containing cells and swirl slowly to spread the reagent.
8. Incubate the cells for 24 h at 37 °C in an incubator with 5 % CO_2.
9. Aspirate the medium and replace with fresh growth medium.
10. Treat with appropriate concentration of E2 (0.1 nM) or vehicle control and incubate the cells for 4 h in the tissue culture incubator.
11. Remove growth medium from the treated and control MCF7 cells.
12. Rinse cells in 1× PBS. Remove as much of the final wash as possible.
13. Dispense a minimum volume of 1× lysis reagent (from the luciferase kit) into each culture vessel (e.g., 400 μL/60 mm culture dish).
14. Rock the culture dishes to ensure complete coverage of the cells with lysis buffer.
15. Scrape lysed/attached cells from the dish, and transfer the entire volume to a microcentrifuge tube; place the tube on ice.
16. Vortex the microcentrifuge tube 10–15 s.
17. Centrifuge at $18{,}000 \times g$ for 1 min at room temperature.
18. Transfer the supernatant to a new tube. This is the cell lysate that will be used for the luciferase assay.

19. Mix 20 μL of cell lysate with 100 μL of Luciferase Assay Reagent [10], and measure the Firefly luminescence (emission filter at 590 nm).
20. Add 100 μL Stop & Glo® Reagent to the plate, measure Renilla luminescence (with emission filter at 590 nm).
21. Normalize Firefly luciferase activities to those of Renilla luciferase.
22. The light intensity of the reaction is nearly constant for about 1 min and then decays slowly, with a half-life of approximately 10 min. Therefore, five readings should be performed at an interval of every 20 s.
23. Plot the normalized Firefly to Renilla luciferase activities.

3.6 Knockdown Protein Synthesis by Antisense Oligonucleotides

The role of proteins such as the estrogen receptors, ERα and ERβ, or of transcriptional coactivators such as mixed lineage leukemia (MLL) histone methylases and other receptor coactivators, can be investigated by knocking down their synthesis with antisense oligonucleotides (*see* **Note 2**). If effective knockdown of ERα or ERβ suppresses E2-induced HOTAIR expression, this would indicate their potential roles in E2-induced HOTAIR expression.

1. Grow MCF7 cells in growth medium for two generations.
2. Seed 2×10^5 MCF7 cells in 60 mm cell culture plates in treatment medium. Incubate the plates in a 5 % CO_2 incubator at 37 °C until the cells reach 55–65 % confluence (approximately 12–14 h).
3. On the day of transfection, prepare antisense oligonucleotide Solution A (containing antisense oligonucleotides complementary to ERα, ERβ, or MLL1-4 mRNA, or other antisense of choice) and Solution B (*see* **Note 3**).
4. Add Solution A to Solution B directly and mix gently by pipetting.
5. Mix the solution by pipetting and incubate the mixture for 35–45 min at room temperature in the dark.
6. Wash the MCF7 cells three times with DMEM-F-12 without serum additives.
7. For each transfection, add 0.8 mL of DMEM-F-12 medium without additives to each tube containing the antisense/iFect transfection reagent complex. Gently spread the diluted complex over the entire surface of the plates containing the washed cells and gently swirl twice.
8. Incubate the cells for 24 h at 37 °C in an incubator with 5 % CO_2.
9. Add 1 mL of DMEM-F-12 media containing twice the normal serum and antibiotic concentration without removing the transfection reagent.

10. Incubate the cells for an additional 24–48 h (varying the time period to identify the efficacy of the antisense-mediated knockdown).
11. At 48 h post-transfection, aspirate out the medium and replace with fresh treatment medium containing E2 (0.1 nM for MCF7 cells) or vehicle and incubate the cells for 4 h in the CO_2 incubator at 37 °C.
12. Harvest the cells by trypsinization and pellet the cells by centrifugation.
13. Make whole cell protein extract: Resuspend the cell pellets in whole cell extraction buffer and vortex.
14. Perform three to four freeze–thaw cycles, by dipping tube in liquid nitrogen to freeze and thaw at room temperature. Once thawed, place the sample on ice.
15. Incubate on ice for 30 min.
16. Centrifuge at 18,000 × *g* for 20 min at 4 °C.
17. Transfer the supernatant (protein extract) into a new tube.
18. Quantify the protein concentration using a standard protein assay. Store at −80 °C until use.
19. Analyze the knockdown of ER or MLL proteins using Western blot with specific antibodies against ER or MLL. The regulation of E2-responsive proteins can also be assessed in vehicle versus E2-treated cells in control cells (no antisense oligonucleotide knockdown) versus those in which estrogen receptors were knocked down.
20. Alternatively, at Subheading 3.6, **step 11**, extract total RNA and analyze the levels of knockdown of ER or MLL RNA, as well as the effect of E2 treatment on the expression of E2-responsive genes such as HOTAIR.
21. Alternatively, at Subheading 3.6, **step 12** cells can be fixed with formaldehyde and then subjected to chromatin immunoprecipitation (ChIP) assays or immunohistochemistry (IHC) for the assessment of the effects of knockdown on cellular responses.

3.7 Chromatin Immunoprecipitation (ChIP) Assay

The binding of E2 to genomic ER activates the receptors to bind to target gene promoter ERE regions. ER coregulators are also recruited to the promoter and induce chromatin remodeling followed by activation of target gene expression. The following protocol is designed to determine whether ERs or other potential coregulators bind to the HOTAIR promoter using the chromatin immunoprecipitation (ChIP) assay (Fig. 3a).

1. Grow MCF7 cells for at least three generations in treatment medium.

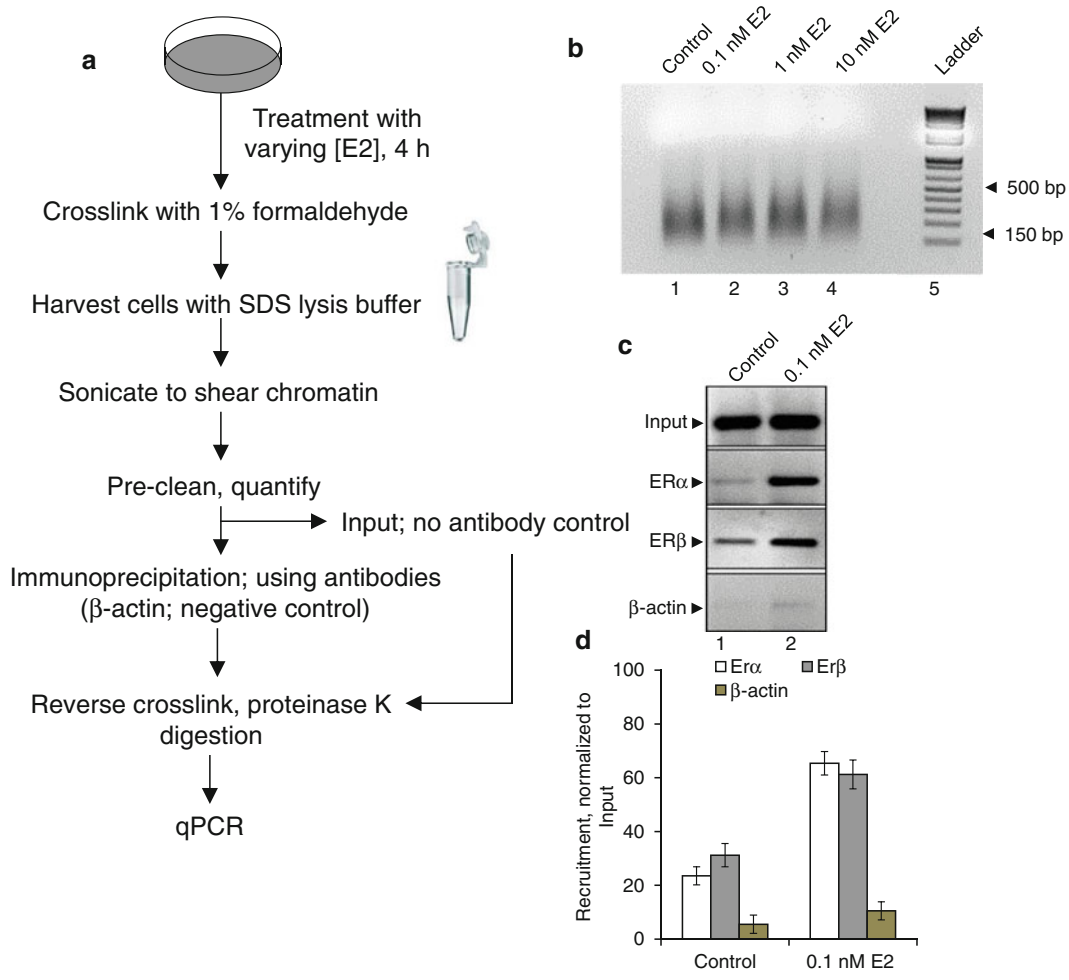

Fig. 3 ChIP assay and analysis of the chromatin via RT-PCR and qPCR. Panel **a** shows the various steps involved in the chromatin immunoprecipitation assay (ChIP). Panel **b** illustrates an agarose gel on which sheared (sonicated) DNA has been separated by electrophoresis, and identifies the optimal sonication level. Panels **c** and **d** show the conventional PCR analysis and qPCR analysis of the purified chromatin obtained by immunoprecipitation using ERα, ERβ, and β-actin (negative control) antibodies. Input refers to the no antibody control

2. When the cells reach to ~80 % confluency in the third generation, passage them into 100 × 20 mm cell culture plates and incubate until confluency reaches 50–60 % (overnight/12 h).
3. Treat the cells with vehicle or 0.1 nM E2 for 4 h.
4. To crosslink proteins to DNA in the cells, add formaldehyde directly to the cell culture medium to a final concentration of 1 % (37 % formaldehyde) into 10 mL treatment medium.
5. Incubate the treated MCF7 cells on a gentle shaking platform for 15 min at 37 °C.

6. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M glycine. Continue to rock at room temp for 10 min.
7. Aspirate the medium.
8. Rinse cells twice with ice-cold 1× PBS containing 0.2 mM PMSF and 10 μL of 200× protease inhibitor mixture.
9. Place the dishes on ice.
10. Add 1 mL of SDS lysis buffer containing 1× Protease Inhibitor mixture.
11. Scrape the cells from each dish and transfer into a separate microcentrifuge tube.
    Centrifuge at 1100 × *g* at 4 °C for 5 min to pellet the cells.
12. Shear the chromatin by sonication: use four to five sets of 15-s pulses of sonication with an ultrasonic processor/sonicator, 50-W model equipped with a 2 mm tip (or equivalent) and set to 30 % of maximum power to obtain the appropriate length DNA fragments. Keep the sample on ice throughout the sonication process (*see* **Notes 4–6**). Centrifuge at 14,000 × *g* at 4 °C for 10 min to remove insoluble material.
13. Centrifuge the sheared DNA samples at 18,000 × *g* for 10–20 min at 4 °C.
14. Transfer the supernatant into a fresh tube; the supernatant contains the sonicated chromatin for ChIP analysis. At this point, chromatin extracts (100 μL aliquots) can be stored at −80 °C until further use.
15. Dilute the sonicated chromatin tenfold in ChIP dilution buffer (to 1 mL).
16. Centrifuge the sonicated samples at high speed in a microcentrifuge for 15 min at 4 °C to pellet the precipitated SDS. Transfer the supernatant to a fresh microcentrifuge tube.
17. Measure the absorbance of the DNA at a wavelength of 260 (A260) with a spectrophotometer (e.g., NanoDrop) to calculate the DNA concentration. Use ChIP lysis buffer that is devoid of SDS as a blank. To assess the quality of the DNA, measure the absorbance at wavelengths of both 260 and 280. The A260/A280 ratio should be ~1.4–1.6. A260/A280 is low at this stage because these are chromatin samples. DNA concentration can be determined by the equation: 1 OD_{260} unit = 50 μg/mL.
18. Aliquot 100 μg of chromatin per antibody to be used into microcentrifuge tubes (*see* **Note 7**).
19. As a negative IgG control, it is important to include an antibody that is irrelevant to nuclear proteins, such as β-actin. Set aside an additional aliquot of chromatin as a no antibody control "input."

20. Dilute the chromatin to a final volume of 300 μL with ChIP dilution buffer supplemented with protease inhibitor mixture.
21. To pre-clear the chromatin, add 50 μL of protein A-agarose beads to each sample of the chromatin and mix by rotation for 2 h at 4 °C.
22. Centrifuge the chromatin samples at 4000 × *g* for 5 min at 4 °C.
23. Transfer the supernatants to fresh 1.5 mL microcentrifuge tubes: these samples comprise the pre-cleared chromatin extract that will be used for immunoprecipitation (IP).
24. Add the appropriate test antibodies (*see* **Note 7**) to each sample of the pre-cleared chromatin solution and mix on a rotating platform overnight at 4 °C.
25. Add 50 μL of protein A-agarose beads (washed and equilibrated with 1× PBS) to chromatin samples and rotate for 2 h at 4 °C (*see* **Note 8**).
26. Centrifuge the samples at 1000 × *g* for 5 min at 4 °C.
27. Carefully pipette out the supernatants from all samples. Take care, not to remove beads that contain the IP.
28. Add 1 mL of high-salt wash buffer (for ChIP) to samples containing agarose beads and mix on a rotating platform for 10 min at room temperature.
29. Centrifuge the samples at 1000 × *g* for 5 min at room temperature.
30. Carefully remove the supernatants and add 1 mL of low-salt wash buffer for ChIP. Mix on a rotating platform for 10 min at room temperature.
31. Centrifuge the samples at 1000 × *g* for 5 min at 4 °C.
32. Carefully remove the supernatants and add 1 mL of LiCl wash buffer for ChIP. Mix on a rotating platform for 10 min at room temperature.
33. Centrifuge the samples at 1000 × *g* for 5 min at 4 °C.
34. Remove the supernatant and wash twice with 1 mL of TE buffer as above.
35. Resuspend the beads (attached to the immunoprecipitated chromatin) and input samples (sonicated chromatin extract for "Input," no antibody control) in 50 μL of ChIP elution buffer supplemented with 1 μL of proteinase K (20 μg/μL), and incubate the samples for 2 h at 55 °C.
36. Resuspend the beads by vortexing the samples.
37. Pellet the agarose beads by brief centrifugation (18,000 × *g* for 1 min) and collect the supernatant into new microcentrifuge tubes.

38. Repeat the elution steps three more times and combine the eluates for a total volume of 200 µL.
39. To reverse the DNA–protein cross-links, add 8 µL 5 M NaCl and incubate at 65 °C for 5 h.
40. Add 1 µL of RNase A and incubate for an additional 30 min at 37 °C.
41. Add 4 µL 0.5 M EDTA, 8 µL 1 M Tris–HCl and 1 µL Proteinase K and incubate at 45 °C for 2 h.
42. Centrifuge the samples at 18,000 × *g* for 5 min at room temperature.
43. Transfer the supernatants to fresh microcentrifuge tubes.
44. Purify the DNA by a phenol–chloroform–isoamyl alcohol extraction (*see* **Note 9**). Mix 1 volume sample and 1 volume of phenol–chloroform–isoamyl alcohol.
45. Vortex for 1 min.
46. Centrifuge at 14,000 × *g* for 10 min at 4 °C.
47. Transfer the upper layer aqueous phase to a fresh tube and add 1 volume of chloroform–isoamyl alcohol (24:1). Vortex for 1 min and centrifuge at 14,000 × *g* for 10 min at 4 °C.
48. Transfer the upper aqueous phase to a fresh tube, add 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95 % ethanol.
49. Mix and place at −80 °C for 2 h.
50. Centrifuge at 18,000 × *g* for 30 min at 4 °C.
51. Remove the supernatant and wash the pellet with 1 mL of 70 % ethanol. Air dry the pellet (20 min).
52. Resuspend DNA in 50 µL of TE buffer.
53. Quantify the concentration of DNA using spectrophotometer (Subheading 3.7, **step 17** above).
54. Use the purified DNA as the template for PCR. Use input chromatin as a loading control. In this case, use pairs of PCR primers that each span one of the ERE sites in the HOTAIR promoter. If E2-stimulated ERα bound to one of the ERE sites in this promoter, then the ERα antibody would immunoprecipitate with the chromatin fragment bearing that ERE site, and the PCR reaction would yield an amplified product. In contrast, proteins that do not bind to DNA, such as β-actin, would not yield a PCR product in this assay. Thus, ChIP technology can be used to differentiate among transcription factors and transcriptional coactivators that bind to specific sequences of DNA (Fig. 2c, d). Similarly, ChIP can be used to analyze the E2 dependence of histone acetyl-transferases and histone methyl-transferases at the HOTAIR promoter or other promoter of interest.

4 Notes

1. Charcoal stripping of the fetal bovine serum using dextran coated charcoal is used to remove low-molecular-weight lipophilic compounds including steroid hormones, and fatty acid ligands of nuclear receptor transcription factors. This helps in analyzing the impact of externally supplemented ligands such as estradiol in a controlled manner. Charcoal-stripped serum is also commercially available from variety of vendors; however, their estrogen response is not always consistent due to inefficient/incomplete stripping and hence presence of some amount of endogenous hormones that interferes with the analysis.
2. Antisense oligonucleotides ultimately reduce the levels of target mRNA in cells. Antisense oligonucleotides must efficiently bind to their target sequences, which depends on antisense oligonucleotide length, sequence content, secondary structure, thermodynamic properties, and target accessibility [22]. The secondary and tertiary structural folding of mRNA/ncRNA molecules may render the RNA inaccessible to an antisense oligonucleotide [22]. Sequences that may appear to be accessible in bioinformatics terms may be involved in intra-molecular hydrogen bonding or stacking interactions which disrupt hybridization of an antisense oligonucleotide [23]. Therefore, predicting the accessible binding sites on an RNA molecule to which the antisense oligonucleotides can hybridize and lead to RNase-H-mediated degradation is important. The length of antisense oligonucleotides may vary from 10 to 25 nt [24]. Most of our antisense oligonucleotides are in the range of ~22 nt and the Tm (melting point) is ~42 °C. Phosphorothioate antisense oligonucleotides against ERα and ERβ can be designed using IDT DNA antisense design software.
3. If more than one sample needs to be knocked down using the same antisense oligonucleotide, one can prepare a master mix containing the components for all the knockdown plates at once.
4. Keep cell lysate ice-cold. Sonication produces heat that can denature the chromatin.
5. In shearing the chromatin by sonication, the goal is to achieve an average chromatin length between 200 and 400 bp. The time and number of pulses will vary depending on sonicator, cell type, and extent of cross-linking.
6. For analysis of the chromatin fragment size, mix 5 μL of DNA sample with 90 μL water and 4 μL 5 M NaCl. Prepare one sample with sheared DNA and a control sample in which the DNA has not been sheared. Incubate the samples for at least 4–5 h to overnight at 65 °C to reverse the DNA–protein cross-links.

Extract the DNA by phenol–chloroform–isoamyl alcohol extraction. Separate the DNA samples by electrophoresis on a 1 % agarose gel (Fig. 3b) and assess the size of fragments in the sheared chromatin.

7. The goal of a ChIP assay is to determine whether a particular protein (for example, a specific transcription factor such as ERα or ERβ, or a coactivator such as MLL1-4) is bound to or associated with a specific DNA sequence (such as one of the ERE sites in the HOTAIR promoter). The antibody chosen for the immunoprecipitation step determines the specificity of the assay as well as the interpretation that can be made.
8. As an alternative, magnetic beads may be used; the separation steps would then use a magnet instead of centrifugation.
9. As an alternative, the sheared DNA can be purified on spin columns designed for DNA purification.

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Chapter 32

Detection and Functional Analysis of Estrogen Receptor α Phosphorylated at Serine 216 in Mouse Neutrophils

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Abstract

Serine 216 constitutes a protein kinase C phosphorylation motif located within the DNA binding domain of estrogen receptor α (ERα). In this chapter we present experimental procedures confirming that mouse ERα is phosphorylated at serine 216 in peripheral blood neutrophils and in neutrophils that infiltrate the uterus, as well as the role of phosphoserine 216 in neutrophil migration. A phospho-peptide antibody (αP-S216) was utilized in Western blot, immunohistochemistry, and double immunofluorescence staining to detect this phosphorylation of an endogenous ERα. Both immunohistochemistry (with αP-S216 or neutrophil marker Ly6G antibody) and double immunofluorescence staining of mouse uterine sections prepared from C3H/HeNCrIBR females revealed that phosphorylated ERα was expressed in all infiltrating neutrophils during hormonal cycles but not in any other of the other uterine cells. Neutrophils infiltrate the uterus from the blood stream. White blood cells (WBC) were prepared from peripheral blood of C3H/HeNCrIBR females or males and double immunostained. Blood neutrophils also expressed phosphorylated ERα but in only about 20 % of cells in both sexes. Only the neutrophils expressing phosphorylated ERα spontaneously migrated in in vitro Transwell migration assays and infiltrated the uterus in mice.

Key words Estrogenreceptorα (ERα), Phosphorylation, Neutrophils, Migration, Infiltration, Mouse uterus, Immunohistochemistry, Immunofluorescencestaining

1 Introduction

Serine 216 of mouse ERα is located on a loop between two zinc fingers and is conserved as serine 212 in human ERα. Mass spectroscopic analysis identified 15 different residues of human ERα that may be phosphorylated in a human breast cancer MCF7 cell line. One of the potential phosphorylation sites is serine 212 [1, 2]. Our cDNA microarray analysis of RNAs, prepared from human hepatoma-derived Huh7 cells, ectopically expressed phosphorylation mimicking ERα S212D or non-phosphorylation mimicking ERα S212A mutant, showed that S212D mutant regulated a distinct set of the genes [3]. While these observations suggest that this phosphorylation may impart a unique biological function to ERα, it has not been confirmed with endogenous ERα in normal

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tissues and/or cultured cells. Therefore, here we describe protocols that utilize an antibody specific to phosphorylated serine 216 of mouse ERα to detect endogenous phosphorylation. These methods demonstrate that ERα is phosphorylated at serine 216 in mouse neutrophils [4]. Furthermore, only a fraction of blood neutrophils were found to express phosphorylated ERα and only those neutrophils were able to migrate in an in vitro assay and also infiltrate the uterus in vivo.

Neutrophils infiltrate the mouse uterus during normal hormonal cycles or in inflammatory conditions. ERα plays an essential role in uterine cells to regulate normal infiltration in response to estrogen [5–7]. However, the presence of ERα and its biological function in infiltrating neutrophils is not well understood at the present time. Our present finding that ERα is phosphorylated at serine 216 in infiltrating neutrophils has provided new insight to investigate the role of neutrophil ERα in uterine functions as well as inflammation-associated development of estrogen-dependent diseases [8–10]. Although not covered in this chapter, knock-in (KI)/knockout (KO) mouse line (*Esr1S216A*) bearing an alanine mutation at residue serine 216 has now been generated. Utilizing these mice, the in vivo roles of phosphorylated ERα will be further investigated.

Serine 216 of mouse ERα is conserved as a phosphorylation motif not only in human ERα but also in 41 out of 48 human nuclear receptors. In fact, phosphorylation of this conserved motif was first confirmed with threonine 38 of nuclear receptor CAR (NR1I3) in hepatocytes and, moreover, its role in the activation of CAR by therapeutics such as phenobarbital [11, 12]. Thus, a unique opportunity to examine whether or not this conserved phosphorylation motif within the DNA binding domains engages a general regulatory mechanism common to the majority of nuclear receptors is presenting itself to us. Our work on serine 216 phosphorylation of ERα have strengthened this opportunity to be realized in future investigations. In this chapter we present experimental protocols, specifically western blot and double label immuno fluorescence staining immunohistochemistry, that confirm that mouse ERα is phosphorylated at serine 216 in peripheral blood neutrophils and in neutrophils that have infiltrated the uterus, as well as an in vitro migration assay protocol to investigate the role of phosphoserine 216 in neutrophil migration and infiltration.

2 Materials

1. Antibody directed against phospho-Ser216 ERα (*see* **Note 1**).
2. Protein kinase C (PKC) (Promega, V5261, *see* **Note 2**): store at −80 °C.

3. Glutathione S Transferase (GST)-mouse ERα and its mutant (Ser216Ala) (*see* **Note 3**): purified ERα proteins purified from bacteria transformed with plasmids carrying these cloned receptors.
4. Tris-buffered saline, pH 7.4 (TBS).
5. Kinase buffer: 41.5 mM TBS, pH 7.4, 6.67 mM $CaCl_2$, 3.3 mM dithiothreitol, 1.67 mM $MgCl_2$, 1.0 mg/mL phosphatidylserine, and 330 μM ATP.
6. 4× SDS-sample buffer: 314 mM Tris–HCl, pH 6.8, 8 % SDS, 50 % Glycerol and 0.02 % Bromophenol Blue.
7. Polyvinylidene difluoride (PVDF) membrane.
8. TBS/Tween 20 (TBS-T) buffer: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20.
9. Bovine serum albumin (BSA).
10. Skim milk (nonfat dry milk).
11. Horseradish peroxidase conjugated goat anti-rabbit antibody (Goat anti-rabbit IgG-HRP). Store at 4 °C.
12. Luminol reagent (Advansta).
13. Xylene.
14. Ethanol: 100, 95, and 70 %.
15. Antigen retrieval reagent and system such as Antigen Decloaker (Biocare Medical).
16. 3 % hydrogen peroxide.
17. Normal goat serum.
18. Biotinylated goat anti-rabbit antibody (ABC kit, Vector Laboratories): store at 4 °C.
19. ExtrAvidin-peroxidase (Sigma-Aldrich).
20. 3,3′-diaminobenzidine solution (DAB+ Chromogen, Dako Cytomation).
21. Hematoxylin solution.
22. Coverslips and mounting solution such as Permount.
23. 3.8 % sodium citrate (*see* **Note 4**).
24. 6 % dextran (*see* **Note 5**).
25. Sterile saline (0.9 % sodium chloride).
26. Phosphate buffered saline (PBS).
27. Cytospin.
28. Glass microscope slides treated for adherence of tissue sections (such as Superfrost Plus, Thermo Scientific).
29. 4 or 10 % formaldehyde in PBS.
30. Avidin/biotin blocking kit (Vector Laboratories).

31. Fluorescein Avidin *D* at a *c*ell *s*orter grade (DCS) (Vector Laboratories).
32. Texas Red® Avidin *D* at a *c*ell *s*orter grade (DCS) (Vector Laboratories).
33. Anti-Ly6G (Lymphocyte antigen 6G) antibody (Clone 1A8, BD Pharmingen) as a neutrophil marker.
34. Biotinylated rabbit anti-rat antibody (ABC kit, Vector Laboratories). Store at 4 °C.
35. Alexa Fluor® 488 Goat anti-rabbit IgG (H + L) antibody (Life Technologies).
36. Alexa Fluor® 594 Goat anti-rat IgG (H + L) antibody (Life Technologies).
37. Mounting medium/DAPI (Vector Laboratories).
38. 24-well transwell plate (3.0 μm pore) (Corning Life Sciences).
39. RPMI conditional medium: RPMI1640 medium (no-phenol red), 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL). Store at 4 °C.
40. Charcoal–dextran stripped fetal bovine serum (FBS).
41. 0.1 M glycine–HCl, pH 2.0.
42. 3 % hydrogen peroxide.
43. Trypan blue.
44. Hemocytometer for cell counting.

3 Methods

3.1 In Vitro Phosphorylation ERαProteins by PKC

1. Add 0.5 μg of GST-mERα protein or its S216A mutant protein to 30 μL of kinase buffer in a 1.5 mL centrifuge tube on ice.
2. Add 1 μL (1.6 units) of PKC to this solution and incubate it at 30 °C for 30 min.
3. Stop the kinase reaction by boiling the reaction solution for 15 min in 4× SDS sample buffer containing β-mercaptoethanol.
4. Separate the phosphorylated mouse ERα proteins in SDS sample buffer (5–15 μL/lane from **step 3** of Subheading 3.1) by electrophoresis on a 10 % SDS polyacrylamide gel. Electrotransfer the proteins from the gel to a PVDF membrane.
5. Incubate the PVDF membrane in TBS-T/1 % BSA for 1 h at room temperature using a rotating shaker.
6. Incubate the membrane with anti-P-S216 antibody (1:1000) in TBS-T/1 % BSA overnight at 4 °C.
7. Wash with TBS-T buffer for 10 min three times.

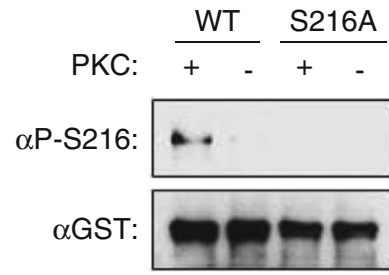

Fig. 1 Western blot analysis to show the specificity of an anti-P-S216 antibody aP-S216. Purified glutathione S transferase (GST)-tagged mERα wild type (WT) and its S216A mutant were incubated with or without protein kinase C (PKC). Subsequent Western blots were performed with αP-S216 or an anti-GST antibody as described in Subheading 3.1

8. Incubate the membrane with a horseradish peroxidase conjugated goat anti-rabbit antibody (1:10,000) in TBS-T/5 % skim-milk for 1 h at room temperature.
9. Wash with TBS-T buffer for 15 min three times.
10. Develop membrane with Luminol reagent and expose the membrane to X-ray film.
11. After detection of phosphorylated ERα, reuse the same membrane for Western blot with an anti-GST antibody. Shake the membrane in 0.1 M glycine-HCl for 10 min at room temperature.
12. Wash the membrane with TBS-T buffer for 15 min three times.
13. Incubate the membrane in TBS-T/5 % skim-milk/anti-GST antibody overnight at 4 °C or for 1 h at room temperature. Hereafter, continue procedures with **steps 7–10** of Subheading 3.1 to develop the anti-GST bands (Fig. 1).
14. Quantitate the P-S216 and GST bands by densitometry.

3.2 Immunohistochemistry of Uterine Sections

1. Fix mouse uterus in 10 % formalin, embed in paraffin, cut 6 μm thick sections, and place them on glass slides.
2. De-paraffinize the sections by submerging the glass slides in xylene for 5 min twice and by sequentially washing with 100, 95 and 70 % ethanol for 3 min at each step and finally with H_2O.
3. Submerge de-paraffinized sections in antigen retrieval solution (decloaker buffer) and heat in the decloaking chamber (*see* **Note 6**).
4. Cool down to room temperature and replace decloaker buffer with H_2O.
5. Incubate with 3 % hydrogen peroxide solution for 15 min to inactivate endogenous peroxidases.

6. Wash twice with TBS-T buffer for 5 min.
7. Incubate with blocking buffer (PBS/1.5 % normal goat serum) for 1 h at room temperature.
8. Incubate with anti-P-S216 antibody (1:25–50 dilution) in blocking buffer for 40 min at room temperature and wash with TBS-T buffer twice for 5 min.
9. Incubate with biotinylated rabbit secondary antibody (1:500 dilution) in blocking buffer for 15 min at room temperature.
10. Wash with TBS-T buffer twice for 5 min.
11. Treat with ExtrAvidin-peroxidase (50-fold dilution in PBS buffer) for 20 min and wash with TBS-T buffer twice for 5 min.
12. React with 3,3′-diaminobenzidine solution for 6 min and wash by flowing water onto the back of glass slides for 3 min.
13. Dip in hematoxylin solution for 10–30 s to counter-stain and wash by flowing water until color disappears from the solution.
14. Wash with TBS-T buffer for 1 min and sequentially dehydrate in 70, 95 and 100 % ethanol for 30 min and finally clear in xylene for 15 min or more.
15. Mount surface of the section with mounting solution and place a cover slip.
16. Observe staining using microscopy.

3.3 Competitive Immunohisto chemistry of Uterine Sections

1. To examine the specificity of the anti-P-S216 antibody, add phosphorylated antigen peptide or the non-phosphorylated peptide counterpart to an anti-P-S216 antibody solution (*see* **Note 7**).
2. Gently shake the mixture for 1 h at room temperature.
3. Incubate uterine sections in one or the other mixture for 40 min at room temperature, then continue the immunohistochemistry procedure from **steps 9** to **16** of Subheading 3.2. If the anti-P-S216 antibody is specific, this step should demonstrate that phosphorylated antigen peptide, but not the non-phosphorylated counterpart, inhibits staining by the anti-P-S216 antibody.

3.4 Preparation of Peripheral White Blood Cell (WBC) Fractions

1. Collect blood from mouse postcaval veins.
2. Add 44 μL of 3.8 % sodium citrate to 0.4 mL of blood in a swing rotor tube and shake gently.
3. Centrifuge the tube at 350 × *g* for 20 min at room temperature (turn off the centrifuge brake).
4. Remove upper layer (platelet rich plasma) from the tube.

5. Add a quarter volume of 6 % dextran solution to the cell pellet and gently mix with the lower layer by pipeting several times (*see* **Note 8**).
6. Add one volume of 0.9 % sodium chloride to the above mixture and gently pipet up and down several times (*see* **Note** 8).
7. Let the tube stand at room temperature for 20–30 min to allow RBC to sediment (*see* **Note 9**).
8. Collect the upper layer (WBC) and place in a new tube.
9. Centrifuge at 220 × *g* for 6 min at room temperature, with centrifuge brake set low.
10. Collect the resulting pellet and resuspend the WBC in less than 0.5 mL of PBS.
11. Dilute an aliquot of the WBC suspension in trypan blue (1/100) and count cells using a hemocytometer (*see* **Note 10**).

3.5 Double Fluorescence Staining with Fluorescein and Texas Red

This section introduces two staining methods using Fluorescein and Texas Red or Alexa.

1. Cytospin mouse WBC (1×10^5 cells per 100 μL) from **step 10** of Subheading 3.4 onto a glass slide. Allow the cells to dry overnight at room temperature.
2. Fix the cells by submerging the slide in 4 % formalin solution for 20 min at room temperature.
3. Incubate with Avidin solution for 15 min at room temperature to block for avidin/biotin.
4. Rinse briefly with PBS and incubate with a Biotin solution for 15 min at room temperature.
5. Wash with PBS twice for 5 min.
6. Incubate with blocking buffer (PBS/1.5 % normal goat serum) for 20 min at room temperature.
7. Incubate with anti-P-S216 antibody (a 1:25–50 dilution) in blocking buffer for 40 min at room temperature and wash with PBS twice for 5 min (Fig. 2).
8. Incubate with a biotinylated goat anti-rabbit antibody (5–10 μg/mL dilution) in blocking buffer for 30 min at room temperature and wash with PBS for 5 min twice.
9. Incubate with Fluorescein Avidin DCS (1:100) in PBS for 8 min and wash with PBS twice for 5 min.
10. To stain the neutrophils in the WBC sample, use Ly6G as a neutrophil marker. Repeat the blocking reactions in **steps 3** and **4** of Subheading 3.5, then continue with **step 11** of Subheading 3.5.
11. Incubate with blocking buffer (PBS/1.5 % normal rabbit serum) for 20 min at room temperature.

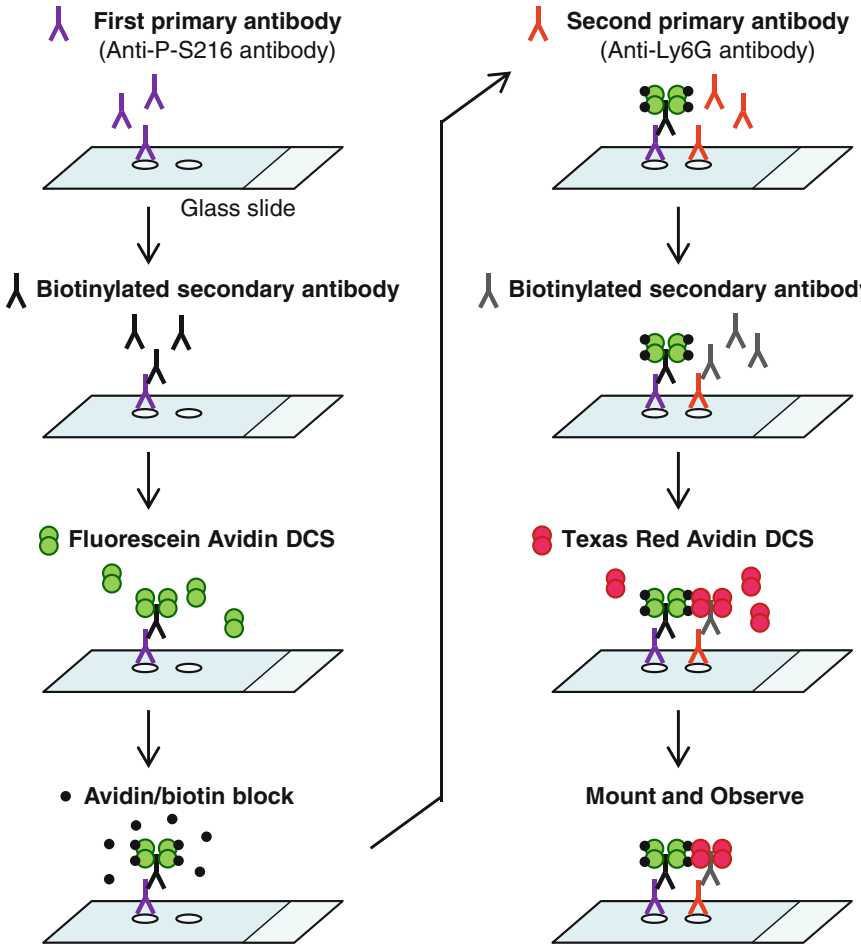

Fig. 2 Flowchart of double immunofluorescent labeling using two primary antibodies from different species

12. Incubate with anti-Ly6G antibody (1:70 dilution) in blocking buffer for 30 min at room temperature.
13. Wash with PBS twice for 5 min.
14. Incubate with a biotinylated rabbit anti-rat antibody (5–10 μg/mL dilution) in blocking buffer for 30 min at room temperature and wash with PBS twice for 5 min.
15. Incubate with Texas Red® Avidin DCS (1:100) in PBS for 8 min and wash with PBS buffer twice for 5 min.
16. Coverslip with mounting medium/DAPI.
17. Observe fluorescence staining using a confocal microscopy.

3.6 Double Fluorescence Staining with Alexa 488 and Alexa 594

1. Fix cells as described in Subheading 3.5, **steps 1** and **2**.
2. Wash cells with PBS twice for 5 min.
3. Incubate with blocking buffer (PBS/1.5 % normal goat serum) for 20 min at room temperature.

4. Incubate the cells with anti-P-S216 antibody (1:25–50 dilution) in blocking buffer for 40 min at room temperature.
5. Wash with PBS twice for 5 min.
6. React with a neutrophil marker anti-Ly6G antibody (1:70 dilution) in blocking buffer for 30 min at room temperature.
7. Wash with PBS twice for 5 min.
8. Incubate with a mixture of Alexa Fluor® 488 Goat anti-rabbit secondary antibody and Alexa Fluor® 594 Goat anti-rat secondary antibody (each 1:500 dilution) in blocking buffer for 2 h at room temperature in the dark.
9. Wash with PBS twice for 5 min.
10. Coverslip with mounting medium/DAPI.
11. Observe fluorescence staining of the cells that are attached to the insert using confocal microscopy.

3.7 In Vitro Migration Assay

1. Add 600 μL of RPMI conditional medium/3 % FBS to each well of a 24-well plate.
2. Dilute WBC (from **step 10** of Subheading 3.4) to 2×10^6 cells per 100 μL in RPMI conditional medium/3 % FBS.
3. Gently add the diluted WBC to a Transwell insert (Fig. 3).
4. Set insert into the well and incubate for 90 min at 37 °C.
5. Collect media from the upper and lower compartments.
6. Attach cells from both compartments on separate glass slides using a Cytospin and dry overnight at room temperature for double fluorescence staining (*see* Subheading 3.5 or 3.6) (*see* **Note 11**).
7. Some neutrophils will migrate through the filter that divides the upper from lower compartments but do not move into the media of the lower compartment. To stain the neutrophils

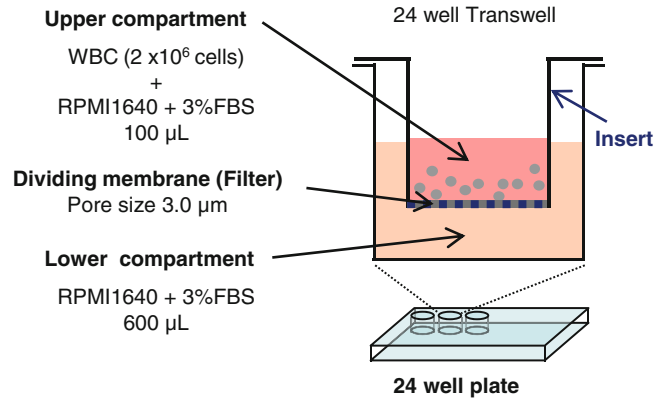

Fig. 3 Schematic of the in vitro Transwell cell migration system

that remain attached to the membrane, employ the following procedures.

8. Wash the insert with PBS to remove cells on the upper surface of the filter.
9. Place the insert in a new well filled with 1 mL of 4 % formalin for 20 min at room temperature to fix cells on the bottom surface of the filter.
10. Perform procedures for double fluorescence staining (*see* Subheading 3.5 or 3.6).
11. Remove the stained filter from the insert and place it on a glass slide with its lower surface facing upwards.
12. Mount this filter with a coverslip and mounting medium/DAPI.
13. Observe staining using confocal microscopy as in Fig. 4.

4 Notes

1. The purified antibody used here (0.29 mg protein/mL) was produced in rabbits by AnaSpec, Inc [4]. This antibody is not commercially available at the present time.
2. Purified fraction from rat brain that consists primarily of α, β and γ isoforms with lesser amount of δ and ζ isoforms.
3. GST-mouse ERα and its mutant (Ser216Ala) were constructed in our laboratory.
4. Weigh directly into bottle (without a spatula) and dissolve in sterile water. Store at 4 °C.
5. Weigh directly into bottle (without a spatula) and dissolve in sterile water by sonicating, warming and shaking. After filtration, store the solution at 4 °C.
6. Set timer of the decloaker apparatus (a type of pressure cooker) for 3 min. However, the entire decloaking process takes a total of 30 min because of the time needed for increasing and decreasing the internal pressure.
7. Dilute anti-P-S216 antibody with PBS/1.5 % normal goat serum to the concentration of 10 μg/mL and add either phospho-peptide or non-phospho-peptide in PBS to the same final concentration as that of the antibody.
8. Pre-warm 6 % dextran solution and 0.9 % sodium chloride in 37 °C water bath just before use.
9. Pipet out bubbles from solution before allowing the tube to stand for 20–30 min.
10. Count only granulocytes based on their smaller size.

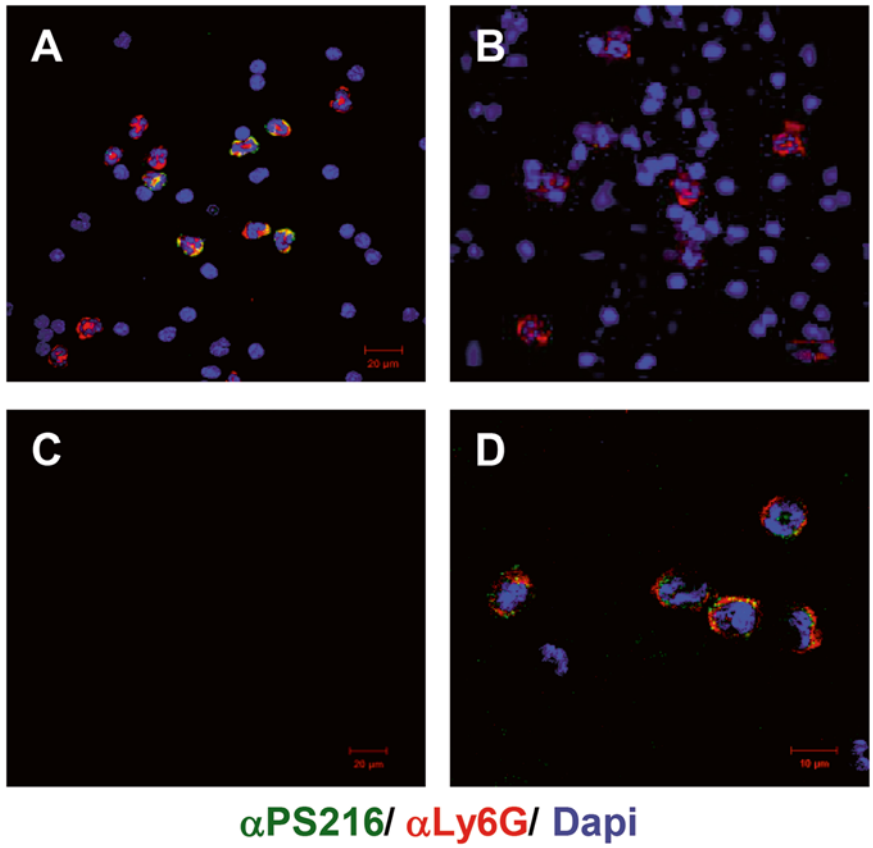

Fig. 4 Migration of phosphorylated ERα-expressing neutrophils in Transwell system. The WBC fraction prepared from peritoneal blood of C3H/HeNCrlBR females was subjected to an in vitro migration assay as described in Subheading 3.7. Panel **a** illustrates the total WBC cell fraction that was added to the *upper* compartment. Panel **b** shows the cells that remained in the *upper* compartment after the migration assay. Panel **c** illustrates the cells that migrated into the *lower* compartment. Panel **d** illustrates the cells that migrated to the lower surface of the dividing filter. These cells were double stained by anti-Ly6G (in *red*) and aP-S216 (in *green*) antibodies. DAPI stains nuclei in *blue*. Neutrophils stained by both antibodies are in *yellow*. The data demonstrate that only those blood neutrophils that expressed phosphorylated ERα were able to migrate in this in vitro Transwell assay

11. Centrifuge medium at 220 × *g* for 6 min at room temperature. Remove the supernatant and resuspend the pellet in 100 μL PBS in preparation for the Cytospin.

