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Regulation of gene expression by estrogen in mammary gland of wild type and estrogen receptor alpha knockout mice

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ABSTRACT

Using serial analysis of gene expression, we examined the effects of estrogen (E_2) replacement in gonadectomized wild type (WT) and E_2 receptor alpha knockout (ERαKO) mice on global gene expression in mammary gland. In WT mice, a total of 429,302 tags were sequenced, representing the expression level of 99,854 tag species. A total of ten transcripts were found to be modulated by E_2, such as sorting nexin 5 and two no match tags. In the ERαKO mice, a total of 459,439 tags were sequenced, representing the expression level of 120,149 tag species. Interestingly, the same three transcripts were inversely regulated by E_2 in ERαKO mice. In total, 78 transcripts were upregulated by E_2, while 29 transcripts were downregulated. In contrast to WT mice, the majority of transcripts related to immunity were repressed in ERαKO mice. Moreover, induction of transcripts involved in cell differentiation, Ca^{2+} response, cytoskeleton, protein biosynthesis and secretion, glycolysis, and oxidative phosphorylation were seen only in ERαKO mice. The current study will provide useful information to understand the cellular mechanisms of E_2-mediated gene regulation in tissues *in vivo* for the development of novel drugs targeting specific ER action in pathological conditions.

1. Introduction

The sex steroid hormone estrogen (E_2) plays an essential role in the development of various tissues and the maintenance of numerous physiological processes such as the menstrual cycle and the functions of reproduction, as well as the regulation of growth by its powerful mitogen effects and proliferation in many target organs including breast, bones, cardiovascular system and genital organs. By its characteristics of growth and proliferation, E_2 is associated with numerous diseases such as breast cancer, Alzheimer's disease, atherosclerosis, osteoporosis and obesity [1]. In fact, the E_2 effects are mediated by two related nuclear hormone receptors (E_2 receptor, ER), namely ERα and ERβ, which are ligand-inducible transcription factors that act by regulating transcriptional processes. Thus, ERs belong to a large superfamily of nuclear hormone receptors sharing a well-conserved DNA-binding domain, a structurally conserved ligand-binding domain (LBD) and N-terminal domain. In addition, there are two activation functions, an N-terminal ligand independent activation function (AF-1) and a C-terminal ligand dependent activation function (AF-2) located within the LBD. Thus, AF-1 and AF-2, which synergically act in a promoter-specific and cell-specific manner, can independently activate transcription and contribute to E_2-mediated transcription. In fact, under identical conditions AF-1 seems more active in ERα on a variety of E_2-responsive promoters, while AF-1 activity in ERβ is minimal [2]. Furthermore, these receptors show a transcription activated by a number of distinct mechanisms. According to classical mechanism, after ligand binding there is dissociation of heat shock proteins from ER, hormone binding in the nucleus or cytoplasm, followed by conformational change within the LBD, dimerization of ERs, and binding to E_2 response elements (EREs) located within the promoters of E_2-responsive genes (ERGs) to finally activate transcription. This activation is achieved through the recruitment of specific transcriptional coregulators (coactivators and corepressors). Thus, coactivators bind to AF-2 or AF-1 and promote transcriptional activity by multiple functions including chromatin remodelling and recruitment of the basal transcriptional machinery, while corepressors inhibit transcriptional activity. In a way distinct of classical model, ERs can regulate the transcription without binding directly to DNA. Thus, ERs are tethered through protein–protein interactions to transcription factor complex that contacts the DNA and regulates the gene expression to large number of ERGs that do not contain EREs. Moreover, other E_2 actions are believed to be mediated through membrane associated ERs. In addition, other stimuli such as the activation of various protein kinase cascades stimulated by various growth factors can activate

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ERs in hormone-independent fashion. Consequently, natural gene promoters can be regulated by many transcription factors that bind to distinct regulatory sites [3].

The mammary gland is a highly E_2-dependent glandular tissue with important regulations of growth and differentiation which participate to the development and progression of breast cancer. In addition, the relative ERα and ERβ expression levels are significantly altered during the development of breast cancer with molecular details of receptors regulation poorly understood [4]. ERα and ERβ have unique roles in E_2-dependent action with different transcriptional activities with certain ligands, cell-types, and promoter contexts [3]. Intriguingly, the ERα-positive cells proliferate in ERα-positive human breast carcinomas and represent two third of all breast cancers [5]. Although there are subtle differences between ERs, ERα requires higher level of E_2 than ERβ for ERE activation. In the cells that have both ERs, the heterodimers are the dominant species in which ERβ acts as transdominant inhibitor of ERα with subsaturing hormone levels. At saturating hormone levels, ERβ does not interfere with transcription activated by ERα [6]. Thus, the effects of E_2 on homodimers and heterodimers ERs in a tissue require to be examined.

Serial analysis of gene expression (SAGE) is a powerful strategy to analyze quantitatively, simultaneously and differentially the expression of all mRNAs in a given tissue, including the expression of known and novel genes [7]. This method may constitute a great tool for the characterization of molecular mechanisms of interests [8]. To identify both known and novel genes regulated by or associated with E_2 action, we have performed the SAGE method in mammary gland of gonadectomized (GDX) wild type (WT) and ERα knockout (ERαKO) mice after injection of E_2.

