Journal of Cancer Therapy, 2013, 4, 70-74
http://dx.doi.org/10.4236/jct.2013.47A011 Published Online August 2013 (http://www.scirp.org/journal/jct)

Angiotensin-(1 - 7) and Human Chorionic Gonadotropin (hCG) Modulate the Nuclear Transcription Factors or Nuclear Receptors Genes in the Tumorigenic Undifferentiated Breast Cancer Cell Line SKBR3*

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Received May 19th, 2013; revised June 20th, 2013; accepted June 29th, 2013

ABSTRACT

Breast cancer is the most common cancer among women. Angiotensin-(1 - 7) [Ang-(1 - 7)] has been correlated with cancer antiproliferative and apoptotic effects, similar properties of the human Chorionic Gonadotrofin (hCG). The aims of this work are to evaluate the role of Ang-(1 - 7) and of hCG in modulating the expression of Nuclear Receptors and Coregulators related genes in the tumorigenic breast cell line SK-BR3. Three experimental groups were created: control, hCG and hCG + Ang-(1 - 7). Cells were treated for 11 days and then had their RNA extracted. Samples were loaded into PCR Array plates containing 84 genes relate to Nuclear Receptors and Coregulators pathways. Gene expression data were used to construct canonical pathways (Metacore™). hCG and hCG + Ang-(1 - 7) treatments markedly modulate the expression of Nuclear Receptors and Coregulators related genes. hCG differentially expressed 17% of the genes, being 29% upregulated and 71% downregulated. Meanwhile, hCG + Ang-(1 - 7) changed the expression of 30% of the genes on the plate, among these genes 56% were upregulated and 44% downregulated. Among these differentially expressed genes, we highlight Esr1, Nr2f2, and Nr2f1, Esr1, Hdac5, and Nr4A1 (>4 fold). Finally MetaCore analysis based on Gene Ontology (GO) generated six networks for hCG and ten networks for the combined treatment. All generated networks are related to regulation of apoptosis or to Programmed Cell Death processes. In summary, our results herein demonstrate that the modulation of sexual hormones and of other nuclear factor genes expression might underlie the tumorigenic protection effect and the induction of cell differentiation caused by the hormones hCG and Ang-(1 - 7), especially in Cancer Stem Cells.

Keywords: Breast Stem Cancer Cells; SK-BR3; hCG; Angiotensin-(1 - 7)

1. Introduction

Breast cancer is the most common cancer among women, accounting for thousands of deaths annually. In 2012, the estimated number of new breast cancer cases is above two-hundred and twenty-nine thousand [1]. Among the various mediators that act in the carcinogenic process, the components of the renin-angiotensin system (RAS) have assumed an important role [2-5]. Angiotensin II (Ang II), better known peptide obtained from the cascade of events of RAS, has vasoconstrictive, angiogenic, hyperplastic, proliferative and metastatic properties [6,7].

Moreover, it has also been demonstrated an association between genetic polymorphisms of some RAS components with breast cancer [8-10]. Many are the evi-

*The work was supported by Grant number 2011/10516-0 and 2008/54383-0 from the Sao Paulo Research Foundation (FAPESP)-Brazil.
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dences that the RAS is related to neoplasia of the breast tissue and also that its disruption may be involved in one or more steps that lead to carcinogenesis [11].

On the other hand, angiotensin-(1 - 7) [Ang-(1 - 7)] another peptide component of the RAS, has been extensively studied lately, for its vasodilator, antiproliferative and apoptotic effects, opposite effects generated by Ang II [12,13].

New breast cancer approaches have identified a small population of highly tumorigenic cells with stem cell properties in the human breast and in other solid tumors. These cells have been considered the source of tumor initiation and of its maintenance. These highly proliferative cells are referred to as cancer stem cells (CSCs) [14].

Altogether, the aims of this work are to evaluate the role of Ang-(1 - 7) and of hCG in modulating the expression of *Nuclear Receptors and Coregulators* related genes in the tumorigenic breast cell line SK-BR3 in order to better understand the molecular mechanisms underlying the effects triggered by these compounds in CSCs.

2. Methods

2.1. Cell Culture and Treatments

The SKBR3 cell line was grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Three experimental groups were created: control, hCG and hCG + Ang-(1 - 7). Cells were treated for 11 days.

2.2. RNA Extraction

Pelleted cells were homogenized in Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was purified with Qiagen RNeasy Mini Kit and subjected to treatment with DNase A. The quantity and quality of extracted RNA were measured by espectrophotometer (Nanodrop Technologies Inc., Rockland, DE).

2.3. Real Time PCR Array

According to the manufacturer's (Qiagen) methodology, reverse transcriptase (RT) was carried out for the synthesis of cDNA. For each sample we used as a template a PCR array plate containing 84 different pairs of primers for studying genes related to Nuclear Receptors and Coregulators pathways (RT2 Profiler^{TM} PCR Array; SABiosciences).

2.4. Analysis of Relevant Biological Processes and Networks by MetaCore

The MetaCore software (GeneGo, St. Joseph, MI) is a computational resource that uses logic operations for identifying altered biological processes based upon gene expression changes. Genes with altered expression were mapped to Gene Ontology (GO) using MetaCore algorithm. GO annotations were used as indicators of biological functions. GO describes gene products in terms of their associated biological processes, cellular components, and molecular functions. The GO entries are hierarchically linked, thus allowing construction of cluster genes of crossed pathways.

2.5. Statistical Analysis

These results were analyzed by descriptive statistics (means and standard deviation) and inferential statistics through the student's t-test, with significance level of 5% ($p < 0.05$). Real-time PCR array reactions were processed through the online software RT2 Profiler^{TM} PCR Array Data Analysis (SABiosciences).