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Chapter 33

Estrogen Receptor-α Knockout Mice

Per Antonson, Patricia Humire, and Jan-Åke Gustafsson

Abstract

Tissue specific knockout mice are valuable tools to study gene function in vivo. The method uses the Cre/loxP system in which loxP sites are cloned into the genome surrounding one or more exons of a gene and the targeted exon(s) are deleted when the Cre enzyme is expressed. Mouse lines that are prepared for the generation of knockout ERα mice have been developed independently by many research groups and the number of available transgenic mouse lines that express Cre under tissue specific promoters is large. Here, we describe how tissue specific ERα knockout mice are generated.

Key words Estrogenreceptor, Knockout mice, Gene targeting, Tissue specific, Transgenic, Cre enzyme, Genotyping, loxP, Floxed

1 Introduction

Estrogenreceptoralpha (ERα) is a nuclear receptor that binds to specific regions in DNA, called estrogen response elements (ERE), and regulates transcription of its target genes in response to its ligands which include estrogens and other estrogenic compounds. The structure of the protein contains an N-terminal transactivation domain (AF1), a zinc finger type DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) [1, 2]. In the mouse ERα gene the DBD is localized in exons 3 and 4.

Conventional ERα knockout mice, in which ERα is inactivated in all cells in the body, have displayed many important functions of ERα and estrogen signaling [3–5]. Nowadays most in vivo studies use the Cre/LoxP system to generate tissue specific knockouts [6, 7]. Cre is a recombinase derived from the bacteriophage P1, which mediates recombination between loxP sites. Mice with loxP sites flanking one or more important exons, so called floxed mice, are created by homologous recombination in embryonic stem cells. When crossed with Cre transgenic mice, recombination between the loxP sites will occur in cells that express Cre, resulting in a deletion of the targeted region in the genome. Thus the gene is knocked out tissue specifically.

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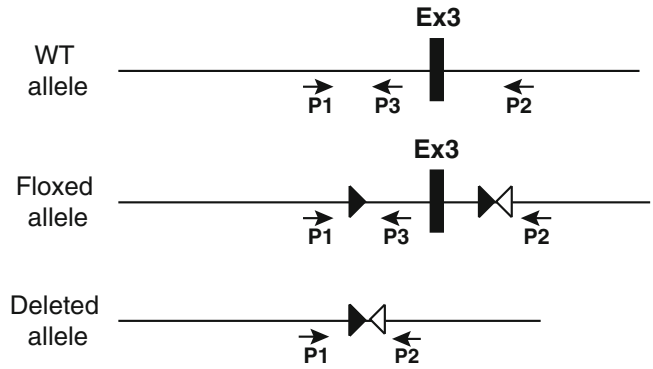

Fig. 1 Structures of the mouse ERα gene exon 3 WT allele, floxed allele, and Cre recombinase deleted allele. Primers for PCR genotyping are indicated as *arrows*. Lox P sites are indicated as *black triangles* and the frt site is indicated as a *white triangle*

FloxedERα mice have been developed independently in several laboratories [8–14]. The same strategy, in which exon 3 is targeted, has been used in all these studies, Fig. 1. After Cre deletion, the DBD is removed and this deletion also causes a frameshift in the coding region which ensures that nothing after exon 2 from the ERα gene is translated. The putative peptide that might be produced from exon 1 and 2 of the targeted ERα gene is not believed to have any function since it lacks both the DBD and LBD.

FloxedERα mice have been used to make tissue-specific knockouts in a variety of tissues including brain [9, 15, 16], mammary gland [10], pituitary gland [17, 18], reproductive organs [19–22], liver [23, 24], bone [12, 25–27], pancreas [28], and adipose [29, 30] and global knockouts [8, 11, 13, 14].

2 Materials

1. $ER\alpha^{flox/flox}$ mice, such as B6.129X1-$Esr1^{tm1Gust}$ [14], are bred with transgenic mice expressing Cre under the control of a tissue specific promoter. A large selection of Cre transgenic mice is available from Jackson labs (*see* **Note 1**).
2. Prepare genomic DNA from tissue removed when mice are labeled for identification using ear punching.
3. Lysis buffer: such as 190 μl DirectPCR® Lysis Reagent (Viagen) supplemented with 10 μl of 2 mg/ml Proteinase K (Fermentas).
4. PCR materials: Use a readymade 2× PCR reaction mix such as REDTAQ Readymix (Sigma-Aldrich), containing buffer, nucleotides, and loading buffer for the PCR reactions. This mix is supplemented with genomic DNA as template, primers, and double-distilled water (ddH_2O).

5. ERαPCRprimers:P1:5′-GGAATGAGACTTGTCTATCTTCGT, P2: 5′-CCTGGCATTACCACTTCTCCT and P3: 5′-GACACATGCAGCAGAAGGTA. *See* Fig. 1 for location of the primers.
6. CrePCRprimers:Cre-sense:5′-CCAATTTACTGACCGTACACC and Cre-antisense:

   5′-GTTTCACTATCCAGGTTACGG (*see* **Note 2**).
7. Analyze PCR products using standard agarose gel electrophoresis.

3 Methods

3.1 Breeding of Mice

Keep a colony of $ERα^{flox/flox}$ mice separately and use offspring from this colony for breeding to generate the tissue specific knockout mouse line.

1. The first breeding step is carried out with Cre transgenic mice together with $ERα^{flox/flox}$ mice. Set up at least two breeding trios with one male and two females.
2. When the litters are separated, use ear punching as an identification system. Use the removed tissue for genotyping. All of the pups are expected to be $ERα^{flox/WT}$ and 50 % should be Cre transgenic.
3. For the second breeding step use Cre-tg/$ERα^{flox/WT}$ and $ERα^{flox/flox}$ mice as the breeders. Set up at least two breeding trios with one male and two females.
4. Genotype the pups when the litters are separated. The possible genotypes of the offspring are: $ERα^{flox/flox}$, $ERα^{flox/WT}$ Cre-tg/$ERα^{flox/flox}$ and Cre-tg/$ERα^{flox/WT}$.
5. In the third breeding step, $ERα^{flox/flox}$ and Cre-tg/$ERα^{flox/flox}$ are used. Use both male and female mice that harbor the transgene to avoid time loss if it turns out that the mutation causes infertility (*see* **Note 3**). All mice are expected to be $ERα^{flox/flox}$ and 50 % should be Cre transgenic.
6. Mice from **step 5** are used to maintain the colony and to scale up the breeding to generate mice for experiments (*see* **Note 4**).

3.2 Genotyping of Mice

Ear punching is used for the identification of individual mice and the tissue removed is used for genotyping (*see* **Note 5**).

1. Place the mouse ear tissue in a 1.5 ml microcentrifuge tube and store at −20 °C for at least 1 h (*see* **Note 6**).
2. Add 200 μl of lysis buffer to each tube and incubate at 55 °C until no tissue clumps are observed.
3. Incubate the tubes at 85 °C for 45 min to inactivate proteinase K, and then place on ice.

4. Use 1 μl of the lysate as the template in a 25 μl PCR reaction.
5. Perform PCR with ERα primers P1 and P3 to detect the floxed and WT alleles (*see* **Note 7**).
6. Perform PCR with Cre sense and antisense primers to detect the Cre transgene (*see* **Note 8**).
7. It is recommended to genotype for the deleted allele regularly using primers P1 and P2, to make sure that no germ line deletion has occurred (*see* **Note 9**).

4 Notes

1. Cre transgenic mice are commercially available from Jackson Laboratories, http://jaxmice.jax.org/.
2. It is recommended to replace the Cre-sense primer with a primer in the promoter region of the transgene. Thus the PCR reaction will be specific for the Cre transgene used ensuring that the correct Cre transgene is analyzed.
3. ERα is known to affect both male and female fertility. Therefore, use both male and female Cre-tg/ERα$^{flox/flox}$ mice for breeding the first time to avoid time loss if it turns out that the ERα deletion causes infertility in one of the sexes.
4. It is well known that expression of Cre in vivo can affect physiological function [31]. Therefore, Cre transgenic mice should be included as controls in addition to ERα$^{flox/flox}$ mice.
5. Tail tips can also be used to extract DNA for genotyping. The same genomic DNA extraction method as described here can be used.
6. It is important to freeze the biopsies that will be used for DNA extraction since this step helps to break down the tissue and improves the yield of genomic DNA.
7. Primers P1 and P3 detect the WT allele as a 205 bp PCR product and the floxed allele as a 310 bp PCR product.
8. The Cre transgene is detected as a 990 bp PCR product. If a promoter-specific primer is used the PCR product will be a bit bigger (*see* **Note 2**).
9. It is well known that some Cre transgenic mice express Cre in germ cells and that can cause germ line deletions [32]. It is therefore important to genotype for that and this could be done by a PCR-based genotyping method using primers P1 and P2 outside the loxP sites. The deleted allele is detected as a 200 bp PCR product and the floxed allele as 700 bp.

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Chapter 34

Detection of Endogenous Selective Estrogen Receptor Modulators such as 27-Hydroxycholesterol

Erik R. Nelson

Abstract

The estrogen receptors (ERs) belong to the nuclear receptor superfamily, and as such act as ligand inducible transcription factors, mediating the effects of estrogens. However, their pharmacology is complex, having the ability to be differentially activated by ligands. Such ligands possess the ability to behave as either ER-agonists or ER-antagonists, depending on the cellular and tissue context, and have been termed Selective Estrogen Receptor Modulators (SERMs). Several SERMs have been identified with clinical relevance such as tamoxifen and raloxifene. Recently, 27-hydroxycholesterol has been characterized as the first identified endogenous SERM leading to the notion that other endogenous SERMs may exist, each having potential pathophysiological functions. This, coupled with the historic pharmaceutical interest as well as growing concern over chemicals in the environment with the ability to behave like SERMs, has increased the demand for assays to detect SERM-like activity. Here, we describe a common, straightforward in vitro assay investigating the induction of classic ER-target genes in MCF7 breast cancer cells, allowing one to identify ligands with SERM-like activity.

Key words Estrogenreceptor, Selective estrogen receptor modulator (SERM), Estradiol, 27-Hydroxycholesterol, Tamoxifen, Gene expression, Quantitative PCR, MCF7 cells, Pharmacology

1 Introduction

The estrogen receptors (ERs) are intracellular transcription factors belonging to the large nuclear receptor superfamily, and are responsible for mediating the majority of the biological activity ascribed to estrogens. Nuclear receptors are characterized by containing a ligand binding domain (LBD) and a DNA binding domain (DBD). There are two forms of the ER, ERα and ERβ [1, 2]. The expression of ERα is fairly ubiquitous, while ERβ is the major form expressed in the ovary, lung and prostate [3]. 17β-Estradiol (E2) is the most potent endogenous agonist for the ERs. However, the well-defined LBD has allowed for the pharmacological development of agonists and antagonists for the treatment of various disorders such as osteoporosis or breast cancer. Intriguingly, it has been

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found that certain ligands have the capacity to antagonize the receptor in some contexts, while behaving as an agonist in other contexts. The best studied example of this is tamoxifen, which behaves as an ER-antagonist in ER-positive breast cancers, but agonizes the receptor in the uterus and bone tissues. These type of dual-activity ligands has been termed Selective Estrogen Receptor Modulators (SERMs) [4].

As a transcription factor, ligand-bound receptor dimers in the nucleus interact directly with DNA on hormone response elements [5, 6], or indirectly via other transcription factors such as AP1, NFκB or Sp1 [7]. Once bound to DNA, the receptors interact with a large assembly of coregulator proteins termed coactivators and corepressors. Different ligands result in the association of unique sets of coregulators, and it is generally understood that it is the composition of these coregulators that determine whether a ligand behaves as an agonist, antagonist, or SERM. With over 300 proteins having the ability of interacting with the ER [8], the potential complexity of the coregulator complex is immense. In a further layer of complexity, the ER can also function to regulate several other signaling pathways. In this regard, the ER has been demonstrated to interact with the c-Src protein kinase complex, the regulatory subunit of phosphoinositide-3 kinase (p85), MAPK, caveolins and the adaptor protein Shc, modulating the activity of the their associated signaling cascades [9, 10]. It is important to note that the assays described below will not be able to distinguish between ligands that directly bind to and activate the ER as opposed to indirect activation of the ER.

Perhaps due to its rather 'flexible' LBD, coupled with the number of signaling pathways that converge on the ER, many ligands have been identified as having SERM activity. These include drugs designed to modulate the ER such as diethylstilbestrol, tamoxifen, raloxifen or fulvestrant, as well as a growing number of anthropogenically derived chemicals such as dichlorodiphenyltrichloroethane (DDT) and its breakdown products, and bisphenol A (BPA). These chemicals, termed xenoestrogens or environmental estrogens, are a class of endocrine disrupting chemicals with SERM activity. Intriguingly, certain cholesterol metabolites such as 27-hydroxycholesterol (27HC) and 25-hydroxycholesterol have been recently identified as endogenous SERMs [11–13]. Importantly, the ability of 27HC to modulate ER activity may have important health consequences as it has been shown to inhibit the cardio-protective effects of estradiol [13], promote atherosclerosis [14], decrease bone quality [15, 16], and promote the growth of ER+ breast cancer [17, 18].

Due to the pharmaceutical interest in the therapeutic development of SERMs, the environmental concerns of endocrine disruptors, and the potential pathological roles of endogenous SERMs, there is strong demand for assays that identify SERM like activity.

There are several approaches that can be taken. While each approach has strong merit in and of itself, in actuality, one would desire a comprehensive workup including several, if not all of these approaches.

The most traditional approach is to determine whether a ligand binds the ER, and/or whether it has the ability to compete for estradiol occupancy [19]. Another conventional method to identify estrogen-like activity is the use of a receptor-reporter assay such as the luciferase reporter assay. A version of this assay was used to identify 27HC as an endogenous SERM [11, 13]. Harnessing the ability of different ligands to shift the ERs into unique conformations, peptide interactions with the ligand-bound ER could potentially be used to predict the SERM like activity of a given ligand [20]. This technique was used to show that 27HC shifted the ER into a conformation similar to, but still distinct from that of tamoxifen or estradiol [11]. Furthermore, this technique has been used to accurately predict the relative agonist or antagonistic properties of different androgen receptor ligands, indicating the powerful nature of this style of assay [21]. One fairly straightforward method to determine whether a ligand modulates ER expression is to determine whether it regulates the gene expression of ER-target genes. This method has the advantage of investigating endogenous gene transcription in a well-defined system. While it can be adapted to high-throughput screening with a one or two gene readout, differential gene expression can also be used to determine the SERM-like nature of ligands [22]. This chapter describes a protocol for assessing ER-regulated gene expression in response to estradiol and known agonists and antagonists of ER, as well as proposed new potential ligands using qRT-PCR as the read out of gene expression.

2 Materials

Prepare all solutions using sterile, ultrapure water (resistivity 18.2 MΩ) and molecular grade reagents.

1. MCF7 cells (*see* **Note 1**).
2. Sterile DMEM/F-12 media with phenol red. This can be purchased from any commercial vendor or made as follows: 1:1 mix of high glucose DMEM (4.5 g/L glucose) and standard Ham's F-12.
3. Sterile, phenol red free DMEM/F-12 (*see* **Note 2**).
4. Fetal Bovine Serum (FBS) (*see* **Note 3**).
5. Culture media: DMEM/F-12 with phenol red, 8 % FBS, 1 mM sodium pyruvate, 1× nonessential amino acid mix, 100

U/mL penicillin–streptomycin (optional). Nonessential amino acid mix can be purchased commercially at 100×.

6. Charcoal stripped FBS (CFBS) (*see* **Note 4**).
7. Experimental media: phenol red free DMEM/F-12, 8 % CFBS, 1 mM sodium pyruvate, 1× non-essential amino acids mix, 100 U/mL penicillin–streptomycin mix (optional).
8. Sterile Phosphate Buffer Saline (PBS).
9. Phenol red free 1× Trypsin–EDTA solution.
10. Trypan blue dye.
11. 10 cm cell culture plates (*see* **Note 5**).
12. 6-well cell culture plates (*see* **Note 5**).
13. 17β-estradiol.
14. 27-hydroxycholesterol (27HC; (25R)-Cholest-5-ene-3β, 26-diol).
15. Fulvestrant (ICI 182,780).
16. Vehicle: dimethyl sulfoxide (DMSO).
17. Alternate vehicle: ethanol.
18. RNA purification kit (*see* **Note 6**).
19. cDNA synthesis kit (*see* **Note 7**).
20. SYBR Green supermix (*see* **Note 8**).
21. siRNA delivery kit and siRNA against ERα.
22. Laminar flow hood (sterile cell culture hood), cell culture incubator, swinging bucket centrifuge, hemocytometer.
23. Real-time thermal cycler capable of reading SYBR Green.

3 Methods

3.1 Establishing MCF7 Cells

1. Remove a vial of MCF7 cells from cryopreservation.
2. Rapidly thaw.
3. Dilute cells in 10 mL of pre-warmed culture media.
4. Plate on 10 cm cell culture plate.
5. Incubate at 37 °C, 5 % CO_2.

3.2 Passaging MCF7 Cells

Passage MCF7 cells approximately twice/week. It is recommended that they are split 1:3 to 1:6 (*see* **Note 9**).

1. Aspirate media.
2. Rinse with PBS.
3. Aspirate PBS.
4. Add 2 mL of warmed trypsin–EDTA mix.

5. Incubate at 37 °C, 5 % CO_2 for approximately 5 min until cells start lifting.
6. Dilute with 8–10 mL of warmed culture media (*see* **note 10**).
7. Split at desired ratio into fresh 10 cm culture plates with 10 mL of culture media.

3.3 Setting Up the Dose Response Experiment

1. Aspirate media from several plates of MCF7 cells.
2. Rinse with PBS.
3. Aspirate PBS.
4. Add 2 mL of warmed phenol red free trypsin–EDTA mix.
5. Incubate at 37 °C, 5 % CO_2 for approximately 5 min until cells start lifting.
6. Dilute with 8–10 mL of warmed Experimental Media and pool cells from different plates.
7. Pellet the cells in a swinging bucket centrifuge (approximately $125 \times g$ for 5 min).
8. Aspirate supernatant and resuspend pellet in approximately 5 mL of Experimental Media (*see* **Note 10**).
9. Count cells:
   (a) Add 10 μL cells to 10 μL trypan blue dye.
   (b) Add 10 μL of the mix to a hemocytometer.
   (c) Count the cells based on trypan blue exclusion.
10. Dilute cells to 300,000/mL in Experimental Media.
11. Plate 1 mL of cells per well in 6-well culture dishes. The total concentration of MCF7 cells should be 300,000 cells/well.
12. Incubate overnight at 37 °C, 5 % CO_2.
13. Treat with ligands such as estradiol and 27HC (*see* **Notes 11–15**).
    (a) Aspirate media.
    (b) Replenish with 1 mL of experimental media already containing diluted ligands.
14. Incubate for 24 h at 37 °C, 5 % CO_2.
15. Aspirate media and lightly rinse with PBS.
16. Aspirate PBS.
17. Freeze at –80 °C, or continue processing as described in Quantitative, Reverse-Transcription PCR.

3.4 Determining Requirement of ER: Pharmacological Approach

Once it has been shown that your ligand of interest modulates the expression of ER target genes (experiment 3.3, 3.6 and 3.7), it is necessary to establish the requirement of the ER. This can be done by use of a pharmacological antagonist of the ER such as fulvestrant (ICI 182,780), or by siRNA against the ER.

1. Repeat Dose Response experiment, **steps 1–12**.
2. Treat with ligands as in Dose Response experiment, **step 13** (*see* **Note 16**).
3. Incubate for 24 h at 37 °C, 5 % CO_2.
4. Aspirate media and lightly rinse with PBS.
5. Aspirate PBS.
6. Freeze at −80 °C, or continue processing as described in Quantitative, Reverse Transcription PCR.

3.5 Determining Requirement of ER: RNAi Approach

Although fulvestrant has been validated as a 'pure' antiestrogen, potential off target effects are still possible. Likewise, RNA interference (RNAi) can also have off-target effects. Therefore, one should make use of both complementary strategies.

1. Repeat Dose Response experiment, **steps 1–9**.
2. Treat with siRNA as described in siRNA delivery kit. Remember to use Experimental Media (no phenol red, charcoal stripped serum) (*see* **Note 17**).
3. Incubate for 24 h at 37 °C, 5 % CO_2 (*see* **Note 18**).
4. Treat with ligands as in Subheading 3.3, **step 13**.
5. Incubate for 24 h at 37 °C, 5 % CO_2.
6. Aspirate media and lightly rinse with PBS.
7. Aspirate PBS.
8. Freeze at −80 °C, or continue processing as described in Quantitative, Reverse Transcription PCR.

3.6 Quantitative, Reverse-Transcription PCR

1. Isolate RNA using a standard RNA column purification kit as described in the instructions provided by the manufacturer (*see* **Note 6**).
2. Quantify RNA by measuring the absorbance at 260 nm.
3. Synthesize complementary DNA (cDNA) as described in the instructions provided by the manufacturer for reverse-transcription (*see* **Note 19**). Remember to include a no-RT negative control.
4. Dilute cDNA with sterile, RNAse and DNAse free water as appropriate (*see* **Note 20**).
5. Set up SYBR Green based quantitative, real-time PCR (qPCR) as described by the kit manufacturer and as optimized by your laboratory (*see* **Note 21**). Include primers for the following genes: pS2, PR, and one or more housekeeping genes (*see* Table 1 and **Notes 22–24**). Remember to create a standard curve to confirm the efficiency of each primer set.

Table 1
Primer sequences for SYBR Green based quantitative PCR

Gene	Forward sequence	Reverse sequence
PS2 (TFF1)	TCCCCTGGTGCTTCTATCCTAATAC	GCAGTCAATCTGTGTTGTGAGCC
PR	GCATCGTTGATAAAATCCGCAG	AATCTCTGGCTTAGGGCTTGGC
36B4	GGACATGTTGCTGGCCAATAA	GGGCCCGACCAGTGTT

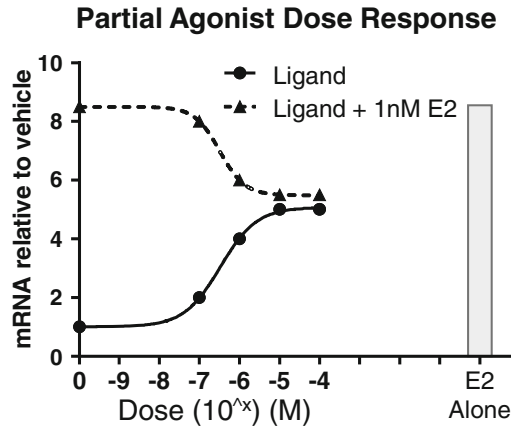

Fig. 1 Example dose response depicting fictitious data from an experiment similar to that in the Dose Response Experiment. This is similar to the results that would be expected from a classic partial ER agonist, such as 27-hydroxycholesterol [11, 13]. The ligand induces ER-target gene expression in a dose related manner, but never reaches the induction level of 1 nM of 17β-estradiol (E2). Furthermore, increasing doses of the ligand decrease the ability of E2 to induce the gene, until the induction reaches the same point as the maximal induction by the ligand alone

3.7 Analysis and Interpretation of Results

1. Ensure that your primers amplify only one product by consulting the melt-curve data generated at the end of the qPCR run.
2. Ensure that each primer set amplified genes in an equally efficient manner.
3. Use the Comparative Ct Method of qPCR analysis (2 – [delta][delta]Ct method) as follows:
   (a) Expression = $2^{(-(\text{Ctgene of interest} - \text{Cthouse keeping gene}))}$.
   (b) Normalize expression to that of vehicle alone (i.e., divide all values by the vehicle expression, so that vehicle becomes set at 1).
   (c) Plot values in graphical format (*see* Figs. 1, 2, and 3).

For dose response data, nonlinear regression can be performed using a 4-parameter fit (variable slope model) (*see* Figs. 1 and 2). If the dose response looks similar to Fig. 1, then it is likely that the

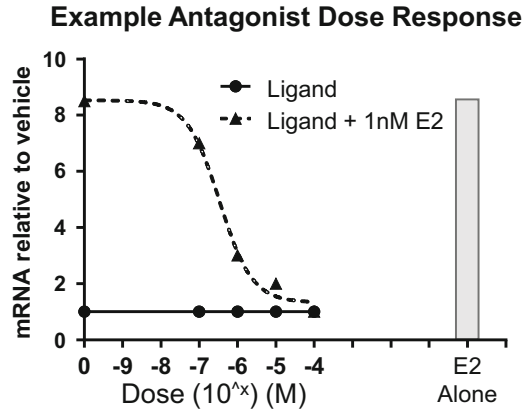

Fig. 2 Example dose response depicting fictitious data from an experiment similar to that in the Dose Response Experiment. This is similar to what would be expected from an antagonistic SERM, such as tamoxifen or fulvestrant. The ligand fails to induce the expression of an ER target gene at any dose tested. However, the ligand antagonizes the activity of estradiol (E2)

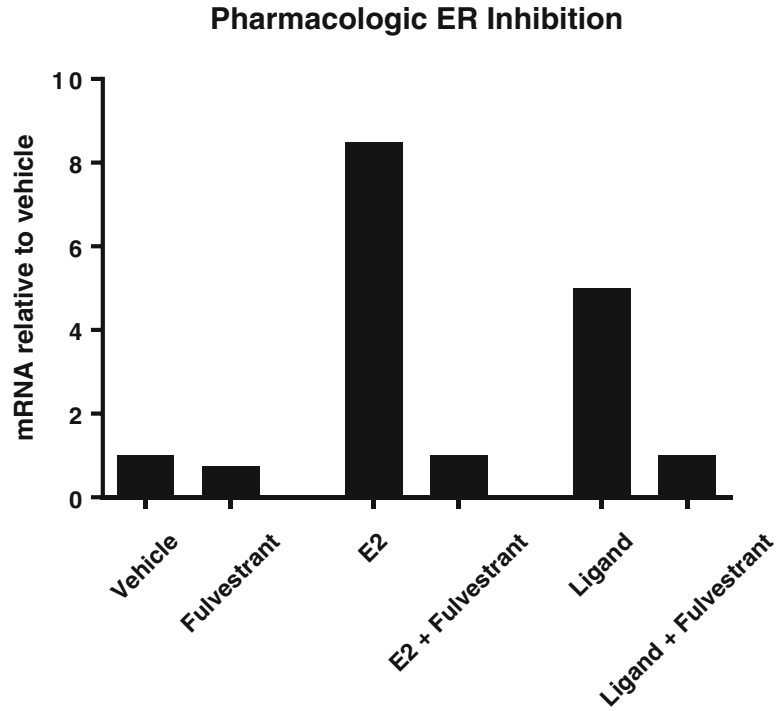

Fig. 3 Example of fictitious data from an experiment designed to determine if the ligand of interest induces gene expression in an ER-dependent manner. Fulvestrant is a "pure" ER antagonist which also leads to the depletion of ER protein. It is common to observe that fulvestrant treatment alone will decrease the expression of ER-target genes slightly. Note that when fulvestrant is co-treated, it attenuates the gene induction by either E2 or the ligand of interest. This is similar to data that would be obtained from a partial ER-agonist such as 27-hydroxycholesterol [11, 17]

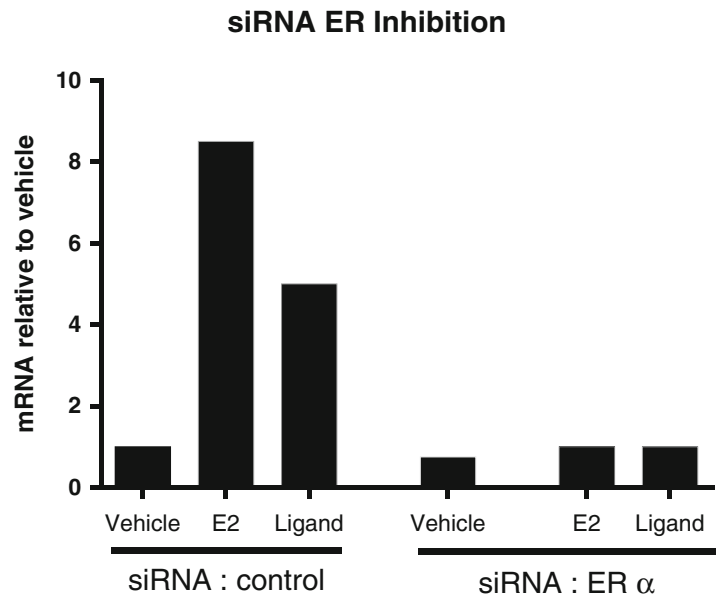

Fig. 4 Example of fictitious data from an experiment designed to determine if the ligand of interest induces gene expression in an ER-dependent manner. Use of siRNA against ERα will deplete the receptor. It is common to observe that siRNA against ERα will decrease the expression of ER-target genes slightly. Note that when siRNA against ERα is present, the E2 or ligand induction of the ER target gene is attenuated. This is similar to data that would be obtained from a partial ER-agonist such as 27-hydroxycholesterol [11, 17]

ligand of interest is a partial ER agonist, similar to 27HC. On the other hand, if the dose response looks similar to Fig. 2, then it is likely that the ligand of interest is an ER antagonist, similar to tamoxifen or fulvestrant. If your data looks similar to Fig. 1, then Determining Requirement of ER experiments should be performed in order to determine if the observed induction of ER target genes requires the presence of the ER. If it is, then you should obtain data similar to Figs. 3 and 4.

4 Notes

1. The Amterican Type Culture Collection (ATCC) supplies MCF7 cells. Many different sublines of MCF7 cells have evolved over time in different labs. While they all typically continue to express ERα, the magnitude of response to estradiol varies significantly between sub-lines. Furthermore, it has been our experience that response to estradiol decreases as MCF7 cells are passaged or if they are overgrown. For more on this, please refer to **Note 9**.

2. Phenol red is a weak 1agonist of the ER. Therefore, to eliminate this confounding effect, and to increase the window of response, use phenol red free media when studying ER gene regulation.
3. FBS lots very greatly with respect to growth factor and steroid concentrations. We test several lots for estrogenic activity using a receptor-reporter assay and select the one with the least activity. We then buy this in bulk. To reduce variation, all experiments should be done with one lot of FBS.
4. FBS is charcoal stripped to remove any steroids, thereby eliminating their confounding effects on the ER, and increasing the window of response. CFBS should be from the same lot of nonstripped FBS. The effectiveness of charcoal stripping should be confirmed in an ER receptor-reporter assay.
5. Although the data is perhaps more anecdotal in nature, plastics can leach chemicals with estrogen like activity. We typically select a high quality plastic and use the same plastic (and manufacturer) throughout all of our studies.
6. A number of commercially available RNA column purification kits are suitable for this assay. We commonly use the Bio-Rad Aurum Total RNA Mini Kit. Guanidinium thiocyanate–phenol–chloroform extraction can also be used [23]. Regardless of the method selected, it is important to include a DNAse step to remove any contaminating genomic DNA. Column purification kits facilitate this by allowing in-column DNAse digestion and subsequent purification from DNAse. If you use the chloroform-extraction method we recommend the Ambion TURBO DNA-free kit (Life Technologies) as this technology completely removes the DNAse prior to subsequent cDNA synthesis.
7. A number of commercially available kits are suitable for this assay. We commonly use Bio-Rad iScript Reverse Transcription Supermix for RT-qPCR. Random hexamer oligo dNTPs are commonly used to anchor the reverse transcriptase, although oligo dT approach can also be used. This step can be done without a kit as well, just be sure to select a reverse transcriptase that can overcome some secondary structure.
8. SYBR green supermix can be purchased from several different vendors. We commonly use Bio-Rad iTaq Universal SYBR Green Supermix. The mix can be made in-house, but requires independent validation and optimization [24].
9. MCF7 cells should be split when they are ~80–90 % confluent. Overcrowding should be avoided. Cells should form a monolayer; cells growing on top of one another are detrimental to this assay. MCF7 cells lose their responsiveness to estradiol

through passaging. We recommend only using MCF7 cells passaged up to maximum of 20–25 times.

10. MCF7 cells are rather sticky and tend to clump together. When diluting from trypsin, run the culture media / cell mixture up and down through a 10 mL pipette several times to help dislodge the cells from one another.
11. To avoid toxicity, it is best to keep the vehicle volume (either DMSO or ethanol) to a minimum. Therefore, we usually keep our ligands at a 1000× stock.
12. Avoid adding ligands directly to the cells. A better approach is to pre-dilute ligands into 1 mL of experimental media, and using this to replace the existing media.
13. Avoid letting cells dry out. One approach is to aspirate and replenish one well at a time.
14. We suggest the following treatment groups:
    (a) Vehicle alone.
    (b) 17β-Estradiol at 1 nM. This will serve as a positive control.
    (c) 4–8 different doses of your ligand of interest.
    (d) 17β-Estradiol in combination with 4–8 different doses of your ligand of interest. This group will allow you to determine whether your ligand of interest is a "partial agonist" or antagonist, with the ability to compete with full agonist potential of estradiol.
15. Remember to control for the total volume of vehicle added (i.e.: the estradiol in combination with your ligand of interest treatment groups will receive vehicle for both treatments. Thus all other groups must have extra vehicle to equal this volume).
16. We suggest the following treatment groups:
    (a) Vehicle alone.
    (b) 17β-Estradiol at 1 nM. This will serve as a positive control.
    (c) Fulvestrant at 1 μM. This is a 'pure' antiestrogen and leads to the degradation of the ER. It is also known as Faslodex or ICI-182,780.
    (d) 17β-Estradiol (1 nM) in combination with fulvestrant (1 μM). This will serve as a positive control for the efficacy of fulvestrant.
    (e) Your ligand of interest at a dose which gives the maximal induction as determined in experiment 3.5.
    (f) Your ligand of interest at the above dose in combination with fulvestrant (1 μM). This dose of fulvestrant has been

chosen as it is sufficient to inhibit the effects of 1 nM estradiol. It may be necessary to optimize this dose, depending on the affinity of your ligand for the ER.

17. ERα is the predominant, if not the only ER subtype expressed in MCF7 cells. Therefore, RNAi knockdown of only this subtype is sufficient to inhibit the estradiol induction of ER target genes.
18. 24 h of siRNA treatment should be enough time to achieve a significant decrease in ER protein. However, this should be confirmed, as different siRNA delivery kits or siRNA targeting sequences may exhibit different kinetics.
19. We typically synthesize 1 μg of total RNA into cDNA.
20. The dilution factor must be optimized for the particular kits and qPCR apparatus you are using. We typically dilute by sixfold.
21. qPCR is a science unto itself and requires optimization and standardization. It is necessary to optimize the conditions for SYBR Green qPCR in your own laboratory as described in the many good manuals, webpages, and advice from the manufacturers of real-time thermocyclers for details.
22. pS2 is also known as Trefoil factor 1 (TFF1), PR is progesterone receptor.
23. Many good housekeeping genes are available. We have found that GAPDH is not reliable and can be regulated by ER. 36B4, RPS18, and tata binding protein have been reliable in our hands. Furthermore, many groups are now advocating that more than one housekeeping gene be used.
24. PS2 and PR have been chosen as they are robustly induced by estradiol in MCF7 cells. However, one can also look at other ER-target genes such as WISP2 and SDF-1. Furthermore, genes specifically regulated by different SERMs in MCF7 cells have been described [22, 25, 26]. We encourage several genes to be included in order to provide a comprehensive overview of the SERM-like activity of the ligand in question.

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Chapter 35

Phytoestrogens Activate the Estrogen Receptor in HepG2 Cells

Lynne A. Kelly

Abstract

Phytoestrogens are popular alternatives to estrogen therapy however their effects on hemostasis in postmenopausal women are unknown. This chapter describes a protocol to determine the effect of the phytoestrogens genistein, daidzein and equol, on the expression of key genes from the hemostatic system in human hepatocyte cell models and to determine the role of estrogen receptors in mediating any response seen using in vitro culture systems and Taqman® gene expression analysis.

Key words Phytoestrogens, Estrogenreceptor, 17β-Estradiol, Genistein, Equol, Daidzein, HepG2, Hep89

1 Introduction

Phytoestrogens are widely used as a 'natural' alternative to hormone therapy for the relief of menopausal symptoms. The isoflavones are generally restricted to legumes, with the highest concentration found in soybeans and soy products. The main isoflavones are genistein, daidzein and equol. There is published concern regarding the risk of cardiovascular disease (CVD) in post-menopausal women using hormone therapy and therefore we chose to investigate the role of phytoestrogens on CVD risk. Metabolism and absorption play a role in defining the effects of estrogens in the body with the first pass liver effect mediating the prothrombotic effects of estrogens and determining the concentration available for systemic circulation [1].

Phytoestrogens bind with greater affinity to ERβ than to ERα and are thought to mediate many of their reported cardioprotective effects through this receptor [2, 3]. However, we have previously shown that ERα, but not ERβ, was detected in rat liver samples [4], indicating that if the effects on haemostatic gene expression which were observed from this study were estrogen receptor mediated, they could not be mediated by ERβ. To

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investigate further the role of the estrogen receptor, a human hepatoma cell line that had been transfected to stably express ERα (Hep89) was used [5]. Treatment effects seen with this cell line were compared with the original ERα-negative cell line (HepG2).

Studies have shown that hormone therapy use by postmenopausal women causes an increase in many of the coagulation and fibrinolytic proteins synthesized in the liver [6–8]. These changes are thought to be implicated in the increased risk of thrombosis observed in hormone users [9, 10]. In this study, the relationship between 17β-estradiol and the phytoestrogens genistein, equol, and daidzein, in haemostatic gene expression and the possible mediating influence of the estrogen receptor was examined [11]. Human hepatocyte cell lines were used in order to study the regulation of the production of these haemostatic markers. HepG2 is a human hepatoma cell line which provides an in vitro model for this study as this cell line expresses many of the genes involved in coagulation activation and fibrinolysis. HepG2 cells are frequently used in studies on the regulation of liver-specific gene expression and they display the majority of haemostatic factors found in the liver. They have been the subject of numerous haemostasis studies including those focusing on the relationship between coagulation FXII and estrogen [12, 13]. We examined the differences between these estrogen receptor negative hepatocytes and those stably transfected with the alpha isoform (ERα) using in vitro techniques and gene expression analysis. Our data showed that genistein and equol increased tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) expression in Hep89 cells with fold changes greater than those observed for estradiol. In HepG2 cells (which do not express ERα), PAI-1 and tPA expression were unchanged and prothrombin was increased. This data suggest that phytoestrogens can regulate the expression of coagulation and fibrinolytic genes in a human hepatocyte cell line; an effect which is augmented by ER. This chapter describes the cell culture techniques and real time RT-PCR protocol used to examine the effects of 17β-estradiol versus phytoestrogens on gene expression in hepatocyte cell lines.

2 Materials

2.1 Cell Culture

1. Human Hepatocarcinoma cells (HepG2) (Health Protection Agency (HPA), Wiltshire, UK).
2. Hep89 (Human Hepatocarcinoma cells): These are HepG2 cells stably expressing ERα created by transfecting the pcDNA3-ERα expression vector into HepG2 cells by electroporation (*see* **Note 1**). Stably expressing cells were selected by resistance to G418 (400 mg/mL). Distinct, well isolated colonies were picked and assessed for the presence of ERα (*see* **Note 2**).

3. Estrogenic compounds for cell culture: 17β estradiol, equol, genistein and daidzein. Prepare stock concentrations of 1 μM in DMSO for each compound; store at −20 °C.
4. Growth medium for both HepG2 and Hep89: eagles minimum essential medium (EMEM) with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 1 % nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. Media can be stored at 4 °C for up to 6 weeks.
5. PRF stimulation medium: phenol red-free (PRF) minimum essential medium with L-glutamine (MEM) (*see* **Note 3**) containing 1 % nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. A final concentration of 10 % dextran charcoal stripped (dcc) fetal calf serum should also be added (*see* **Note 4**). Media can be stored at 4 °C for up to 6 weeks.
6. Trypsin–EDTA: Prepare stock concentration at 0.25 % Trypsin–EDTA. Aliquot and store at −20 °C.
7. Dulbecco's phosphate buffered saline (PBS).
8. Freezing Medium: EMEM as in **step 4** of Subheading 2.1 with 5 % dimethylsulfoxide.
9. Hemocytometer and tally counter for cell counting.
10. Filtered trypan blue solution.

2.2 Gene Expression

1. Qiagen RNeasy Total RNA mini kit for column purification of RNA.
2. Qiagen QIA Shredder system.
3. Qiagen RNase-Free DNase Set (optional, see manufacturer's instructions).
4. High-Capacity cDNA Archive Kit (Applied Biosystems).
5. Taqman® Universal PCR mastermix.
6. RNase-free water.
7. Optical 96-well reaction plates.
8. Optical adhesive covers.
9. Taqman® gene expression assay (*see* **Note 5**).

3 Methods

3.1 In Vitro Culture System

Wipe down all surfaces with 70 % ethanol. Adhere to aseptic technique at all times and carry out all work in the laminar flow cabinet.

1. Add cell culture growth medium to two T75cm^2 flasks at a volume of 200 μL/cm^2. Incubate these flasks at 37 °C, 5 % (V/V) CO_2 and water saturated atmosphere for approximately 30 min.

2. Take a cryovial of frozen cells from the liquid nitrogen container to the laminar flow cabinet and slightly open the screw-top to reduce the overpressure and then close again.
3. Bring this vial of cells to a 37 °C pre-warmed water bath and agitate the tube carefully and continuously until ~90 % of the contents are thawed.
4. Remove the vial from the water bath and agitate gently until all the contents are completely thawed.
5. Transfer the vial to the laminar flow cabinet for the addition of the cells to the culture flasks.
6. Pipette the cell suspension into 5 mL pre-warmed medium and perform a cell count (*see* Subheading 3.2).
7. Seed the equilibrated cell culture flasks with 18,000 cells/cm^2 and distribute evenly across the base of the flask (*see* **Note 6**).
8. Place the flasks in the incubator overnight. Check the cells the following day (*see* **Note 7**). After adherence has been checked, replace the medium and place the flasks back in the incubator (*see* **Note 8**).
9. Replace the growth culture medium twice per week; the cells are deemed ready for subculturing when 70–90 % confluent.
10. Remove the Trypsin–EDTA from the –20 °C freezer and thaw at 37 °C. Depending on the number of experiments and replicates required, the required number of new cell culture flasks should be filled with fresh growth medium and left in the incubator to equilibrate at 37 °C for 30 min (*see* **Note 9**).
11. Examine the growing cells under the microscope for 70–90 % confluence.
12. Bring these two flasks back into the laminar flow cabinet and remove the growth medium.
13. Wash the monolayers once with PBS (50 μL/cm^2) by gently swirling the culture flask with the buffer inside.
14. Remove the PBS from both flasks and then cover the monolayers with Trypsin–EDTA (40 μL/cm^2).
15. Incubate the flasks at room temperature for 2 min followed by 5 min at 37 °C to trypsinize, after which the cells should be examined under the microscope to observe their separation from the wall of the flasks.
16. When the cells are completely detached, add 106 μL/cm^2 of growth medium to each flask, gently agitate the flasks, and transfer the cell suspensions (~11 mL/flask) to two sterile 50 mL centrifuge tubes.

17. Centrifuge the cells at 125 × *g* (Eppendorf 5804R) for 5 min and return to the laminar flow cabinet.
18. Check the tubes for a cell pellet attached to the bottom and carefully remove the supernatant from both tubes without disrupting the cells.
19. Add 1 mL of pre-warmed fresh growth medium to each cell pellet and resuspend the cells by gently drawing the cells and medium slowly and carefully up and down with a sterile pipette tip, avoiding the introduction of air bubbles.
20. Now perform a cell count for each flask using the trypan blue stain (Subheading 3.2).
21. Distribute the cell suspensions in the newly equilibrated flasks at the recommended seeding density of 20,000–30,000 cells/cm^2 and place these flasks back into the incubator.
22. After 24 h, check the cells for adherence under the microscope then change the medium.

3.2 Cell counting with Trypan Blue

1. Clean the hemocytometer using 70 % ethanol.
2. Moisten the shoulders of the hemocytometer and affix a coverslip using gentle pressure and small circular motions (*see* **Note 10**).
3. Following trypsinization and centrifugation of the cells, resuspend the cell pellet in 1 mL of fresh medium.
4. Using filter sterilized trypan blue staining solution, add 20 μL of the cell suspension to 180 μL of trypan blue in a clean sterile 1 mL tube.
5. Using the pipette, draw up 10 μL of the cell suspension containing trypan blue/cell suspension and carefully fill the hemocytometer by gently resting the end of the tip at the edge of one of the chambers (*see* **Note 11**). Focus on the grid lines of the hemocytometer using the 10× objective of the microscope. Focus on one set of a 16 corner square for initial counting.
6. Using a hand tally counter, count the number of cells in this area of 16 squares (*see* **Note 12**). When the counting of this square is complete, move your focus to another set of 16 corner squares and continue the counting until all 4 sets of 16 corner squares are counted.
7. The hemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells × 10^4/mL. The total count from 4 sets of 16 corners = (cells/mL × 10^4) × 4 squares from one hemocytometer grid, therefore; the count should be divided by 4 and multiplied by 10 to adjust for the 1:10 dilution in trypan blue. These two steps are also equivalent to multiplying the cell count by 2.5.

3.3 Stimulating the Cells with Phytoestrogens

1. Change the medium in the flasks to PRF medium 24 h prior to plating the cells.
2. On the day of plating the cells, prepare each 6/12-well culture plate with 2 mL PRF medium/well and leave to equilibrate to 37 °C in the incubator.
3. Trypsinize each flask required as per the "subculturing" method for HepG2/Hep89 (**steps 9–22** of Subheading 3.1).
4. Resuspend the cell pellets in 1 mL of PRF medium and perform a cell count.
5. Plate the cells at a seeding density of 20,000–30,000/cm^2 (*see* **Note 13**).
6. Incubate each plate at 37 °C for 24 h.
7. Stimulate the cells with 100 μL genistein, daidzein, or the daidzein metabolite, equol, to give a final concentration of 50 nM (*see* **Note 14**).
8. Cells should also be treated with 50 nM 17β-estradiol and 100 μL DMSO (as vehicle) (*see* **Note 15**).
9. Incubate the plates for 24 h at 37 °C.

3.4 Taqman® Gene Expression Analysis

1. Following 24 h incubation, extract the RNA from the cells using the RNeasy mini kit as per the manufacturer's instructions (*see* **Notes 16–21**).
2. Measure the quantity and quality of the RNA obtained with a spectrophotometer (*see* **Note 22**).
3. Reverse transcribe 2 μg of RNA into a final volume of 50 μL cDNA using the cDNA archive kit as per the manufacturer's instructions.
4. TaqMan® real time PCR is used to determine the effects of each phytoestrogen or estradiol treatment on hemostatic gene expression (*see* **Notes 23** and **24**).
5. Prepare the samples and mastermix on ice in the laminar flow cabinet.
6. Dilute each cDNA sample with RNase-free water to yield a concentration of 8 ng/μL per well.
7. For each gene to be measured, 3 μL of sample at 8 ng/μL is required. In this case, each sample of 20 ng/μL was diluted 1:2.5 with RNase-free water to yield a concentration of 8 ng/μL to a total volume of 5 μL (which is the 3 μL needed plus 2 μL excess).
8. The appropriate amount of master mix for the number of samples to be assayed should be prepared according to Table 1.

3.5 Loading the TaqMan® Plate

1. Add 1 μL of sample or control in triplicate to each well of a 96-well plate by pipetting into the bottom of the well.

Table 1
Volumes of each component required for a Taqman assay in one well of a 96-well plate

Component	1× (μL)	Manufacturer
TaqMan® Universal PCR buffer mix	5.0	Applied Biosystems
TaqMan® gene expression assay (predesigned primer and probe set against target gene)	0.5	Applied Biosystems
RNase-free water	3.5	Qiagen
Total	9.0	

2. Pipette 1 μL of the first sample into the first three wells (A1–A3), followed by 1 μL of the second sample into the next three wells (A4–A6) and so on until each sample for the first gene has been loaded.
3. Repeat this for the next gene and continue until all samples have been loaded.
4. Next, add the first gene probe mastermix to the first set of samples (*see* **Note 25**).
5. Continue this with the next few probes until all wells have sample plus probe loaded.
6. Seal the plate with a plastic thermostable cover (optical adhesive film) ensuring that all sides and corners are well adhered with no gaps for evaporation.
7. Remove the side tabs and centrifuge the plate at 41 × *g* for 30 s.
8. Analyze gene expression using a real-time PCR platform set with the following thermal cycler conditions: 2 min at 50 °C, 10 min at 94.5 °C, and for 40 cycles, 30 s at 97 °C and 1 min at 59.7 °C.

3.6 Calculation of Results

Retrieve the data file in Excel format and calculate the mean Ct value of each triplicate sample. Express the results as the ratio of target gene cDNA to the internal control using the $2^{-\Delta\Delta CT}$ method [14]. Use unpaired *t*-tests to determine the significance of fold changes relative to DMSO as vehicle. $P < 0.05$ should be considered significant (*see* **Note 26**).

4 Notes

1. Limitations of transfection have been reported including diversions from the normal growth of the cells. However, using very low passage numbers for these experiments to compensate for possible growth problems can prevent these issues arising.

2. This cell line was a kind gift from Dr Harnish; please refer to this author for further information if required [5].
3. Phenol red was also omitted before treating the cells as it also has been known to interfere with steroid-receptors in in vitro studies [15] and could therefore also influence experimental results.
4. Replace FCS with dextran-coated charcoal stripped serum before stimulating the cells as this treatment depletes any hormones present that may interfere with estrogen receptor binding.
5. TaqMan® Gene Expression Assays consist of a pair of unlabelled PCR primers and a TaqMan® probe with a FAM™ or VIC® dye label on the 5′ end, and a nonfluorescent quencher on the 3′ end. The primer is a sequence of DNA which targets the gene of interest and the probe allows for fluorescent identification on the PCR platform.
6. Before incubation, quickly check the cells for any obvious defects by using an inverted microscope; for example, check that these cells have an epithelial morphology.
7. Successfully cultured cells show an 80 % adherence rate, they lack contamination, cells are uniform and display the typical cell morphology and possibly mitosis.
8. When replacing growth medium, prior to the addition of fresh medium to the cells, the medium should be prewarmed to 37 °C for 20 min.
9. Depending on the number of replicate experiment and replicate tests, cell numbers must be calculated in advance to work out the number of T75 flasks needed for the stimulation experiments
10. Newton's rings (a ringed pattern of rainbow colors) should be observed when the coverslip is correctly affixed, thus the depth of the chamber is ensured.
11. Care should be taken so as not to overfill the chamber. Capillary action draws the sample out of the pipette, with the fluid running to the edges of the grooves only.
12. Only live cells that look healthy (unstained by trypan blue) should be counted. The cells that are within the square and any positioned on the right hand or bottom boundary line are included.
13. Excess cells can be cryopreserved. When preparing to freeze down cells in liquid nitrogen, trypsinize the cells according to the subculturing method in Subheading 3.1. Following centrifugation and a cell count, resuspend the cells at a concentration of 3×10^6 cells/mL growth medium containing 5 % DMSO. Transfer each milliliter of cell suspension to a cryovial

and freeze using a CoolCell container (Biocision) at −80 °C. Alternatively, gradual freezing can be used as follows: 4 °C for 45 min, −20 °C for 1 h, −70 °C overnight, followed by liquid nitrogen for long term storage.

14. This concentration was chosen to reflect the expected serum levels of phytoestrogens following supplementation [16]. Each treatment was performed in triplicate for biological replicates.
15. The vehicle control is the solution in which the treatment of interest is diluted. The DMSO used was biological grade >99.9 %.
16. Qiagen RNA kit; Buffer RLT: Prepare a working solution of Buffer RLT by adding 10 μL of 14.3 M β-mercaptoethanol per mL of RLT stock buffer. Mix the solution in a 50 mL centrifuge tube. Cap the tube and wrap it with foil; the working solution can be kept at room temperature for up to 4 weeks. Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of 96 % ethanol to the stock to obtain the working RPE solution.
17. DNase kit: Prepare the DNase I stock solution before using the RNase-Free DNase kit for the first time. Dissolve lyophilized DNase I (1500 Kunitz units) in 550 μL of the RNase-free water provided, securing that no DNase I is lost when opening the vial. Gently invert the vial to mix as it is extremely sensitive to physical denaturation. For long term storage of the DNase I, aliquot the stock solution and store at −20 °C for a maximum of 9 months. Thawed aliquots may be kept at 4 °C for up to 6 weeks.
18. QIA Shredder system: Clean the working space for RNA extraction at this step using alcohol, RNase Zap and DEPC water.
19. RNA extraction: Lyse monolayers of cultured cells directly in the plate. Completely aspirate the culture medium and add 600 μL of RLT buffer to the well. Transfer the lysate to a microcentrifuge tube and pipette up and down to mix the sample. Ensure that no cell clumps are visible before proceeding to the homogenization step. Following the manufacturers procedure, pipette the lysate directly onto the purple shredder column in a 2 mL collection tube and centrifuge at maximum speed (Eppendorf 5418R) for 2 min to homogenize. Transfer the supernatant to a new 2 mL collection tube and discard the pellet. Follow the procedure from the RNeasy mini kit to purify the RNA.
20. Prepare diethylpyrocarbonate (DEPC)-treated water as follows; autoclave 500 mL of double distilled H_2O, then add 500 μL of DEPC, incubate at room temperature for 1 h without mixing, and autoclave again.

21. Ethanol: Prepare 70 % EtOH with DEPC water and filter through a 20 μm membrane with syringe into a sterile tube.
22. Measurement was carried out using the Thermo Scientific NanoDrop 2000 spectrophotometer. The NanoDrop is calibrated with RNase-free water and 1 μL sample is used for measurement.
23. A positive control sample known to express the gene of interest should also be included in triplicate.
24. An endogenous control (housekeeping gene) must also be included so that any skewed results can be corrected. This control must be expressed at a similar level in all study samples, it must also yield similar PCR efficiencies when using the comparative C_T method and finally it must be more abundantly expressed than the target gene. 18S was the internal control gene used in all experiments, it was abundant in the tissues and its expression is not affected by treatment with estrogens.
25. Angle the pipette down to the side of the well and fully expel the tip contents. Change the pipette tip for each new probe.
26. Statistical analysis showed that genistein and equol increased tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) expression in Hep89 cells with fold changes greater than those observed for estradiol. In HepG2 cells (which do not express ERα), tPA and PAI-1 expression was unchanged. For further results and graphical data please refer Kelly et al. [11].

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Chapter 36

Detection of the Phosphorylation of the Estrogen Receptor α as an Outcome of GPR30 Activation

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Abstract

Phosphorylation of the serine residues in estrogen receptor (ER) α is important in transcriptional activation. Hence, methods to detect such posttranslational modification events are valuable. We describe, in detail, the analysis of the phosphorylated ERα by electrophoretic separation of proteins and subsequent immuno-blotting techniques. In particular, phosphorylation of the ERα is one possible outcome of activation of the putative membrane estrogen receptor (mER), GPR30. Hence, phosphorylation represents a cross talk event between GPR30 and ERα and may be important in estrogen-regulated physiology.

Key words Phosphorylation, Serine, Nuclear receptor, Antibody, Tissue, Lysate, Cell line, GPR30, ERα

1 Introduction

Steroid hormones bind to nuclear receptors, which then act as ligand-dependent transcription factors, typically termed as a slow, genomic mode of action [1, 2]. However, steroid hormone ligands can also modify nuclear receptors rapidly [3] though the consequences of such modification are often unclear [4–6]. For example, estrogen, or the natural ligand, 17β-estradiol (E2), can induce phosphorylation of the estrogen receptor (ER)α [7, 8] or reduce palmitoylation of ERα in CHO cells [9]. The majority of phosphorylation sites on ERα are on the serines in the N-terminal AF-1 region; mutation of this region leads to a loss in phosphorylation of ERα [10]. Two such serine residues are serine at 118 (S118), thought to be phosphorylated by ERK [11, 12] or cdk7 and at serine 167 (S167), thought to be phosphorylated by Rsk [13] or Akt [14]. Phosphorylation at serine S118 directs coactivator recruitment and increases gene transcription [15]. Phosphorylation at this site has been implicated in endocrine therapy to breast cancer [16, 17].

Most of the previous work has focused on the E2-mediated phosphorylation of ERα, which is presumed to occur subsequent

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to the binding of the ligand to the ERα [11, 18]. Alternatively, ERα could be phosphorylated in a ligand-independent manner by growth factors [19–21]. We have shown previously that the phosphorylation of the ERα at serine 118 occurs rapidly within 10 min on the administration of 10^{-9} M E2 in the SK-N-BE(2)C neuroblastoma cell line [22]. In addition, we have also shown that a membrane-limited conjugate, E2-BSA (10^{-8} M), can rapidly increase phosphorylation of the ERα at S118 in this cell line within 20 min of administration, suggesting that (a) plasma membrane-initiated signaling is sufficient to phosphorylate the ERα, and (b) that the ERα can be phosphorylated in a ligand-independent manner in a neural cell line [22]. Such membrane-initiated actions by E2 could occur via a putative membrane ER, the GPR30 [23, 24], which is a former orphan G-protein coupled receptor, localized widely in the rodent central nervous system [25]. The actions of GPR30 can be studied with a selective agonist, G-1 [26], in tandem with the selective antagonists, G-15 [27] or G-36 [28], that do not bind the full-length ERα. Our study has shown that a single injection of G-1 (~10 μg/kg body weight) administered subcutaneously to gonadectomized male mice can induce the phosphorylation of the ERα at S118 in the ventral hippocampus within 30 min [29]. A number of investigators have used polyacrylamide gel electrophoresis and subsequent transfer onto membranes for detection of phosphorylated ERα, followed by detection of total ERα [30–32]. However, though detection of ERα at S118 is fairly routine [33], it has been fairly difficult to detect total full-length (66 kDa) ERα in the rodent brain due to the quality of the antibodies [34]. We present here a protocol for detection of phospho-ERα from cell lines or from brain tissue that utilizes western blotting.

2 Materials

2.1 Components for Creating Lysates

1. 5× RIPA buffer (Radioimmunoprecipitation assay buffer: 50 mM Tris–HCl, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, and 0.1 % SDS, pH 7.4 ± 0.2).
2. Protease Inhibitor Cocktail (Sigma Chemical Co).
3. Phosphatase Inhibitor Cocktail 2 (Sigma Chemical Co).
4. Phosphatase Inhibitor Cocktail 3 (Sigma Chemical Co).
5. Complete Lysis Buffer: (*see* **Note 1**) Combine 1 mL of 5× RIPA Buffer, 50 μL Protease Inhibitor Cocktail, 50 μL Phosphatase Inhibitor Cocktail 2, and 50 μL Phosphatase Inhibitor Cocktail 3 with enough double-distilled water (ddH_2O) to bring the final volume to 5 mL. Store at 4 °C for up to 1 week.

6. Phosphate-Buffered Saline (*see* **Note 2**).
7. Cell Scrapers for making lysates from cell cultures (*see* **Note 3**).
8. Kimble-Chase Kontes™ Pellet Pestle™ for making lysates from tissue (*see* **Note 4**).
9. Laemmli's SDS-Sample Loading Buffer (4×, reducing) (Boston BioProducts): 375 mM Tris–HCl, 9 % SDS, 50 % glycerol, 9 % β-mercaptoethanol, and 0.3 % bromophenol blue, pH 6.8.

2.2 SDS Polyacrylamide Gel Components

1. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8. Weigh out 18.17 g of Tris Base and place in a beaker. Add 80 mL of ddH_2O and mix. Place buffer into refrigerator until it reaches 4 °C. Adjust using HCl (*see* **Note 5**) until pH is 8.8 while the buffer is 4 °C. Bring volume to 100 mL using ddH_2O. Store at 4 °C.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Weigh out 6.06 g of Tris Base and place in a beaker. Add 80 mL of ddH_2O and mix. Place buffer into refrigerator until it reaches 4 °C. Adjust using HCl until pH is 6.8 while the buffer is 4 °C. Bring volume to 100 mL using ddH_2O. Store at 4 °C.
3. 30 % Acrylamide/Bis solution (29:1).
4. 10 % SDS: Weigh out 10 g of sodium dodecyl sulfate and place in a beaker. Add 80 mL of ddH_2O and mix. Adjust volume to 100 mL with ddH_2O. Store in a closed container at room temperature (*see* **Note 6**).
5. 10 % APS: Weigh out 10 g of ammonium persulfate and put into a beaker. Add 80 mL of ddH_2O and mix. Adjust volume to 100 mL with ddH_2O. Aliquot and store at −20 °C. Only use freshly thawed aliquots and replace all aliquots after 6 months of storage.
6. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine.

2.3 Immunoblotting Components

1. Immobilon-P PVDF Membrane, 0.45 μm (*see* **Note 7**) (EMD Millipore).
2. Running Buffer: To make 10× Running Buffer stock, weigh out 288 g glycine, 60 g Tris base, and 20 g sodium dodecyl sulfate. Add to a flask containing 1.5 L ddH_2O and mix. When all components are dissolved, bring volume up to 2 L with ddH_2O. Store 10× Running Buffer in a closed container at room temperature.
3. To make 1× Running Buffer, add 100 mL of 10× Running Buffer stock to 900 mL of ddH_2O. 1× Running Buffer may be filtered after use and reused up to four times and stored at room temperature.
4. Transfer Buffer: To make 10× Transfer Buffer stock, weigh out 288 g glycine and 60 g Tris base. Add to a flask containing

1.5 L ddH_2O and mix. When all components are dissolved, bring volume up to 2 L with ddH_2O. Store 10× Transfer Buffer in a closed container at room temperature.

5. To make 1× Transfer Buffer, combine 100 mL of 10× Transfer Buffer stock with 200 mL of methanol and 700 mL of ddH_2O. Store and use 1× Transfer Buffer at 4 °C.
6. TTBS (Tween-Tris buffered saline): To make 5× TBS stock, weigh out 87.7 g of NaCl and 12.1 g of Tris Base. Mix with 1.5 L ddH_2O. Adjust pH to 7.4–7.5 with HCl. Bring volume to 2 L with ddH_2O. Store at room temperature.
7. To make 1× TTBS, combine 800 mL ddH_2O, 200 mL of 5× TBS, and 1 mL Tween 20.
8. Blocking Solution: Weigh 5 g of bovine serum albumin and mix into 90 mL of 1× TTBS. Adjust volume to 100 mL. Store at 4 °C when not in use. Do not keep for more than 3 days.
9. Mini PROTEAN 3 or Tetra Running and Blotting Modules (Bio-Rad).
10. Plastic Containers (*see* **Note 8**).
11. Gel Blot Paper: 15×20 cm sheets can be quartered to create 4 perfectly sized piece of blot paper.
12. Pre-stained Protein Standards/Ladder.
13. 1.0 mm glass spacer plates, short plates, and casting module (Bio-Rad).
14. Water-saturated isobutanol.

2.4 Antibodies and Stripping Buffer

1. Rabbit anti-phospho-ERα (Ser 118) antibody (SC-12915-R, Santa Cruz Biotechnology).
2. Rabbit anti-phospho-ERα (Ser 167) antibody (sc-101676, Santa Cruz Biotechnology).
3. Rabbit anti-ERα antibody (sc-7207, Santa Cruz Biotechnology).
4. Goat anti-Rabbit IgG, HRP-linked antibody (*see* **Note 9**) (7074S, Cell Signaling Technologies).
5. Harsh Stripping Buffer as described by Abcam. To make 100 mL, use 20 mL of 10 % SDS, 12.5 mL of 0.5 M Tris–HCl, pH 6.8, 67.5 mL ultrapure water. Add 0.8 mL β-mercaptoethanol in the hood. (http://www.abcam.com/ps/pdf/protocols/stripping%20for%20reprobing.pdf).

2.5 Cell and Animal Treatments

1. Vehicle (sesame oil), E2 (200 μg/mL or 10 μg/50 μL), G-1 (agonist of GPR30, 6 μg/mL or 0.3 μg/50 μL), G-15 (antagonist of GPR30, 30 μg/mL or 1.5 μg/50 μL). Dissolve E2, G-1, and G-15 slowly in sesame oil at 55 °C for 24 h. Store in aliquots at −20 °C and remove aliquots for use; avoid frequent freeze–thaw cycles.

3 Methods

3.1 Tissue Procurement

1. Gonadectomize adult mice (male or female) (~27–30 g). Allow 1 week for the mice to recover from the surgery and to ensure that gonadal hormones have cleared the circulation. Make sure that all animal protocols conform to ethical procedures and are
2. Inject gonadectomized mice with 50 μL of vehicle, E2 (300 μg/kg), G-1 (10 μg/kg) subcutaneously. (It may be necessary to perform dose-response analyses with selected agents to determine the most appropriate treatment.) To determine whether the effect of G-1 or of E2 is specific, pretreat the animals with a fivefold molar excess of the GPR30 antagonist, G-15, (250 μg/kg) 30 min before the administration of either E2 or G-1.
3. Euthanize the animals 30 min after injection.
4. Quickly remove the brain.
5. Wash the brain in cold 1× PBS.
6. Dissect the brain region of interest (e.g., ventral hippocampus) using a brain block.

Alternatively, samples can be obtained from cultured primary hypothalamic neonatal male mouse cells or from neural cell lines. We have used final concentrations of 10^{-9} M E2 and 10^{-8} M G-1 in cell culture. In order to block the effect of G-1, we have used both 10- and 100-fold excess of the antagonist, i.e., 10^{-7} or 10^{-6} M G-15. In all cases, the drugs or hormones are dissolved in DMSO and the concentration of DMSO added to wells in 0.1 % or less of the volume of the media present in the well containing the cells.

3.2 Making the Lysate

1. Follow instructions to make complete lysis buffer. Make sure lysis buffer is ready at 4 °C or on ice prior to use.
2. To prepare lysate from cell culture:
   (a) Remove media from cells and rinse two times with 0.5–1 mL of ice-cold PBS.
   (b) After removing the last rinse, add 50–100 μL of complete lysis buffer to cells (*see* **Note 10**).
   (c) Allow complete lysis buffer to sit on cells for 1–3 min.
   (d) Using a pipette tip or a cell scraper, disrupt the cells and scrape them from the bottom of the dish. Limit the number of bubbles generated.
   (e) Pipette out buffer and cell mixture and transfer to a microcentrifuge tube.
   (f) Spin tubes with cells for 10 min at 13,000–16,000 × *g* in a refrigerated centrifuge set to 4 °C.

(g) Remove the supernatant and transfer to a new microcentrifuge tube.

(h) Store lysate at −20 °C for up to a week or at −80 °C for long-term storage.

3. To prepare lysate from brain tissue:

(a) Dissect out the brain region of interest.

(b) Place tissue into a microcentrifuge tube containing 50–200 µL of complete lysis buffer (*see* **Note 11**).

(c) Homogenize the tissue manually using a hand-held pestle. Alternatively, a motorized pestle or tissue sonicator can be used. These methods will provide a higher protein yield.

(d) When the tissue is dissociated, centrifuge the microcentrifuge tubes for 30 min at 13,000–16,000 × *g* in a refrigerated centrifuge set to 4 °C.

(e) Pipette out the supernatant and transfer to a new microcentrifuge tube.

(f) Store lysate at −20 °C for up to a week or at −80 °C for long-term storage.

3.3 Making Two Gels

1. Place short plates and 1.0 mm spacer plates into casting module. Secure with clip.
2. Temporarily place the 10-well plastic comb in place between the plates. Using a wax pencil or marker, draw a small line on the short glass plates where the end of the teeth will be for the well comb. This mark serves as the fill level for the separating gel. Remove the comb.
3. To make 10 % resolving gel (*see* **Note 12**), combine 5 mL 1.5 M Tris–HCl (pH 8.8), 6.6 mL 30 % acrylamide (29:1), 200 µL 10 % SDS, and 8.2 mL ddH_2O in a 50 mL conical tube. De-gas with suction for 15 min (*see* **Notes 13** and **14**).
4. Add 100 µL 10 % APS and 10 µL TEMED to initiate polymerization of the gel. Make sure to use a fresh aliquot of 10 % APS each day. Gently rock to mix.
5. Fill the plates with the separating gel up to the line drawn earlier.
6. Pour or pipet enough water saturated isobutanol onto the separating gel to make a complete layer across the gel.
7. Allow the gel to polymerize for 30–45 min. If the isobutanol is on the gel any longer than 45 min it starts drying out the gel.
8. During the last 15 min of the polymerization of the separating gel, begin making the stacking gel. Combine 1.25 mL 0.5 M Tris–HCl (pH 6.8), 670 µL 30 % acrylamide (29:1), 50 µL 10 % SDS, and 3 mL ddH_2O in a 15 mL conical tube. Degas as described above.

9. Once the separating gel has polymerized, pour out the isobutanol from the top of the gels. Rinse two times with ddH_2O (*see* **Note 15**).
10. Immediately before use, add 25 μL 10 % APS and 5 μl TEMED to the stacking gel. Gently rock to mix.
11. Pour the stacking gel on top of the separating gel.
12. Insert the combs into the liquid stacking gel gently in a slight left to right movement. Make sure there are no bubbles under the comb.
13. Allow to polymerize for 45 min to an hour.
14. The gel can be used immediately after it has completely polymerized. Alternatively, it can be wrapped in paper towels soaked with running buffer, wrapped in plastic wrap or in a plastic bag, and stored at 4 °C until use, up to 2 weeks.

3.4 Gel Electrophoresis and Blotting

For more information on preparing protein samples for electrophoresis, please *see* **Note 16**.

1. Place two gels, well-comb side in, into the running apparatus. If using only one gel, place the plastic dam on the second side.
2. Place the gel apparatus into the tank and fill it with 1× Running Buffer (*see* **Note 17**).
3. Gently remove the combs from the wells. To do this, you may have to shift the combs back and forth a little to help release the wells.
4. Wash out the wells by flushing each well with running buffer a few times using a pipette.
5. Heat protein samples at 95 °C for 8 min. After boiling, quickly centrifuge sample tubes to ensure that all liquid has returned to the bottom of the tube.
6. Load 10 μL of protein standards/ladder (*see* **Note 18**).
7. Load protein samples into respective wells.
8. In any remaining unfilled wells, add a proportionate amount of loading dye.
9. Place the lid on the apparatus making sure to match red to red connectors and black to black connectors.
10. Run the gel at 120 V until there is enough separation between the ladder bands to properly see your band of interest.
11. When the gel is approximately halfway through the run (the dye front is halfway down the gel), cut as many pieces of PVDF membrane as needed to the size of the gel (*see* **Note 19**).
12. Soak the membranes in methanol for one min. Rinse off with ddH_2O. Place in 1× Transfer Buffer for at least 15 min prior to use (*see* **Note 20**).

13. Place the sponges and blot paper (2 each per gel) in a box of 1× Transfer Buffer to allow them to soak for at least 15 min prior to use. Make sure remaining 1× Transfer Buffer stays in the refrigerator until use.
14. After the electrophoresis run has completed, remove the gel apparatus and the glass plates. Carefully separate the short plate from the spacer plate using a plastic tool.
15. Remove the stacking gel from the running gel with a piece of plastic and scrape the stacking gel away.
16. Gently remove the gel from the glass plates keeping track of the top of the gel. Place the gel in a small box filled with ddH_2O to rinse off excess SDS. Swish briefly, remove water, and replace with 1× Transfer Buffer.
17. Remove running buffer and rinse out the tank with ddH_2O to remove excess SDS.
18. Insert the blotting module and an ice pack into the tank and place it on a stir plate. Insert a small magnetic stir bar and a small amount of 1× Transfer Buffer. Make sure the stir bar can spin freely before continuing any further.
19. Assemble the transfer sandwich on the open transfer cassette with the black side down. Start building the sandwich on the black side. Place down one sponge and top it with one piece of blot paper. Gently transfer the gel to the top of the filter paper keeping track of the top of the gel. It is preferable to place the protein marker/ladder at the top, with the top portion where the wells were facing the inner side of the cassette. Make sure to pour liberal amounts of 1× Transfer Buffer over and between every layer to ensure that the sandwich remains moist and air bubbles do not form. It is recommended to do this step over a secondary plastic container to catch excess buffer overflow.
20. Carefully place a piece of presoaked PVDF membrane over the gel, ensuring that no bubbles form between the membrane and the gel.
21. Top with another piece of blot paper and another sponge.
22. Close the sandwich cassette and insert it with the black side of the cassette facing the black side of the transfer apparatus.
23. Fill the tank with 1× Transfer Buffer. Replace the lid. Transfer at a 300 mA for 60–90 min.
24. Do not allow the transfer to get too warm. Check the transfer every 15 min to ensure that it does not overheat. The entire transfer apparatus can be placed into a bucket of ice on the stir plate to keep the temperature down longer to allow for more complete transfers (*see* **Note 21**).

3.5 Blocking and Antibody Incubation

1. After the transfer is complete, disassemble the sandwiches and remove the membrane. Place the membrane into a small plastic box containing 7–10 mL of 5 % BSA/TTBS (*see* **Note 22**).
2. Place the box on orbital shaker for 1 h (*see* **Note 23**).
3. When the blocking step is near completion, begin making the primary antibody mixtures to image phospho-ERα on the membrane. 5 % BSA/TTBS is used to make all primary antibody mixtures. Allow 7–10 mL per blot. (If using systems like the LiCOR Odyssey system with primaries of different species, co-incubation of primary antibodies of different species is possible.)

   The antibody dilutions, incubation times, and incubation temperatures are as follows:

   (a) Rabbit anti-phospho-ERα (Ser 118): Dilute 1:4000. Incubate for 1 h at room temperature on orbital shaker.

   (b) Rabbit anti-phospho-ERα (Ser 167): Dilute 1:750. Incubate overnight at 4 °C on orbital shaker.
4. Following incubation in primary antibody, rinse the blot in TTBS four times, 10 min per wash on an orbital shaker. The box should be approximately one-third full with TTBS.
5. Prepare the secondary antibody cocktail using 5 % BSA/TTBS. Dilute Goat anti-Rabbit HRP conjugated secondary antibody to 1:50,000 (*see* **Note 9**).
6. Incubate blots in secondary antibody cocktail for 1 h at room temperature on an orbital shaker.
7. Following incubation in secondary antibody, rinse the blot in TTBS six times, 10 min per wash on orbital shaker. Box should be approximately one-third full with TTBS.
8. Visualize the blot as desired (*see* **Note 24**).
9. Strip the anti-phospho-ERα antibodies from the membrane using protocol and buffer from Abcam (*see* **Note 25**) in preparation for imaging nonphosphorylated ERα.
10. Following the required rinses in water, wash the blot with TTBS four times, 10 min per wash on orbital shaker.
11. Re-block membrane using 5 % BSA in TTBS (*see* **Note 26**).
12. When the blocking step is near completion, begin making the ERα primary antibody mixture using 5 % BSA in TTBS.

    (a) If the protein source was cell culture lysates: Dilute ERα antibody 1:4000. Incubate the membrane in this primary antibody overnight at 4 °C on an orbital shaker.

    (b) If the protein source was tissue lysates: Dilute ERα antibody 1:1000. Incubate the membrane in this primary antibody overnight at 4 °C on orbital shaker.

13. Rinse in TTBS four times, 10 min per wash on orbital shaker.
14. Prepare the secondary antibody mixture using 5 % BSA/TTBS. Dilute Goat anti-Rabbit HRP conjugated secondary antibody to 1:50,000 when the protein source is cell culture lysates or 1:20,000 when the protein source is tissue lysates.
15. Incubate blots in secondary antibody cocktail for 1 h at room temperature on orbital shaker.
16. Rinse with TTBS four times, 10 min per wash on orbital shaker.
17. Develop blot as desired (*see* **Notes 24** and **27**).
18. The expected results may vary depending on the dose. However, we have seen an increase in phosphorylated ERα at S118 in ventral hippocampal tissues treated with the GPR30 agonist, G-1, as compared to the vehicle treated tissue. In addition, we expect that this increase is blocked in tissues treated with the GPR30 antagonist, G-15. Though the phospho-S118 ERα antibody detects a strong band of 66 kDa, the antibodies that detect total ERα often detect bands of other sizes such as 52, 46, 60 kDa and sometimes higher molecular weight bands of about 72 kDa in the mouse brain. The 66 kDa band detected by this antibody in tissue is often lower in intensity to the other bands. This is in contrast to the scenario in uterine tissue from the rat or mouse, where the 66 kDa band detected by the total ERα antibody is the predominant band.

4 Notes

1. Ultrapure water (~18 MΩ) should be used whenever ddH_2O is required.
2. PBS made in the lab from a standard recipe works just as well as premade PBS for these purposes.
3. Whether or not cell scrapers are useful will have to be determined by the experimenter and the method in which the cells are cultured. Small areas of cells (e.g., 24-well plates) are easily scraped using a 200 μL pipette tip instead of a scraper.
4. These pestles can be used to mechanically dissociate the tissue. Other methods of tissue homogenization can be used.
5. A 6 N HCl solution can be used for adjusting pH.
6. Stirring the 10 % SDS solution can create a large amount of bubbles. It is recommended to stir this slowly or wait for the bubbles to settle down before adjusting with ddH_2O to final volume.
7. PVDF membranes work well for the protocol described. However, the membrane should be chosen based on methods of developing the final product. For instance, a low fluorescence

membrane is recommended when using infrared secondary antibodies.

8. Plastic containers should be as close to the size of the blot as possible to reduce waste of reagents. Our lab has found that the boxes used to sell push pins are of the perfect size for blots made using a Mini-PROTEAN 3 or Tetra system.
9. The method described here is based on using HRP substrates and film to develop the final product. However, there are alternative methods of developing a Western blot and the secondary antibody chosen should match the developing method.
10. The exact amount of complete lysis buffer used is highly dependent on cell density and the surface area of the culture dish. It is recommended to run a few trials to find the optimal amount of lysis buffer for an experiment.
11. Similar to making cell culture lysates, the amount of complete lysis buffer needed varies greatly on brain region and sample size. It is recommended to run test trials to determine the optimal amount of lysis buffer for the sample. Particularly large samples of tissue may be subdivided into multiple tubes to facilitate homogenization.
12. Both phosphorylated versions of ERα described in this protocol work well with either 7.5 or 10 % resolving gels. If another protein needs to be probed on the same blots as phospho-ERα, a different percentage gel can be used to optimize the other protein without causing problems in phospho-ERα detection.
13. Degassing a gel improves the quality of results, but is not necessary if no methods of degassing are available.
14. To make a 7.5 % resolving gel, combine 3 mL 1.5 M Tris–HCl (pH 8.8), 3 mL 30 % acrylamide (29:1), 120 μL 10 % SDS, and 5.75 mL ddH_2O. Add 120 μL 10 % APS and 12 μL TEMED to initiate polymerization of the gel.
15. Take care to remove as much of the isobutanol as possible.
16. The volume of each component that is used depends on the quantity (in μg) of protein you want to load and the total volume of the mixture you want to load. The amount of protein loaded is determined through optimization work. We have found that 20 μg is sufficient for detection of most proteins we use but this can be adjusted as needed. As far as total volume of the mixture, you can load an absolute maximum volume of 30 μL into a 10 well gel. However, it is easier and more desirable to load smaller volumes to minimize spillover between lanes. The ideal loading volume is 20 μL for 10 well gels. The amount of Laemmli Sample loading buffer (SLB) used is 1/3 of the total volume of the mixture and the amount of RIPA used is the volume required to dilute each sample to the same total volume.

17. The fill line will be labeled on the tank if using a Mini-PROTEAN Tetra system or to the top of the gels if using a Mini-PROTEAN 3 system.
18. Specialized loading tips are recommended. These tips facilitate complete transfer of sample to wells. However, 200 μL tips are an acceptable substitute.
19. It is recommended to have a cardboard template cut for this purpose.
20. Old pipette tip boxes make excellent containers for this part of the protocol.
21. Constant voltage can be used instead of constant amperage for the transfer. This choice is dependent on time and other variables. The selection of either constant voltage or constant amperage should not alter the end result when working with the described antibodies.
22. Coomassie solution can be used to counterstain the gels to check for transfer of protein or quality of remaining protein.
23. It is not recommended to block the blots for an extended period of time. Extended blocking can enhance background or lead to protein transfer from the blots. Additionally, the use of 5 % BSA/TTBS may be inappropriate for different developing methods and cause exogenous background (e.g., infrared detection methods).
24. We recommend using SuperSignal West Femto Chemiluminescent Substrate and developing the blot with film.
25. The harsh stripping protocol by Abcam is very effective. Perform this procedure in a fume hood and make sure to rinse the blot adequately with TBST afterwards since traces of β-mercaptoethanol may damage the antibody.
26. We recommend checking the effectiveness of the antibody strip by reincubating the blot in secondary antibody and visualizing. If old primary antibodies were stripped from the membrane, no signal should be detected. We have not seen any loss of protein from the membrane using this protocol.
27. A control protein should be probed to allow for normalization of protein bands to control for any variations in loading.

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Chapter 37

GPER Mediates Non-Genomic Effects of Estrogen

Marco Pupo, Marcello Maggiolini, and Anna Maria Musti

Abstract

Estrogens are important modulators of a broad spectrum of physiological functions in humans. However, despite their beneficial actions, a number of lines of evidence correlate the sustained exposure to exogenous estrogen with increased risk of the onset of various cancers. Mainly these steroid hormones induce their effects by binding and activating estrogen receptors (ERα and ERβ). These receptors belong to the family of ligand-regulated transcription factors, and upon activation they regulate the expression of different target genes by binding directly to specific DNA sequences. On the other hand, in recent years it has become clear that the G protein-coupled estrogen receptor 30 (GPR30/GPER) is able to mediate non-genomic action of estrogens in different cell contexts. In particular, GPER has been shown to specifically bind estrogens, and in turn to functionally cross-react with diverse cell signaling systems such as the epidermal growth factor receptor (EGFR) pathway, the Notch signaling pathway and the mitogen-activated protein kinases (MAPK) pathway. In this chapter we will present some of the different experimental techniques currently used to demonstrate the functional role of GPER in mediating non-genomic actions of estrogens, such as the dual luciferase assay, assessment of the involvement of GPER in the stimulation of cell migration in breast cancer cell lines and in cancer-associated fibroblasts, and chromatin immunoprecipitation assay. Overall, the experimental procedures described herein represent key instruments for assessing the biological role of GPER in mediating non-genomic signals of estrogen.

Key words Estrogen, Breast cancer, GPR30/GPER, Non-genomic signaling, MAPK phosphorylation

1 Introduction

Estrogens regulate many aspects of human physiology and influence diverse pathological processes, including the growth of hormone-dependent tumors [1]. The biological actions of estrogens are generally attributed to the genomic estrogen receptors, ERα and ERβ. They act as ligand-activated transcription factors and regulate the transcription of different genes by binding to estrogen responsive elements (ERE) located within their promoter regions [2]. However, in the late 1990s a novel transcript encoding for a G-protein coupled estrogen receptor associated with estrogen expression was identified [3]. This receptor was first named GPR30, but recently its name was changed in G-Protein coupled

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estrogen receptor 1 (GPER/GPER1) because of its ability to bind estrogen and mediate estrogenic signals [4]. Indeed, several studies demonstrated that GPER is able to transduce rapid non-genomic signals triggered by estrogen in both normal and cancer cells [5–17]. In particular, it has been shown that once activated, GPER induces a series of intracellular events such as the rapid phosphorylation of mitogen-activated protein kinases (MAPK) ERK1/2, the activation of PI3-kinase (PI3K) and phospholipase C (PLC), increase in cAMP concentrations, as well as the intracellular calcium mobilization [18]. In addition, it has now become evident that in different cellular contexts GPER stimulates ERK1/2 phosphorylation through transactivation of the epidermal growth factor receptor (EGFR) and induction of the matrix metalloproteinase activity, which in turn triggers the extracellular release of heparan-bound epidermal growth factor (HB-EGF) [19, 20]. Moreover, we have recently observed that a functional crosstalk between the Notch signaling pathway and GPER mediates certain non-genomic estrogenic signals. In particular, we showed that estrogens, through a crosstalk between GPER and Notch signaling pathways, are able to induce proliferative and migratory effects not only in breast cancer cells but also in cancer-associated fibroblasts (CAFs) [21]. In the last few years, indeed, it has become clear that the tumor microenvironment plays a key role in tumorigenesis, cancer progression and therapeutic failure [22, 23]. CAFs represent the principal cellular component of the microenvironment and therefore are the most important mediator of biological responses in this compartment. Once recruited and activated by the tumor mass, CAFs acquire different morphological and functional features [24]. Hence, understanding the molecular pathways that are active in CAFs is important to characterize their roles in cancer onset, progression and chemoresistance. This is further emphasized by recent findings showing that CAFs may contribute to the proliferative stimulus of the steroid hormone estrogen in breast cancer [25]. It is worth noting that estrogenic signals are mediated by GPER in CAFs [26]. Moreover, recent studies have demonstrated that estrogen stimulates migratory effects through GPER in CAFs [27].

A variety of techniques are currently used to demonstrate that GPER is able to mediate estrogenic non-genomic signals through the aforementioned signaling mechanisms. For example, methods such as evaluation of MAPK phosphorylation by western blotting and GPER-target genes [28] expression analysis through real-time PCR and semiquantitative RT-PCR [29] are useful. In this chapter we will describe the techniques of dual luciferase assay, wound healing cell migration assay, Boyden chamber migration assay, and chromatin immunoprecipitation assay. Moreover, since studies have demonstrated that estrogenic signals are mediated by GPER

in cancer associated fibroblasts from breast cancer patients [26, 27], we briefly describe the currently used method to isolate these cells from tumor biopsies. Overall, the experimental procedures described below represent useful tools for assessing the biological role of GPER in mediating non-genomic signals of estrogen in breast cancer.

2 Materials

2.1 Dual Luciferase Assay

1. 24-well culture plates.
2. SkBr3 ER-negative breast cancer cell line.
3. Penicillin/streptomycin (Pen/Strep) stock solution: 10,000 U penicillin, 10 mg/mL streptomycin.
4. SkBr3 Growth medium: RPMI 1640 without phenol red supplemented with 10 % fetal bovine serum (FBS) and 1 % Pen/Strep.
5. SkBr3 serum-free medium: RPMI 1640 without phenol red or FBS, supplemented with 1 % Pen/Strep.
6. Plasmid constructs: CTGF luciferase reporter plasmid p(−1999/+36)-luc [30], c-FOS-luc plasmid encoding a −2.2-kb 5′ upstream fragment of human c-FOS [31], Egr-1-Luc plasmid (pEgr1A), containing the −600 to +12 5′-flanking sequence from the human EGR-1 gene [32], Renilla luciferase pRL-CMV plasmid.
7. X-treme Gene 9 transfection regent (Roche).
8. AG-1478, 10 μM diluted in DMSO (EGFR inhibitor).
9. PD98059, 10 μM diluted in ethanol (MEK inhibitor).
10. 17-β-Estradiol, 10 nM solubilized in ethanol.
11. 1× Phosphate saline Buffer (PBS).
12. 5× Passive lysis Buffer (PLB) (Promega).
13. Luciferase Assay Reagent II (LAR II) (Promega).
14. Luciferase Assay Buffer II (Promega).
15. 50× Stop & Glo® Substrate (Promega).
16. Stop & Glo® Buffer (Promega).
17. Siliconized polypropylene tube or small glass vial.
18. Luminometer.

2.2 Isolation of Cancer Associated Fibroblasts (CAFs) Isolation

1. Breast cancer biopsies.
2. CAF collection medium: Medium 199 and Ham's F12 (1:1) with phenol red and supplemented with 10 % FBS, 1 % Pen/Strep, and 1 % Fungizone.

3. CAF digestion medium: CAF collection medium plus 300 U/mL collagenase, 100 U/mL hyaluronidase.
4. CAF growth medium: CAF collection medium without Fungizone.
5. 10 cm petri dishes.
6. Scalpel and scissors.
7. Collagenase from *Clostridium histolyticum*.
8. Hyaluronidase from bovine testes.
9. Rotating shaker.

2.3 Wound Healing Assay

1. 12-well culture plate.
2. SkBr3 breast cancer cells and cancer-associated fibroblasts (CAFs).
3. SkBr3 growth medium: as in Subheading 2.1, **item 4**.
4. CAFs growth medium: as in Subheading 2.2, **item 4**.
5. SkBr3 charcoal-treated medium: RPMI 1640 without phenol red, supplemented with 5 % Charcoal treated FBS and 1 % Pen/Strep.
6. CAFs charcoal-treated medium: DMEM without phenol red, supplemented with 5 % Charcoal-treated FBS and 1 % Pen/Strep.
7. p200 pipette tip.
8. 17-β-estradiol, 100 nM.
9. Phase-contrast light microscope.
10. Microscope camera.

2.4 Boyden Chamber Assay

1. SkBr3 breast cancer cells and cancer associated fibroblasts.
2. SkBr3 growth medium: as in Subheading 2.1, **item 4**.
3. CAFs growth medium: as in Subheading 2.2, **item 4**.
4. SkBr3 serum-free medium: as in Subheading 2.1, **item 5**.
5. CAFs serum-free medium: DMEM without phenol red, supplemented with 1 % Pen/Strep.
6. SkBr3 2.5 % charcoal-treated medium: RPMI 1640 without phenol red, supplemented with 2.5 % charcoal-treated FBS and 1 % Pen/Strep.
7. CAFs 2.5 % charcoal-treated medium: DMEM without phenol red, supplemented with 2.5 % Charcoal-treated FBS and 1 % Pen/Strep.
8. TrypLE™ Express (1×) (Life Technologies).
9. 24-well cell culture plate.
10. Corning® Transwell® polycarbonate membrane cell culture inserts.

11. 17-β-estradiol, 100 nM.
12. 4 % formaldehyde in PBS.
13. 1× phosphate buffer saline (PBS).
14. 100 % methanol.
15. Giemsa stain, modified solution.
16. Cotton swabs.
17. Phase-contrast light microscope.
18. Microscope camera.

2.5 Chromatin Immunoprecipitation Assay

1. SkBr3 breast cancer cells.
2. Growth medium: as in Subheading 2.1, **item 4**.
3. Serum-free medium: as in Subheading 2.1, **item 5**.
4. γ-secretase inhibitor: cbz-Leu-Leu-Nle-CHO (GSI) (Calbiochem), 100 nM.
5. 17-β-estradiol, 10 nM.
6. 11 % formaldehyde solution: 50 mM Hepes–KOH, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 11 % formaldehyde.
7. 2.5 M glycine solution in sterile distilled water.
8. 1× Phosphate buffer saline (PBS).
9. Protease inhibitor cocktail 100×.
10. 1× PBS.
11. Lysis buffer: 1 % sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris–HCl, pH 8.1, distilled sterile water.
12. Sonicator.
13. IP (Immunoprecipitation) buffer: 0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 16.7 mM NaCl, sterile distilled water.
14. Primary antibody: Notch 1 goat polyclonal antibody (C-1, Santa Cruz).
15. Protein A Agarose/Salmon Sperm DNA.
16. Wash A solution: 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl, sterile distilled water.
17. Wash B solution: 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl, sterile distilled water.
18. Wash C solution: 0.25 M LiCl, 1 % NP40, 1 % sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1, sterile distilled water.
19. Tris–EDTA (TE) buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, sterile distilled water;

20. Elution buffer: 1 % SDS, 0.1 M $NaHCO_3$, sterile distilled water.
21. 1 mg/mL RNaseA.
22. NaCl 5 M.
23. EDTA 0.5 M.
24. Tris–HCl, 1 M, pH 6.5.
25. 20 mg/mL proteinase K.
26. Phenol–chloroform–isoamyl alcohol solution.
27. 10 ng/mL yeast tRNA.
28. 95 % ethanol.
29. 70 % ethanol.
30. Tris–EDTA buffer, pH 8.
31. Software Primer Express version 2.0 (Life Technologies) (*see* **Note 1**).
32. Specific primers that amplify RBP-J binding site corresponding to −167 to +6 located in the 5′-flanking region of Hes-1 gene: Fwd: 5′-CAGACCTTGTGCCTGGCG-3′ and Rev: 5′-TGT GATCCCTAGGCCCTG-3′.
33. PCR Master mix, Power SYBR Green (Life Technologies).
34. MicroAmp 48-well plate (Life Technologies).
35. Step One (TM) sequence detection system (Life Technologies).

3 Methods

3.1 Dual Luciferase assay

Estrogen stimulates the promoter activity of GPER target genes through the EGFR/MAPK pathway in ER-negative breast cancer cells.

The dual luciferase assay is commonly used to test the promoter activity of specific genes. In this section we will present how this simple assay can be used to assess whether estrogen treatment activates the promoter regions of three different GPER target genes (c-fos, EGR-1 and CTGF) [22] in GPER-positive breast cancer cells not expressing both isoforms of the classical estrogenic receptor (ERα and ERβ) [12]. In particular, we used this assay to demonstrate that the promoter of these genes in ER-negative SkBr3 breast cancer cells is activated by 17-β estradiol (E2) treatment, whereas activation is reduced in cells pretreated with inhibitors of the epidermal growth factor receptor EGFR (AG1478) and MEK (PD98059). These observations further confirm that GPER is able to mediate estrogenic signals through a non-genomic pathway that involves transactivation of EGFR and MAPK activation (Fig. 1) (*see* **Note 2**).

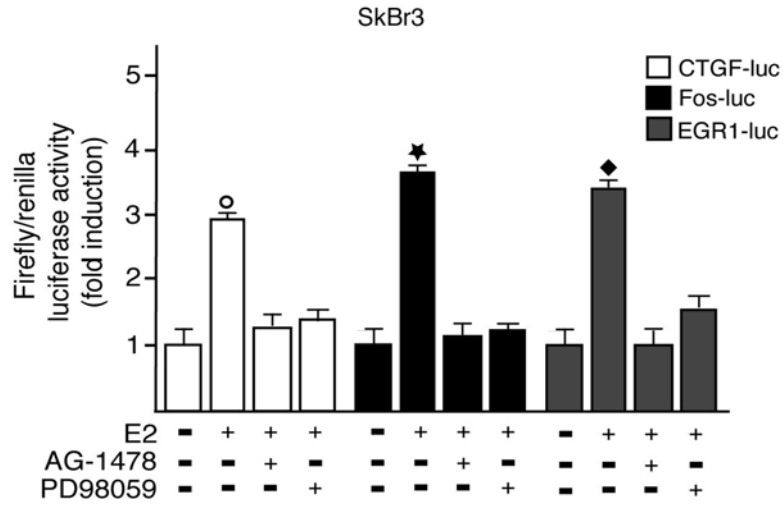

Fig. 1 Estrogen treatment transactivates GPER target genes through the GPER/EGFR/MAPKs pathway in SkBr3 breast cancer cells. The treatment of SkBr3 cells with estrogen induces the luciferase activity of CTGF, Fos and EGR-1 reporter plasmids transfected in these cells. This induction is abrogated when cells are co-treated with inhibitors of EGFR (AG-1478) and MEK (PD98059). Luciferase activities were normalized to the internal transfection control, and values of cells receiving vehicle (–) were set as onefold induction upon which the activity induced by treatment was calculated. Bar graphs represent the mean ± SD of three independent experiments each performed in triplicate. Symbols (*star*, *circle*, *square*) indicate $P < 0.05$ for cells receiving vehicle (–) versus treatment

1. Plate SkBr3 breast cancer cells in 10 cm petri dishes in SkBr3 Growth Medium and incubate in a humidified incubator at 37 °C and 5 % CO_2.
2. Split the SkBr3 breast cancer cells in a 24-well culture plate and leave them to attach (*see* **Note 3**).
3. When the cells are attached prepare the transfection mix.
4. Put in a sterile tube 50 μL of SkBr3 Serum-free Medium for each well.
5. Add to the medium 3 μL of X-treme Gene 9 transfection reagent for each μg of DNA that you will transfect (*see* **Note 4**).
6. Leave the mixture of medium and transfection reagent for 5 min at room temperature.
7. After 5 min add 0.5 μg of each reporter plasmid directly to the mixture. Include also in each mix 1 ng of the internal transfection control pRL-CMV.
8. Leave the mixture containing the plasmids and the transfection reagent at room temperature for 30 min.

9. Dispense 50 μL of the transfection mix into each well and put the plate in a humidified incubator at 37 °C and 5 % CO_2 for 6–8 h.
10. After 6–8 h, pretreat the cells with the EGFR inhibitor, 10 μM AG-1478, or the MEK inhibitor, 10 μM PD98059, for 1 h without removing the transfection mix. Then treat the cells with 100 nM 17-β-estradiol and incubate the cells for 16–18 h in a humidified incubator at 37 °C and 5 % CO_2.
11. Wash the cells twice with 1× PBS.
12. Dilute the Passive Lysis Buffer from 5× to 1× with sterile distilled water.
13. Dispense into each culture well 100 μL of 1× Passive Lysis buffer (*see* **Note 5**).
14. Place the plate on a rocking platform or orbital shaker with gentle rocking/shaking to ensure complete and even coverage of the cell monolayer with 1× PLB. Rock the culture plates at room temperature for 15–30 min (*see* **Note 6**).
15. Resuspend the lyophilized Luciferase Assay Substrate (LARII) in 10 mL of the Luciferase Assay Buffer II. Prepare working aliquots of the substrate. Put one aliquot on ice and store the remaining aliquots at –80 °C (*see* **Note 7**).
16. Dilute a working aliquot of the Stop & Glo® Substrate from 50× to 1× in Stop & Glo® Buffer. Put the diluted aliquot on ice.
17. Program the luminometer to perform a 2-s premeasurement delay, followed by a 10-s measurement period for each reporter assay.
18. Dispense 10 μL of the LAR II substrate into each siliconized polypropylene tube or small glass vial.
19. Add 20 μL of the cell lysate directly to the tube containing LAR II, mix by pipetting two or three times and perform the first measurement of luminescence.
20. Remove the sample tube from the luminometer, add 100 μL of Stop & Glo® Reagent and mix by pipetting.
21. Replace the sample in the luminometer, and start the second reading.
22. Discard the reaction tube, and repeat the same steps for all of the samples.
23. Analyze the data provided from the first and the second luminescence measurements. In particular, the first measurement will give indications on the activity of the promoter and must be normalized to the second, which is the internal control.

3.2 CAFs Isolation

Estrogen stimulates migration of breast cancer cells and cancer associated fibroblasts (CAFs) through the GPER-mediated non-genomic pathway.

1. For the isolation of cancer associated fibroblasts (CAFs): bring breast cancer tissue biopsies from the operating theater to the laboratory on ice in CAFs Collection Medium as quickly as possible.
2. Put the tissue in a 10 cm petri dish under a laminar flow hood and rinse it twice with 1× PBS.
3. Mince the tissue in small pieces using scalpel and scissors.
4. Collect the minced tissue fragments in a sterile 15 mL tube and add 5–10 mL of CAFs Digestion Medium.
5. Digest the minced tissue for 16–18 h at 37 °C (*see* **Note 8**).
6. After enzymatic digestion, centrifuge the digested mixture at 150 × *g* for 2 min.
7. Transfer the supernatant, containing fibroblasts, to a new sterile 15 mL tube and centrifuge it at 800 × *g* for 8 min. Discard the supernatant that is highly enriched for epithelial organoids (*see* **Note 9**).
8. Resuspend the pellet, containing fibroblasts, in CAFs growth medium (*see* **Note 10**).
9. Place the cells in a 10 cm petri dish and leave the cells to attach and grow.
10. Check the spindle shaped morphology of the attached cells under a light microscope and continue to culture them in the mixture of CAFs Growth Medium (*see* **Note 11**).

3.3 Wound Healing Assay

The wound healing assay is a commonly used method to evaluate whether a specific compound is able to induce migration of cultured cells. In this section we will describe how this method can be used to demonstrate that estrogen induces migratory effects in both ER-negative and GPER-positive cells, as SkBr3 cells and CAFs, respectively [12] (Fig. 2) (*see* **Note 12**).

1. Plate SkBr3 cells or CAF cells in their regular growth medium on 12-well culture plates to create a confluent monolayer (*see* **Note 13**).
2. Leave the cells to adhere and spread on the well for approximately 6 h at 37 °C.
3. Scrape the cell monolayer with a sterile pipet tip in a straight line to create a “scratch”.
4. Remove the cell debris by washing the cells once with 1 mL of the growth medium and then replace the regular growth medium with 5 mL of 5 % charcoal-treated medium.

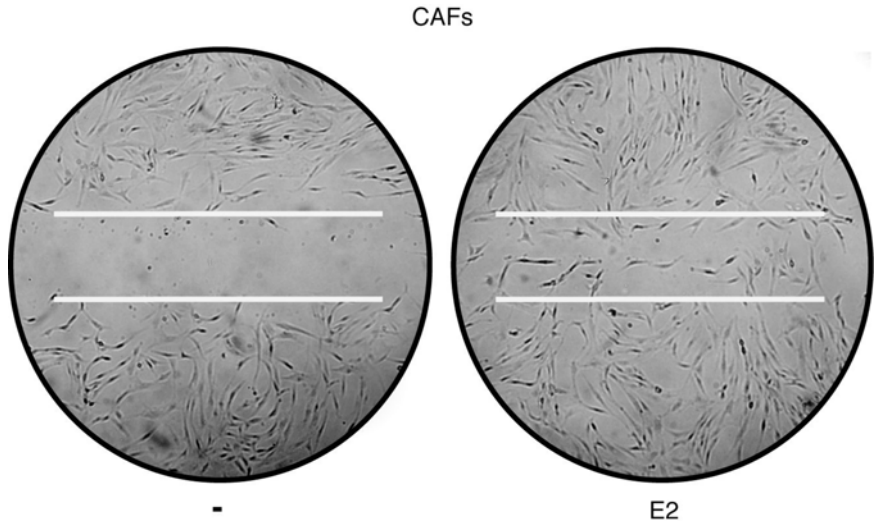

Fig. 2 Estrogen stimulates the migration of ER-negative and GPER-positive CAFs. Representative photographs from wound healing assay showing that the migration of CAFs is stimulated after a 24 h treatment with 100 nM E2

5. To obtain the same field during image acquisition, create a mark on the outside of the dish close to the scratch with an ultrafine pen to be used as reference point.
6. Treat the cells with vehicle or 100 nM 17-β-estradiol and incubate the cells at 37 °C for 24 h.
7. After the incubation, place the dish under a phase-contrast microscope, match the reference point and acquire images.
8. Analyze the acquired images, comparing the size of the scratches/migration of cells back into the scratched area in the estrogen-treated wells with that of the control wells.

3.4 Boyden Chamber Invasion Assay

The Boyden chamber assay is widely used to measure the ability of cultured cells to pass through a porous membrane under the stimulation of a specific compound acting as chemoattractant. In this section we will describe how to use this method to demonstrate that estrogen stimulation induces the aforementioned effect in SkBr3 cells and in CAFs which are both ER-negative but GPER-positive cells [12] (Fig. 3) (*see* **Note 14**).

1. Trypsinize the SkBr3 cells or CAFs with 1 mL of TrypLE™ Express (1×).
2. Transfer the detached cells to a sterile 15 mL tube and add 1 mL of regular growth medium.
3. Pellet the cells by centrifugation for 5 min at 2000 × *g*.
4. Resuspend the cell pellet with 1 mL of regular growth medium.

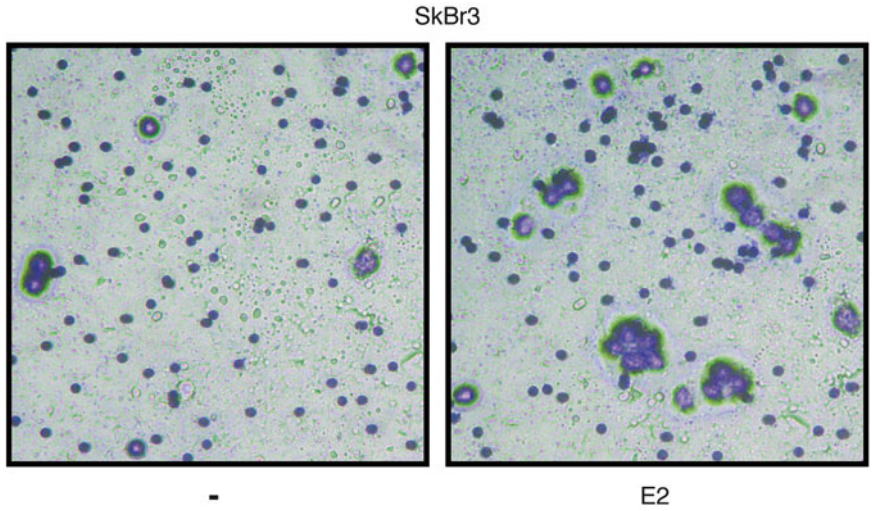

Fig. 3 Estrogen treatment induces the invasion of ER-negative and GPER-positive breast cancer cells SkBr3. Representative photographs from Boyden chamber assays showing that the migratory effects are stimulated in SkBr3 cells by a 24 h treatment with 100 nM E2

5. Count the cells with a hemocytometer or an automatic cell counter and prepare a solution containing 4×10^5 cells/mL in serum-free medium.
6. Distribute 750 μL of 2.5 % charcoal-treated medium into the wells of the 24 well-cell culture plate.
7. Put the Corning® Transwell® polycarbonate membrane cell culture inserts into the wells.
8. Transfer 200 μL of the solution containing cells in serum-free medium to the upper part of the Corning® Transwell® polycarbonate insert.
9. Treat each well with 17β-estradiol placing the treatment in the bottom of the well.
10. Incubate the cells for 16–18 h at 37 °C.
11. Aspirate the medium from the top of the filter.
12. Fix the cells on the filter placing each filter in 1 mL of 4 % formaldehyde in PBS for 10 min.
13. Wash the filter twice with 1× PBS.
14. Permeabilize the fixed cells by placing each filter in 1 mL of 100 % methanol for 45 min.
15. Wash each filter twice with 1× PBS.
16. Stain the cells on the filter immersing each filter in 1 mL of the Giemsa stain solution for 30 min (*see* **Note 15**).
17. Wash each filter twice with PBS and remove the cells from the top of the membrane by wiping with a cotton swab.

18. Cut the membrane free from the well.
19. Use a phase-contrast microscope with a camera to acquire images of 10–20 random fields for each insert.
20. Analyze the images by counting the number of cells in each field and normalize the average of the counted cells in the treated wells against the control wells.

3.5 Chromatin Immunoprecipitation Assay (ChIP)

Estrogen activates Notch signaling pathway through GPER.

1. Plate the cells in 10 or 15 cm cell culture dishes and grow them in regular growth medium.
2. When the cells reached 80–90 % confluence, switch them to serum-free medium for 24 h.
3. After 24 h, pretreat the cells with 100 nM of γ-secretase inhibitor (GSI) for 1 h to block the activation of Notch receptor by inhibiting cleavage of the receptor by γ-secretase. After 1 h treat the cells with 17-β-estradiol for 8 h to activate GPER.
4. Add to each plate 1/10 volume of fresh 11 % formaldehyde solution.
5. Gently shake the plates to distribute the formaldehyde and incubate them for 10 min at room temperature.
6. Add 1/20 volume of 2.5 M glycine solution to quench formaldeyde.
7. Rinse the cells twice with 1× PBS plus 10× protease inhibitor cocktail.
8. Scrape the cells in 1× PBS with protease inhibitor and transfer them to a 15 mL sterile tube.
9. Pellet the cells by centrifuging the tubes at 2000 × *g* for 5 min.
10. Cell lysis and sonication: Remove the supernatant and dissolve the cell pellet in 200 μL of lysis buffer.
11. Transfer the lysates in a new 1.5 mL tube.
12. Incubate on ice for 10 min.
13. Sonicate the samples on ice four times for 10 s at 20–25 % of the maximum sonicator power.
14. Vortex the sonicated samples.
15. Centrifuge the tubes at 16,000 × *g* for 10 min at 4 °C.
16. Immunoprecipitation and elution: Prepare one new sterile tube for each sample and add 1.3 mL of IP buffer with 10× antiprotease cocktail to each of them.
17. Transfer the supernatant from the centrifuged tubes to the new tubes containing the IP buffer. Save 25 μL of supernatant from each sample as whole-cell extract DNA (input). Store at −20 °C.

18. Mix the cell lysates with the IP buffer by inversion.
19. Add 15 μg of the primary antibody, anti-Notch 1, for each sample and incubate the tubes overnight on a rotating shaker (*see* **Note 16**).
20. Add 50 μL of Protein A Agarose/Salmon Sperm DNA for each sample and incubate the tubes for 2 h at 4 °C on the rotating shaker.
21. Centrifuge the samples at 1500 × *g* for 4 min at 4 °C.
22. Discard the supernatant and wash each pellet with 1 mL of wash A solution.
23. Vortex the samples and place them on the rotating shaker for 5 min at 4 °C.
24. Centrifuge at 1500 × *g* for 4 min at 4 °C.
25. Discard the supernatant and wash the pellet with 1 mL of wash B solution.
26. Vortex the samples and place them on the rotating shaker for 5 min at 4 °C.
27. Centrifuge the tubes at 1500 × *g* for 4 min at 4 °C.
28. Discard the supernatant and wash the pellet with 1 mL of wash C solution.
29. Vortex the samples and place them on the rotating shaker at 4 °C for 5 min.
30. Centrifuge the tubes at 1500 × *g* for 4 min at 4 °C.
31. Discard the supernatant and add to the pellet 1 mL of TE buffer.
32. Vortex the tubes and place them on the rotating shaker for 5 min at 4 °C (*see* **Note 17**).
33. Centrifuge at 1500 × *g* for 4 min at 4 °C.
34. Discard the supernatant and add 250 μL of Elution buffer directly to the pellet (*see* **Note 18**).
35. Vortex the tubes and place them on the rotating shaker for 30 min at room temperature.
36. Centrifuge the samples at 1500 × *g* for 4 min at 4 °C.
37. Transfer 250 μL of the supernatant into new sterile tubes.
38. Once again, add 250 μL of elution buffer to the pellet.
39. Vortex and place the tubes on the rotating shaker for 30 min at room temperature.
40. Centrifuge the samples at 1500 × *g* for 4 min at 4 °C.
41. Transfer the supernatant to the same tube in which the first 250 μL of elution buffer was collected to obtain a volume of 500 μL in each tube.

42. Also add 500 μL of elution buffer to the tubes containing the input.
43. Crosslink reversal and digestion of protein and RNA: Starting from this point, process the samples and the input together.
44. Vortex all of the tubes and add to each of them 20 μL of 5 M NaCl.
45. Incubate the tubes overnight at 65 °C.
46. Spin down the tubes by centrifugation at maximum speed for 30 s and add to each sample 8 μL of 1 mg/mL RNaseA.
47. Incubate at 37 °C for 30 min.
48. Spin down the tubes by centrifugation at maximum speed for 30 s and prepare a mixture containing: 10 μL 0.5 M EDTA, 20 μL 1 M Tris–HCl, pH 6.5, 2 μL of 20 mg/mL Proteinase K.
49. Add 32 μL of this mix to each sample.
50. Vortex, spin down, and incubate the tubes at 45 °C for 1 h.
51. DNA extraction: Add to each tube 500 μL of phenol–chloroform–isoamyl alcohol solution.
52. Vortex the samples for 30 s and leave them for 1 min at room temperature.
53. Centrifuge the tubes at 18,000 × *g* for 5 min at 4 °C (*see* **Note 19**).
54. Save the transparent fraction of the supernatant and transfer it into a new sterile 1.5 mL tube. Discard the remaining fractions in the tubes.
55. Add to each sample 2 μL of 10 ng/mL yeast tRNA and mix the tubes by inversion.
56. Add 500 μL of ice-cold 95 % ethanol to each tube and mix by inversion.
57. Precipitate the DNA at −20 °C overnight.
58. Centrifuge the samples for 1 h at 18,000 × *g* and 4 °C.
59. Discard the supernatant and wash the pellet with 1 mL of ice-cold 70 % ethanol.
60. Vortex the tubes and centrifuge at 18,000 × *g* for 30 min at 4 °C.
61. Discard the supernatant and leave the pellet to dry at room temperature.
62. Add 20 μL of TE buffer to resuspend the DNA pellet.
63. Store the pellet at −20 °C until real time quantitative PCR is performed.
64. For the real time PCR reaction, use the following specific primers that amplify an RBP-J binding site corresponding to

−167 to +6 located in the 5′-flanking region of Hes-1 gene: Fwd: 5′-CAGACCTTGTGCCTGGCG-3′ and Rev: 5′-TGT GATCCCTAGGCCCTG-3′. Use unprocessed lysates (input DNA) to normalize the data obtained.

65. Real time PCR: Dilute the DNA samples and specific primers 1:10.
66. Prepare a mix with 1.5 μL of each primer and 15 μL of PCR Master mix (Power SYBR Green) for each sample.
67. Dispense 2 μL of DNA into each well of the 48-well plate and add to each well 18 μL of the mix containing primers and Master Mix.
68. Analyze the plate using the Step One™ sequence detection system machine and set ΔΔCt method for relative quantifications. Perform the analysis in triplicate for each sample and use water instead of DNA as a negative control.
69. Normalize the quantity of DNA respect to a control gene expression such as GAPDH or 18S.

4 Notes

1. This software designs specific primers to be used for Real time PCR. However, specific primers can be designed using other bioinformatic tools available online. Moreover, the database PrimerBank, available on-line at the address: http://pga.mgh.harvard.edu/primerbank/ offers a collection of sequences of primers for different genes already tested for Real Time PCR.
2. Although the cells that we suggest to use (SkBr3) are ER-negative, we suggest to confirm that the observed responses are mediated by GPER by pretreating cells (1 h before E2 treatment) with 100 nM of the commercially available GPER inhibitor, G-15 [33].
3. The SkBr3 cells can be transfected the day after being plated when they are attached. Cells that are more difficult to transfect must be resuspended.
4. We use X-treme Gene transfection reagent. This transfection reagent is a very well tested method to knockdown GPER. This transfection reagent is a non-liposomal multicomponent reagent. To silence GPER it is also possible to use other transfection reagents. However, the toxicity of transfection reagents should be taken into consideration when performing transfection experiments. When using Xtreme gene 9, avoid touching the plastic walls of the tube with the pipette tip and be careful to pipette the reagent directly into the liquid medium.

5. Passive lysis buffer is specifically designed to be used with the Dual Luciferase kit. An alternate lysis buffer could be used but the use of PLB is always suggested.
6. Normally, 15 min are enough to allow a complete dissolution of cell structure. However, some types of cultured cells may exhibit greater resistance to lysis, and optimizing the quantity of passive lysis buffer to use and the time of lysis buffer action may be required.
7. The components of LAR II are heat-labile. Hence, is important to avoid freeze-thawing cycles and prepare working aliquots of the dissolution of the powder. In addition, it is recommended to thaw the reagent in a room temperature water bath.
8. For breast cancer tissue, a digestion overnight is typically enough to destroy the tissue integrity. However, longer digestion times may be required for tough fibrous tissue, shorter digestion times for softer tissue. In addition, cover the tube with aluminum foil if the rotary shaker can be placed directly in the 37 °C, 5 % CO_2 incubator. If the shaker is not in a 5 % CO_2 incubator the tubes should be sealed.
9. Different protocols exist that allow isolation and culture of epithelial tumor cells from this step [34].
10. The best medium for culturing the isolated fibroblasts is Medium199/Ham's F-12 (1:1) mixture. However, is also possible to use DMEM/F-12 (1:1) medium.
11. To ensure that the isolated cells are cancer-associated fibroblasts, the expression of markers such as Vimentin and Fibroblast activation protein (FAP) should be evaluated.
12. To highlight the involvement of GPER in the stimulation of this biological effect by estrogen we suggest to use cells like SkBr3 or CAFs that do not express either isoform of the classical estrogenic receptor (ERα and ERβ) but express GPER. However, as positive control, the same experiment can be performed treating cells with 100 nM of the GPER inhibitor, G-15, 1 h before E2 treatment or transfecting them with shRNA against GPER 24 h before the E2 stimulation.
13. The required number of cells for a confluent monolayer depends on both the particular cell type and the size of dishes and needs to be adjusted appropriately.
14. The Boyden assay can be carried out pretreating cells for 1 h with G-15, 100 nM, or by transfecting them for 24 h with an shGPER to confirm the involvement of the GPER receptor in the biological response induced by E2.
15. The SkBr3 cells and CAFs can be stained with fluorescent cell tracker dyes such as 5-chloromethylfluoresceindiacetate (CMFDA, green fluorescent) or a rhodol-based fluorophore

(CMRA, red fluorescent) before starting the assay. Staining can be done by incubating them in growth medium containing the dyes at 2 μM for 30 min. After washing and incubation with dye-free medium for an additional 30 min, cells can be trypsinized and used for the Boyden chamber assay. If the staining is carried out using this method, at the end of the experiment it is possible to evaluate the number of migrated cells by using fluorescence microscopy.

16. This amount of antibody is appropriate for the immunoprecipitation of Notch-1. However, the amount of antibody to use can be experimentally determined in a range from 2 to 15 μg.
17. After each of the aforementioned washes be very careful in removing the supernatant. In particular, be sure not to disturb the pellet. A small volume (50–100 μL) of the supernatant may be left in the tube before adding the subsequent washing solution;
18. At this step, remove of all the supernatant from the tube and be very careful to not disturb the immunoprecipitated pellet.
19. Repeat the centrifugation if the separation between the different phases is not completely evident.

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Chapter 38

GPER/GPR30 Knockout Mice: Effects of GPER on Metabolism

Geetanjali Sharma and Eric R. Prossnitz

Abstract

Endogenous estrogens, predominantly 17β-estradiol (E2), mediate various diverse effects throughout the body in both normal physiology and disease. Actions include development (including puberty) and reproduction as well as additional effects throughout life in the metabolic, endocrine, musculoskeletal, nervous, cardiovascular, and immune systems. The actions of E2 have traditionally been attributed to the classical nuclear estrogen receptors (ERα and ERβ) that largely mediate transcriptional/genomic activities. However, more recently the G protein-coupled estrogen receptor GPER/GPR30 has become recognized as an essential mediator of certain, and particularly rapid, signaling events in response to E2. Murine genetic knockout (KO) models represent an important approach to understand the mechanisms of E2 action in physiology and disease. Studies of GPER KO mice over the last years have revealed functions for GPER in the regulation of obesity, insulin resistance and glucose intolerance, among other areas of (patho) physiology. This chapter focuses on methods for the evaluation of metabolic parameters in vivo and ex vivo with an emphasis on glucose homeostasis and metabolism through the use of glucose and insulin tolerance tests, pancreatic islet and adipocyte isolation and characterization.

Key words Estrogen, GPER, GPR30, Metabolism, Obesity, Adipose, Adipocyte, Insulin resistance, Insulin sensitivity, Glucose tolerance, Type 2 diabetes

1 Introduction

Obesity and its pathophysiological consequences, including type 2 diabetes and cardiovascular diseases, are becoming ever more prevalent, leading to their designation as epidemics [1–3]. In mouse models as in humans, there is a clear sex difference with respect to metabolism [4–8]. In women and female mice, estrogen (E2) is protective against obesity, diabetes, and cardiovascular disease [9, 10]. This protection is less evident in males, and is lost in females after menopause or following oophorectomy/ovariectomy. Thus, the function of estrogen and its receptors in regulating metabolism has been of great interest [11]; however, with the recent identification of a 7-transmembrane estrogen receptor (GPR30/GPER) [12–14], unrelated to the classical estrogen receptors (ERα and

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ERβ), many questions arose as to the role of this newest estrogen receptor in the regulation of metabolism [15–18]. In addition to selective pharmacological agents [19–21], knockout mice represent an important tool to examine GPER function in vivo [22]. GPER KO mice exhibit increased adiposity and impaired insulin secretion from pancreatic islets [23, 24]; however, only female mice are glucose intolerant at 6 months of age [25], whereas males become progressively glucose intolerant with advanced age [23]. These phenotypes suggest that GPER plays an important role in metabolism and merits further investigation.

Obesity not only results in increased overall fat but also remodeling of existing adipose tissue. In addition, adipose tissue functions not as an inert storage tissue, as long thought, but rather an active endocrine gland [26, 27]. Furthermore, adipose tissue in the obese exhibits increased leukocyte infiltration that in turn alters the cytokine profiles of the macrophages/adipocytes giving rise to a pro-inflammatory phenotype [28]. Increased pro-inflammatory cytokines and circulating lipids (arising from dyslipidemia) result in metabolic dysfunction due to multiple effects on various tissues (Fig. 1) [29]. In prediabetes, insulin resistance sets in and the primary insulin responsive tissues (adipose, skeletal muscle, and liver) become less responsive to insulin; with adipose and skeletal muscle displaying diminished insulin-stimulated glucose uptake along with increased glucose production from liver. Since the body fails to respond to insulin, in order to maintain normal blood glucose, insulin production and secretion increase. This leads to the compensatory formation of new pancreatic islets but eventually, as the islets become exhausted due to continued overload, normoglycemia cannot be maintained and fasting blood glucose levels rise. To further compound these problems, lipotoxicity interferes with hepatic function and results in a failure to control gluconeogenesis in spite of already high blood glucose levels [30]. Usually in a healthy animal, blood glucose changes are highly dynamic and can be measured using very simple and effective methods, such as intraperitoneal glucose tolerance tests (IPGTT) and insulin tolerance tests (IPITT). These tests can reveal the dynamics of blood glucose homeostasis and are important tools to investigate glucose metabolism. As estrogen and its receptors play important roles in glucose and lipid metabolism as well as metabolic dysfunction, employing knockout mice with a deficiency in a single estrogen receptor has been highly informative regarding the roles of individual estrogen receptors. The procedures and assays described in this chapter (Table 1) provide important information regarding normal metabolic function as well as dysfunction under both fasting and non-fasting conditions in the mouse.

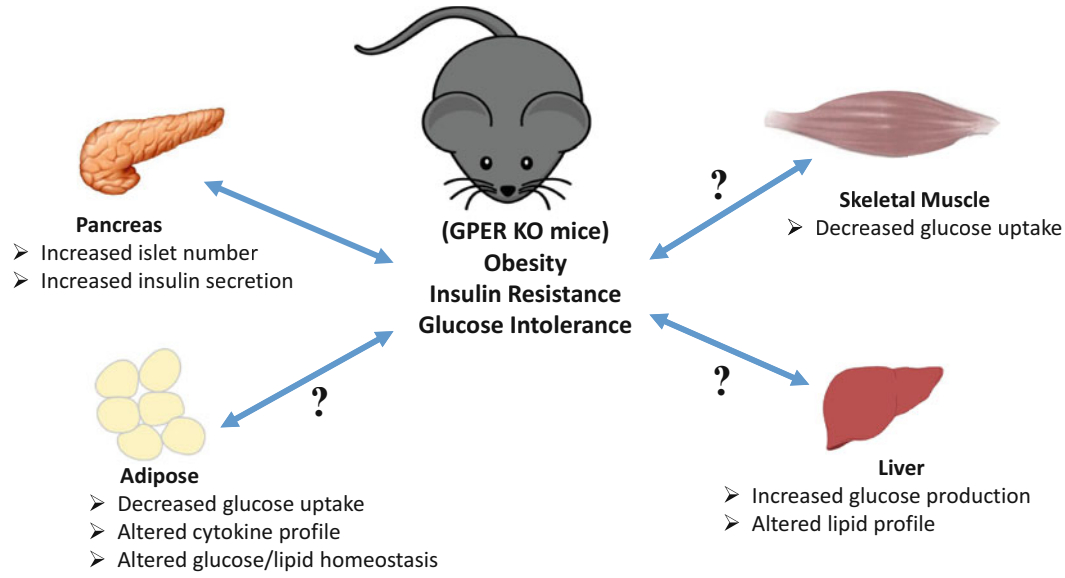

Fig. 1 Schematic representation of the possible metabolic dysfunctions leading to or resulting from obesity in GPER-deficient mice. Increased adiposity leads to insulin resistance with ensuing glucose intolerance. Obesity may also result in multiple defects in metabolically active tissues such as the pancreas, adipose, liver, and muscle. In the pancreas, insulin resistance leads to increased insulin secretion and a compensatory increase in the number of islets, resulting in hyperinsulinemia. In adipose and muscle, insulin resistance may lead to decreased insulin-stimulated glucose uptake, which along with concomitantly increasing hepatic glucose production, results in hyperglycemia. In addition, altered lipid homeostasis in the adipose and liver leads to hyperlipidemia. Together with hypertension, these pathophysiological metabolic effects (abdominal obesity, elevated fasting plasma glucose, high serum triglycerides, and low high-density cholesterol levels) comprise the condition referred to as metabolic syndrome. Whereas GPER is known to stimulate insulin secretion and survival of pancreatic β-cells, little is known regarding the role(s) of GPER in other insulin-responsive peripheral organs/tissues (indicated by *question marks* (?) in the figure)

Table 1
Procedures and assays to study functions of GPER in metabolism

Assay	Tissue/cells	Function
Adipocyte isolation	Adipose tissue	Metabolic assays/adipokine secretion
Adipogenesis	Preadipocytes	Triglyceride accumulation
Fasting glucose/insulin	Fasting mouse blood	Insulin resistance
Glucose tolerance/insulin tolerance	Fasted mouse blood	Glucose homeostasis
Glucose uptake	Adipocytes/adipose tissue	Insulin sensitivity
Islet isolation	Pancreas	Insulin secretion

2 Materials

2.1 Isolation of Adipocytes and Stromal Vascular Fraction

1. Mouse perigonadal fat pads.
2. Growth medium: DMEM/F12, 10 % fetal bovine serum, penicillin–streptomycin–glutamine (100 U/mL; 100 μg/mL, and 0.292 mg/mL, respectively).
3. Krebs Ringer solution, HEPES buffered (KRH buffer): 25 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.3 mM KH_2PO_4.
4. Collagenase solution (100 mg/mL): 100 mg collagenase per mL KRH buffer.
5. RBC lysis buffer: 155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA.
6. Cell strainers (40/100/400 μm).
7. Sterile petri plates.
8. Sterile 50 mL conical tubes.
9. Shaking water bath.
10. Centrifuge and microfuge.
11. Pentobarbital.

2.2 Differentiation of Preadipocytes

1. 3T3-L1 preadipocytes or primary preadipocytes.
2. Insulin, 10 mg/mL stock (1000×).
3. 3-isobutyl-1-methylxanthine (IBMX), 50 mM stock (100×).
4. Dexamethasone, 1 mM stock (1000×).
5. Differentiation medium: DMEM, 10 % FBS, Pen/Strep/glutamine (100 U/mL, 100 μg/mL, and 0.292 mg/mL, respectively), 10 μg/mL insulin, 500 μM IBMX, 1 μM dexamethasone.

2.3 Glucose Uptake

1. Differentiated adipocytes as above or minced adipose tissue pieces (ex vivo).
2. 2-Deoxy-D-[2,6-^{3}H]-glucose.
3. 2-Deoxy-D-[2,6]-glucose.
4. KRH buffer containing radioactive glucose: KRH buffer, 2-deoxy-D-[2,6-^{3}H]-glucose in 200 μM non-radioactive 2-deoxy-D-[2,6]-glucose.
5. RIPA buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 % SDS, 140 mM NaCl.
6. Reagents for standard protein assay.
7. Perchloric acid.
8. Hydrogen peroxide.

2.4 Glucose and Insulin Testing

1. Animal scale.
2. Mouse restrainer.
3. Timer.
4. Blood glucose meter (ReliOn Confirm Micro, or similar) and glucose strips (ReliOn, or similar).
5. Insulin syringes (½ or 1 cc) with 30½ G needle.
6. Glucose stock (20 % w/v in saline).
7. Insulin (Humulin R, Eli Lilly & Co).
8. Fine scissors.
9. Mercodia Ultrasensitive Mouse Insulin Elisa kit (Calibrators, Standards, 96-well plate coated with capture antibody, Antibody-enzyme conjugate, Substrate TMB, Stop solution, Wash buffer).
10. Plasma samples of interest.
11. Plate reader with capacity to read at absorbance 450 nm.

2.5 Islet Isolation

1. Hank's Balanced Salt Solution (HBSS).
2. Collagenase (Fraction V) in HBSS.
3. DMEM supplemented with 3 or 11 mM glucose, 10 % FBS, Pen/Strep/glutamine (100 U/mL, 100 μg/mL, and 0.292 mg/mL, respectively).
4. Sodium diatrizoate (such as Histopaque).

3 Methods

3.1 Adipocyte and Stromal Vascular Fraction Isolation from Adipose Tissue

Adipose tissue consists of a variety of cells such as preadipocytes, adipocytes, macrophages, and endothelial cells, etc. To understand the differentiation process of preadipocytes or physiological function of adipocytes, such as cytokine secretion (without the input from macrophages), in response to a specific stimulus or condition, isolation of individual cell types is essential. Ensure that all procedures are carried out in accordance with local institutional policies and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

1. Sacrifice mice by injecting pentobarbital or by cervical dislocation.
2. Isolate the desired fat pads (perigonadal/subcutaneous/brown) in sterile DMEM/F12 medium.
3. Mince the tissue in a sterile petri plate and add sterile collagenase solution (~5 mL/g tissue) and transfer to a sterile 50 mL conical tube.
4. Incubate for 1 h at 37 °C in a shaking water bath at 150 rpm.

5. After digestion, add an equal volume of cold DMEM/F12 to stop digestion.
6. Pass the digested fat slurry through a sterile cell strainer (100–400 μm) attached on top of a 50 mL tube by gravity. The undigested tissue on the cell strainer can be discarded.
7. To the flow through, add 20 mL DMEM/F12.
8. Centrifuge at 50 × *g* (or 500 rpm) for 5 min to remove (pellet) any debris.
9. After centrifugation, adipocytes will float.
10. The adipocytes can be collected from the top and transferred to a new tube containing in DMEM/F12 medium supplemented with 10 % FBS, Pen/Strep/Glutamine (100 U/mL; 100 μg/mL and 0.292 mg/mL, respectively).
11. The adipocytes remain floating but can be cultured further for 48–72 h for various experimental procedures (*see* **Note 1**).
12. Transfer the medium below the floating adipocytes to a new 50 mL tube and centrifuge at 500 × *g* for 15 min to pellet the stromal vascular (SV) cells.
13. Remove most of the supernatant.
14. Resuspend the cells in 10 mL RBC lysis buffer.
15. Incubate at room temperature for 5 min.
16. Add an equal volume of growth medium.
17. Filter the cell suspension through a 40 μm cell strainer into a new tube to remove endothelial cell clumps.
18. Centrifuge the sample at 500 × *g* for 5 min.
19. Remove most of the supernatant and resuspend the pellet (stromal vascular fraction) in 10 mL growth medium.
20. Dispense the appropriate number of cells into the desired plate, dish or flask.

3.2 Differentiation of Preadipocytes (3T3-L1 or Primary Cells)

Preadipocytes can either be cultured from a cell line such as 3T3-L1 murine fibroblasts, committed to an adipocytic fate, or can be isolated from the stromal vascular fraction of adipose tissue ex vivo as discussed above.

1. After the preadipocytes reach confluence (day 0), replace the growth medium and let cells grow for another 2 days (day 2).
2. Induce differentiation by adding differentiation medium for 48 h (days 3–4).
3. Following differentiation, adipocyte maturation is induced in growth medium supplemented with only insulin for another 3 days with medium changes every other day (days 5–7).
4. By day 7–10, lipid droplets will be visible in the adipocytes (*see* **Note 2**).

5. Adipocytes can be used for various assays at any time between 5 and 10 days following the initiation of differentiation.

3.3 Glucose Uptake in Adipocytes/Adipose Tissue

Once preadipocytes are committed to an adipocytic fate, they upregulate and express glucose transporter 4 (GLUT4). This renders them responsive to insulin, which results in the stimulation of glucose uptake and conversion to fat in the presence of insulin and excess glucose. However, under conditions of cellular insulin resistance, adipocytes fail to respond to insulin and exhibit diminished glucose uptake in response to insulin. Glucose uptake is measured in the adipose tissue ex vivo to assess insulin responsiveness.

1. Once preadipocytes (3T3-L1 or primary cells) are differentiated into adipocytes in 24-well plates, serum-starve the cells in KRH buffer for 3 h with appropriate treatments or controls (*see* **Note 3**).
2. Wash the cells with KRH buffer and incubate with 1 mL of KRH supplemented with 100 nM insulin for 15 min (*see* **Note 4**).
3. To the solution above, add 1 μCi 2-deoxy-D-[2,6-3H]-glucose (in non-radioactive 2-deoxy-D-[2,6]-glucose, 200 μM, 50 μL total volume) to each well for 10 min.
4. Wash the cells with KRH buffer three times to remove excess tritiated glucose.
5. Lyse the cells in 1 mL of RIPA buffer and mix an aliquot (800 μL) with scintillation fluid in a vial and leave overnight (*see* **Note 5**). Read in a scintillation counter the next day.
6. Measure the protein content in the RIPA lysate using a standard protein assay.
7. Normalize the values obtained from the radioactive counts to the protein concentration in the wells.
8. For glucose uptake in ex vivo adipose tissue, mince the tissue into small pieces and weigh.
9. Incubate the tissue fragments in KRH buffer for 2 h followed by **steps 2** to **4** as above.
10. After washing, solubilize the tissues in 400 μL perchloric acid and 200 μL H_2O_2 at 60 °C overnight.
11. After dissolution, place an aliquot in scintillation fluid overnight, then count as above in **step 5**. Normalize the values to the tissue weight.

3.4 Intraperitoneal Glucose (IPGTT/GTT) and Insulin Tolerance Test (IPITT/ITT)

One of the earliest hallmarks of metabolic dysfunction is impaired glucose tolerance. In the glucose tolerance test, impaired glucose clearance in response to a bolus of glucose reflects either a defect in insulin secretion or ineffective insulin function in peripheral tissues. In contrast, the insulin tolerance test determines whether the metabolic dysfunction is due predominantly to insulin resistance in peripheral tissues (insulin responsiveness) (*see* **Notes 6** and **7**).

1. Fast the mice for 4 h prior to the assay. Weigh each mouse and place it in an individual fresh cage with only water (without food) for the 4 h period. Care should be taken to keep the mice calm (*see* **Note 8**).
2. Prior to the assay, calibrate the glucometer as per the manufacturer's instructions. Although we use a ReliOn glucometer, any other commercial glucometer for human use, such as Accu-Check Active (Roche Diagnostics) and One Touch Ultra (LifeScan), will be adequate.
3. At the start of the experiment, place the mouse in a restrainer and gently nick the tail 1–2 cm from the tip. Discard the first drop of blood and record the basal blood glucose (0 min reading) by inserting the test strip in the glucometer and directly touching it to the drop of blood on the tail (test strip uses about 3–5 μL of blood).
4. Immediately after the basal glucose recording, inject the mouse intraperitoneally with the glucose solution (2 g/kg body weight).
5. Proceed with subsequent mice, keeping the time interval between mice constant. Take additional glucose readings at regular intervals (15, 30, 60 and 120 min) after the injection. The blood glucose first rises rapidly, and then decreases over time due to clearance from the blood (*see* **Notes 9** and **10**).
6. For the insulin tolerance test, mice are similarly prepared as for the GTT, except that instead of injecting glucose, insulin is injected (0.5 U/kg body weight). Subsequently, mice display an initial decrease in blood glucose that rises back to basal levels over time.
7. Graph blood glucose readings as a function of time and analyze the area under the curve (AUC) to reveal the nature of any metabolic dysfunction/differences between groups of mice (*see* **Notes 11** and **12**).

3.5 Fasting Plasma Glucose and Insulin Levels and Calculation of Insulin Resistance Index

Fasting levels of glucose reveal whether or not mice are normoglycemic in the resting or basal state. In some instances however, even though mice exhibit normal fasting glucose levels, their fasting insulin levels might be higher due to insulin resistance, where the body requires higher insulin levels to maintain normal blood glucose levels.

1. For measuring fasting blood glucose, mice are prepared exactly as for GTT/ITT above and fasting blood glucose is measured directly from a blood drop at the tail tip using the glucometer.
2. For fasting insulin measurements, plasma is separated from the fasting blood (either obtained from terminal sacrifice or tail bleed) and insulin is measured using a mouse ultrasensitive

insulin ELISA kit following the manufacturer's instructions. The assay is typically based on a sandwich ELISA where two antibodies against different epitopes are utilized. The assay range for such a kit is typically 0.025–1.5 μg/L.

3. Pipet calibration standards, controls and plasma (25 μL each) into microtiter plate wells coated with capture antibody.
4. Add 100 μL antibody-enzyme conjugate to each well and incubate on a shaker at room temperature (18–25 °C) for 2 h. This antibody is the detection antibody and is conjugated to horseradish peroxidase (HRP).
5. Wash 3 times with 350 μL wash buffer and add 200 μL substrate 3,3′,5,5′-tetramethylbenzidine (TMB) and incubate at room temperature for 15 min. Stop the reaction by adding 50 μL of stop solution. Mix the plate contents on a shaker for a few seconds and place in a plate reader set to measure absorbance at 450 nm.
6. If samples fall out of range and are saturated, dilute and read again. However, linearity may be compromised through dilution of off-scale samples.
7. Plot the graph on a log-log scale and calculate the insulin concentration in the unknown samples using a cubic spline regression analysis.
8. Paired glucose and insulin readings can be used to assess the insulin resistance index using the Homeostatic Model Assessment (HOMA-IR). The formula for calculating HOMA-IR = (Glucose × Insulin)/405 (glucose in mg/dL and insulin in mU/L). If glucose is expressed in molar units (mmol/L), then the formula for calculating HOMA-IR = (Glucose × Insulin)/22.5 (insulin in mU/L).

3.6 Islet Isolation

Islets play a critical role in the pathophysiology of metabolic dysfunction during obesity and diabetes. Islet isolation is an important technique to generate islets for ex vivo studies, such as addressing endocrine function, the viability of cells, or in vivo transplantation. The method is based on collagenase infusion into the common bile duct of the euthanized animal; enzymatic degradation of the pancreatic architecture, separation of islets from other tissues and culturing viable islets. Although the procedure appears simple, it can be very challenging to obtain a pure and viable islet preparation.

1. Euthanize the mouse and surgically expose the abdomen (*see* **Note 13**).
2. Carefully locate the entry site of the bile duct into the duodenum and block the common bile duct opening by clamping it. To isolate islets, slowly inject the collagenase solution into the common bile duct opening to maximize solution entry into

the pancreas. Since the opening into the small intestine is blocked, this will force the collagenase solution to enter the pancreas and it will expand like a balloon (*see* **Note 14**).

3. Perfusion of the pancreas from the inside through the common bile duct in this manner provides efficient access to collagenase compared to other methods such as dissection of pancreas, cutting small pieces and incubating in the collagenase solution (*see* **Note 15**).
4. The type of Collagenase used for digesting the pancreas affects the yield of islets. We routinely use collagenase fraction V from Sigma, which provides good yield as well as good viability.
5. Carefully remove the pancreas and incubate in collagenase solution in a 50 mL conical tube for 8–11 min. It is important to incubate for the optimum time as longer incubation in collagenase solution will increase the yield of smaller islets and may also decrease the viability of islets.
6. Apply the digested slurry on a discontinuous gradient of Histopaque (sodium diatrizoate; 1.109, 1.096, 1.070, and 0.570 g/mL) and isolate the islets from the interface of the 1.070/1.096 and 1.096/1.109 g/mL layers.
7. Further clean the fractions obtained from the Histopaque interfaces by individually handpicking the islets from a petri plate under a dissecting microscope. Pool islets of similar size. For experiments, islets are mixed from each group in similar numbers (typically ~10).
8. Islets can be cultured in DMEM with 11 mM glucose and 10 % FBS overnight to let them recover from the isolation procedure.
9. To measure insulin secretion, first wash the islets and incubate in DMEM with 3 mM glucose for 90 min and subsequently incubate in DMEM with 3 mM or 16 mM glucose (± treatments) for 60 min.
10. Recover the medium and measure insulin using the Ultrasensitive Mouse Insulin Elisa kit (as above) (*see* **Note 16**).

4 Notes

1. The adipocytes isolated from the adipose tissue ex vivo should be used for experiments as soon as possible as they may start producing increased pro-inflammatory cytokines following collagenase treatment that may induce experimental artifacts.
2. After adipogenesis, fat in the adipocytes can cause them to come off the plate so assays should be performed as soon as possible after differentiation and maturation. Data should be normalized to the protein or DNA content of the well.

3. In glucose uptake assays, starvation times and conditions can play a key role. Unlike other cells, adipocytes enter senescence when starved overnight in low glucose medium. Thus, we starve cells for 3 h in KRH buffer directly and bypass the low glucose medium step.
4. Insulin responses in adipocytes peak within 30 min so it is advisable to plan the treatment time such that the reaction is stopped at 30 min after the insulin stimulation to achieve a good positive control.
5. In glucose uptake experiments, the final lysate should be left in the scintillation fluid overnight to disperse evenly before placing in the scintillation counter.
6. Since mice display significant variation, care should be taken to perform experiments with adequate numbers of animals to achieve statistical significance. A minimum of 6–8 animals should be used for each of the in vivo experiments. If large variations are observed, outlier analysis using statistical software such as GraphPad Prism can be performed.
7. Reference values for metabolite and hormone levels in a number of mouse strains are available at the Jackson Laboratories website (http://www.jacksonlaboratory.com).
8. A very important prerequisite for the mice that will undergo GTT/ITT is that they should be kept as calm as possible (no extraneous noises, smells, personnel, etc.). Also, trained personnel should handle the animals. Care should be taken to ensure that mice are not stressed between readings. Only a limited number of mice should be planned for this experiment that can be handled effectively given the constraints of the assay time points.
9. For GTT assay, we sometimes also take a 7.5 min glucose measurement post glucose challenge. This reading is a good measure of the first phase of the pancreatic response. However, if employing a 7.5 min time point, care should be taken to use a small group of animals as handling larg numbers of animals in such a short time span can lead to missing time points.
10. For GTT or ITT experiments, determining the area under the curve (AUC) may provide statistically significant differences between groups, where effects at individual time points may not. In GTT, glucose levels first peak, then recover over time. Early time points reveal the dynamics of the first phase of insulin response from the pancreas and later time points provide information about the combined effect of insulin secretion from pancreas and insulin responsiveness in peripheral tissues. This combined effect can be further delineated by analyzing the ITT graph, which provides information regarding insulin responsiveness.

11. In the ITT analysis, two phases are evident: the first when glucose levels decrease in response to insulin, and the second when the glucose levels return to normal levels in response to glucagon and other liver enzymes. Plotting these two phases separately by AUC can provide information regarding responsiveness of these two contributing pathways.
12. Insulin sensitivity may vary between the strains or sex of mice used for the study. The insulin dose recommended above may be too high and may lead to hypoglycemia or even death in certain cases. Thus, performing a small pilot experiment to determine the appropriate dose of insulin for the animals under study is helpful. A hypoglycemic mouse approaching death can be "rescued" by injecting glucose, avoiding the loss of an animal from an experimental cohort, and allowing further studies at later times.
13. For islet isolation, the procedure should be performed as soon as possible after euthanasia as the pancreas is prone to autolysis.
14. To obtain a pure and viable islet preparation, it is important to perfuse the pancreas with collagenase solution through the common bile duct. This procedure is challenging and should be performed by trained and experienced personnel.
15. During the islet isolation procedure, the incubation time of the perfused pancreas in the collagenase solution is critical. Too much or too little time will lead to an overdigested or underdigested slurry that will eventually affect the quantity and quality of the islets. It is advisable to standardize the incubation time with each new lot of Collagenase V and also the animal model.
16. The number of islets in the insulin secretion assays should typically be ~10–12. If small islets are obtained (e.g., in insulin-resistant obese mice where new islets form resulting in hyperinsulinemia), then the number of islets can be increased accordingly.

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Chapter 39

Analysis of G-Protein Coupled Receptor 30 (GPR30) on Endothelial Inflammation

Subhadeep Chakrabarti and Sandra T. Davidge

Abstract

The female sex hormone estrogen (the most common form 17-β-estradiol or E2) is known to have both anti-inflammatory and pro-inflammatory effects. Given the diversity of estrogen responses mediated through its three distinct receptors, namely, estrogen receptor α (ERα), ERβ, and the G-protein coupled receptor 30 (GPR30), it is plausible that different receptors have specific modulatory effects on inflammation in different tissues. We have shown that activation of GPR30 exerted anti-inflammatory effects as demonstrated by significant attenuation of tumor necrosis factor (TNF)-mediated upregulation of adhesion molecules in isolated human umbilical vein endothelial cells. Interestingly, estrogen alone had no such effect and blockade of classical ERs restored the anti-inflammatory effect, suggesting that this effect was dependent on GPR30 and opposed to classical ERs. These findings were further validated by the negation of anti-inflammatory GPR30 effects by classical ER agonists. This chapter focuses on multiple pharmacological options to activate GPR30 and the use of TNF activated endothelial cells as a model system for inflammatory response as assessed by adhesion molecule detection through western blotting.

Key words Estrogen, Endothelium, Inflammation, GPER, GPR30, TNF, HUVEC, PPT, DPN, G-1, G-15

1 Introduction

The female sex hormone estrogen has been long considered to exert anti-inflammatory and anti-atherogenic effects on the cardiovascular system [1, 2]. Despite evidence of its anti-inflammatory roles under experimental conditions, clinical trials of hormone replacement therapy with estrogen have had decidedly mixed results [3, 4]. These results suggest a more complex regulatory role for estrogen on the inflammatory processes, which may be differentially modulated by coexisting factors and signaling through distinct receptors [5]. The estrogen responses are mediated by the three different receptors, namely, estrogen receptor α (ERα), ERβ and the G-protein coupled receptor 30 (GPR30, also called G-protein coupled estrogen receptor (GPER)) [6]. While the signaling actions of the classical receptors (ERα, ERβ) are better known, the

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functional significance of the novel estrogen receptor GPR30 is only recently being investigated [7–10]. A number of recent studies have suggested GPR30 can exert anti-inflammatory effects under experimental conditions involving both in vitro and in vivo animal studies [11–15]. Given the complex effects of estrogen on vascular inflammation, we specifically investigated the anti-inflammatory potential of GPR30 in the human vascular endothelium using cultured human umbilical vein endothelial cells (HUVECs) as a model system. Our studies showed that GPR30 was indeed capable of exerting anti-inflammatory effects in the context of tumor necrosis factor (TNF) induced inflammatory responses [16]. The upregulation of protein expression of ICAM-1 and VCAM-1 in HUVECs in response to stimulation by TNF is taken as a functional readout of inflammation [16–18]. This chapter describes the protocols for isolation and culture of primary HUVECs and for western blot (immunoblot) analysis of inflammatory markers in response to TNF and GPR30.

2 Materials

2.1 Isolation and Culture of HUVECs

1. Growth medium: M199 cell culture medium with phenol red supplemented with 20 % (v/v) heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 1 % penicillin–streptomycin (5000 U/mL).
2. Resting medium: M199 cell culture medium without phenol red, supplemented with 1 % (v/v) heat-inactivated FBS, 2 mmol/L L-glutamine, and 1 % penicillin–streptomycin (5000 U/mL).
3. 1× phosphate buffered saline (PBS).
4. Deionized distilled water (ddH_2O).
5. Heparin solution: dissolve 10 mg (500K units) heparin per mL of PBS.
6. Growth supplement: Commercially available endothelial cell growth supplement (such as ECGS, from Corning Inc): dissolve ECGS at a concentration of 5 mg/mL in PBS containing 10 mg/mL heparin. Use at 1 % (v/v) in growth medium to allow for optimal proliferation of HUVECs.
7. Type I collagenase (Worthington Biochemical): dissolve in PBS at 150 U/mL.
8. Trypsin–EDTA: 0.25 % Trypsin–EDTA is added to equal volume of 1× PBS to obtain 0.125 % Trypsin–EDTA for splitting cells.
9. 0.1 % Gelatin solution: Heat (37 °C) to dissolve 0.1 % w/v porcine gelatin in ddH_2O.

10. Plasticware including cell culture flasks, 48-well culture plates and 50 mL tubes.
11. Gelatin coated 48-well culture plates: Add 150 μL of 0.1 % gelatin solution to each well. Rock gently to ensure even coating of the well surface. After 30–60 min, aspirate the excess liquid and let dry for approximately 30 min. Store plates in a sealed bag at 4 °C.
12. Hemostatic clamps, forceps, needles, syringes, sterile scissors, sterile cannula, 70 % ethanol.
13. Standard laboratory equipment such as tissue culture flow hood, floor centrifuge, 37 °C water bath and cell culture incubator (95 % air, 5 % CO_2).

2.2 GPR30 Activation and Induction of Inflammation

1. Selective GPR30 agonist, G-1: 0.1–3.0 μM (Calbiochem).
2. Selective GPR30 antagonist, G-15: 2 μM (Calbiochem).
3. Water-soluble form of estradiol, 17-β-estradiol encapsulated in cyclodextrin (E2): 10–100 nM (Sigma Aldrich).
4. Antagonist of classical ERs, ICI 182780: 1 μM (Tocris Bioscience).
5. Selective agonist for ERα, Propylpyrazole triol (PPT): 10 nM (Tocris Bioscience).
6. Selective agonist for ERβ Diaryl propionitrile (DPN): 10 nM (Tocris Bioscience).
7. Pro-inflammatory cytokine, TNF: 5 ng/mL (Peprotech).

2.3 Preparation of Cell Lysates

1. 5× Laemmli buffer: 312 mM Tris–HCl, pH 6.8, 10 % (w/v) glycerol, 10 % (w/v) sodium dodecyl sulfate (SDS), 0.1 % bromophenol blue.
2. Lysis buffer: 25 mM Tris–HCl, pH 6.8, 0.5 % Triton-X-100.
3. Preparation of 2× Laemmli's buffer: Mix 5× Laemmli buffer and lysis buffer (25 mM Tris and 0.25 % Triton-X-100) in 2:3 ratio. Boil for 2 min. Add DTT to a final concentration of 25 μM.
4. Dithiothreitol (DTT): 1 M aqueous stock solution.
5. Microfuge tubes, electric kettle.

2.4 Western Blotting: SDS-PAGE

1. 4× separating gel buffer: 1.5 M Tris–HCl, pH 8.7, 0.4 % (w/v) SDS in water.
2. 4× stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.4 % (w/v) SDS in water.
3. 10× running buffer: 0.25 M Tris, 1.92 M glycine, 1 % SDS (w/v) in water.
4. 1× running buffer: Thoroughly mix 90 mL 10× running buffer with 810 mL ddH_2O.

5. Separating gel (9 %): 5 mL 4× separating gel buffer, 6 mL 30 % acrylamide, 8.785 mL ddH2O, 15 μL tetramethyl ethylenediamine (TEMED), and 200 μL ammonium persulfate (APS), for a total volume of 20 mL (enough for two gels) (*see* **Note 1**).
6. Ammonium persulfate (APS): 100 mg/mL.
7. Stacking gel: 1.25 mL 4× stacking gel buffer, 580 μL 30 % acrylamide, 3.115 mL ddH2O, 5 μL TEMED, and 50 μL APS for a total volume of 5 mL.
8. Apparatus for gel electrophoresis of mini-gels and protein transfer: this protocol describes the Bio-Rad Mini-PROTEAN® Cell for vertical slab gels and the Bio-Rad Mini-TransBlot® electrophoretic cell tank transfer system, but equivalent mini-gel systems can also be used.
9. Prestained protein molecular weight markers such as the PageRuler Plus pre-stained ladder (Fisher Scientific).

2.5 Western Blotting: Immunoblotting

1. Bio-Rad Mini Trans-Blot® Cell Tank Transfer System or equivalent.
2. 1× transfer buffer: 0.025 M Tris, 0.192 M glycine, 20 % (v/v) methanol in water, stored at 4 °C.
3. Nitrocellulose membrane (0.22 μm).
4. Blotting filter paper.
5. 1× TPBS: 0.05 % Tween 20 in PBS.
6. Blocking Buffer for Fluorescent Western Blotting.
7. Heat sealable plastic pouches and pouch sealing apparatus.
8. Primary antibodies: Anti-VCAM-1 (rabbit polyclonal antibody); Anti-ICAM-1 (mouse monoclonal antibody from Santa Cruz Biotechnologies); Anti-α-tubulin (rabbit polyclonal antibody) (*see* **Note 2**).
9. Secondary antibodies: IRDye® conjugated donkey anti-mouse and goat anti-rabbit secondary antibodies (LiCor Biosciences, Lincoln, NE)-red (IRDye® 680RD) and green (IRDye® 800CW).
10. Odyssey Infrared imager and software [v3.0] for imaging western blots (LiCor Biosciences).

3 Methods

Overview of experimental approach: Briefly, HUVECs were stimulated for 4 h with the pro-inflammatory cytokine TNF (5 ng/mL) to induce inflammatory changes such as upregulation of endothelial expressed leukocyte adhesion molecules like ICAM-1 and VCAM-1. GPR30 was activated in the endothelial cells by two

different means, either by its selective agonist G-1, or by administration of E2 in the presence of classical ER blocker ICI 182780 (which would allow the E2 to only activate GPR30 as other ERs remain blocked). GPR30 activation by G-1 significantly (but not completely) inhibited TNF induced ICAM-1 and VCAM-1 protein expression (Fig. 1a, b). These effects were completely

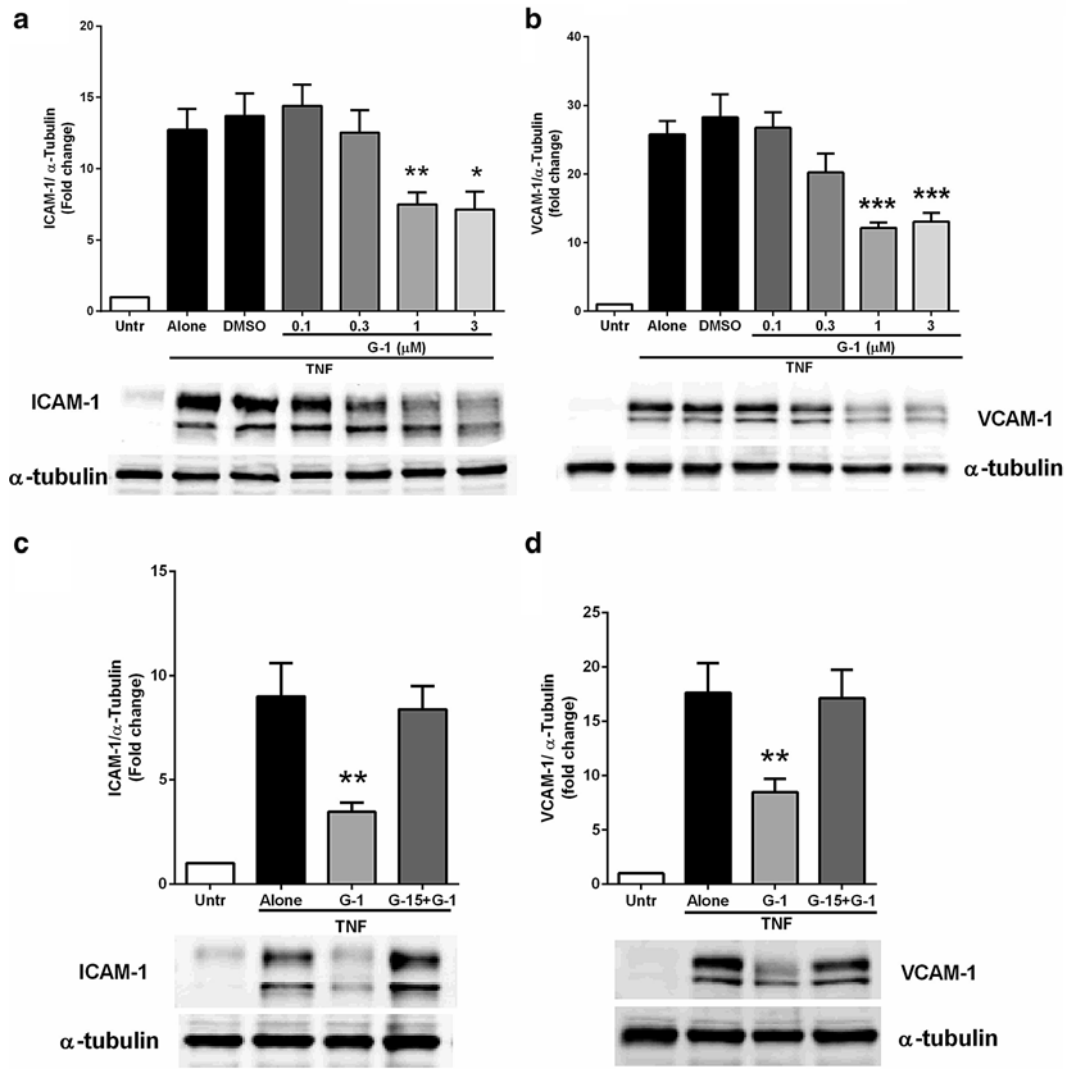

Fig. 1 GPR30 agonist G-1 attenuates TNF-mediated ICAM-1 and VCAM-1 expression in the endothelium. (**a** and **b**) Endothelial cells were treated with G-1 (0.1–3 μM) for 45 min before 4 h stimulation with TNF (5 ng/ml). Cell lysates were immunoblotted with antibodies against ICAM-1 (**a**), VCAM-1 (**b**) and α-tubulin (loading control). DMSO (1:5000) was used as a solvent control for G-1. (**c** and **d**) Endothelial cells were treated for 30 min with/without the GPR30 inhibitor G-15 (2 μM) followed by treatment with G-1 (1 μM) for 45 min prior to 4 h stimulation with TNF (5 ng/ml). Cells lysates were immunoblotted for ICAM-1 (**c**), VCAM-1 (**d**) and α-tubulin. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively, compared to the cells treated with TNF alone (Adapted from [16])

prevented by the selective GPR30 antagonist G-15 (Fig. 1c, d). Similar effects were also noted with E2 in presence of classical ER inhibition but not by E2 alone (Fig. 2a, b) suggesting different roles for classical ERs and GPR30 in this situation. Indeed, concomitant activation of ERα or ERβ abrogated the anti-inflammatory effects of G-1 (Fig. 2c, d), indicating that GPR30 alone could

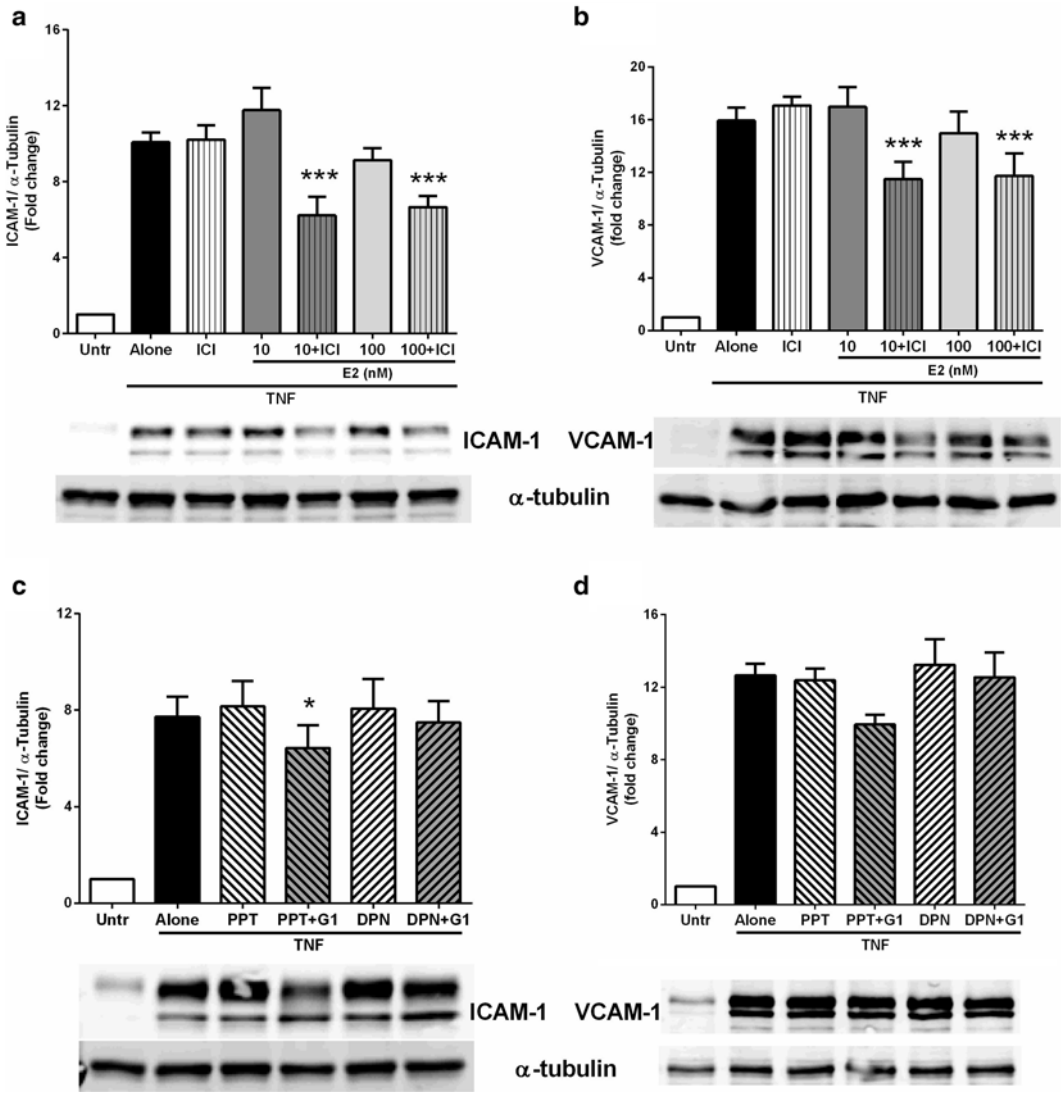

Fig. 2 Classical estrogen receptors (ERs) antagonize anti-inflammatory effects of GPR30. (**a** and **b**) Endothelial cells were treated with/without the classical ER blocker ICI 182780 (ICI, 1 μM) for 30 min prior to 45 min treatment with E2 (10–100 nM). The cells were then stimulated with TNF (5 ng/ml) for 4 h. Cell lysates were immunoblotted with antibodies against ICAM-1 (**a**), VCAM-1 (**b**) and α-tubulin. (**c** and **d**) Endothelial cells were treated with/without the ERα agonist PPT (10 nM) or ERβ agonist DPN (10 nM) for 30 min followed by 45 min with/without the GPR30 agonist G-1 (1 μM). Cells were then stimulated with TNF (5 ng/ml) for 4 h, lysed and immunoblotted with antibodies against ICAM-1 (**c**), VCAM-1 (**d**) and α-tubulin. * and *** indicate $p<0.05$ and $p<0.001$ respectively, compared to cells with TNF alone (Adapted from [16])

mediate the anti-inflammatory effects of estrogen and the classical ERs were counteracting these anti-inflammatory functions in our system. These findings have been published in a previous research article from our laboratory [16] and the current communication provides detailed descriptions of the various experimental procedures involved in such studies.

3.1 Isolation and Culture of HUVECs

1. Ensure that all protocols for the collection of human tissues, including human umbilical cord, have prior approval by the ethics committee of your institution. For example, our protocols are approved by the University of Alberta Ethics Committee. This procedure also conforms to the principles mentioned in the Declaration of Helsinki as well as Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, effective December 13, 2001. Ensure that all subjects provide informed consent in writing before participation in the study.
2. Collect placenta and umbilical cords immediately after birth from both sexes. Clean the umbilical cord with a paper towel soaked in 70 % ethanol.
3. Separate the cord from the placenta by cutting with a sterile scissors and allow the cord blood to drain out.
4. Place the cord in ice cold sterile 1× PBS in a sterile plastic container for transportation to the laboratory. Typically the cord needs to be at least 5 in. (12.5 cm) long in order to obtain a sufficient yield of HUVECs.
5. Wipe the cord clean with 1× PBS.
6. Identify the umbilical vein and cannulate.
7. Pass 1× PBS through the vein to remove blood clots, follow with infusion of air to remove excess PBS.
8. Clamp the distal end of the cord and fill the vein filled with type I collagenase solution.
9. Clamp the proximal end of the cord.
10. Place the cord in a 37 °C water bath for 11–12 min.
11. Collect the collagenase solution containing dislodged endothelial cells in a 50 mL tube containing 5 mL FBS.
12. Centrifuge the sample at 1400 × *g* for 5 min.
13. Discard the supernatant and resuspend the pellet with 5 mL of growth medium with 1 % ECGS (*see* **Note 3**).
14. Transfer the resuspended cells to a T-25 culture flask. Incubate the cells in the incubator at 37 °C. These cells are to be considered as passage 0.
15. Split and passage cells after reaching 90–100 % confluence. This usually occurs within 72 h of isolation from cord. These are

passage 1 cells. To split the cells, remove the culture medium from the confluent cells and add the 0.125 % Trypsin–EDTA solution. Incubate at room temperature for 1–2 min. Check the cells under a light microscope to observe detachment of cells. This can be accelerated by tapping the flask on a hard surface. Once the cells are detached, immediately add an equal volume of Growth Medium to inactivate the Trypsin-EDTA. Transfer the cell suspension to a 50 mL tube, centrifuge at 1400 × *g* for 5 min, remove the supernatant and resuspend the cell pellet in fresh Growth Medium containing 1 % ECGS for plating onto cell culture flasks and/or plates.

16. Split Passage 1 cells upon reaching 80–100 % confluence and plate the cells onto gelatin coated 48-well plates.
17. Second passage cells grown to 80–90 % confluence on gelatin-coated 48-well plates are used for all experiments (*see* **Note 4**). All experimental procedures are carried out in resting medium only (*see* **Note 5**). Experiments are performed 4–8 times using cells obtained from different cords ($n = 4–8$).
18. The purity of HUVEC preparations can be confirmed by immunostaining for the endothelium specific marker von Willebrand's factor (vWF) [17].

3.2 GPR30 Activation and Induction of Inflammation

Inflammatory changes are induced in cultured HUVECs by incubation with TNF (5 ng/mL, in resting medium) for 4 h. Based on our previous studies, both the concentration and time period for TNF stimulation is sufficient to upregulate protein expression of ICAM-1 and VCAM-1 which is taken as a functional read-out of inflammation [16–18].

1. For pharmacological activation of GPR30: Treat isolated HUVECs with the GPR30 selective agonist G-1 (0.1–3.0 μM) for 45 min prior to 4 h stimulation with TNF (*see* **Note 6**). To demonstrate the specificity of the agonist, treat the cells first with the GPR30 antagonist G-15 (2 μM) for 30 min followed by administration of G-1 (45 min) and TNF (4 h).
2. To evaluate the GPR30 mediated E2 effects: Treat HUVECs with the ER blocker ICI 182780 (1 μM) for 30 min, followed by a 45 min incubation with E2 at 10 or 100 nM and then stimulated with TNF for 4 h.
3. To determine the interaction between GPR30 and classical ERs: Treat HUVECs with either PPT or DPN (both at 10 nM) for 30 min followed by 45 min incubation with G-1, the GPR30 agonist. Afterwards, stimulate the cells with TNF for 4 h.

3.3 Preparation of Cell Lysates

1. Remove the culture medium from the cells at the end of TNF stimulation.
2. Add 50 μL of hot 2× Laemmli buffer to each well.

3. Scrape the cells clean from the well.
4. Collect the cell lysate in a microfuge tube.
5. Boil the cell lysate 3–4 min in order to reduce the constituent proteins.
6. Use the cell lysates immediately for western blotting or store at −20 °C until further usage.

3.4 Western Blot: SDS-PAGE, Preparation and Loading of Protein Samples

1. Clean the glass plates first with ddH_2O and then with 70 % ethanol (*see* **Note 7**).
2. Attach the glass plates together in the casting frame and place them into the gel casting stand.
3. Check for leaks by adding water between the two plates. Make adjustments to ensure a tight seal.
4. Prepare a 9 % separating gel.
5. Pipette 8.0–8.5 mL of the separating gel into each set of the assembled glass plates.
6. Layer a small volume of isopropanol on top of the gel to remove any bubbles.
7. Check for polymerization of the separating gel by observing changes to the surplus gel mixture remaining in the beaker. Typically, the gel is formed within 10 min.
8. Pour off the isopropanol from the top of the gel and allow to dry.
9. Prepare the stacking gel.
10. Pipette the preparation of stacking gel on top of each separating gel up to the top of the shorter glass plate.
11. Immediately insert a15-well comb into each stacking gel (*see* **Note 8**). The stacking gel should polymerize within 10 min. Confirm polymerization by observing changes to the remaining amount of stacking gel in the beaker.
12. Insert the glass plates containing the gels into a gel electrode assembly and set the assembly into running buffer tank.
13. Pour 1× running buffer into the inner chamber of the gel electrode assembly to overflow into the running buffer tank such that it covers the top of the comb.
14. Remove the comb. Check the wells for damage and to ensure the wells have filled with 1× running buffer.
15. Prior to loading, boil the cell lysate samples for 3–4 min. (This second boiling step can be avoided if the samples are loaded onto a gel immediately after initial preparation (**step 6** of Subheading 3.3).)
16. To the first well (from the left) of the gel, add 2.5 μL of pre-stained protein ladder.

17. Load 15 μL of each cell lysate sample to the subsequent wells of the gel (*see* **Note 9**).
18. Connect the power supply by securing the lid onto the running buffer tank, and ensure that the color coded plugs and jacks are properly aligned with the gel electrode assembly.
19. Run the SDS-PAGE at 140 V at room temperature. Allow the proteins in the samples to separate until the leading blue dye front has reached the bottom of the gel.

3.5 Western Blot: Transfer and Detection of Proteins

This protocol describes wet transfer of resolved proteins from the separating gel to a nitrocellulose membrane prior to antibody mediated detection.

1. For each gel, cut to size two pieces of blotting filter paper and a single piece of nitrocellulose membrane such that each would cover the entire gel. Before assembling the transfer stack, place the nitrocellulose membrane in 1× transfer buffer for 5 min on a rocker.
2. Separate the glass plates containing the gel, and discard the stacking gel. Place the separating gel in 1× transfer buffer for 5 min on arocker.
3. Place an open transfer cassette with its black panel side down in a large dish. Fill the dish with enough 1× transfer buffer to cover the transfer cassette.
4. Prepare the transfer stack in the following order (from the bottom up) on the black panel side of transfer cassette: fiber pad, blotting filter paper, separating gel, nitrocellulose membrane, blotting filter paper, fiber pad. Ensure that all components are submerged in the buffer and no bubbles are present in between various layers.
5. Close the transfer cassette by bringing the clear side of the cassette down onto the stack. Avoid bubbles in between the layers.
6. Insert the transfer cassette into the electrophoresis transfer module, such that the black panel of the transfer cassette is toward the black side of the blotting module.
7. Set the blotting module holding the transfer cassette/s and an ice pack into a buffer tank.
8. Fill the buffer tank to the top with 1× transfer buffer.
9. Connect the power supply by securing the lid to the buffer tank. Ensure that the color coded plugs and jacks are aligned correctly.
10. Run the transfer at 100 V for 1 h at 4°C.
11. When the transfer is complete, disassemble the transfer assembly and stacks. Incubate the membranes individually in freshly

prepared blocking solution (25 % in PBS) on a rocker for 1 h at room temperature (*see* **Note 10**).

12. Probe for VCAM-1, ICAM-1, and α-tubulin on the same membrane. Prepare a solution of anti-VCAM-1 and anti-ICAM-1 antibodies (each at 1 μg/mL) in 1× TPBS (~600 μL per membrane).
13. Place the membrane in a heat sealable plastic pouch. Add 600 μL of the dual antibody solution to the pouch and heat seal. Take care to avoid air bubbles.
14. Incubate the membranes overnight on a rocker at 4°C.
15. Remove the membranes from the plastic pouches. Wash the membranes 2× in TPBS (each wash for 4–5 min on a rocker).
16. Incubate the membranes in 10 mL of anti-α-tubulin antibody solution (1:6000 dilution in TPBS) for 1 h on a rocker at room temperature.
17. Discard the antibody solution and wash the membranes 2× in TPBS (each wash for 4–5 min on a rocker).
18. Incubate the membranes with anti-mouse and anti-rabbit secondary antibodies (1:10,000 dilutions in 25 % blocking solution in TPBS) on a rocker for 30 min at room temperature. Avoid exposing the membranes to light. The anti-mouse and anti-rabbit secondary antibodies used are tagged with infrared fluorescent dyes of different excitation wavelengths to enable visualization of different proteins upon binding to their respective primary antibodies (*see* **Note 11**).
19. Discard the secondary antibodies and wash the membranes (2×; 4–5 min each with rocking) in TPBS.
20. Place the membranes in ddH_2O before visualization of the protein bands on the membranes.
21. Lift each membrane with forceps and place it face-down on the glass imaging surface of the Odyssey Infrared Imaging System. Acquire the image/s using the requisite software program (*see* **Note 12**).
22. Analyze the protein bands with the Odyssey imaging processing software. Quantify the bands by densitometric analysis. Normalize each band of VCAM-1 (or ICAM-1) to its corresponding band of α-tubulin (loading control).
23. Analyze the data using commercially available statistical software (such as PRISM from GraphPad) and express the data as mean ± SEM (standard error of mean). Use a one-way analysis of variance (ANOVA) with an appropriate post hoc test (Dunnett's for comparison to untreated control, Tukey's for multiple comparisons) to determine statistical significance. A p-value of less than 0.05 is taken as significant.

4 Notes

1. The combination of TEMED with APS initiates the polymerization of the gels. TEMED should always added before APS. Doing otherwise may result in uneven polymerization and distorted bands in western blots.
2. Some antibodies do not work well for western blot as they may be optimized for alternative applications. For the proteins analyzed here, we have had good experience with anti-VCAM-1 (Santa Cruz Biotechnologies, #sc-8304), anti-ICAM-1 (Santa Cruz Biotechnologies, #sc8439), and anti-α-tubulin (Abcam, #ab15246). (Catalog numbers may vary depending on your location.)
3. Once pelleted, HUVECs should be resuspended as soon as possible to prevent their activation and consequent cell damage.
4. HUVECs should be used between Passages 1–3 for optimal results. In our experience, the cells tend to lose their characteristics and show alterations in morphology and decreased inflammatory responses from Passage 4 onwards.
5. Since the growth medium has a high content (20 %) of FBS that may activate the cells, overnight incubation in resting medium (with only 1 % FBS) prior to the actual experiment is required for quiescing and cell cycle synchronization.
6. To ensure proper dissolution of reagents (agonists, antagonists or TNF), it is recommended to mix them with the culture medium by pipetting up and down for 3–4 times.
7. The Bio-Rad apparatus uses glass plates with integrated spacers. Use plates with integrated spaces with a thickness of 1.5 mm.
8. The comb should be inserted into the (still unpolymerized) stacking gel at an angle to prevent bubbles.
9. Since confluent HUVEC cultures are used, each well of a 48-well culture plate should have approximately the same number of cells which would be solubilized in the same volume (50 μL) of 2× Laemmli buffer. Hence, using the same volume of lysate for each data point should result in loading approximately equal amounts of cellular protein per well of an SDS-PAGE during western blotting. Alternatively, carry out a protein assay and load equal quantity of protein per well.
10. PBS but not TPBS should be used for making blocking buffer for the membranes. Presence of Tween 20 causes excess background fluorescence during imaging.
11. For detection of two proteins with similar (or close) molecular weights, it is preferable to use primary antibodies generated in different species and probe them with species-specific second-

ary antibodies (e.g., anti-mouse or anti-rabbit) containing fluorescence tags with different excitation and emission wavelengths (for red or green colorization). When imaging, bands for the 2 proteins would be clearly visualized in their respective channels (channels 700 and 800 for red and green, respectively, in the Odyssey imager). For proteins with widely different molecular weights (e.g., VCAM-1 and α-tubulin), primary antibodies generated in the same species can be used without any difficulty.

12. Troubleshooting western blots: If the desired bands are light, check your transfer conditions; make sure that the transfer of proteins from gel to membrane was run for at least 1 h. Also check that the appropriate antibody was used (*see* **Note 2**). If the molecular weight markers appear to indicate the wrong size for your band of interest, check whether all colors and sizes of the markers are visible. Molecular weight markers may transfer variably depending on the concentration (% acrylamide) of the running gel.

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Chapter 40

Atherosclerosis and Vascular Biologic Responses to Estrogens: Histologic, Immunohistochemical, Biochemical, and Molecular Methods

Thomas C. Register, Susan E. Appt, and Thomas B. Clarkson

Abstract

Atherogenesis is a multifactorial pathologic process influenced by genetics and environmental factors such as diet, exercise, stress, and other exposures. Estrogen receptors (ER) are expressed in cells of the arterial wall, suggesting that estrogen receptor ligands (estradiol, natural and pharmacologic ligands) may directly affect arterial biology and atherogenesis. Ligand bound estrogen receptor alpha and beta (ERα, ERβ) can influence physiology through direct binding to estrogen response elements in the DNA, through interactions with other transcription factors such as NF-κB, or through rapid effects not dependent on gene expression changes but instead through non-nuclear membrane sites involving ERα, ERβ, or G-coupled protein ER (GPER1).

Elucidation of potential direct effects of estrogens on the artery wall requires careful evaluation of arterial biologic responses to estrogens. We have developed a comprehensive approach to understand the mechanisms of estrogen action which employs histologic measures of the size and other characteristics of atherosclerotic lesions, immunohistochemical assessments of cellular composition, evaluation of chemical, molecular, and genomic changes in the arterial environment, and determination of the relationships between arterial estrogen receptor expression and atherogenesis. This approach can provide important insights into the mechanisms of action of estrogen and other mediators of atherogenesis.