2. Materials and methods

2.1. Sample preparation

Female C57BL6 mice ($n = 24$, 12–15 weeks old) were obtained from Charles River Laboratories (St. Constant, Canada). ERαKO mice ($n = 19$) were produced as previously reported [9]. The GDX mice had access to Lab Rodent Diet No. 5002 and water *ad libitum*. E_2 (50 ng) was injected 3 h (E_2 3 h) and 24 h (E_2 24 h) by a single subcutaneous injection preceding the sacrifice. The control group (GDX) received vehicle solution (0.4% (w/v) Methocel A15LV Premium in 5% ethanol). Although the ERαKO mice were heavier than WT mice, the bolus dose of E_2 was not adjusted to body weight, since the total blood volume was similar considering that the mice had few grams of difference in each group. Moreover, it is well established that a certain range of E_2 dose induces the same physiologic effect.

The mammary gland of *inguinal region* was dissected, and the samples from all mice of the same group were pooled to eliminate interindividual variations and to extract sufficient amount of mRNA. The samples were stored at −80 °C until the analysis. All animal experimentation was conducted in accord with the requirements of the Canadian Council on Animal Care.

2.2. Transcriptome analysis

The SAGE method was performed as previously described [7,10]. Total RNA was isolated by Trizol (Invitrogen Canada Inc., Burlington, Canada). The quality of total RNA was monitored by micro-capillary electrophoresis (Bioanalizer 2100, Agilent Technologies, Mississauga, Canada). Polyadenylated RNA was extracted with the Oligotex mRNA Mini Kit (Qiagen Inc., Mississauga, Canada), annealed with the biotin-5′-T_{18}-3′ primer and converted to cDNA using the cDNA synthesis kit (Invitrogen Canada Inc.). The resulting cDNA library was digested with NlaIII (New England BioLabs Ltd., Pickering, Canada), and the 3′ restriction fragments were isolated with streptavidin-coated magnetic beads (Dynal Biotech LLC, Brown Deer, USA) and separated into two populations. Each population was ligated to one of the two annealed linker pairs and extensively washed to remove unligated linkers. The tag beside the most 3′ NlaIII restriction site (CATG) of each transcript was released by digestion with BsmFI (New England BioLabs Ltd.).

The blunting kit from Takara Bio Inc. (Otsu, Japan) was used for the blunting and ligation of the two tag populations. The resulting ligation products containing the ditags were amplified by PCR with an initial denaturation step of 1 min at 95 °C, followed by 22 cycles of 20 s at 94 °C, 20 s at 60 °C and 2 s at 72 °C with 27 bp primers [10]. The PCR products were digested with NlaIII and the band containing the ditags was extracted from the 12% polyacrylamide gel with Spin-X microcentrifuge tube (Fisher, Pittsbergh, USA). The purified ditags were self-ligated to form concatemers using T4 ligase (Invitrogen Canada Inc.). The concatemers ranging from 500 bp to 1800 bp were isolated by agarose gel and extracted with Gene-Clean Spin (Qbiogene, Montreal, Canada). The resulting DNA fragments were ligated into the SphI site of pUC19 (Invitrogen Canada Inc.) and cloned into UltraMAX DH5αFT competent cells (Invitrogen Canada Inc.). White colonies were picked up with a Q-Bot colony picker (Genetix Ltd., Hampshire, UK). Concatemer inserts were sequenced by the Applied Biosystems 3730 (Foster City, USA).

2.3. Bioinformatic analysis

Sequence files were analyzed using the SAGEana program, a modification of SAGEparser [11]. In brief, SAGE tags corresponding to linker sequences were discarded and replicate concatemers were counted only once. Identification of the transcripts was obtained by matching the 15 bp (sequence at the last CATG + 11 bp tags) with SAGEmap, UniGene and GenBank databases. A minimum of one expressed sequence tag (EST) with a known polyA tail had to be in the UniGene cluster to identify the last NlaIII site on the corresponding cDNA. We have previously shown that the SAGE method is very reproducible with $r^2 = 0.96$ between two SAGE libraries generated from two cDNA libraries constructed from the same total RNA pool [11]. Classification of the transcripts was based upon the updated information of the genome directory [12] found at the TIGR web site (http://www.tigr.org/), the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and the OMIM (http://www.ncbi.nlm.nih.gov/) as well as previously published literatures.

2.4. Validation by the quantitative real-time PCR (Q_RT-PCR)

First-strand cDNA was synthesized using 5 μg of pooled RNA of each WT experimental group (GDX, E_2 3 h and E_2 24 h) in a reaction containing 200 U of Superscript III RNase H-RT (Invitrogen Canada Inc.), 300 ng of oligo-dT_{18}, 500 mM deoxynucleotides triphosphate, 5 mM dithiothreitol and 34 U of human RNase inhibitor (Amersham Pharmacia, Piscataway, USA) in a final volume of 50 μl. The resulting products after treating with RNase A were purified with Qiaquick PCR purification kits (Qiagen Inc.). The cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based real-time PCR quantification using the LightCycler real-time PCR apparatus (Roche Inc., Nutley, USA). Reagents were obtained from the same company and were used as described by the manufacturer. Reading of the fluorescence signal was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signal. Oligoprimer pairs that allow the amplification of approximately 250 bp were designed by GeneTools software (Biotools Inc., Edmonton, Canada) and their specificity was verified by blast in GenBank database. Gene name, GenBank accession numbers and regions used for the primer pairs were the following: