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Estradiol-independent modulation of breast cancer transcript profile by 17beta-hydroxysteroid dehydrogenase type 1

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ABSTRACT

17beta-hydroxysteroid dehydrogenase type 1 (17β-HSD1) is a steroidal enzyme which, in breast cancer cells, mainly synthesizes 17-beta-estradiol (E2), an estrogenic hormone that stimulates breast cancer cell growth. We previously showed that the enzyme increased breast cancer cell proliferation via a dual effect on E2 and 5α-dihydrotestosterone (DHT) levels and impacted gene expression and protein profile of breast cancer cells cultured in E2-contained medium. Here, we used RNA interference technique combined with microarray analyses to investigate the effect of 17β-HSD1 expression on breast cancer cell transcript profile in steroid-deprived condition. Our data revealed that knockdown of 17β-HSD1 gene, *HSD17B1*, modulates the transcript profile of the hormone-dependent breast cancer cell line T47D, with 105 genes regulated 1.5 fold or higher ($p < 0.05$) in estradiol-independent manner. Using Ingenuity Pathway Analysis (IPA), we additionally assessed functional enrichment analyses, including biological functions and canonical pathways, and found that, in concordance with the role of 17β-HSD1 in cancer cell growth, most regulated genes are cancer-related genes. Genes that primarily involved in the cell cycle progression, such as the cyclin A2 gene, *CCNA2*, are generally down-regulated whereas genes involved in apoptosis and cell death, including the pro-apoptotic gene *XAF1*, *IFIH1* and *FGF12*, are on the contrary up-regulated by 17β-HSD1 knockdown, and 21% of the modulated genes belong to this latter functional category. This indicates that 17β-HSD1 may be involved in oncogenesis by favoring anti-apoptosis pathway in breast cancer cells and correborates with its previously shown role in increasing breast cancer cell proliferation. The gene regulation occurring in steroid-deprived conditions showed that 17β-HSD1 can modulate endogenous gene expression in steroid-independent manners. Besides, we tested the ability of estrogen to induce or repress endogenous genes of T47D by microarray analysis. Expression of a total of 130 genes were found to increase or decrease 1.5-fold or higher ($p < 0.05$) in response to E2 treatment (1 nM for 48 h), revealing a list of potential new estrogen-responsive genes and providing useful information for further studies of estrogen-dependent breast cancer mechanisms. In conclusion, in breast cancer cells, in addition to its implication in the E2-dependent gene transcription, the present study demonstrates that 17β-HSD1 also modulates gene expression via mechanisms independent of steroid actions. Those mechanisms that may include the ligand-independent gene transcription of estrogen receptor alpha (ERα), whose expression is positively correlated with that of the enzyme, and that may implicate 17β-HSD1 in anti-apoptosis pathways, have been discussed.

1. Introduction

Breast cancer is the most frequently diagnosed cancer in women with projected 256,840 total new cases in 2015 in Canada and the United States (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2015; Siegel et al., 2015). It is well known that 17-beta-estradiol (E2), the most potent estrogen, plays a crucial role in the development of hormone-dependant breast cancer. The hormone exerts its mitogenic effects on breast cancer cells principally through the mechanisms of its cognate receptor, estrogen receptor alpha (ERα). These mechanisms involve the binding of E2 to ERα, the activation of ERα, and the interaction of ERα with specific enhancers known as estrogen-responsive elements (ERE) present in

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the promoters of estrogen-responsive genes (ERGs), leading to the expression of these estrogen-regulated genes as well as stimulation of cell growth and proliferation by the alteration of the expression of genes responsible for controlling cell cycle and proliferation (Mense et al., 2008; Perillo et al., 2000). ERGs are genes for which expression is regulated by E2 and generally undergo stimulation from a physiological concentration of E2. For example, in the breast cancer cell line MCF7, physiological concentrations of E2 range from 10 pM to 1 nM have been shown to stimulate a total of 50 ERGs, leading to the stimulation of cell growth (Lippman et al., 1976; Kodama and Kodama, 2001; Garvin and Dabrosin, 2008). In breast cancer cells, E2 is majorly synthesized by the steroid-converting enzyme 17beta-hydroxysteroid dehydrogenase type 1 (17β-HSD1) with NADPH as cofactor, and we previously showed that 17β-HSD1 expression is positively correlated to the E2 level and the growth of breast cancer cells (Aka et al., 2009, 2010; Lin et al., 2010). We have also reported the first observation that 17β-HSD1 gene knockdown by small interfering RNA (siRNA) modulates endogenous pS2 expression (Aka et al., 2010). The latter is a well-known ERG which has been used as a marker of estrogen responsiveness in ER-containing breast cancer cells (Kim et al., 2000). Thus, it would be of particular importance to study the relation between 17β-HSD1 expression and that of other ERGs, notably those involved in breast cancer cells growth, in order to better understand its role in these cells.

Previously, in a proteomics study, we demonstrated for the first time that 17β-HSD1 modulates breast cancer protein profile, including important proteins that are relevant to cell growth control (Aka et al., 2012). It would be of particular importance to study the impact of 17β-HSD1 expression on breast cancer transcript regulation. Overexpression experiment was used to generate the previous proteomics data from the estrogene-sensitive breast cancer cell line MCF7 cultured in E2-containing medium. As MCF7, T47D is a human hormone-dependent breast cancer cell line, expressing estrogen receptor, that was previously reported to express some ERGs (Inoue et al., 2002; Wang et al., 2004), and which was widely used for breast cancer studies. But contrary to MCF7, T47D cell line expressed high level of endogenous 17β-HSD1 (Aka et al., 2010, 2012; Laplante et al., 2009; Aka and Lin, 2012). DNA microarray is the most popular tool to investigate global changes in gene expression following biological treatments. We thus used this technique in combination with RNA interference technique to study the effect of 17β-HSD1 gene knockdown on transcription profile regulation of T47D cultured in steroid-deprived as well as in estradiol-containing media. Additionally, the gene lists revealed by microarray data were further analysed for functional enrichment through Ingenuity Pathway Analysis (IPA) in order to understand the biological functions, gene networks, canonical pathways, and potentially upstream regulators of the regulated genes.

2. Material and methods

2.1. Cell culture

T47D cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were propagated in phenol red-free DME high glucose medium containing 7.5 mg/l bovine insulin (Sigma, Oakville, Ontario, Canada) and 10% fetal bovine serum (FBS). When indicated, FBS was treated overnight at 4 °C with 2% dextran-coated charcoal to remove the remaining steroids present in the serum. Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.2. siRNA transfections and steroid treatment

The sense and antisense sequences of three 17β-HSD1 siRNAs were selected and synthesized as previously described (Aka et al., 2010). Scramble siRNA was used as negative control siRNA. Two days before transfection, T47D cells in T75 flasks were cultured in steroid deprived medium (dextran-coated charcoal-treated medium). On the transfection day, cells in T75 flasks were trypsined and resuspended in a fresh charcoal-treated medium. 3×10^5 cells were then reverse-transfected in 6-well plates with 200 nM mixed 17β-HSD1 specific siRNAs (si17B1) or with negative control siRNA (NC) using Lipofectamine siRNAMax (Invitrogen, Burlington, Ontario, Canada), and cells were incubated. Two days (48 h) after transfection, cell culture media were replaced by fresh charcoal-treated medium containing either the steroid E2 (1 nM) or ethanol as a vehicle control, and cells were incubated for two more days before RNA extraction. The RNA samples included two independent biological replicates, coming from two independent cell culture experiments, for a total of eight RNA samples.

2.3. RNA preparation

Total RNA was isolated from T47D cells using Trizol Reagent (Invitogen) in 6-well plates. RNA samples were treated with DNase1, purified using RNeasy Mini Kit column (Qiagen, Mississauga, ON, Canada), and RNA quality was assessed on the Agilent Bioanalyser 2100 (Agilent Technologies, Mississauga, Ontario, Canada). Analysis using the RNA 6000 Nano Chips (Agilent Technologies) showed good qualities for all RNA samples with the RNA Integrity Numbers (RIN) greater than 8/10 for all the samples. RNA samples for subsequent microarray analyses and reverse transcription quantitative real-time PCR (RT-qPCR) comprised of eight samples composed of two biological repetitions for each of the four treatment conditions.

2.4. Microarray processing

RNA samples were processed according to the manufacturer's recommended procedures on GeneChip Whole Transcript (WT) Sense Target Labeling Assay from Affymetrix (http://www.affymetrix.com/support/downloads/manuals/wt_sensetarget_label_manual.pdf). The assay was started with 0.2 μg of each T47D cells RNA samples and the protocol is based on the principle of performing one cycle of cDNA synthesis and in vitro transcription (IVT) for target amplification to generate cRNA following by reverse transcription reactions to synthesis the WT cDNA. About 2.7 μg sample of fragmented cDNAs was used to hybridize human oligonucleotide array Gene 1.0 ST (Genechip; Affymetrix). The array comprised more than 750,000 unique 25-mer oligonucleotides constituting over 28,000 gene-level probe sets of the human genome. The cDNA probe corresponding to each biological repetition for each condition was hybridized on separate arrays. After hybridization, chips were processed using the Affymetrix GeneChip Fluidic Station 450 (protocol F450_0007). Chips were scanned with a GeneChip scanner 3000 7G (Affymetrix) and images were extracted with the GeneChip operating software (Affymetrix GCOS v1.4). The microarray processing was performed at the DNA Biochip Platform service at CHU de Québec - CHUL Research Centre (Québec, Canada).

2.5. Microarray analysis

Quantified Affymetrix image files ("CEL" files) for each of the treatment conditions (including two independent replicates per treatment condition) were exported into the statistical software environment R where the microarray analyses were performed using the Bioconductor package OneChannelGUI (Gentleman et al., 2004; Sanges et al., 2007). Three contrasts were made for the