Key words Atherosclerosis, Inflammation, Vascular biology, Gene expression, Macrophage, T cell, Estrogen receptor alpha and beta

1 Introduction

The presence of immune cells, particularly macrophages and T cells, is a common characteristic of developing atherosclerotic lesions [1]. Activated macrophages produce inflammatory cytokines that mediate pro-inflammatory effects through both innate and adaptive immunity. $CD4^+$ T cells account for the majority of activated T cells in atheroma [2]. $CD8^+$ T cells are less common and their role in

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atherogenesis is less well understood. T cells influence adaptive immunity by producing pro- and/or anti-inflammatory cytokines. T helper-1 (Th-1) cells, major activated T cells in lesions, produce pro-atherogenic cytokines such as interferon-γ (IFN-γ), TNF-α, and IL-2, whereas T helper-2 (Th-2) cells secrete anti-inflammatory cytokines IL-4 and IL-10 [1]. During early T cell development, and under certain circumstances, Th-1 and Th-2 cells can switch their cytokine expression patterns [3].

Estrogen appears to influence atherogenesis and cardiovascular disease through multiple mechanisms, since it alters inflammation, vasoactive molecules, lipid concentrations, antioxidant, coagulation, and fibrinolytic systems [4]. Estrogen therapy may produce beneficial effects on plasma lipoprotein concentrations, but these alterations explain less than half of the atheroprotective effects of estrogen [5, 6], suggesting beneficial effects which are independent of plasma lipids and involving direct effects on arterial biology. Anti-inflammatory effects of estrogens include reducing circulating levels of monocyte chemoattractant protein-1 (MCP-1), and vascular cell adhesion molecule-1 (VCAM-1) [7, 8], likely in part through transcription factor cross-talk involving estrogen receptor (ER) antagonism of NF-kB activity [9, 10]. ERα and ERβ are both present within the arterial wall, and there is evidence for direct effects of estrogens on arteries and arterial cells [11–13]. ERs are also expressed by immune cell populations [14].

Observational studies in women and experimental studies in animals provide support that estrogen protects against coronary heart disease (CHD) and atherosclerosis [5, 13, 15–17], and there is evidence from ER knockout models that the presence of ERα is important for this effect [18]. Evidence that atheroprotective effects of estrogens might be attenuated when initiated in late menopause arose from our own work in the ovariectomized nonhuman primate model [19, 20], later supported by data from the Women's Health Initiative (WHI) which demonstrated lack of a beneficial effect of estrogen plus progestogen therapy (EPT) overall and potential adverse effects on clinical coronary heart disease outcomes in the first year of treatment of older postmenopausal women [21, 22]. The interval between menopause and initiation of estrogen therapy is important; women assigned to hormone therapy (HT) within 10 years of menopause had a hazard ratio of 0.76 (95 % CI 0.50, 1.16) compared to 1.28 (95 % CI 1.03, 1.58) for women assigned to HT 20 years or later after menopause [23]. The "timing hypothesis" regarding cardioprotective benefits of ET emerged from these and other data. Our recent work suggests that this may relate to a reduced ability of estrogen to influence macrophage accumulation and behavior in more advanced atherosclerotic plaques [24]; however, the exact mechanism(s) underlying this remain unclear. Thus, there are important questions to be answered regarding the relationships between risks and benefits of estrogen therapy and the timing of the initiation of therapy.

This chapter was developed to provide guidance to investigators in the evaluation of atherosclerosis and the effects of hormones and other agents in randomized preclinical studies in animal models. This chapter contains four methodological sections: (1) tissue collection and preservation, (2) morphologic assessments of fixed tissues, (3) immunohistochemical assessment of fixed and frozen tissues, and (4) biochemical assessments of frozen tissues. These general approaches and newer molecular approaches have evolved over decades of research studies at Wake Forest School of Medicine (formerly named Bowman Gray School of Medicine) and is broadly useful in studies of the effects of diet and other interventions on atherogenesis and other processes influenced by diet and hormones [13, 24–32]. Observations in the nonhuman primate within our group [13, 24, 31, 32] and in human subjects by others [33–35] suggest that estrogen receptor alpha is important in the atheroprotective effects of estrogens. Ongoing research should help to reveal the exact mechanisms by which this occurs.

2 Materials

2.1 Plasma or Serum Lipids

1. Blood draw supplies, EDTA Vacutainer (Becton, Dickinson & Co., BD) for plasma preparation, or red top serum collection Vacutainer (BD).
2. Clinical centrifuge.
3. Clinical Chemistry Autoanalyzer with reagents and validated calibrators for appropriate species (ACE ALERA autoanalyzer, Alfa Wasserman Diagnostic Technologies).
4. Total Cholesterol Reagent (ACE cholesterol, Alfa Wasserman Diagnostic).
5. HDL Cholesterol Reagent (ACE HDL-C, Alfa Wasserman Diagnostic Technologies).
6. Triglyceride Reagent (ACE Triglycerides, Alfa Wasserman Diagnostic Technologies).

2.2 Tissue Preparation

1. Lactated ringers or phosphate buffered saline (0.1 M phosphate, pH 7.4, PBS) for perfusion of the cardiovascular system and isolated arterial segments.
2. Ice cold PBS (4 °C) for tissue hydration and protection.
3. Scalpels, fine instruments including scissors, tweezers, weigh boats, thin cardboard strips.
4. OCT (Optimized Cutting Temperature Compound, Tissue-Tek®).
5. 10 % neutral buffered formalin (NBF).
6. 4 % paraformaldehyde buffered with 0.1 M $NaPO_4$, pH 7.4.

7. Cryoprotectant buffer: 15 % sucrose-PBS.
8. 70 % alcohol for receiving tissue transfers from fixative.
9. Dry ice and liquid nitrogen.
10. Tissue cassettes and cryomolds (Tissue-Tek).
11. Parafilm and aluminum foil for wrapping cryopreserved tissue sections.
12. Ultralow freezer for preservation of OCT and frozen tissues.

2.3 Histology/Immunohistochemistry

1. Chemical safety hood.
2. Microtome and blades.
3. Slides, slide covers, Permount.
4. Water bath, pressure cooker, and/or steamer.
5. Graded alcohols.
6. Histomorphometric software such as ImageJ (NIH) or Image-Pro Plus software22 (Media Cybernetics, Inc).

2.4 Immunohistochemistry (Frozen Sections)

1. Cryostat (−20 °C) for cutting 7–10 μm frozen sections from OCT embedded tissues.
2. Slides prepared for frozen sections: VECTABOND (Vector Laboratories)-coated slides or Fisher-Plus (Fisher Scientific) slides.
3. Solvents, acetone.
4. Appropriate primary and secondary antibodies and detection reagents.
5. Automation buffer (Tris buffered saline with non-ionic detergent, pH 7.4) commercially available (Genetex and other vendors).
6. PBS buffer (0.1 M phosphate, pH 7.4).
7. Monoclonal antibodies (MAb) to specific targets. MAbs that have been successfully used to detect cell type specific antigens in frozen sections of macaque tissue include: mouse anti-human CD4 (1:100 L200 clone, BD Pharmingen), mouse anti-human CD8 (1:10 RPA-T8 clone, BD Pharmingen), mouse anti-human Ham56 (1:1 Ham56, DAKO), and mouse anti-human CD68 (1:100 Y1/82A clone, BD Pharmingen).
8. Biotinylated secondary antibodies (Vector Laboratories, BioGenex, Inc., Southern Biotechnology), streptavidin-alkaline phosphatase and Vector Red substrate (VECTASTAIN-AP, Vector Laboratories). If sensitivity is an issue, horseradish peroxidase (HRP) conjugated secondary Ab and reagents can be used (VECTASTAIN Elite ABC, Vector Laboratories).
9. 10 mM citrate, pH 6.0 for antigen retrieval.

2.5 Biochemical Assessments

1. Chemical safety hood for drying procedures.
2. Oven capable of steady temperatures of up to 110 °C.
3. Vacuum desiccator.
4. 3:1 chloroform–methanol for lipid extraction.
5. Glass test tubes with Teflon coated caps for extractions and acid hydrolysis.
6. Kits or chemicals for assessment of cholesterol, cholesterol ester, hydroxyproline.

3 Methods

3.1 Plasma or Serum Lipids

1. Obtain blood from fasted animals in EDTA vacutainer tubes (for plasma) or red top tubes (for serum).
2. Centrifuge the blood sample at 1100–1300 × *g* for 10 min to separate plasma from circulating cells. Samples may be analyzed within 24 h or frozen at −70 °C for later analysis. Avoid freeze thawing of samples for most evaluations of plasma/serum biomarkers.
3. Use an automated clinical chemistry autoanalyzer to measure total plasma cholesterol, HDL cholesterol, and triglyceride (*see* **Notes 1** and **2**). Alternatively, kits are available for the measurement of plasma lipids from many commercial vendors (e.g., Sigma-Aldrich, Thermo-Fisher).
4. Calculate the concentration of non-HDLc, which approximates the sum of low-density lipoprotein (LDL) cholesterol and very-low-density lipoprotein cholesterol, by subtracting HDLc from TPC.

3.2 Tissue Preparation

Approaches to tissue preparation vary with the primary intent for the use of the tissues and the species being studied. Historical approaches to evaluation of the extent, distribution, and pathologic characteristics of atherosclerotic plaques relied on whole body perfusion of an appropriate fixative at a pressure similar to the mean blood pressure of the species being studied; for monkey this is approximately 100 mmHg. This approach provides quality tissue preservation for histologic evaluation of the whole cardiovascular tree, but is not ideal for studies of the biochemistry or molecular biology and genomics of the tissues. We have largely adapted this approach so that some arterial sites are fixed for histology while others are preserved for molecular approaches by slam freezing, by preserving in RNAlater, by preserving in OCT (*see* below), or other methods. The arteries of the greatest clinical and translational interest are the epicardial coronary arteries (important for coronary heart disease risk), and the carotid bifurcation and

internal carotid arteries because of their association with ischemic changes in brain associated with declining cognitive function and stroke. We have also focused on iliac arteries as a key tissue due to the similarities in atherosclerosis and responses to treatment between the iliac arteries and the coronary arteries. The abdominal aorta can also be evaluated as a key arterial site with propensity for aneurysm development.

1. Only pigs and Old World monkeys share with human beings the anatomical feature of primary epicardial arteries (left main, left circumflex, left anterior descending and right epicardial arteries). The coronary arteries of lower animals (New World monkeys, rabbits, and mice for example) begin branching at the aortic coronary ostia into multiple intramyocardial arteries. For that reason, mouse researchers often use the coronary ostia and subclavian arteries as surrogates of epicardial arteries. For species with epicardial arteries, the heart with its epicardial arteries should be evaluated. For other species, the aortic root, subclavian, innominate arteries, carotids, and aorta may be the focus.
2. Perfusion fixation of the heart and coronary arteries with 10 % neutral buffered formalin (or less commonly 4 % paraformaldehyde) for 1 h at 100 mmHg pressure (the approximate mean arterial pressure for nonhuman primates such as the cynomolgus macaque) helps to maintain the approximate in vivo geometry of the arteries for evaluation (*see* **Note 3**).
3. For the major epicardial coronary arteries, obtain five serial blocks, each 3 mm in length perpendicular to the long axis of the artery. These blocks of tissue will contain the segment of artery along with adjacent epicardial fat and myocardium from each of the left circumflex, the left anterior descending, and the right coronary arteries (Fig. 1).
4. Remove and process other arterial sites such as the aorta, iliac and mesenteric arteries, common carotid arteries, carotid bifurcations, and internal carotid arteries in accordance with desired approaches to analysis. Non-coronary arteries may be cleaned of adherent connective tissues in situ or after removal from the animal.
5. To best evaluate the atherosclerosis in carotid bifurcation and internal carotid arteries, the specimens should be unopened and processed like blocks of coronary artery. We generally take one block of carotid bifurcation and 2–3 blocks of internal carotid artery. For other arterial sites from longer less geometrically complex blood vessels such as aorta, common carotid, or iliac-femoral arteries, we generally open the excised arteries longitudinally, section into desired segments, and fix segments of artery flat on cardboard in 10 % neutral buffered formalin or 4 % paraformaldehyde, freezing other adjacent sections for

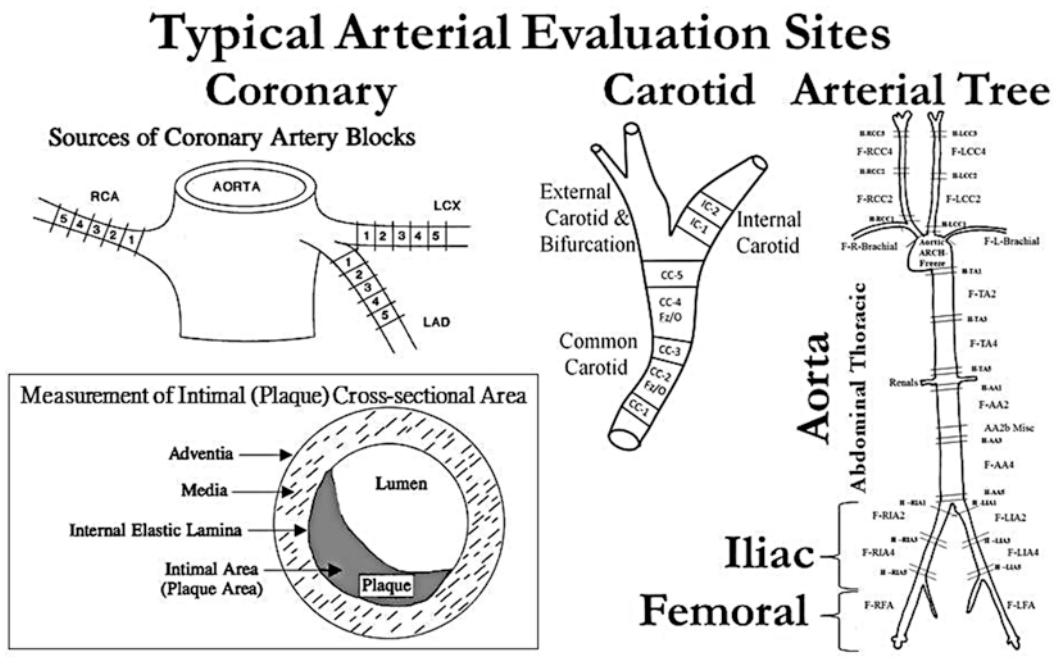

Fig. 1 Diagram of typical arterial regions of interest in the heart (coronary arteries), neck (carotid arteries), and trunk (aorta and iliac arteries) and a representative sampling regimen from an experiment investigating the effects of hormones on atherosclerosis in coronary, central (aorta), and peripheral arteries. In addition, the *inset* contains a description of basic measurements of perfusion fixed coronary artery and atherosclerotic plaques

angiochemical and molecular studies, and other sections in OCT for specialized IHC studies.

6. Subdivide arterial sites into segments in order to preserve arteries for maximal utilization by a broad array of methods. Convention can vary; one approach is to designate one segment for standard fixation for histology, another segment for embedding in OCT for future immunohistochemical or other analyses such as laser capture microdissection (LCM), and then one or more segments for freezing and/or preservation in RNAlater® for angiochemical or molecular/genomic analyses (*see* Figs. 1 and 2).
7. For freezing in OCT, fill a prelabeled plastic cryomold partly with OCT. Remove excess moisture from tissue section by touching on filter paper or a paper towel. Select best orientation for the tissue and place in tissue mold with OCT. Let tissue settle to the bottom of the mold (where sectioning will be initiated). Add OCT to cover tissue sections completely and fill the mold. Hold the mold with a hemostat and dip only the most bottom part into liquid nitrogen. It is important to not submerge. The OCT will turn white as it freezes. Once all of the OCT is frozen, wrap the mold in parafilm and aluminum foil, label, and store at −80 °C until use.

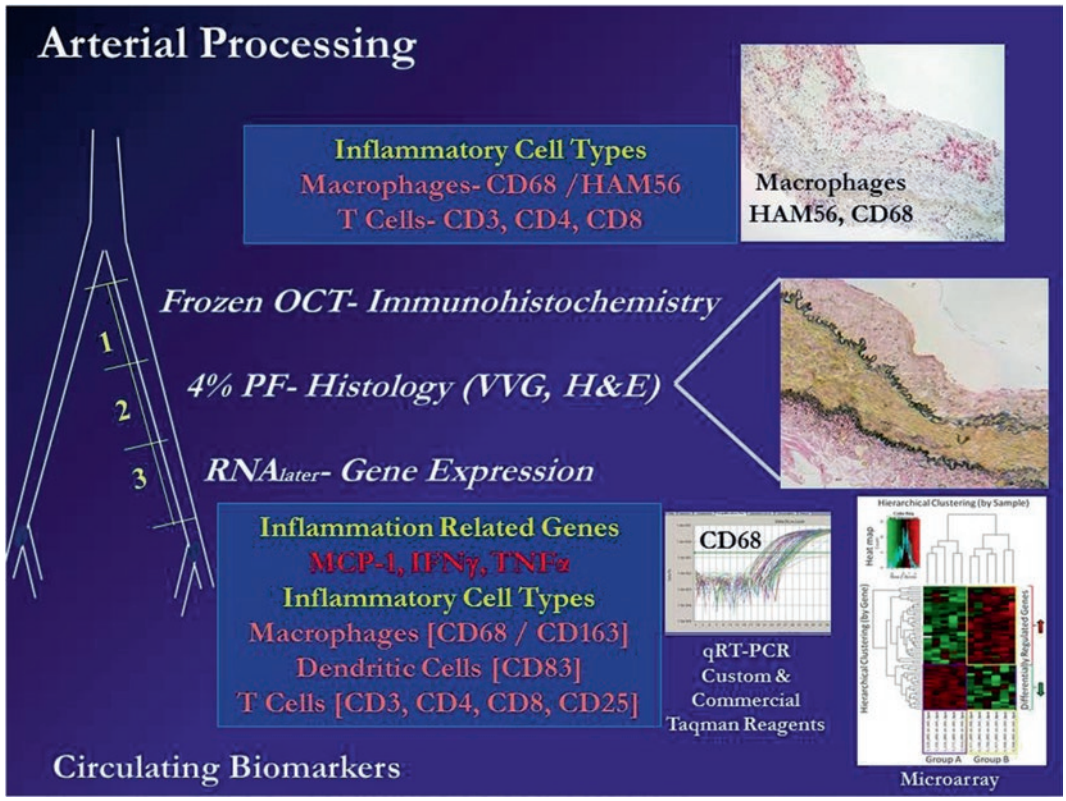

Fig. 2 Diagram of approaches to evaluation of cellular, morphologic, and molecular phenotypes in an iliac artery biopsy or another arterial specimen obtained ante or post mortem in response to diet and interventions. A biopsy of the iliac artery can be obtained surgically and prepared for immunohistochemistry (image 1, cryopreserved in OCT embedding medium), histology (image 2, fixed with 4 % paraformaldehyde, moved to 70 % alcohol, and then embedded in paraffin), and for assessment of gene expression by qRT-PCR, microarray, or RNAseq (images 3 and 4, prepared in RNA_{later}® or slam frozen). *Abbreviations*: *OCT* Optimal Cutting Temperature freezing embedding medium, *VVG* Verhoeff-van Gieson stain, *H and E* hematoxylin and eosin. *Macrophage markers* CD68 and/or HAM56 (monoclonal antibody to human alveolar macrophages), CD163 (*M2 marker* hemoglobin-haptoglobin receptor), *T cell markers* CD3 (T cell lineage), CD4 (T helper cells), CD8 (NK cells), CD25 (Tregs). *Dendritic cells* CD83 [24, 31, 32]. In general, estrogen treatment initiated soon after the loss of estrogen (through ovariectomy) causes a 50–70 % inhibition of diet induced atherosclerotic plaque size in the coronary arteries on monkeys relative to untreated controls. Molecular and cellular responses to estrogen (reduced levels of arterial expression of inflammation and inflammatory cell associated markers) can be detected well before changes in lesion size can be observed; therefore, molecular approaches can significantly reduce sample sizes required for appropriate statistical power to see an effect

3.3 Histology

1. Transition formalin or paraformaldehyde fixed vascular tissues through graded alcohols. Tissues fixed in 4 % paraformaldehyde should be transitioned to 70 % alcohol within 24 h.
2. Embed the tissues in paraffin blocks in the appropriate orientation for later sectioning.
3. Cut 5 μm sections using a microtome. Float sections in a ~45 °C flotation bath to allow appropriate placement on glass slides.

4. Stain sections with Verhoeff-van Gieson (VVG) stain for accurate measurement of the cross-sectional area of the atherosclerotic lesion and other histologic parameters in the artery. VVG allows the identification of the internal and external elastic lamella (stained black), cell nuclei (stained black), and collagen (stained red). Sections can also be stained with hematoxylin and eosin (H and E), which stains cell nuclei blue and cytoplasm and extracellular matrix (ECM) various shades of pink. Mineral appears as blue on H and E, but if mineralization is a target it can be better detected with Von Kossa or Alizarin Red stains.
5. Determine plaque area/extent of atherosclerosis (mm^2) of the artery section as the cross-sectional area of intimal lesions in each of the VVG stained sections of the artery segments (Fig. 1 [6]) by computer assisted histomorphometric methods using software such as ImageJ or Image-Pro Plus software22. For coronary arteries, digitally trace around the areas occupied by lesion, lumen, and media and guide the software to calculate the cross-sectional area. For peripheral arteries that are opened longitudinally, trace the areas of the lesion, lumen, and media as for coronary arteries. Also measure the length of the internal elastic lamella (IEL) of the sections (*see* **Note 4**).
6. Evaluate the length of the *external* elastic lamella (EEL) in arterial sections as a measure of artery size (although this does not appear to provide significant information above that of the IELA). Additional measures include the maximum intimal thickness (MxIT), the minimal intimal thickness (MinIT), and maximum and minimum medial thickness (MxMT, MinMT).
7. Differentiate fatty streaks from plaques by the following rule of thumb which relates to the proportions of lesion size to sublesion media thickness; plaques are defined as sites where the intimal thickness is at least 2× the medial thickness at that site.
8. For the determination of coronary artery atherosclerosis, make measurements using 15 blocks (each 3 mm in length) cut perpendicular to the long axis of the arteries, five serial blocks each from the left circumflex, the left anterior descending, and the right coronary arteries. From those sections each arterial site can be evaluated independently, or the plaque size can be averaged across some or all coronary artery beds to obtain mean coronary artery atherosclerosis. For the iliac arteries, a biopsy of one common iliac artery may be obtained to establish baseline measures before randomization to treatment and the contralateral iliac artery taken at necropsy for terminal measures [32, 36, 37]. Five micrometer sections from one to five blocks have generally been used to assess atherosclerosis in the iliac arteries (*see* **Note 5**). Carotid artery atherosclerosis can be assessed in both

the left and right common carotid arteries (three blocks each), carotid bifurcations (one block each), and left and right internal carotid arteries (two blocks each) (Fig. 1).

9. To evaluate plaque complications histologically, take individual sections from blocks representative of the length of a specific artery site. For example, prepare four sections from each of five blocks of the three coronary arteries to be stained by VVG or H and E staining (described above) to provide a reasonable representation of the lesion composition in that artery.
10. Stain the sections with H and E and adjacent sections with VVG stains [7].
11. Count the density of leukocytes that have accumulated both within the adventitia and within the intimal plaque as an estimation of the degree of inflammation associated with the atherosclerotic plaque. Establish a subjective scoring system of 0–4 for the laboratory or for an experimental dataset, in which 4 is assigned to the most extensively involved of those cases within the data set.
12. Estimate atheronecrosis as a percent of the cross-sectional area of the plaque.
13. Measure the fibro-muscular caps overlying the plaques histomorphometrically at points of maximum and minimum thickness.
14. Use trichrome reagent to stain for collagen, which can be estimated using color based segmentation of slides with ImageJ or ImageProPlus.

3.4 Immunohistochemistry

Immunohistochemical staining can be performed on fixed tissue embedded in paraffin or OCT-embedded tissues.

3.4.1 Fixed Tissues

1. For immunostaining of fixed tissues, cut 5 μm sections from the arterial block of interest.
2. Mount the sections on Superfrost Plus slides.
3. Deparaffinize the sections in xylenes.
4. Rehydrate the sections in graded alcohols.
5. To perform antigen retrieval, place the slides in 10 mM citrate (pH 6.0) in a 95–100 °C steamer, water bath, or pressure cooker for 20–30 min to expose epitopes of interest (*see* **Note 6**).

3.4.2 Frozen Tissues

1. Cut sections at 5–10 μm thickness on a Cryostat at the appropriate temperature. Generally the temperature should be close to −15 to −20 °C ±5 °C; colder temperatures may crack the OCT block.
2. Dry the slides overnight at room temperature in a vacuum desiccator.

3. Store the slides at –20 °C in a desiccated slide box inside a sealed zip-lock bag.
4. To prepare for immunohistochemical staining, bring the sections to room temperature while still in the sealed and desiccated bag to prevent condensation on tissue sections.
5. Fix the sections in cold acetone for 10 min, transfer to deionized water.

3.4.3 Staining of Fixed or Frozen Sections

1. Preincubate tissue sections with a blocking agent (normal serum or a protein solution) for 30 min at room temperature to prevent nonspecific Ab binding and reduce background noise.
2. Detect the epitopes of interest using commercially available primary monoclonal antibodies and associated reagents. Incubate the slides in primary antibody for 1.5 h at room temperature or overnight at 4 °C then wash with cold PBS or TBS buffer for 5 min.
3. To localize the primary antibodies, incubate tissue sections with appropriate biotinylated secondary antibody for 20 min at room temperature then wash with cold PBS or TBS buffer for 5 min.
4. Incubate sections with streptavidin-alkaline phosphatase for 20 min at room temperature, then wash with cold PBS or TBS buffer for 5 min.
5. Incubate sections with Vector Red substrate chromogen for 3 min (or until desired staining intensity is obtained) at room temperature then wash with cold PBS or TBS buffer (*see* **Note 7**).
6. Counterstain the sections with Mayer's Hematoxylin or an alternative counterstain and examine by light microscopy.
7. Capture images with a motorized stage and tiling software (Image-Pro Plus) or obtain digital images at a core facility equipped with appropriate digital image capture equipment.
8. Quantify immunohistochemical cell staining using computer assisted morphometry (Image-Pro Plus Software, Media Cybernetics, Inc. Silver Springs, MD). A grid filter (in this case 8.5 μm^2) can be applied to digitize images and each crosshatch within the intima evaluated for positive or negative staining. Contrast and color settings should be kept constant for all slides. Each point of intersection can then be evaluated according to staining character and location. Location should be defined as intima, media, or adventitia, and staining character classified as positive if immunostained or negative if not immunostained. Express staining as the percentage of intima occupied by positive cell staining, or as a percentage of total cells (nuclei), depending on the staining pattern observed.

3.5 Biochemical Assessments

Arteries can be assessed biochemically for content of lipids, calcium, collagen, and elastin [19]. Likewise, content of specific proteins can be assessed by Western blotting or proteomic approaches and RNA content can be examined by real time RT-PCR, microarray analysis, or RNAseq. These protein and RNA technologies are beyond the scope of this manuscript, but each hold the promise of providing additional insights into broad effects of estradiol and other endocrine modulators on cardiovascular and other diseases.

3.5.1 Arterial Lipid Composition

1. Pin tissue segments that will be used for chemical composition studies flat on a dissection board and photograph them for subsequent determination of lumen surface area.
2. Blot the surface of the tissue to remove surface liquid and obtain the wet weight of the tissue. Sections may then be extracted directly or frozen for future analysis.
3. Extract lipids from tissues with 20 volumes (vol/wt) of chloroform-methanol (3:1, vol/vol).
4. Rinse the tissues with additional chloroform: methanol and pool the extracts.
5. Determine the lipid free dry weight (LFDW) of the tissue by drying *in vacuo* until consistent weights are achieved.
6. Tissue cholesterol content (free and esterified) can be determined in the lipid extracts by the method of Rudel and Morris [38]. Kits employing this methodology are available commercially from Sigma-Aldrich (MAK043-1KT) and other vendors.

3.5.2 Arterial Calcium Content

1. Take the lipid-free dry artery from **step 4** above and incubate it in 0.1 M HCl at 4 °C for 7 days to rehydrate and decalcify the tissue.
2. Determine the calcium content of the acid extract using Arsenazo III reagent and protocols supplied with Roche Reagents' "Reagent for calcium" (Roche Diagnostic Systems) using a microtiter plate reader at 630 nm with background correction at 450 nm.
3. Prepare calcium standards and assay them under the same conditions as the samples (in 0.1 M HCl).
4. Dry the decalcified, delipidated tissue to a constant (reproducible) weight (decalcified LFDW).

3.5.3 Aortic Collagen and Elastin Content

1. Take the decalcified tissues from the above steps and rehydrate them in deionized water at 4 °C for several days.
2. Solubilize the collagen from the tissue by hot alkali extraction at 98 °C in 0.1 N NaOH for 50 min in a shaking water bath [39].

3. Separate insoluble material (elastin) from the soluble collagen by centrifugation at 1100 × *g* for 30 min.
4. Wash with 0.1 N NaOH and pool with original extract, and then with deionized water to remove salts, centrifuging between each wash as described above.
5. Dry insoluble material under vacuum to constant mass. This will give you arterial elastin content.
6. Acid hydrolyze the extracted collagen fraction in 6 M HCl at 110 °C for 17 h to produce free amino acids.
7. Determine the hydroxyproline content using an appropriate method such as that of Bergman and Loxley [40] to estimate collagen content. Kits employing this methodology are available commercially from Sigma-Aldrich (MAK008-1KT) and other vendors.

3.5.4 Expression of Data

Express angiochemical measurements on a concentration basis (milligrams per gram of wet or lipid-free dry weight) and on an area basis (mg/cm^2 of lumen) (*see* **Note 8**).

3.6 Statistical Analysis

All data should be assessed for normality and transformed if necessary. Generally atherosclerosis data is not normally distributed and requires transformation prior to analysis. Since there are frequently animals which have no detectable lesion (0), we add a constant, usually 1, to the intimal area and take the log of that [log(Intimal Area + 1)].

4 Notes

1. HDL cholesterol concentrations can also be determined using the heparin-manganese precipitation procedure reported [41] as described in detail in the Manual of Laboratory Operations of the Lipid Research Clinics Program (1974). For cynomolgus macaques, the protocol should be modified to include the use of 2 M $MnCl_2$ rather than the 1 M $MnCl_2$ originally suggested for the Lipid Research Clinics. This modification facilitates the complete precipitation of low density lipoproteins, portions of which are resistant to precipitation in certain hyperlipoproteinemic monkeys when 1 M $MnCl_2$ is used. The use of 2 M $MnCl_2$ does not result in loss of HDL.
2. Cholesterol, HDLc, and triglycerides for the NHP work are standardized to calibrated controls from the Centers for Disease Control and Prevention-National Institutes of Health Lipid Standardization Program using reagents from Soloman Park Research Laboratory (Kirkland, WA) in conjunction with Northwest Lipid Metabolism and Diabetes Research

Laboratories (NWLMDRL) at the University of Washington (Seattle) [42]. Intra- and inter-assay coefficients of variation should be less than 5 %.

3. For pressure perfusion fixation, we have developed special perfusion chambers which can be pressure regulated with applied air pressure. Alternatives include elevation of the perfusion buffers to a level above the tissue perfused which produces the desired perfusion pressure (for 10 % formalin at 100 mmHg, this is approximately 132 mm above the heart).
4. Artery size in longitudinally opened segments of peripheral arteries can be estimated by using the IEL length (IELL) as a circumference for mathematical derivation of the area encompassed by the IEL, the IEL area: $\text{IELA} = \text{IELL}2/(4 \times \pi)$. This is an estimate, as distortion of the actual size of the arterial segment may occur when arteries are fixed without the perfusion fixation required to preserve arterial in situ shape and size. Lumen area can then be estimated by subtracting the intimal area from the IELA.
5. In the nonhuman primate, the common iliac arterial site has been shown to have similar plaque sizes in the left and right arteries ($r = 0.97$) and to be highly associated with coronary artery plaque extent ($r = 0.86$) [6].
6. Antigen retrieval methods are required for many targets and may need to be specialized depending on the antigen of interest. For citrate buffer antigen retrieval, *see* http://www.ihcworld.com/_protocols/epitope_retrieval/citrate_buffer.htm.
7. Alternatively, for difficult to detect antigens one can use streptavidin-horseradish peroxidase and DAB substrate for immunohistochemical detection.
8. In general, the use of wet weight of the tissue tends to underestimate amounts of a component per unit tissue, as increases in lipid and cell contents of the atherosclerotic aorta increase the wet weight of the tissue. The weight of the tissue after lipid extraction (LFDW, in grams) provides a more accurate estimate of the unit of tissue. However, tissue weight is dependent on the amount of connective tissue in the artery which is altered in response to atherogenesis as well as regression of atherosclerosis. Both of these lead to variable accumulation and loss of specific ECM components. The surface area of a tissue section (mm^2) is less likely to be altered by chemical changes but is affected by vascular remodeling (shrinkage or enlargement) and is less accurately measured, especially in tissue that has been previously frozen.

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Chapter 41

Assessing Direct Vascular Actions of Estrogens

Douglas S. Martin and Kathleen M. Eyster

Abstract

Estrogens are known to affect vascular function. In order to decipher the underlying mechanisms, it is essential to study the direct actions of estrogenic substances on blood vessels. There are two widely used approaches to assess the effects of estrogenic substances directly on blood vessels, the isolated perfused intact mesenteric vascular bed (McGregor preparation) and the isolated perfused/pressurized vessel approach. The McGregor preparation relies on constant flow with vascular reactivity assessed as changes in perfusion pressure. The isolated perfused/pressurized vessel approach uses a single vessel mounted on glass micropipettes. The main readout in this approach is vascular diameter. This chapter describes these approaches which remain cornerstones in the investigation of direct vascular actions of estrogenic substances.

Key words Isolated perfusedmesentery, Isolated perfused/pressurized vessel, Vascular response, Perfusion pressure, Vascular diameter

1 Introduction

The vasculature expresses genomic estrogen receptors [1–3] as well as non-genomic estrogen receptors [1, 2, 4]. Consequently the vasculature is recognized as an important target of estrogen and phytoestrogen action [5, 6]. Nonetheless, the actions of estrogenic compounds in the cardiovascular system remain unclear, complex, and sometimes contradictory [5, 7, 8]. The effects of estrogenic substances on the vasculature represent an intricate interaction between the endothelium, vascular smooth muscle and neural inputs which may not be fully captured in approaches such as cell culture. Moreover, these interactions may be dynamic and affected by physical factors such as pressure and flow that are difficult to mimic in isolated cell systems. Conversely, study of estrogenic effects in whole animal systems represents the aggregate effects on vasculature and other cardiovascular control systems such as the kidney, heart, and neurohumoral control mechanisms. Accordingly, ex-vivo whole organ or isolated blood vessel systems play critical roles in deciphering the direct influences of estrogenic

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substances in the vasculature. This chapter describes two such protocols for measuring the vasoactive effects of estrogenic compounds.

The first protocol describes the isolated perfused mesenteric vascular tree as described by McGregor [9] and since used extensively to assess estrogenic modulation of vascular bed responses [10–13]. In this system, the flow of the perfusate is maintained at a constant rate; therefore, perfusion pressure can be used as a direct index of changes in mesenteric vascular resistance. Since the entire vascular bed is perfused, the overall behavior of the vascular bed can be assessed. Moreover, basal perfusion pressure in this system represents the intrinsic state of contraction of the vasculature which may be affected by both active smooth muscle changes and passive structural changes.

The second protocol describes the isolated perfused/pressurized single vessel preparation, sometimes referred to as the pressurized arteriograph. In this preparation a single blood vessel from a target organ of interest is mounted on glass micropipettes and imaged. This system allows control of both flow and perfusion pressure. Consequently, very accurate flow–pressure, flow–diameter, and pressure–diameter relationships, as well as concentration-diameter relationships, can be evaluated allowing assessment of both vascular reactivity and vascular diameter/vascular structure changes. This system has been used to assess the effects of estrogenic compounds on vascular responses to pharmacological [14, 15] as well as physical stimuli [16, 17].

Both systems allow the assessment of long term genomic as well as short term non-genomic vascular responses to estrogens and their effects on vascular responses to other pharmacological (vasodilators, vasoconstrictors) or to physical (e.g., shear stress) challenges. Moreover, both systems are applicable to animals of different species, sizes and sexes by merely changing the sizes of attachments to the system. Accordingly these systems are ideal for the comprehensive assessment of direct effects of estrogens on vascular function.

2 Materials

2.1 Materials for the Perfused Mesenteric Vascular Bed Protocol

1. Animals: 4-week-old female rats (Sprague Dawley or strain of choice) that have been gonadectomized and treated with vehicle or estrogenic compounds (*see* **Notes 1** and **2**).
2. Isoflurane anesthetic (3 % isoflurane in 100 % oxygen), isoflurane vaporizer unit, oxygen source, and scavenging unit (*see* **Note 3**).
3. Estrogens and Selective Estrogen Receptor Modulators: vehicle for gavage treatment (20 % 2-hydroxypropyl-β-cyclodextrin), ethinyl estradiol (0.15 mg/kg), estradiol benzoate (0.15 mg/kg), tamoxifen (3 mg/kg), raloxifene (3 mg/kg).

4. Dissecting instruments, suture (3-0 silk or other similar strong suture).
5. Polyethylene cannula (PE 50), normal saline, polyethylene T connectors, heated water bath, perfusate reservoir (4 L beaker or Erlenmeyer flask)
6. Low volume transducer (e.g., Ohmeda P23XL) connected to a computer and software for acquisition of blood pressure and heart rate (e.g., Acknowledge software, BIOPAC Corporation).
7. Perfusate buffer (modified Krebs-Henseleit): 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 2.6 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.1 mM glucose, pH 7.4, oxygenated with 95 % O_2–5 % CO_2 gas.
8. Vasoconstrictors: potassium chloride (KCl, 25–125 mM), norepinephrine (NE, 0.1–100 nmoL), serotonin (5-HT, 0.1–30 nmoL), methoxamine (MTX, 75 mM), dissolved in Krebs-Henseleit.
9. Vasodilators: acetylcholine (ACh, 1–3000 pmoL), papaverine (PPV, 0.01–30 nmoL), oxymetazoline (OXY, 0.01–3 nmoL), nitroprusside (SNP, 0.1 nmoL to 10 μmoL) dissolved in Krebs-Henseleit.
10. Components of the isolated perfused mesenteric bed apparatus: Perfusate reservoir (4 L beaker), gas dispersion tube (Pyrex 39533-12C), general connector tubing (Masterflex 6424-16 for the main connecting lines) to connect sections of the apparatus, bubble trap, and injection ports, roller pump tubing with luer lock tips (e.g., Tygon 8-3503; the precise tubing will depend on the desired flow rates and types of roller pumps), 2 roller pumps capable of flow in the 2–10 mL/min range pumps (We use Ismatec 4 channel roller pumps), and a low pressure transducer (e.g., Ohmeda P23XL) linked to a computer with analytical software (Acknowledge Software, BIOPAC corp.) (Fig. 1).

2.2 Materials for the Isolated Perfused/Pressurized Blood Vessel Protocol

1. Animals: 4-week-old female rats as in **item 1** in Subheading 2.1 (*see* **Notes 1** and **2**).
2. Isoflurane anesthesia system as in **item 2** in Subheading 2.1 (*see* **Note 3**).
3. Petri dish (10–14 cm diameter) with bottom covered in wax or silicone (Sylgard) to pin out mesentery for identification and harvest of individual vessels. The petri dish should be deep enough to allow sufficient perfusate to cover the tissue and to be bubbled with gas (95 % O_2, 5 % CO_2).
4. Dissecting pins or 30 gauge needles to pin out the mesentery.
5. Surgical materials: large dissecting scissors, large toothed forceps, eye dressing forceps, fine forceps with no teeth or

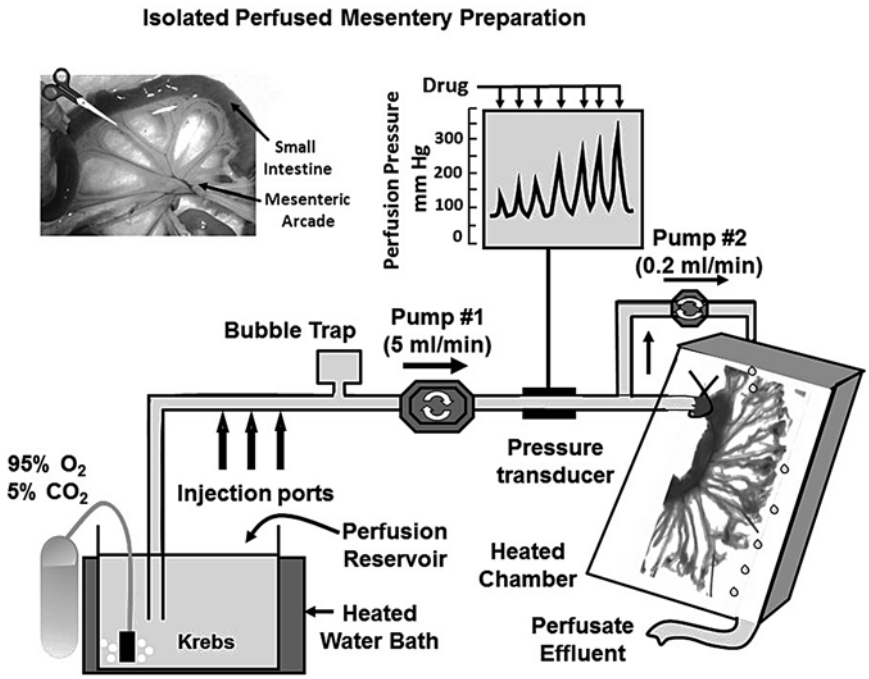

Fig. 1 This schematic illustrates the basic features of the isolated perfused mesentery preparation. The superior mesenteric artery is cannulated and the entire mesenteric vascular tree is removed beginning at the superior mesenteric artery and ending at the intestinal border. The cannula is connected to a perfusion apparatus that consists of a pump that continuously draws oxygenated physiological salt solution (perfusate buffer) which is continuously oxygenated and pumps through the mesentery at a constant rate. Upstream from the pump are injection ports for delivery of drugs; since they are upstream of the pump, injections do not affect flow or pressures in the system. Downstream from the pump is a pressure transducer that measures perfusion pressure. Since flow is held constant in this preparation, changes in perfusion pressure directly reflect changes in arteriolar resistance in the mesentery. This is a flow through system so perfusate drains off the mesentery and is not recycled. Dose-response curves for arteriolar reactivity are constructed by injecting increasing doses of drugs into the injection ports

serrations (to prevent damage to vessels) (e.g., Dumont #5 forceps), 2×2 gauze, physiological salt solution, fine suture (e.g., 6-0 Vicryl), 70 % ethanol, razor.

6. Microscopy: A good binocular stereo microscope (0.7–30×) for dissection of the mesentery. A second similar microscope with the added capability of video microscopy is needed for the perfused vessel setup (Fig. 2) such as an Olympus SZ-11 stereo microscope coupled to a Hitachi CCD camera (KP-M1U).
7. Glass Pipette Puller (World Precision Instruments PUL-1) capable of accepting micropipette glass of 1–2 mm outer diameters. This will allow pulling glass pipettes of varying sizes (50–300 μm) to match vessel of interest.
8. Flow through Perfusion Chamber: A custom built chamber or a commercially available chamber (e.g., Living Systems model

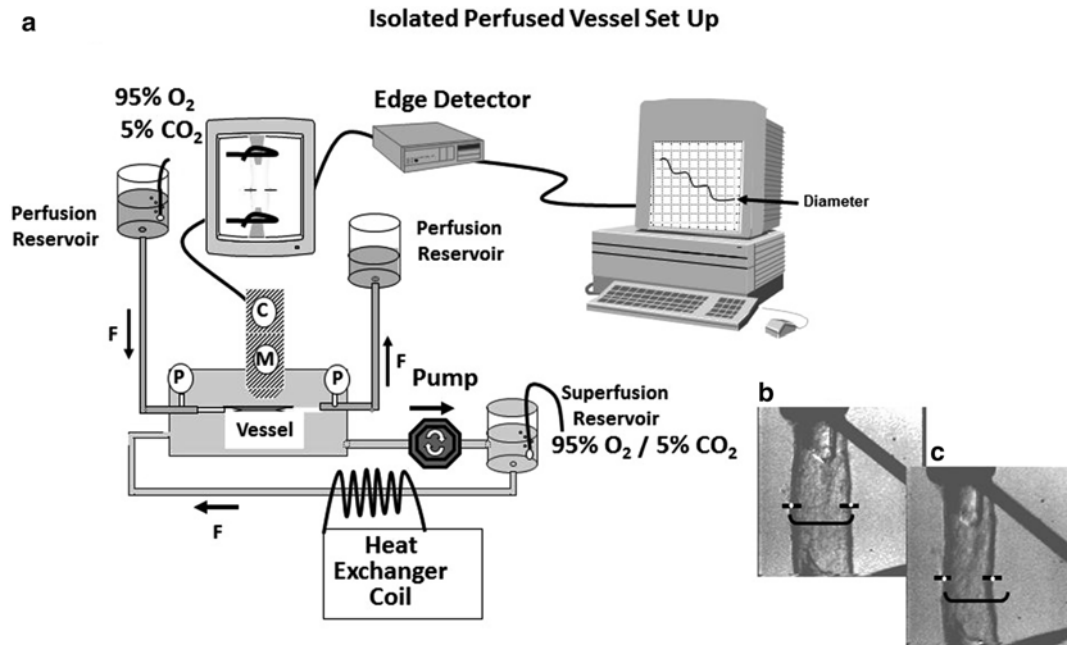

Fig. 2 Panel **a**: This schematic illustrates the basic features of the isolated perfused/pressurized vessel (pressure arteriography) preparation. In this approach, small mesenteric veins or arteries are removed from the mesenteric vascular bed and suspended between two glass micropipettes in a chamber that allows both superfusion and perfusion of the vessel. Superfusion is provided by pumps that constantly circulated oxygenated perfusate through the chamber. Perfusion is provided by adjustable height reservoirs. Flow (F) and pressure (P) are monitored and can be manipulated by changing the heights of the reservoirs. The vessel is imaged via a microscope (M) coupled with a CCD video camera (C). Vessel diameter is tracked by an edge detector. Panels **b** and **c** show representative vessel images under baseline conditions (**b**) and following perfusion with a vasoconstrictor agent (**c**)

CH-1-Lin) can be used. The essential components of the chamber are a bath of sufficient volume for the vessel to be fully covered by perfusate, holders for glass micropipettes, and inflow and outflow tubing to connect to the glass pipettes. The distance between the glass pipette holders should be adjustable with a micrometer to allow fine adjustments of vessel longitudinal tension.

9. Apparatus for superfusion of the vessel: Use a Masterflex roller pump (model 7520) with Masterflex tubing (6424-16) to continually supply the perfusion chamber with oxygenated physiological salt solution as in **item 7** in Subheading 2.1 maintained at 37 °C.
10. Use an in-line heat exchanger (e.g., GHE-LSI Living Systems Instrumentation) to maintain a constant bath temperature.
11. Apparatus for luminal perfusion of the vessel: The perfusion system consists of two reservoirs (e.g., 50 cc syringes, plungers

removed, attached to height adjustable stands) connected to the glass micropipettes via tubing (Tygon or Masterflex). Commercially available systems use servo controlled pumps to regulate flow and pressure. However, it is equally feasible to regulate flow and pressure by adjusting the heights of the inflow and outflow reservoirs while measuring inflow and outflow pressure and flow through the vessel. Place in-line pressure transducers (e.g., APT300 Pressure Transducers) on the inflow and outflow sides of the circuit to monitor pressure. Pressure drop (perfusion pressure) across the vessel (measured as the difference between inflow and outflow pressures) is adjusted by changing the height of the reservoirs (Fig. 2).

12. Measurement and Acquisition of data: Video images of the vessels are displayed on a monitor (Sony PVM 137). The video signal is fed into an edge detector (Crescent Electronics model VED104 or Living Systems model V94). Analog output from the edge detector is captured with an analog-to-digital data acquisition system (e.g., Acknowledge software, BIOPAC Corp.). Although the edge detector output provides vessel diameter data in real time, an analog to digital video capture system is also recommended to save the video images of the vessel. This allows reanalysis of the experiments if needed.
13. Test agents: vasoconstrictors and vasodilators as in **items 8** and **9** in Subheading 2.1. This system is amenable to testing virtually any substance either luminally or abluminally.

3 Methods

3.1 Isolated Perfused Mesenteric Bed Protocol

1. Ovariectomize rats at 4 weeks of age to prevent exposure to the large increase in circulating estrogen that occurs at puberty. Allow 2 weeks of recovery from the surgery, and then begin treatments with vehicle or estrogenic compounds.
2. Treat the animals for 3 weeks or more with vehicle, estrogen, or estrogenic compounds (*see* **Note 4**).
3. After treatment is complete, anesthetize the animals with isoflurane using the anesthesia machine so that the appropriate plane of anesthesia can be maintained.
4. Isolate the left carotid artery and insert a polyethylene cannula containing normal saline.
5. Connect the cannula to a low pressure transducer and allow a 30 min equilibration period.
6. Link the pressure transducer to the computer acquisition system software and collect blood pressure and heart rate data.
7. Attach a 10 mL syringe filled with perfusate buffer to a 20 gauge needle hub connected to a polyethylene (PE 90) cannula.

8. Clear the superior mesenteric artery of connective tissue and nerves for a space of approximately 1–1.5 cm near the aorta.
9. Loop three 3-0 sutures around the artery. Working quickly place traction on the distal suture and tie off the proximal suture.
10. Cannulate the superior mesenteric artery by making a small incision in the mesenteric artery. Introduce the catheter into the artery for a distance of about 1 cm and then tie the catheter in place using both the middle and distal sutures.
11. Separate the mesenteric arcade from the intestine using scissors to cut along the intestinal-mesenteric border.
12. Flush the mesentery with perfusate and quickly transfer the entire arcade to the perfusion apparatus.
13. Remove the uterus and record its wet weight (*see* **Note 5**), as well as any other tissues of interest.
14. Collect a 2 mL blood sample through the arterial catheter or via cardiac puncture.
15. Centrifuge the blood sample and collect and freeze the plasma for later determination of estrogen concentrations or other biochemical assays.
16. Euthanize the animal.
17. Assemble the isolated perfused mesenteric bed device (Fig. 1); make sure the system is prepared before collecting the tissue (**steps 3–12** in Subheading 3.1). Fill the perfusate reservoir with perfusate buffer. Bubble 95 % O_2, 5 % CO_2 through the perfusate buffer in the reservoir. Place the reservoir in a heated water bath adjusted to appropriate temperature so that perfusate temperature is 37 °C when measured at the outflow cannula to which the mesentery will be connected. Connect the perfusate reservoir to Pump #1 with tubing; include injection ports between the beaker and the pump. For each channel, insert a low pressure transducer in line after Pump #1, then connect a T connector with one branch going to Pump #2 and the other branch connected to the superior mesenteric artery (*see* **Note 6**). The line from Pump #1 perfuses the mesenteric arcade (*see* **Note 7**); the line from Pump #2 superfuses the surface of the mesentery and prevents it from drying out.
18. Attach the mesenteric arcade to the isolated perfused mesentery apparatus such that Pump #1 perfuses the mesentery with perfusate buffer at 5 mL/min and Pump #2 superfuses the tissue with the same buffer at 0.2 mL/min. The perfusate will drip from the edges of the mesenteric tree. Allow the tissue to stabilize for 30 min after it is placed in the perfusion circuit. Record basal perfusion pressure.
19. After the stabilization period, challenge the mesenteric vascular bed with a series of vasoconstrictors. Using the injection

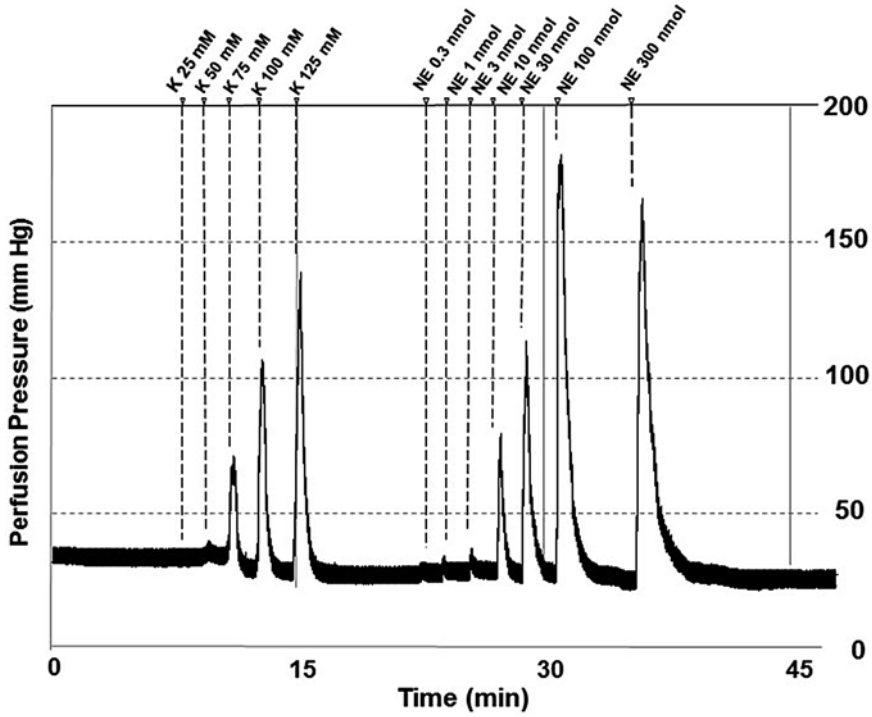

Fig. 3 This chart tracing illustrates the types of responses obtained during stimulation of the perfused mesenteric arterial bed with increasing doses of either potassium chloride (KCl) or norepinephrine (NE). Note that because flow is maintained constant by the perfusion pump, there are large changes in perfusion pressure in response to these constrictor agents. Since flow is held constant, the perfusion pressure responses directly represent changes in vascular resistance

ports, inject the drugs into the perfusion system. Build concentration–response curves for KCl, NE, and 5-HT by injecting increasing concentrations of each drug, waiting 5 min between each concentration. The order of drug injection should be randomized, but low doses should be administered before higher doses. Allow 15 min between different drugs to allow basal perfusion pressure to return to control levels. Record the perfusion pressure after injection of each concentration of drug (*see* **Note 8**) (Fig. 3).

20. To examine the effects of vasodilators, preconstrict the mesenteric vascular tree with a vasoconstrictor agent (e.g., methoxamine or phenylephrine). If this is the first time the vasoconstrictor agent is used, it will be necessary to determine an effective concentration. Inject increasing concentrations of the vasoconstrictor agent until the desired perfusion pressure is achieved (e.g., 80–120 mmHg). Make up perfusate buffer containing that concentration of vasoconstrictor and switch out the baseline perfusate for that containing the vasoconstrictor agent. Allow the vasoconstrictor-treated mesentery to

equilibrate for 30 min. This should result in a stable perfusion pressure in the desired range.

21. Perform concentration-response curves for ACh, PPV, SNP, and OXY by injecting increasing doses at 5 min intervals. Record the perfusion pressure after injection of each concentration of drug. Allow a 15 min equilibration period when switching from one vasodilator to the next, randomize the sequence of drugs, and always treat with lower concentrations of drugs before higher concentrations (*see* **Notes 8–12**).
22. At the end of an experiment it is critically important to thoroughly clean each of the circuits. Glucose present in the perfusate can promote the growth of bacteria or molds that will release toxins and compromise vessel function in subsequent experiments (*see* **Note 13**).

3.2 Isolated Perfused/Pressurized Vessel Protocol

1. Rats should be ovariectomized at 4 weeks of age as in **step 1** in Subheading 3.1 (*see* **Notes 1** and **2**).
2. Treat the animals with vehicle, estrogen or estrogenic substances for 3 weeks or more (*see* **Note 4**).
3. After 3 or more weeks of treatment, anesthetize the animals with isoflurane (3 % isoflurane in 100 % oxygen) using the anesthesia machine so that the appropriate plane of anesthesia can be maintained (*see* **Note 3**).
4. Isolate the left carotid artery and insert a polyethylene cannula containing normal saline. Connect the cannula to a low pressure transducer and allow a 30 min equilibration period.
5. Link the pressure transducer to the computer acquisition system software and collect blood pressure and heart rate data.
6. When hemodynamic data collection is complete, deeply anesthetize the rat.
7. Shave and clean the abdomen with 70 % ethanol. Perform a midline laparotomy to expose the mesentery and gastrointestinal tract.
8. Carefully clear the superior mesenteric artery of connective and nerve tissue near its origin at the aorta. Place two ligatures loosely around the superior mesenteric artery.
9. Fill both the superfusion and perfusion reservoirs with oxygenated perfusate buffer. Bring the perfusate and system to operating temperature (37 °C) and start the flow in both the superfusion and perfusion circuits. Check for bubbles (*see* **Notes 7** and **14**).
10. Ligate the superior mesenteric artery by tying off both sutures with sufficient space between to allow the artery to be cut. Cut the artery and quickly, but carefully, cut along the mesenteric-intestinal border until the mesentery is free.

11. Quickly place the mesentery in a silicone bottomed petri dish containing perfusate and place on ice.
12. Collect and weigh the uterus (*see* **Note 5**) and any other tissues targeted for harvest.
13. Collect a 2 mL blood sample through the arterial catheter or via cardiac puncture.
14. Centrifuge the blood sample and collect and freeze the plasma for later determination of estrogen concentrations or other biochemical assays.
15. Euthanize the rat.
16. Place the petri dish containing the mesentery on the dissecting microscope stage.
17. Using the dissection pins or 30 gauge needles carefully spread out and pin the mesentery to the silicone bottom to display the mesenteric blood vessels. It should be relatively easy to distinguish between arteries and veins at this point (arteries are generally much smaller in diameter).
18. Identify the general area(s) where you wish to harvest vessels (try to pick areas without branches) (*see* **Note 15**). To harvest the blood vessels of interest, carefully use fine dissecting forceps (e.g., Dumont #5) to tease apart the vessel from the surrounding tissue until it is clear of fat, nerve and other vessels for a length of approximately 1.5–2 cm (*see* **Note 16**). Be gentle during this process and especially try to avoid stretching the vessel (*see* **Note 17**). Examine the segment carefully under relatively high power for any small branches or holes in the vessel. Then using fine dissecting scissors (e.g., Vanna scissors) cut the ends of the cleared segment and using fine forceps place the vessel segment into a separate small container of perfusate and place on ice. Repeat these steps until as many vessels as needed (plus a few "spares") are collected. The vessels can remain on ice for several hours and still retain good function. The remaining mesentery can be collected for biochemical assays such as Western blot or real time RT-PCR.
19. Make sure that the perfusion apparatus is functioning and up to operating temperature (37 °C). Stop flow through the glass micropipettes as this will make mounting of the vessel to the inflow side much easier. Tie sutures loosely in place on both glass micropipettes. The suture used is a matter of personal preference; we prefer 6-0 Vicryl. The goal is to achieve a secure seal with the glass micropipette and vessel (*see* **Note 18**). Transfer the vessel from the storage container to the perfusion chamber using forceps. Carefully slide the vessel onto the inflow glass micropipette for a distance of 2–5 mm then secure it in place with the previously placed suture (*see* **Note 19**). Repeat the process for the outflow micropipette. Once the vessel is tied in

place, examine the vessel under pressure. If the vessel is not straight (e.g., shows some bends) use the micro manipulators to move the glass pipettes to place some tension on the vessel. Use only enough tension to make the vessel straight. Establish flow through the vessel, check for leaks and allow the vessel to equilibrate for a minimum of 30 min (*see* **Note 20**).

20. During the equilibration time, adjust the edge detector raster lines to the center of the vessel. Adjust lighting to provide the best contrast between vessel and perfusate and make sure the raster lines "lock" onto the vessel edge (*see* **Note 21**).
21. Following the equilibration time, test vessel viability by perfusing the vessel with high KCl perfusate (e.g., 60 mM). This should cause a marked constriction of the vessel. Conduct this test 3 times to establish consistency. If the vessel fails to respond or is not consistent, replace it with another vessel (*see* **Notes 22** and **23**).
22. After recovery from the KCl challenge, cumulative concentration-response curves to targeted vasoactive agents can be constructed (*see* **Note 12**). We routinely use norepinephrine as a vasoconstrictor (Fig. 4). To test vasodilators, it is

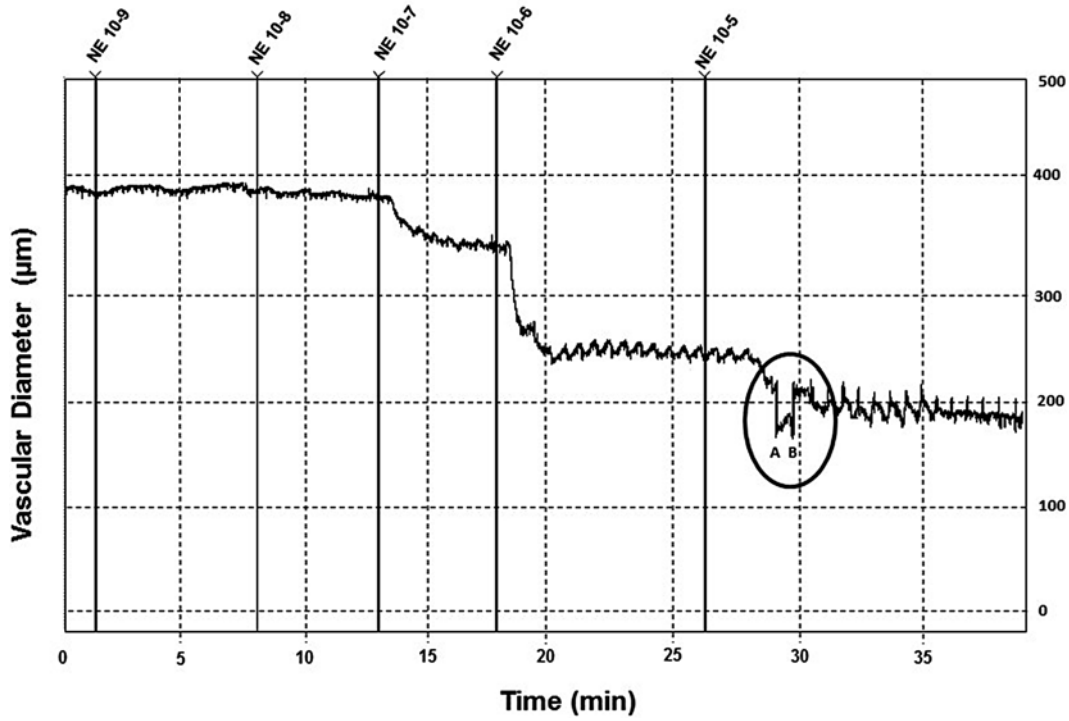

Fig. 4 This figure shows the changes in diameter obtained during stimulation of a larger mesenteric vein with norepinephrine (NE). Note that at higher concentrations of NE vasomotion is observed which appears as oscillations in the diameter. The circled area shows a point (A) where the edge detector lost its "lock" on the vessel. This appeared as a sudden dramatic change in vessel diameter. Reestablishment of the "lock" (B) restored the response to a more normal profile

necessary to preconstrict the vessel. This can be accomplished by superfusing the vessel with norepinephrine, phenylephrine or other vasoconstrictor agent to produce the desired diameter. Since the constrictor is in the superfusate, it is possible to apply vasodilators luminally via perfusion. We routinely use acetylcholine as an endothelium dependent vasodilator and nitroprusside as an endothelium independent vasodilator. We prefer to construct cumulative concentration curves wherein the vessel is allowed to achieve a plateau response to the first concentration, and then the solution is changed to the next highest concentration without washout or return of the vessel to baseline diameter. Perform a washout with perfusate buffer. The time of washout will vary depending on the agent; however, it is advisable to allow the vessel to recover to baseline diameter between different drugs. If determination of classic pharmacological parameters (e.g., Effective Dose 50 % (ED50) or maximum attainable response (Emax)) is a goal, it is essential to choose a concentration range that spans from below threshold to at least one or two concentrations above the maximal response of the vessel (*see* **Notes 24** and **25**).

23. At the end of an experiment it is critically important to thoroughly clean each of the circuits. Glucose present in the perfusate can promote the growth of bacteria or molds that will release toxins and compromise vessel function in subsequent experiments (*see* **Note 13**).

4 Notes

1. Carefully consider the animals' diet. In many cases suppliers will use the most economical source of protein available. In some cases this may be soy protein or other protein sources that may contain substantial amounts of phytoestrogens which may affect the variables being studied.
2. All animal use must be performed in accordance with ethical standards that have been established by the appropriate body (e.g., the National Institutes of Health Council on Animal Care in the US) and approved by the appropriate Animal Care and Use Committee at the user's institution.
3. Gaseous anesthesia with isoflurane is convenient. However, most forms of anesthesia are acceptable for this application. It is important to note, however, that if the mesentery is the vascular bed of interest, intraperitoneal injection of anesthetics (a frequent route of administration) may affect the mesentery due to physical damage (e.g., needle pierce) or exposure to concentrated vehicle or drug solutions (e.g., pentobarbital is dissolved in ethanol and polyethylene glycol).

4. Estrogen and estrogenic compounds can be delivered by various approaches. To simulate long-term oral administration in humans, treat the animals by gavage at the same time daily for 3 weeks with vehicle or estrogenic compounds (ethinyl estradiol, estradiol benzoate, tamoxifen, raloxifene). The 3 week treatment allows time for vascular remodeling that may occur in response to the treatment to develop. To produce sustained blood levels that can mimic depot injections, the rats can be treated with subcutaneously implanted slow release pellets. These can be obtained commercially (Innovative Research of America) or can be made by packing silicone (Silastic) tubing with estrogen [18].
5. In an ovariectomized animal, the uterus will atrophy. Estrogen treatment of ovariectomized animals will increase the uterine wet weight significantly over that of vehicle-treated control ovariectomized animals. Failure of uterine growth in response to estrogen treatment indicates failure of the treatment protocol so uterine wet weight is a useful bioassay for the efficacy of estrogen treatment.
6. This method is applicable to animals of different species, sizes and sexes. For the McGregor preparation, the primary consideration is the size of the main feeder vessel in the vasculature under study. The method can be adapted to accommodate different sizes of vasculature by changing the sizes of the polyethylene tubing used to cannulate the main feeder artery. Also, it is critical to match the pump flow rate with the known flow rate of the vascular bed or vessel under study.
7. Bubbles that pass through the vasculature will compromise the function of the endothelial cells with consequent changes in vascular reactivity. It is critical to ensure that the entire perfusion system (tubing, cannulae, connectors) is free of bubbles, thus a bubble trap is an integral part of the perfused tissue system. Bubbles can form spontaneously as the perfusate is warmed from room temperature to 37 °C. Therefore fill the system early and allow it to run periodically until the complete system is free of bubbles and has reached the appropriate operating temperature (37 °C).
8. Each concentration of NE or 5-HT can be injected into the perfusion system as a bolus of 100 μL; however, the KCl should be infused into the system over a 30 s time span. Similarly, each concentration of ACh, PPV, or OXY can be administered as a 100 μL bolus. It is important to maintain the temperature of the perfusate and mesentery near 37 °C since temperature can have a marked effect on vascular reactivity [19].
9. In interpreting the responses it is best to focus on the plateau response to the drug injection rather than the initial transient response.

10. This is a pass through system, so with only small modifications to the perfusion chamber it is possible to collect the effluent from the mesentery for analysis of substances released from the tissue. For example, analysis of catecholamine release in response to tyramine injections can be measured in the perfusate.
11. While this approach is primarily used to assess the effects of pharmacological agents, it is also possible to employ field stimulation in this setting to assess neurally mediated responses.
12. Non-genomic effects of estrogens can be assessed by including estrogen or estrogenic compounds in the perfusate.
13. Bacterial or mold growth can release toxins that can alter vascular function, thus it is essential to thoroughly clean the circuits between uses. There are several procedures available for this. We recommend that the system be run (in the absence of any tissue or vessel) with distilled water for 5–10 min using pass through mode so that none of the perfusate is recirculated. Follow this with perfusion of an acetic acid solution (10 %) for 5–10 min. Stop the perfusion for another 5–10 min, and then flush the system with sterile water for 15–20 min. Finally, leave the system filled with sterile water.
14. Pay particular attention to bubbles that may appear in the joints and the glass micropipettes of the apparatus. Conversely, bubbles can be used to advantage in the study of endothelial mediated responses. A bubble (1–2 mL) can be injected to pass through the vessel to effectively impair endothelial function. The integrity of the endothelium and endothelial function can be assessed by recording the response to administration of acetylcholine which should produce an increase in diameter if the endothelium remains intact.
15. This method is applicable to animals of different species, sizes and sexes. For the isolated vessel preparation, the primary variable is the size of the vessel to be isolated. It is important to match the size of the glass pipette tips to the size of the vessel and anticipated flow rates. From a practical standpoint the lower limit of vessel diameter is in the range of 40–50 μm. Also, it is critical to match the pump flow rate with the known flow rate of the vascular bed or vessel under study.
16. Place the petri dish on a bed of ice while cleaning and isolating the vessels as cooling the mesentery facilitates fat removal from the vessels.
17. Speed is important; work quickly but carefully. During the vessel cleaning process, be careful not to apply undue stress to the vessels. This can damage the vascular smooth muscle with consequent effects on vascular reactivity.
18. It is important to match the size of the pipettes to the vessel diameter. While smaller pipettes allow easier mounting of the

blood vessel, they may introduce an artificial fixed resistance which will affect the intraluminal pressure experienced by the vessel.

19. Mount the vessel to the inflow cannula first and briefly start flow through the vessel. This will open the outflow side of the vessel and make mounting to the outflow pipette easier.
20. It is important to check for leaks in the vessel before starting an experiment; vessels must not be leaky. Leaks may come from small branch vessels or may be introduced during vessel preparation. During the preparatory and mounting stages, handle the vessels only by the ends, never by the mid sections. Leaks can usually be detected by clamping both the inflow and outflow tracts for the vessel and then observing the pressure or diameter in the vessel. If the pressure or diameter gradually decreases during stop flow, there is a leak.
21. The edge detectors used to measure vessel diameter rely on the change in contrast between the vessel (dark) and perfusate (light). In some circumstances the contrast of the image may change sufficiently to cause the edge detector raster lines to lose their "lock" on the edge of the vessel. This will show up as a very sudden sharp change in the diameter readout (Fig. 4). If vessel diameter readout is seen to change very rapidly and unexpectedly (especially if this does not correspond to the vessel image), check to make sure that the raster lines are still locked on the edge of the vessel.
22. At the outset of an experiment, vessel viability can be tested by depolarizing the vascular smooth muscle with KCl. Similarly, the general health of the vessel can be assessed during long experiments by periodically rechecking the response to KCl. The caveat to this test is it may not be valid if the manipulations used might be expected to affect potassium channel function.
23. When initially setting up the system, it is useful to estimate the time lag for delivery of substances to the vessel. This can be done using dye front or simply an air bubble in the perfusion line. Recording the time required to move dye or a bubble from the perfusion reservoir to the inflow pipette (with no vessel in place) can then be used as a rough time lag for the delivery of the drug to the vessel. This is useful when constructing concentration response curves where the initial concentrations may produce little or only minimal vessel response. Assuming the length of the circuit does not change, once this time lag is known it should remain consistent.
24. Abnormal behavior of the vessel (e.g., sudden loss of contraction or spontaneous vasomotion) can signal an improper temperature, pH or oxygen/carbon dioxide balance. These values should be checked immediately if abnormal vessel behavior is encountered. Alternatively a bubble may have passed though

the vessel unnoticed. The integrity of the endothelium can be tested with an Ach challenge.

25. Norepinephrine is quite prone to oxidization. We routinely incorporate ascorbic acid (100 μM) into our norepinephrine solutions to prevent oxidization. However, this approach is not applicable to all situations. For example if one was interested in reactive oxygen species, inclusion of ascorbic acid might introduce a confounder.

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Chapter 42

Molecular Design, Synthesis, and Evaluation of SNIPER(ER) That Induces Proteasomal Degradation of ERα

Keiichiro Okuhira, Yosuke Demizu, Takayuki Hattori, Nobumichi Ohoka, Norihito Shibata, Masaaki Kurihara, and Mikihiko Naito

Abstract

Manipulation of protein stability using small molecules has a great potential for both basic research and clinical therapy. Based on our protein knockdown technology, we recently developed a novel small molecule SNIPER(ER) that targets the estrogen receptor alpha (ERα) for degradation via the ubiquitin–proteasome system. This chapter describes the design and synthesis of SNIPER(ER) compounds, and methods for the evaluation of their activity in cellular system.

Key words Estrogenreceptor, SNIPER, Proteinknockdown, Ubiquitin–proteasome system, Cell death

1 Introduction

Temporal and spatial control of cellular protein expression and function using small molecules is a challenge in the field of drug discovery. Recently, we developed a protein knockdown system using a series of hybrid small molecules named SNIPER (*S*pecific and *N*on-genetic *I*AP-dependent *P*rotein*ER*aser), which induces target protein degradation via the ubiquitin-proteasome system (UPS) [1–6]. SNIPER is composed of two distinct ligands that are chemically linked as a single molecule: one is bestatin ((−)-*N*-[(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-l-leucine, BS) that interacts with a ubiquitin ligase cIAP1, and the other is a ligand for the target protein. Accordingly, SNIPER cross-links the target protein and the ubiquitin ligase, thereby inducing cIAP1-mediated ubiquitylation of the target protein (Fig. 1).

Because most primary breast cancers express estrogen receptor α (ERα) and exhibit estrogen-dependent proliferation [7], the inhibition of estrogen signaling by downregulating ERα protein is an attractive strategy to treat breast cancer patients [8–10]. By using 4-hydroxytamoxifen (4-OHT) as an ERα ligand, we developed a

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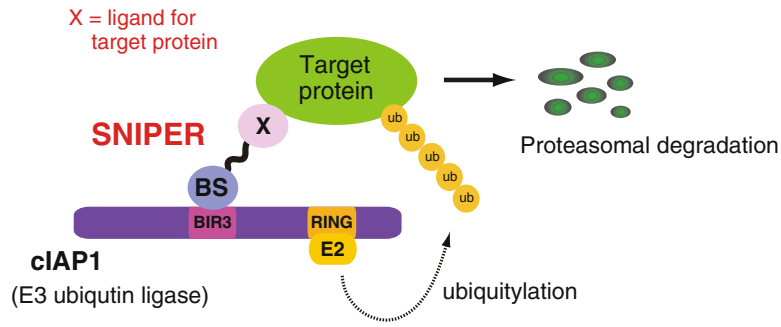

Fig. 1 Scheme of SNIPER(ER)-induced target protein degradation

SNIPER(ER) that targets ERα protein for degradation [11, 12]. The SNIPER(ER) induces degradation of ERα without inducing estrogen-regulated gene expression, and causes rapid cell death in ERα-positive breast cancer MCF-7 cells [12]. In this chapter, we describe the experimental methods used in these studies, especially focusing on the following points: (1) design and synthesis of SNIPER(ER), (2) evaluation of ERα protein degradation, and (3) measurements of cell death in ERα-positive breast cancer cells.

We designed ligated molecules with different lengths of spacers, SNIPER(ER)-1, -2 and -3, which have the ability to induce ubiquitylation and proteasomal degradation of ERα. SNIPER(ER)s are composed of two biologically active scaffolds: one is a tamoxifen derivative binding to the ERα, and the other is a BS molecule binding to cIAP1 to induce ubiquitylation of the ERα. These molecules have been designed based on the basis of the X-ray co-crystal structure (PDB: 3ERT) between 4-hydroxytamoxifen and ERα. Because the dimethylamino moiety of 4-hydroxytamoxifen was exposed on the ERα surface (Fig. 2a, b), the bestatin was ligated to this moiety via an alkyl spacer (Fig. 2c). A scheme for the synthesis of SNIPER(ER)s is shown in Fig. 3.

The protein knockdown activity of SNIPER(ER)s was evaluated by Western blot analysis (Fig. 4a–c). In-depth protocols for Western blotting are described below. Since ERα is activated by endogenous estrogens, it is constantly degraded by the UPS even in normal culture media supplemented with 10 % FBS. When protein knockdown activity is not obvious due to the estrogen-induced degradation of ERα, cells are depleted of estrogens prior to the treatment with SNIPER(ER)s in some experiments. To deplete the endogenous estrogen, cells are precultured more than 4 days in a media supplemented with estrogen-depleted serum (*see* **Note 1**). In this condition, ERα protein expression levels are increased due to the lack of endogenous estrogen, and the SNIPER(ER)-induced downregulation of ERα can be evaluated more easily.

Various assays can be used to evaluate cell viability and cell death. The methods used in our laboratory are described below. Crystal violet staining is used to determine the number of adhesive

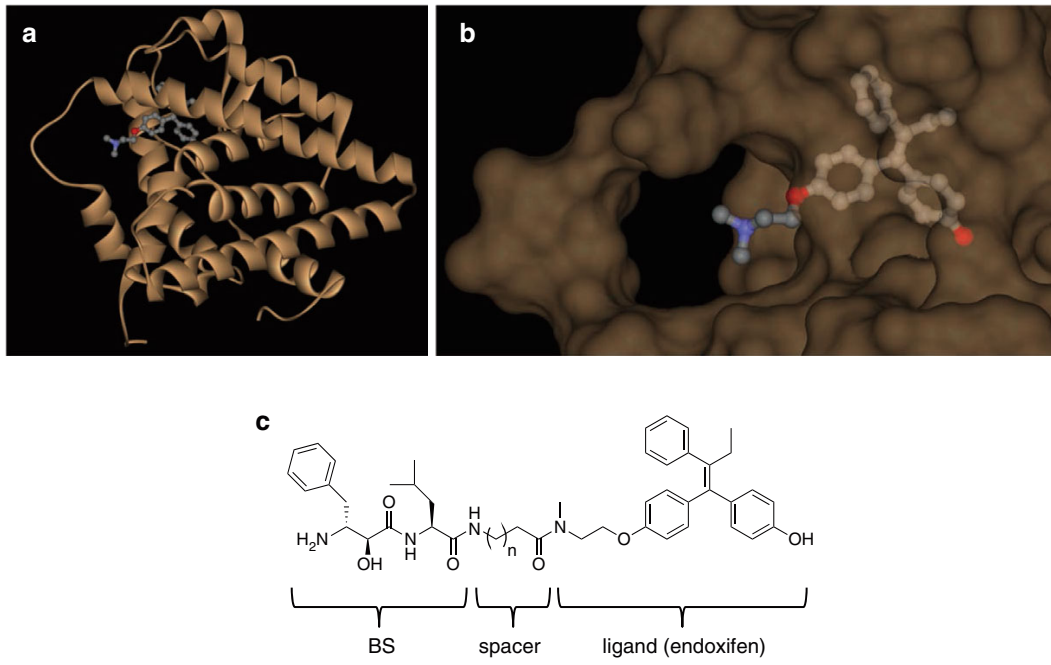

Fig. 2 (**a**, **b**) X-ray structure of the complex formed between 4-hydroxytamoxifen and ERα. (**c**) Design of the ERα degradation inducer

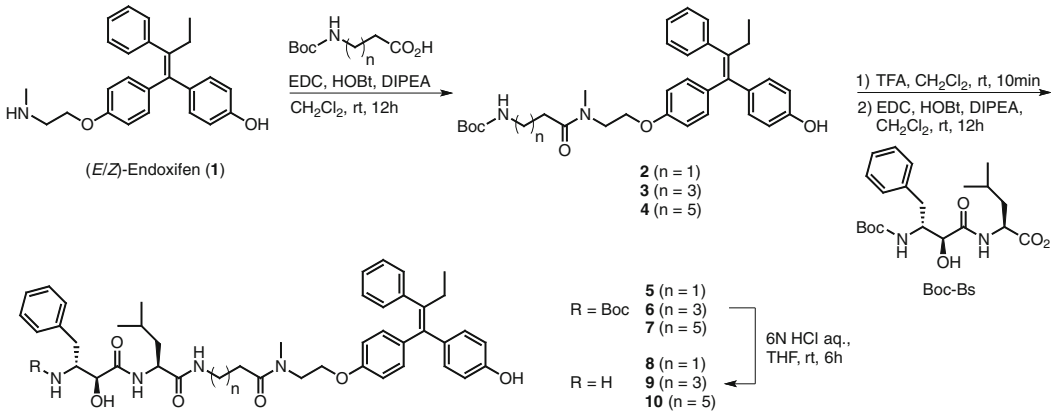

Fig. 3 Synthesis of ligated compounds **8**, **9**, and **10**

and living cells (Fig. 5b). WST-8 is a water-soluble tetrazolium salt and is reduced by NADPH dehydrogenase activity of mitochondria in cells to produce a yellow-color formazan dye, the intensity of which is directly proportional to the number of living cells. Propidium iodide (PI) staining can be used to monitor cells undergoing late apoptosis or necrosis (Fig. 5c). High mobility group box protein 1 (HMGB1), a chromatin-binding nuclear protein is released from cells undergoing necrosis but not apoptosis (Fig. 5d). The protocols for each of these four cell viability/cell death assays are described in this chapter.

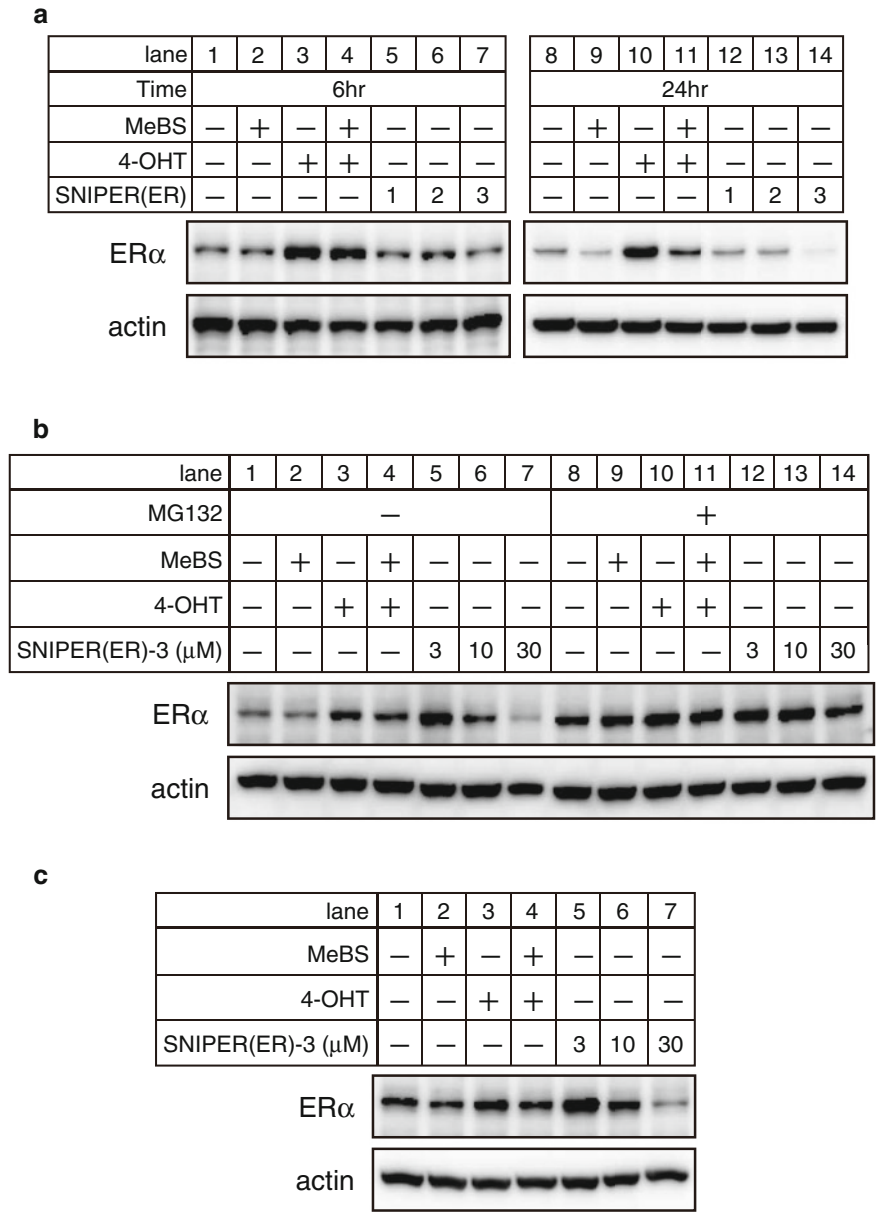

Fig. 4 Degradation of ERα protein by SNIPER(ER)s. (**a**) MCF-7 cells cultured in the media containing 10 % FBS were treated with 10 μM of compounds (MeBS, 4-OHT, SNIPER(ER)-1, -2, -3) for 6 h or 24 h. Since FBS contains a significant concentration of estrogens, 4-OHT increased the ERα protein level (lanes 3, 4, 10, and 11). When compared with the 4-OHT (lanes 3 and 10) or the combination of 4-OHT and MeBS (lanes 4 and 11), SNIPER(ER)s with different linker length reduced the ERα protein levels (lanes 5–7, 12–14). Although there were no apparent differences in the activities of these three SNIPER(ER)s at 6 h, SNIPER(ER)-3 most potently reduced the ERα level at 24 h (lane 14). (**b**) MCF-7 cells were treated with compounds for 6 h in the presence or absence of 10 μM MG132. Proteasome inhibitor MG132 inhibited the ERα degradation. (**c**) SNIPER(ER)-3 degraded ERα in T47D breast cancer cells. T47D cells were treated with compounds for 12 h. This figure was modified from ref. [12] with permission of John Wiley & Sons Ltd

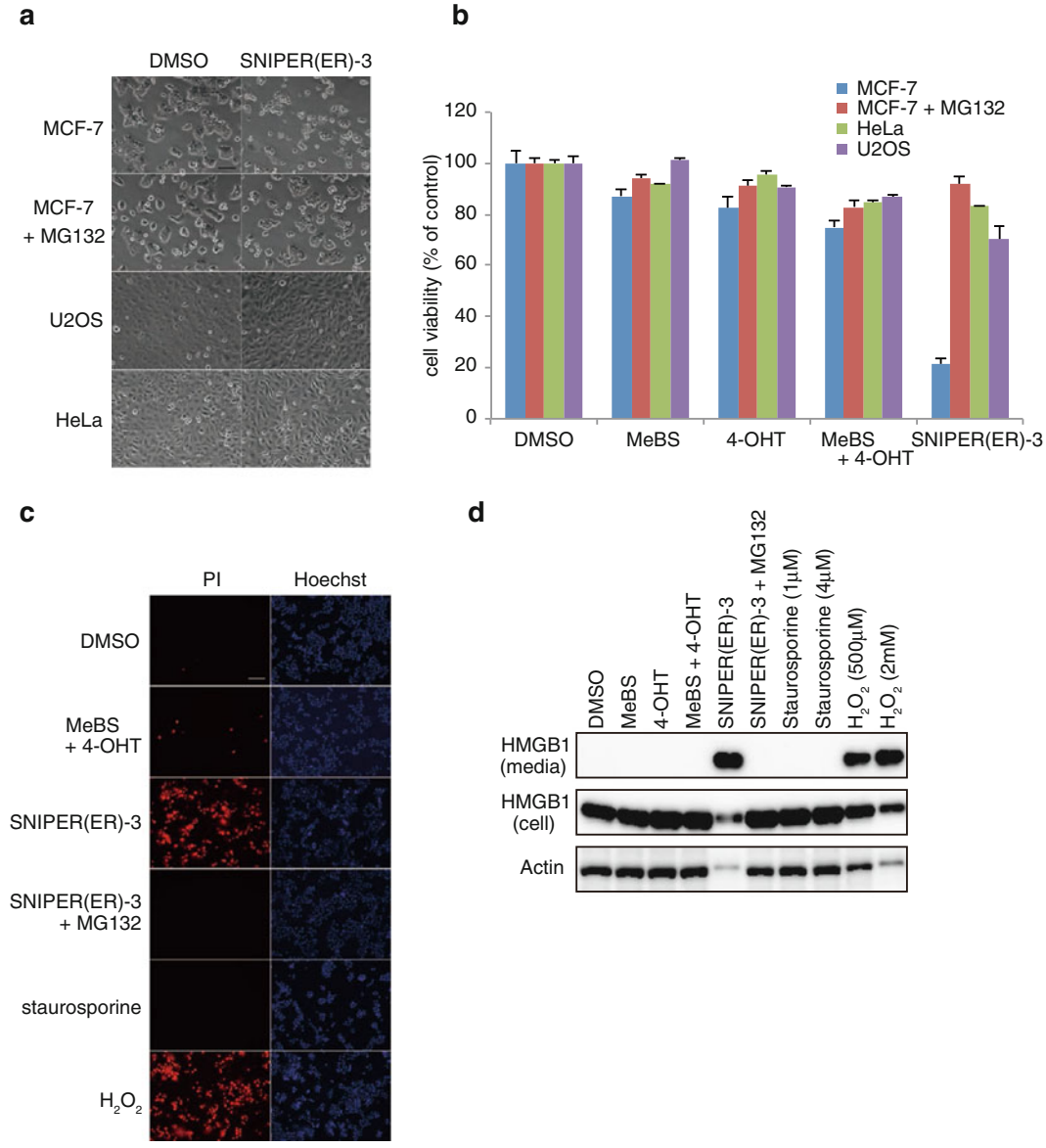

Fig. 5 SNIPER(ER) induced necrotic cell death in MCF-7 cells. (**a**) MCF-7, U-2OS or HeLa cells were treated with 30 μM of SNIPER(ER)-3 for 6 h in the presence or absence of 10 μM MG132. Bar: 100 μm. (**b**) After the treatment as in (**a**), living cells were stained with crystal violet and the cell viability was calculated as values relative to control cells. SNIPER(ER)-3 reduced the cell viability in MCF-7 cells expressing ERα, but not in U2OS and Hela cells that do not express ERα protein. A proteasome inhibitor MG132 protected the SNIPER(ER)-3 treated MCF-7 cells, suggesting that ERα degradation is crucial for the SNIPER(ER)-induced cell death. Values are the means ± S.D. of triplicate samples. (**c**) MCF-7 cells were treated with MeBS plus 4-OHT (30 μM), SNIPER(ER)-3 (30 μM), staurosporine (1 μM), or hydrogen peroxide (500 μM) for 6 h. Cells were stained with PI (necrotic and late apoptotic cells) or Hoechst 33342 (nuclear), and analyzed by fluorescence microscopy. Staurosporine induced apoptosis, whereas hydrogen peroxide induced typical necrosis. The SNIPER(ER)-3-treated cells were stained with PI, suggesting necrotic cell death. Bar: 100 μm. (**d**) MCF-7 cells were treated with MeBS (30 μM), 4-OHT (30 μM), MeBS plus 4-OHT (30 μM), SNIPER(ER)-3 (30 μM), staurosporine (1 μM or 4 μM), hydrogen peroxide (500 μM or 2 mM) for 9 h. Shown are immunoblots of cell lysates and culture media stained with indicated antibodies. SNIPER(ER)-3 induced the release of HMGB1 protein into the media, indicating that cells are dying by necrosis. This figure was reproduced from ref. [12] with permission of John Wiley & Sons Ltd

2 Materials

2.1 Design and Synthesis of SNIPER(ER)

1. Reagents for the synthesis of compounds including SNIPER(ER): N-Boc-β-alanine, N-Boc-5-aminovaleric acid, N-Boc-7-aminoheptanoic acid, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt), *N*,*N*-diisopropylethylamine (DIPEA), dichloromethane (DCM), trifluoroacetic acid (TFA), *n*-hexane, ethyl acetate (AcOEt), sodium hydrogen carbonate ($NaHCO_3$), sodium sulfate (Na_2SO_4), hydrochloric acid (HCl), tetrahydrofuran (THF).
2. Silica gel 60 N, spherical, particle size 40–100 μm.
3. (*E*/*Z*)-Endoxifen [13].
4. Boc-bestatin [3].

2.2 ERα Protein Degradation

1. Cells: MCF-7 (human breast adenocarcinoma cell line), T47D (human ductal breast epithelial tumor cell line), HeLa (human cervical epithelial carcinoma cell line), U-2OS (human osteosarcoma cell line) (American Type Culture Collection).
2. RPMI 1640 medium: RPMI 1640, 10 % heat-inactivated fetal bovine serum (FBS), and 60 μg/mL of kanamycin (used for MCF-7 and T47D cells).
3. DMEM medium: DMEM, 10 % fetal bovine serum (FBS), and 60 μg/mL kanamycin (used for HeLa and U-2OS cells).
4. Charcoal/dextran-treated fetal bovine serum (FBS) and phenol-red free media (used when cells are treated with SNIPER(ER)s in serum-depleted conditions).
5. 6-well cell culture plates.
6. Synthesized compounds: SNIPER(ER)s (compound **8**, **9**, and **10** in Fig. 3), 10 mM in DMSO stock solution.
7. 4-Hydroxytamoxifen (4-OHT): 10–30 μM.
8. Bestatin Methyl Ester (MeBS, Millipore): 10–30 μM.
9. MG-132 (proteasome inhibitor, Z-Leu-Leu-Leu-H (aldehyde)): 10 μM (Peptide Institute).
10. Staurosporine: 1–4 μM.
11. Hydrogen peroxide: 500 μM to 2 mM.
12. Phosphate-buffered saline (PBS).
13. Lysis buffer: 0.1 M Tris–HCl, pH 7, 1 % SDS, 10 % Glycerol.
14. Materials for BCA assay: 96-well plate, BCA Protein Assay Reagent Kit, microplate reader.
15. SDS loading buffer (5×): 0.5 M Tris–HCl, pH 7, 5 % SDS, 50 % Glycerol, 0.5 M dithiothreitol, 0.05 % bromophenol blue.

16. Pre-stained Protein Marker, Broad Range (7–175 kDa).
17. Pre-cast gel: Perfect NT Gel A (10–15 %, 18-well) (DRC).
18. SDS-PAGE (polyacrylamide gel electrophoresis) apparatus.
19. SDS-PAGE running buffer: 25 mM Tris, 192 mM Glycine, 0.1 % SDS.
20. Filter paper.
21. PVDF (polyvinylidenedione) membrane.
22. Transfer buffer: 25 mM Tris, 192 mM glycine, 20 % (v/v) methanol.
23. Transfer apparatus for Western blotting including power supply.
24. Tris buffered saline (TBS): 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, with 0.05 % Tween-20 (TBS-T).
25. Blocking solution: 5 % nonfat dried skim milk in TBS-T.
26. Antibodies: anti-estrogen receptor α (ERα), anti-β-actin, anti-rabbit IgG HRP conjugate, anti-mouse IgG HRP conjugate.
27. Chemiluminescent substrate for HRP: SuperSignal West Dura or equivalent.
28. Digital imaging equipment for chemiluminescence.

2.3 Cell Death

1. Crystal violet staining: 96-well plate, 10 % crystal violet in methanol, 1 % SDS solution, microplate reader.
2. Propidium iodide (PI) staining: 12-well plate, 2 mg/mL Propidium iodide, 2 mg/mL Hoechst 33342 (Life Technologies), fluorescence microscope.
3. WST-8 assay: 96-well plate, Cell Counting Kit-8, microplate reader.
4. HMGB1 (high mobility group box protein 1) release: 12-well plate, Western blotting components, anti-HMGB1.

3 Methods

3.1 Design and Synthesis of SNIPER(ER)

1. To prepare compounds **2**, **3**, and **4**: Stir a mixture of EDC (0.6 mmol), HOBt (0.6 mmol), DIPEA (0.6 mmol), (*E/Z*)-endoxifen (**1**; 0.5 mmol), and N-Boc-β-alanine or N-Boc-5-aminovaleric acid or N-Boc-7-aminoheptanoic acid (0.6 mmol) in DCM (5 mL) at room temperature for 12 h. Wash the solution with 3 % aqueous HCl, saturated aqueous $NaHCO_3$, brine, and dry over Na_2SO_4. After removal of the solvent, purify the residue by column chromatography on a silica gel (*n*-hexane–AcOEt = 2:1) to produce compounds **2** (88 %), **3** (90 %), and **4** (76 %), respectively (Fig. 3).

2. To prepare compounds **5**, **6**, and 7: Stir a mixture of compound **2**, **3** or **4** (0.3 mmol) and TFA (0.5 mL) in DCM (5 mL) at room temperature for 10 min. Concentrate the solution in vacuo to produce crude amines, which are used for the next reaction without further purification. Stir a mixture of EDC (0.36 mmol), HOBt (0.36 mmol), DIPEA (0.36 mmol), Boc-bestatin (0.36 mmol), and the abovementioned amines in DCM (5 mL) at room temperature for 12 h. Wash the solution with 3 % aqueous HCl, saturated aqueous $NaHCO_3$, brine, and dry over Na_2SO_4. After removal of the solvent, purify the residue by column chromatography on a silica gel (AcOEt) to produce compounds **5** (82 %), **6** (86 %), and 7 (77 %), respectively (Fig. 3).
3. To prepare compounds **8**, **9**, and **10**: Stir a mixture of compounds **5**, **6** or 7 (0.2 mmol) and 6 M aqueous HCl (0.1 mL) in THF (2 mL) at room temperature for 6 h. Concentrate the solution in vacuo to produce compounds **8** (>99 %), **9** (>99 %), and **10** (>99 %), respectively (Fig. 3).

3.2 Analysis of ERα Protein Degradation by Immunoblot

1. Maintain MCF-7 and T47D cells in RPMI 1640 medium. Maintain HeLa and U-2OS cells in DMEM medium.
2. Plate cells at a density of 400,000 cells/well in 6-well cell culture plates. In preparation for the experiments under estrogen-depleted conditions, switch the culture medium to phenol red-free medium (RPMI 1640 for MCF-7 and T47D cells or DMEM for HeLa and U-2OS cells) containing 4 % charcoal/dextran-treated FBS and kanamycin for more than 72 h. Twenty-four hours before beginning the treatments, change the medium to the cell-appropriate medium containing 0.2 % charcoal/dextran-treated FBS and kanamycin (*see* **Note 1**).
3. Treat the cells with 10 μM bestatin methyl ester, 10 μM 4-hydroxytamoxifen, or SNIPER(ER)-1, -2, or -3 for 6, 12 or 24 h in the absence and presence of 10 μM of the proteasome inhibitor MG-132. When MG-132 is used to block the degradation of ERα by SNIPER(ER), treat the cells with MG-132 for 30 min before adding the SNIPER(ER)s.
4. After incubation, aspirate the medium, add 1 mL/well of cold PBS, and scrape adherent cells from each well with a cell scraper and transfer the cells to 1.5 mL tubes.
5. Centrifuge the cells at 800 × *g* for 5 min.
6. Aspirate the PBS from the pellet and add 100 μL of lysis buffer.
7. Vortex the sample to dissolve the cell pellet.
8. Boil the sample at 95 °C for 10 min.
9. Place the cell lysate on ice for 2 min.

10. Centrifuge the cell lysate at 8000 × *g* at 4 °C for 10 min to remove undissolved debris.
11. Transfer the supernatant (~90 μL) to a new 1.5 mL tube.
12. Determine protein concentrations by the BCA method [14] using the BCA assay kit. Add diluted cell lysate or BSA standard and working reagent for BCA assay (50:1 mixture of solution A and B) to each well in a 96-well plate, mix briefly on a plate shaker and incubate at 37 °C for 30 min. Read the absorbance at 550–570 nm by a microplate reader (*see* **Notes 2** and **3**)
13. To prepare the sample for SDS-PAGE, dilute equal amounts of protein from each cell lysate to equal volumes with lysis buffer. Add 5× SDS sample buffer and boil the samples at 95 °C for 5 min.
14. Load the samples onto a polyacrylamide gel and electrophorese for 0.5–1 h at 200 V in SDS-PAGE running buffer.
15. Transfer the proteins electrophoretically onto a PVDF membrane for 10–24 h at 50–120 V (1200 V.h) in transfer buffer (*see* **Note 4**).
16. Block the membranes in blocking solution for 1 h on a shaker.
17. Immunoblot the membranes with the primary antibody (for ERα or β-actin) for 2 h at room temperature in blocking solution (*see* **Note 5**).
18. Remove the primary antibodies.
19. Wash the PVDF membranes three times with TBS-T for 5 min each.
20. Incubate the membranes with the respective secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature in blocking solution.
21. Remove the secondary antibodies.
22. Wash the PVDF membranes three times with TBS-T for 5 min each.
23. Visualize the protein signals using chemiluminescent substrate and the appropriate imaging system (Fig. 4).

3.3 Analysis of Cell Death by Crystal Violet Staining

Crystal violet staining is used to determine the number of adhesive and living cells.

1. Plate the cells at a density of 15,000 cells/well in 96-well plates.
2. The next day, treat the cells with 30 μM SNIPER(ER)-3 for 6 h in the absence or presence of 10 μM MG132.
3. Aspirate the medium and add 50 μL of PBS containing 0.1 % crystal violet.

4. Incubate the cells on a gentle shaker at room temperature for 15 min.
5. Wash the cells thoroughly with distilled water.
6. Dry the cells at 37 °C for 2 h.
7. Add 100 μL of 1 % SDS solution.
8. Incubate the cells on a shaker at room temperature for 1 h.
9. Read the absorbance at 600 nm using a microplate reader (Fig. 5b).

3.4 Analysis of Cell Death by Propidium Iodide Staining

Propidium iodide (PI) staining can be used to monitor cells undergoing late apoptosis or necrosis.

1. Plate the cells at a density of 150,000 cells/well in 12-well cell culture plates.
2. The next day, treat the cells with 30 μM bestatin methyl ester plus 4-hydroxytamoxifen, 30 μM SNIPER(ER)-3 in the absence or presence of 10 μM MG132, 1 μM staurosporine, or 500 μM hydrogen peroxide for 6 h.
3. After the treatment, incubate the cells in medium containing 2 μg/mL PI and 2 μg/mL Hoechst 33342 in the CO_2 incubator for 5–15 min.
4. Generate cell images using fluorescence microscopy (Fig. 5c).

3.5 Analysis of Cell Survival by WST-8 Assay

WST-8 is a water-soluble tetrazolium salt and is reduced by NADPH dehydrogenase activity of mitochondria in cells to produce a yellow-color formazan dye, the intensity of which is directly proportional to the number of living cells.

1. Plate the cells at a density of 15,000 cells in 100 μL/well in 96-well plates.
2. The next day, treat the cells with 30 μM bestatin methyl ester plus 4-hydroxytamoxifen, 30 μM SNIPER(ER)-3 in the absence or presence of 10 μM MG132, 1 μM staurosporine, or 500 μM hydrogen peroxide for 6 h.
3. Add 10 μL Cell Counting Kit-8 reagent to each well of the plate followed by plate incubation for 1–4 h in the CO_2 incubator.
4. Read the absorbance at 450 nm using a microplate reader.

3.6 Analysis of Cell Necrosis by HMGB1 Immunoblot

High mobility group box protein 1 (HMGB1) is a chromatin-binding nuclear protein that is released from cells undergoing necrosis but not apoptosis.

1. Plate the cells at 150,000 cells/well in 12-well plates.
2. The next day, treat the cells with 30 μM bestatin methyl ester, 30 μM 4-hydroxytamoxifen, 30 μM bestatin methyl ester plus

4-hydroxytamoxifen, 30 μM SNIPER(ER)-3 in the absence or presence of 10 μM MG132, 1 or 4 μM staurosporine, or 500 μM or 2 mM hydrogen peroxide for 9 h.

3. After the treatment of the cells with compounds, collect the medium and centrifuge at 15,000 × *g* at 4 °C for 10 min.
4. Transfer the supernatant to a new tube. Mix an equal amount of medium (5–10 μL) with 5× SDS sample buffer and boil at 95 °C for 5 min.
5. After removing the medium from the cells (**step 3** in Subheading 3.6), lyse the cells as in **step 4** in Subheading 3.2 and process the lysate as in **steps 5–13** in Subheading 3.2 in preparation for immunoblot.
6. Perform SDS-PAGE with samples both from the medium as well as from the cell lysates (Subheading 3.2).
7. Transfer proteins to PVDF membrane (Subheading 3.2).
8. Perform immunoblot using primary antibody against HMGB1.
9. Visualize the protein signals as described in **step 23** in Subheading 3.2 and compare the levels of HMGB1 protein found in the media to that inside the cells (from the lysate) (Fig. 5d) (*see* **Note 6**).

4 Notes

1. Upon estrogen binding, ERα translocates to nuclei and activates gene expression, and then it is degraded via the ubiquitin proteasome system. To differentiate the SNIPER-induced ERα degradation from physiological degradation by estrogen, cells are cultured in a medium containing estrogen-depleted (charcoal/dextran-treated) serum. A phenol-red free medium is also used because phenol red has a weak estrogenic activity.
2. The BCA protein assay is compatible with the detergents used in the experiment. The absorbance of 1 % SDS lysis buffer reacting with the reagents for the BCA assay is negligible.
3. According to the instructions, reaction complexes exhibit strong absorbance at 562 nm. Measurement of absorbance in this assay is compatible between 540 nm and 590 nm.
4. The PVDF membrane is immersed in 100 % methanol and then transferred into transfer buffer before use.
5. Alternatively, CanGet Signal or similar reagents can be used to enhance the signal detected by antibodies.
6. When the treated cells undergo necrosis, the HMGB1 proteins released into the culture media are usually detectable by immunoblotting without concentrating the proteins. However, the

secreted proteins are extremely diluted in the culture media and may be difficult to detect in some cases. The diluted proteins can be concentrated by filtering devices such as Centricon (Millipore) or by immunoprecipitation with specific antibodies.

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Chapter 43

Regulation of Activation Induced Deaminase (AID) by Estrogen

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Abstract

Regulation of Activation Induced Deaminase (AID) by the hormone estrogen has important implications for understanding adaptive immune responses as well as the involvement of AID in autoimmune diseases and tumorigenesis. This chapter describes the general laboratory techniques for analyzing AID expression and activity induced by estrogen, focusing on the isolation and preparation of cells for hormone treatment and the subsequent analysis of AID responsiveness to estrogen at the RNA level and for determining the regulation of AID activity via estrogen by analyzing Ig switch circle transcripts and mutations in switch region loci.

Key words Activation induced deaminase, AID, Estrogen, Adaptive immune responses, Splenic B-cells, Ig switch circle transcripts, Somatic hypermutations, Autoimmune diseases

1 Introduction

Infection triggers an immune response, which aims to specifically target and remove the source of the infection from the organism while keeping its own tissues unharmed. Interestingly, it has been noted that female mice produce more antibody than male mice after immunization [1, 2] and that the immune responses in humans display a similar sexual dimorphism that is characterized by a quicker and stronger humoral immune response in females than in males [3].

The differences in immune responses seem to be partly mediated by hormone levels in vivo, since systemic sex hormone treatment or castration in mice converts the immune responses more similar to that of the opposite sex [4, 5]. Furthermore, an important factor in the susceptibility of various human autoimmune diseases is the hormonal status of the patient, which can affect the activity of B-lymphocytes [6–8]. Female sex biases of over 80 % have been

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reported in various autoimmune diseases including Sjogren's syndrome, lupus and scleroderma [8], while other common diseases such as myasthenia gravis, rheumatoid arthritis (RA) and multiple sclerosis (MS) have a sex distribution of 60–75 % women [8, 9]. Altogether, the increased prevalence of autoimmune disease in women, the sexual dimorphism of the immune response, and the modulatory effects of sex steroids such as estrogen on the immune function in vitro suggest their involvement in mediating these sex differences. Indeed, estrogen receptors (ERα and β) are expressed not only in reproductive tissues but also in multiple other cell types, including cells of the immune system such as B-lymphocytes [10–13].

One of the activative effects on B-cells by the hormone estrogen is mediated via its stimulatory effects on Activation Induced DNA deaminase (AID) [14], an enzyme that is necessary for diversification of the antibody repertoire by inducing somatic hypermutation and Ig class switch recombination via DNA deamination [15, 16].

Estrogen has been shown to induce the expression of AID in B-cells and thereby enhance AID-mediated downstream processes including Ig class switching. Hence, AID induction by estrogen is important for understanding adaptive immune responses in physiological conditions but also suggests its involvement in harmful autoimmune processes [14]. Due to the mutagenic activities of AID on DNA, these regulatory mechanisms could also have important implications for tumorigenesis in estrogen-responsive tissues other than B-cells [17]. This chapter describes the application of basic methods for studying the interconnection of estrogen and AID expression and activity in mouse splenic B-cells at the RNA level as well as switch circle transcripts and mutations in Ig switch region loci. This chapter also highlights some of the technical nuances in working with estrogen, which are important to keep in mind for a successful application of these techniques for determining the function of estrogen as a regulator of AID in B-cells.

2 Materials

2.1 Reagents

Reconstitute all reagents using sterile diethylpyrocarbonate (DEPC)-treated water or sterile DMSO.

1. Lipopolysaccharide (LPS): 25 μg/mL.
2. Mouse interleukin 4 (IL4): 50 ng/mL.
3. Human transforming growth factor β1 (TGFβ1): 20 ng/mL.
4. 17β-estradiol: concentrations of estrogen range between 1 and 100 ng/mL (*see* Table 1) and depend on the experiment. Tamoxifen (antagonist of estrogen): 50 nM. Hormone stocks are prepared in dimethylsulfoxide (DMSO) at a concentra-

Table 1
Treatment of cells with hormones

Experiment	Cells	Depletion (h)	[nM]	Treatment
AID mRNA	B-cells	24	0.1–1000	1–8 h
Mutations in switch regions	B-cells	24	10–50	6 days
CSR (switch circle)	B-cells	24	1–50	48 h

tion of 100 mM. Dilute the stock solution in DMSO to yield 1000× stock solutions; final dilutions are made in media (final DMSO concentration is less than 0.1 %). Store hormone stocks at −80 °C.

5. 0.1 % BSA in PBS; filter sterilize the solution prior to use.
6. RPMI 1640, GlutaMAX, fetal calf serum (FCS), 70 % ethanol.
7. Hormone depletion medium: Opti-MeM Reduced Serum Medium supplemented with charcoal stripped fetal bovine serum, nonessential amino acids 8.9 mg/mL L-alanine, 15.0 mg/mL L-asparagine, 13.3 mg/mL L-aspartic acid, 14.7 mg/mL L-glutamic acid, 7.5 mg/mL glycine, 11.5 mg/mL proline, 10.5 mg/mL L-serine. Filter-sterilize the media before use (*see* **Notes 1** and **2**).
8. cDNA synthesis reagents: 2.5 μg of total RNA, 5× first strand buffer, dithiothreitol to a final concentration of 5 mM, dNTPs to a final concentration of 0.4 mM each, 0.1 μg oligo dT primer, and/or 2.5 μg $d(N)_{10}$ random primers, 8 U RNaseOUT (Invitrogen), 200 U Superscript III reverse transcriptase, and the appropriate quantity of RNase-free H_2O to a 20 μL reaction.
9. PUREGENE genomic DNA isolation kit (Gentra Systems).
10. Tris–EDTA buffer, pH 8.0.
11. Phosphate buffered saline (PBS) with 0.1 % bovine serum albumin (BSA).
12. Mouse B-cell Negative Isolation Kit (Dynal Biotech, Norway).
13. Glasstic slide 10 with grids hemocytometer (Kova International).
14. SYBRGreen PCR reaction mixture and RNase-free water.
15. qPCR primers for gene regulated by breast cancer 1 (GREB1): GREB1_forward 5′-TCCGAGTTCAGAGGTCGGC-3′ and GREB1_reverse 5′-GTCCTACCTGTTGAGCTCCCACT-3′.
16. qPCR primers for activation induced deaminase (AID): Aid_Forward 5′-AACCCAATTTTCAGATCGCG-3′ and Aid_Reverse 5′-AGCGGTTCCTGGCTATGATAAC-3′.

17. qPCR primers for the housekeeping gene, GAPDH: GAPDH_Forward 5′-GCACAGTCAAGGCCGAGAAT-3′ and GAPDH_Reverse 5′-GCCTTCTCCATGGTGGTGAA-3′.
18. PCR primers for the Sγ3 switch region: forward primer for Sγ3 region g3.2F (5′-gcgaattcTTGCAACTCC-TAAGAGGAAA-GATCCC-3′) and reverse primer for Sγ3 region g3.2R (5′-gcggatcCAGCCTGGTCC-CTACACTCC TAAC|AAC-3′) [18].
19. 10× buffer, 10× PCR enhancer, and Pfu Turbo R DNA polymerase.
20. Zero Blunt TOPO PCR Cloning Kit, including TOPO vector (Invitrogen).
21. LB plates and liquid medium with kanamycin.
22. RNeasy Mini kit (Qiagen) or equivalent for RNA extraction.
23. Sybr Gr een qPCR reaction mixture.
24. QIAprep Spin MiniPrep kit (Qiagen) or equivalent for plasmid purification.
25. Lympholyte M (Cedarlane Laboratories Limited) cell separation medium.

2.2 Mouse Tissue

Obtain mouse spleen samples from freshly euthanized 8–12 week old female nonpregnant BALB/c mice. All experiments using animals need prior approval by IACUC or a corresponding Animal Ethics Committee depending on the research location. The described research was conducted in Cancer Research UK and approved by Cancer Research UK Animal Ethics Committee and the UK Home Office.

3 Methods

3.1 Isolation of Mouse Splenic B-Cells

1. Transfer freshly isolated mouse spleens to a tube containing 30 mL of RPMI 1640 + GlutaMAX with 10 % FCS prewarmed to 18–25 °C.
2. Sterilize the spleens by placing them twice into 70 % ethanol for 1 s and then into sterile media.
3. Transfer the tissues to a wet 70 μm cell strainer on top of a 50 mL tube.
4. Macerate the cells through the filter using the back of a syringe plunger.
5. Rinse the filter twice with 5 mL RPMI 1640 containing 10 % FCS solution.
6. Fill the tube with cold RPMI 1640 containing 10 % FCS and centrifuge at 90 × *g* at 4 °C for 10 min.

7. Discard the supernatant and resuspend the cells in 5 mL room temperature RPMI 1640 with 1 % FCS.
8. Carefully pipette the cell suspension onto 7 mL Lympholyte M in a 15 mL tube.
9. Centrifuge the sample at 90×*g* for 20–30 min to remove erythrocytes.
10. Collect the interphase with B-cells into a fresh tube (*see* **Note 3**).
11. After filling the tube with RPMI 1640 1 % FCS, centrifuge the cells at 90×*g* for 10 min and remove the supernatant, leaving 2–3 mL of media on the cells (*see* **Note 4**)
12. Add 120 U of DNAse I per 1 mL of cell suspension, mix gently, and incubate on a rotator for 15 min at room temperature. Filter the suspension through a 70 μm cell strainer.
13. Count for leucocytes with a hemocytometer to obtain the total cell number. Use 12 μL of cell suspension on Glasstic slide 10 with grids hemocytometer, count 5 squares and calculate the average cell number. Multiply this number by 10,000 and the dilution coefficient of your cell suspension to obtain the number of leucocytes per mL.
14. Fill the tube with cold RPMI 1640 with 1 % FCS and centrifuge at 90×*g* at 4 °C for 10 min.
15. Resuspend the leucocytes in PBS with 0.1 % BSA (1×10^7 per 100 μL) and transfer the cells to a 15 mL tube.
16. Isolate mouse splenic B-cells with reagents from the Mouse B-cell Negative Isolation Kit. Add 20 μL heat inactivated FCS per 1×10^7 leucocytes, followed by the addition of 20 μL of Antibody Mix (from the kit) per 1×10^7 cells.
17. Incubate the mix for 20 min at 4 °C (*see* **Note 5**).
18. Wash the cells in 2 mL sterile PBS with 0.1 % BSA per 1×10^7 leucocytes and centrifuge at 90×*g* at 4 °C for 10 min.
19. Remove the supernatant and resuspend the cells in 800 μL PBS with 0.1 % BSA per 1×10^7 leucocytes.
20. Wash the Mouse Depletion Dynabeads in PBS with 0.1 % BSA before adding 200 μL per 1×10^7 leucocytes. Incubate the cells for 15 min at room temperature with gentle rotation.
21. Resuspend the cells in 1 mL PBS with 0.1 % BSA per 1×10^7 leucocytes.
22. Place the tube in the magnet for approximately 2 min. This will bind all the undesired leucocytes to the magnet while only the B-cells will remain freely floating in the solution.
23. Transfer the supernatant with isolated mouse B-cells into a new tube.
24. Centrifuge the cells at 90×*g* at 4 °C for 5 min and remove the PBS with 0.1 % BSA from the cell pellet.

3.2 Hormone Treatment

1. Pre-warm hormone depletion medium to 37 °C before using it to resuspend the cells.
2. Gently resuspend the cells in sterile hormone depletion medium.
3. Incubate the cells for 24–72 h at a concentration of 250,000 cells/mL (*see* **Notes 6** and 7).
4. A list of different concentrations of estrogen and the time of cell treatment for various experiments is indicated in Table 1 (*see* **Note 8**). It is recommended to add estrogen freshly to media (*see* **Note 9**).

3.3 Analysis of AID mRNA Induction in Response to Estrogen

1. Extract RNA from splenic B-cells using the RNeasy Mini kit according to the manufacturer's instructions. Elute RNA with 30 μL of RNase-free water by centrifuging the column at 16,000 × *g* for 1 min (*see* **Note 10**).
2. Measure the RNA concentration and store the samples at −80 °C until cDNA synthesis (*see* **Note 11**).
3. Perform cDNA synthesis as follows: denature 2.5 μg of total RNA at 70 °C for 2 min and place on ice. Add 5× first strand buffer, DTT (to a final concentration of 5 mM), dNTPs (to a final concentration of 0.4 mM each), 0.1 μg oligo dT primer, and/or 2.5 μg $d(N)_{10}$ random primers, 8 U RNaseOUT, 200 U Superscript III reverse transcriptase, and the appropriate quantity of RNase-free H_2O to a 20 μL reaction. Incubate the reaction at 37 °C for 10 min, 50 °C for 40 min and then 55 °C for 10 min. Add 80 μL of sterile H_2O to the cDNA and incubate at 100 °C for 2 min.
4. Analyze gene expression by quantitative real time PCR (qPCR). First, confirm the responsiveness of cells to estrogen by qPCR of GREB1 (gene regulated by breast cancer 1), a known estrogen-responsive gene [19] using the primers from **item 15** in Subheading 2.1 (*see* **Note 12**). Design the qPCR reaction as follows: add 10 μL of SYBRGreen PCR reaction mixture to 9 μL of RNase-free water and primers for GREB1 in a final concentration of 0.5 μM. Add 1 μL of cDNA to the PCR reaction volume. The qPCR reaction parameters are incubation at 50 °C for 2 min, then 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and at 72 °C for 30 s.
5. Analyze the expression of AID by qPCR using the primers from **item 16** in Subheading 2.1.
6. Analyze the expression of the housekeeping gene, GAPDH, by qPCR using the primers from **item 17** in Subheading 2.1 to normalize gene expression across all samples (Fig. 1).

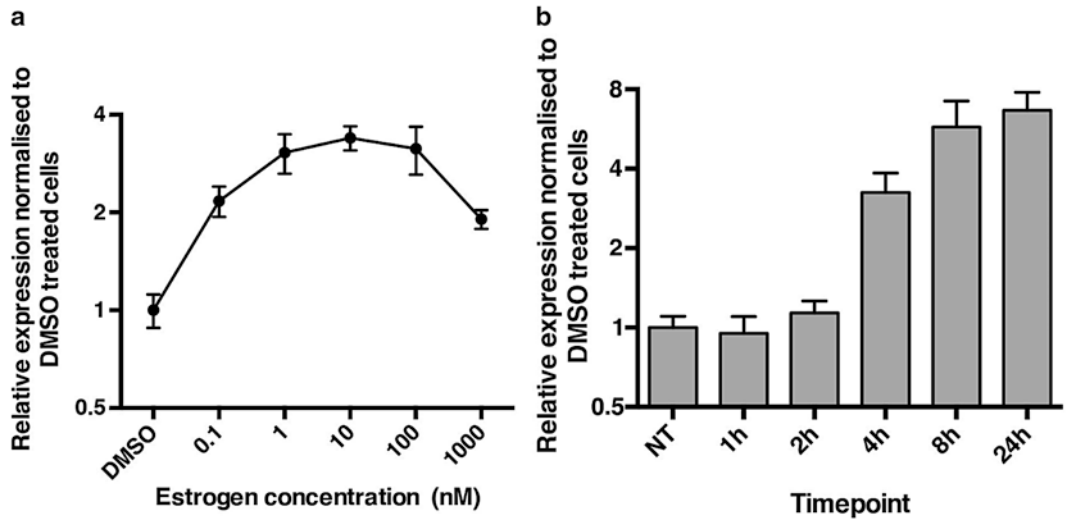

Fig. 1 The effects of estrogen on AID mRNA in splenic B-cells. Both estrogen and AID modulate the strength and duration of normal adaptive immune responses but have also been implicated in pathological processes including gender-biased autoimmune diseases and cancers. The stimulatory effects of estrogen on AID expression through its transcriptional regulation could play an important role in these processes. The induction of mRNA in B-cells is a direct readout of the potential interplays between estrogen and AID. (**a**) Changes in AID mRNA in response to estrogen treatment in stimulated B-cells. Isolated mouse splenic B-cells were stimulated with LPS and IL4 and treated with different physiological concentrations of estrogen for 8 h. (**b**) AID mRNA in response to estrogen treatment in un-stimulated B-cells, after 8 h treatment with physiological concentrations of estrogen. Data are representative of three independent experiments and error bars indicate standard deviations from the mean. DMSO is set to 1, and treatments are represented as relative change to DMSO. *NT* not treated

3.4 Circle Transcript Detection in B-Cells After Estrogen Treatment

1. Place isolated mouse splenic B-cells into 12-well plates in 1 mL of hormone depletion medium.
2. Stimulate isolated splenic B-cells for up to 72 h with LPS + IL4 for inducing switching to IgG1 and IgE, LPS + TGF-β1 for switching to IgA, and LPS for switching to IgG3.
3. Add hormones (estradiol ± tamoxifen) to the cells together with LPS and cytokines in fresh media.
4. After hormone treatment, collect cells, isolate RNA, and synthesize cDNA as described above.
5. Use qPCR to analyze the relative amounts of circle transcripts (Fig. 2). Primers that are used for detecting IgG1 [20], IgG3, IgA [21] and IgE [22], are listed in Table 2.
6. Use qPCR to analyze circle transcripts as follows: add 10 μL of SYBRGreen PCR reaction mixture to 9 μL of RNase-free water and forward/reverse primers with a final concentration of 0.5 μM. Add 1 μL of cDNA to the PCR reaction. Perform the qPCR reaction under the same conditions as in **step 4** in Subheading 3.3.

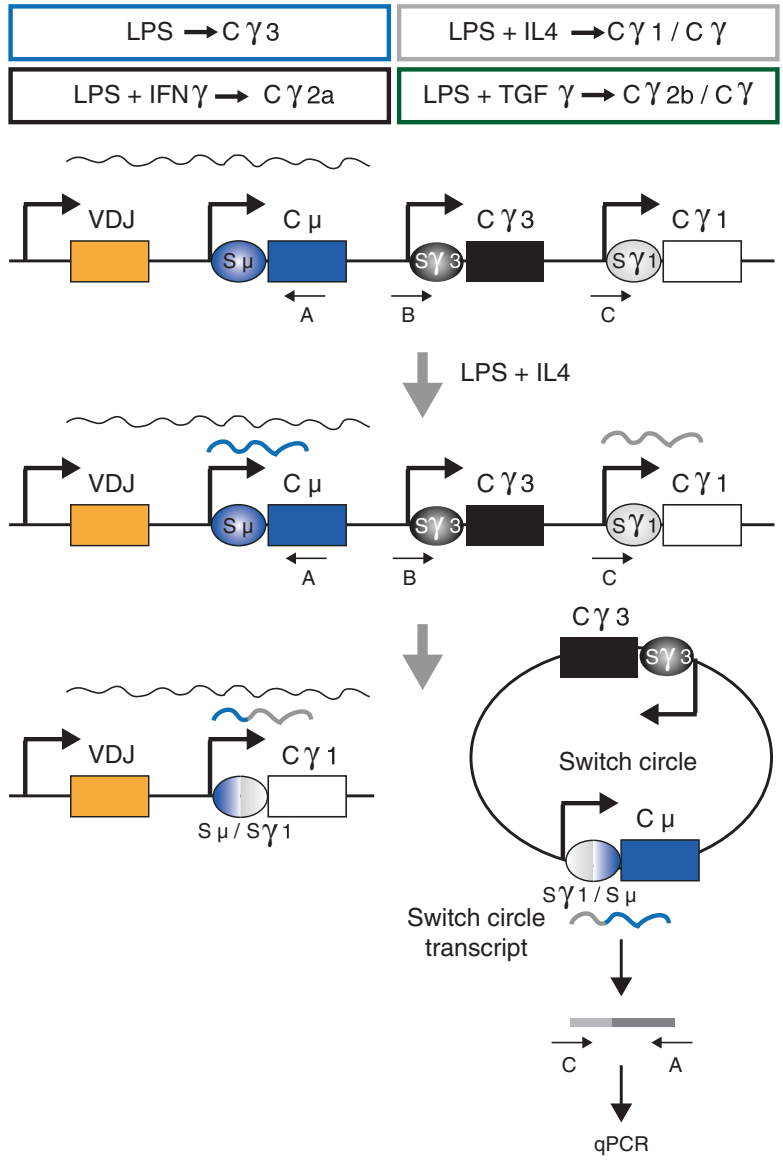

Fig. 2 A schematic drawing of class switch recombination (CSR) and the analysis of switch circle transcripts by qPCR. CSR occurs between a Sμ switch region (*dark oval*) and a S region located upstream of another C region, such as S γ1 (*white oval*), which will result in the switching from IgM to IgG1. The intervening DNA sequence is predominantly released as a circular episome called a switch circle. The combinations of cytokines and LPS that lead to the switching of Ig isotypes are indicated in the *top panel. Ovals* indicate switch regions, *arrows mark* the promoters and *rectangles* are different constant region exons (Cμ, Cγ3, Cγ1). Class-switching can result in the formation of a switch circle, from which a hybrid transcript (switch circle transcript—*wavy line*) is expressed [10]. These transcripts can be reverse-transcribed for cDNA, and using specific primers (e.g., *arrows* marked with C and A for detecting the switching to Cγ1 isotype or with B and A for detecting the switching to Cγ3 isotype), quantitate switch circle formation by qPCR. Since AID is essential for inducing class switch recombination andFig. 2 (continued) hence the formation of switch circle transcripts, the regulatory effect of estrogen on AID activity can be studied by qPCR analysis of switch circle transcripts. Since AID activity in response to estrogen treatment is also reflected in the accumulation of mutations in the switch regions due to AID-induced DNA deamination, these can be detected by sequencing of the switch regions. The stepwise protocols for analyzing switch circle transcripts and mutations in switch regions are described in this chapter. Adapted from ref. [14]

Table 2
Primers used for detecting circle transcripts

Subclass	Orientation	Sequence
IgG1	Forward	5′-TCGAGAAGCCTGAGGAATGTG-3′
IgG1	Reverse	5′-GAAGACATTTGGGAAGGACTGACT-3′
IgG3	Forward	5′-TGGGCAAGTGGATCTGAACA-3′
IgG3	Reverse	5′-AATGGTGCTGGGCAGGAAGT-3′
IgA	Forward	5′-CCAGGCATGGTTGAGATAGAGATAG-3′
IgA	Reverse	5′-AATGGTGCTGGGCAGGAAGT-3′
IgE	Forward	5′-TTGGACTACTGGGGTCAAGG-3′
IgE	Reverse	5′-CAGTGCCTTTACAGGGCTTC-3′

3.5 Analysis of Mutations in Switch Regions in Response to Estrogen

1. Isolate genomic DNA from cells using PUREGENE products for genomic DNA isolation according to manufacturer's recommendations. Add 50 μL of TE to DNA pellets and allow to rehydrate overnight at room temperature before storing the samples at −20 °C.
2. The switch region loci (the Sγ3 region is described here as an example) from genomic DNA are amplified by PCR as follows: add 50 ng of DNA to a 50 μL PCR reaction that includes the forward and reverse primers for Sγ3 region g3.2 (**item 18** in Subheading 2.1) at 20 μM, 10× buffer, 10× PCR enhancer, dNTP's each at 0.4 mM, and 2.5 U of Pfu Turbo R°DNA polymerase.
3. Denature the reaction at 94 °C for 1 min, followed by nine cycles of denaturation at 94 °C for 30 s, decreasing annealing temperature from 68 °C by 1 °C every cycle, extension at 72 °C for 2 min. Thereafter, 35 cycles denaturation at 94 °C for 30 s, annealing at 63 °C and extension at 72 °C for 2 min is performed, before a final extension at 72 °C for 5 min to generate a 750 bp PCR product.
4. Clone the PCR fragments into TOPO vector using Zero Blunt TOPO PCR Cloning Kit according to manufacturer's guidance. Transform the One Shot competent cells according to manufacturer's instructions, plate them on LB plates with kanamycin and grow at 37 °C overnight.
5. Individual bacterial colonies from LB plates with antibiotics are picked into tubes containing 1.2 mL of LB medium.
6. Incubate the tubes overnight at 37 °C with constant shaking at 1300 rpm.

7. Pellet the bacteria by centrifugation at 6000 × *g* for 15 min. Decant the supernatant and store the tubes at −80 °C until mini-preparation and sequencing.
8. Extract the plasmid DNA with mini-prep columns (QIAprep Spin Miniprep Kit) according to manufacturer's guidelines.
9. Perform sequencing with M13 forward and M13 reverse primers, followed by sequence alignment to the parental switch region sequence (*see* **Note 13**).
10. Mutations in the switch region sequence are validated by their presence in both forward and reverse sequencing reactions.

4 Notes

1. Do not use phenol red as a pH indicator in the media, because this reagent can activate estrogen receptors and hence will mask the specific effects of estrogen stimulation.
2. Do not use conventional serum in your media, because it will contain hormones including estrogen that will mask the specific effects of estrogen stimulation in your samples. It is recommended to use charcoal stripped fetal bovine serum for analyzing the effects of estrogen stimulation in B-cells and other tissues.
3. The efficiency of separating erythrocytes from other cells can be visually assessed by the color of the interphase cell pellet, which will be white in the absence of erythrocytes, while the inefficient separation of erythrocytes will maintain its red color.
4. After collecting the cells from the interphase, it is important to wash the cells with media as described, to remove the Lympholyte M. Otherwise cell survival will be decreased.
5. Rotating at 4 °C improves antibody binding to the cells and hence the total purity of splenic B-cells.
6. Hormone depletion of mouse splenic B-cells should usually be performed for 24 h, followed by treatment with estrogen in combination with LPS, IL4 or other cytokines for activating B-cells. A convenient format for such experiments is a 24-well plate and use of 250,000–500,000 cells/well in 1 mL of media.
7. If hormone depletion is performed on samples other than primary cells, such as mouse or human cell lines, the cells can be depleted from hormones for up to 72 h prior to estrogen treatment. Since each cell line has different characteristics of estrogen receptor alpha and beta expression levels, a longer than 24 h hormone depletion is recommended particularly for cancer lines with high levels of estrogen receptor expression. This should be determined with experimental optimization of the treatment conditions.

8. Aliqout estrogen stocks to smaller volumes (e.g., 25 μL) in separate tubes and store them at –80 °C. Avoid repeated freezing and thawing of the stocks.
9. Add estrogen to media freshly every day since estrogen in not stable in media for a prolonged time.
10. To avoid any possible degradation of RNA in samples, wipe the bench and pipettes with RNase Away or equivalent prior to starting the process. Use RNase-free tubes and RNAse-free water. The integrity of RNA can be confirmed by running it on a 1 % agarose gel and visualizing with ethidium bromide with UV light.
11. Avoid repeated freezing and thawing of RNA samples, which will decrease RNA integrity.
12. The primers for qPCR should be designed using 60 °C as the Tm and 150 bp as the length of the PCR product.
13. The switch region locus should be sequenced also in the isolated splenic B-cells prior to cytokine stimulation, to obtain the unmutated parental sequence that can be used as a template for sequence alignments.

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ERRATUM TO

Chapter 43
Regulation of Activation Induced Deaminase (AID) by Estrogen

Slim Pauklin

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The work, and the data and information represented in Figure 1 of this paper, come from the lab of Dr. Svend Petersen-Mahrt (now DNA Editing in Immunity and Epigenetics, IFOM-Fondazione Istituto FIRC di Oncologia Molecolare, Via Adamello 16, 20139 Milano IT, svend.petersen-mahrt@ifom.eu). It was generated with the help of Dr. Heather Coker, and jointly belonging to Cancer Research UK. Data are used under permission. The work was supported by CRUK and a CRUK/LRI Group Leader Grant to Dr. Svend Petersen-Mahrt.

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Erratum to: Bioinformatics Analysis of Estrogen-Responsive Genes

Adam E. Handel

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Erratum to: Atherosclerosis and Vascular Biologic Responses to Estrogens: Histologic, Immunohistochemical, Biochemical, and Molecular Methods

Thomas C. Register, Susan E. Appt, and Thomas B. Clarkson

Erratum to:
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In this chapter, in note number 4, the equation appears in the wrong form: EL, the IEL area: IELA = π (IELL/π)2.

The original chapter has been corrected. The correct form of the equation is IEL, the IEL area: IELA = IELL2/(4×π).

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Sara Della Torre and Paolo Ciana

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The first name and surname of the author (Sara Della Torre) appeared incorrectly on all websites. This has been corrected.

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