3. Results

hCG and hCG + Ang-(1 - 7) treatments markedly modulates the expression of Nuclear Receptors and Coregulators related genes (**Figures 1** and **2**). hCG differentially expressed 17% of the genes, being 29% upregulated and 71% downregulated. Meanwhile, hCG + Ang-(1 - 7) changed the expression of 30% of the genes on the plate, among these 56% were upregulated and 44% downregulated. In general, the combined treatment generates a more downregulated expression profile of these genes than hCG itself (**Figures 1** and **2**).

Among these differentially expressed genes, we highlight Esr1, Nr2f2, and Nr2f1, Esr1, Hdac5, and Nr4A1 (>4 fold) (**Figure 3**). Finally MetaCore analysis based on Gene Ontology (GO) generated six networks for hCG treatment and ten networks for the combined treatment. All generated networks are related to regulation of apoptosis or to Programmed Cell Death processes (**Figure 4**).

4. Discussion

The search for possible new molecular targets to treat or to early detect breast cancer is of paramount importance. Nowadays many researchers are focusing on the actions elicited by the hormone hCG, which has been shown to decrease proliferation of mammary tumor cells [15,16].

The results here presented clearly demonstrate that hCG alters the expression profile of many genes encoding for proteins that act as nuclear transcription factors or as nuclear receptors.

Surprisingly both treatments restored the expression of the estrogen receptor 1 (ESR1) gene in this originally estrogen receptor negative cell line. The ESR1 is a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and

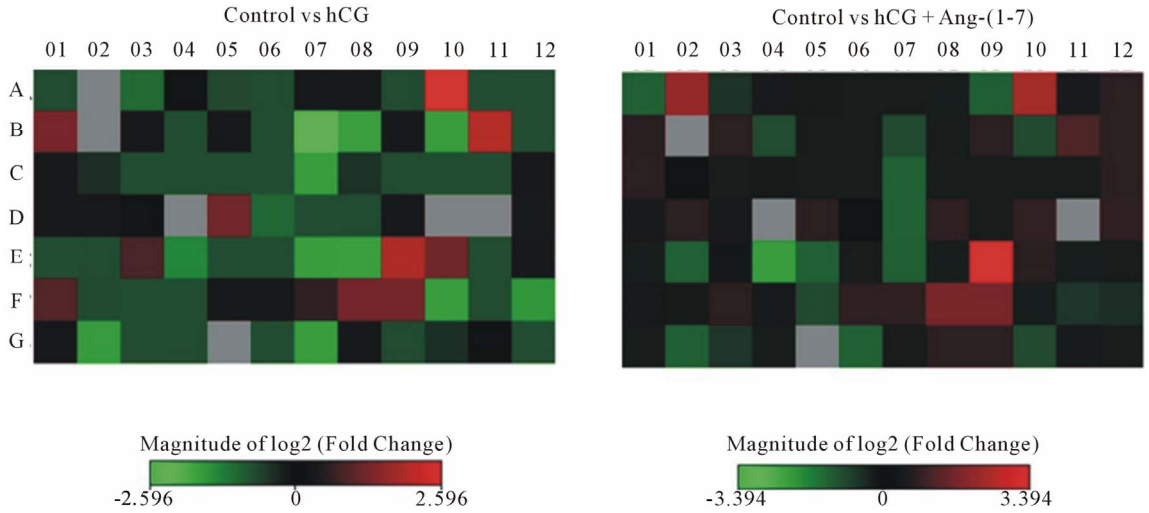

Figure 1. Heat map of SKBR3 cells gene expression after treatment.

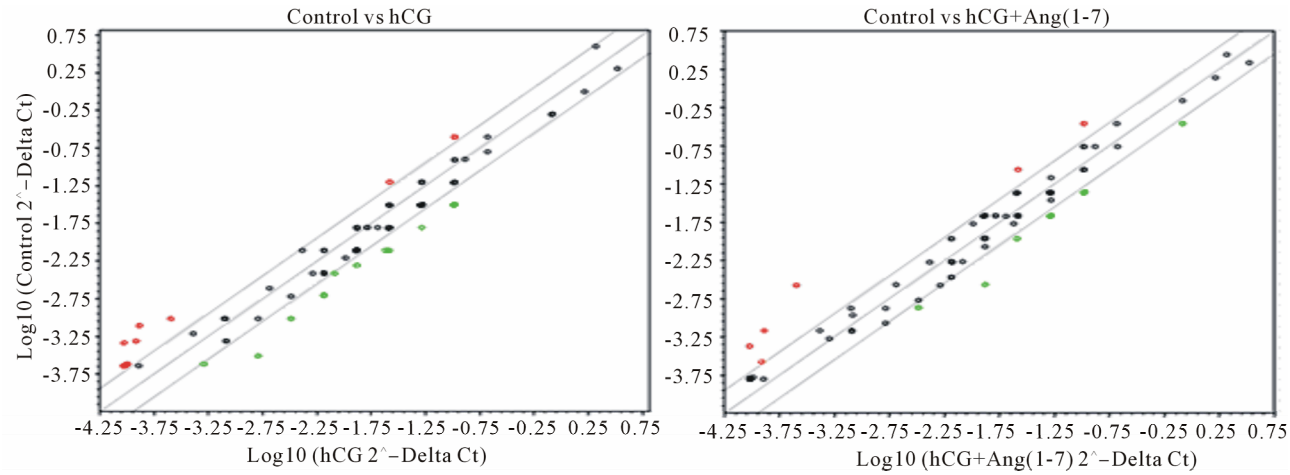

Figure 2. Gene expression analysis presented by scatter plot graphs.

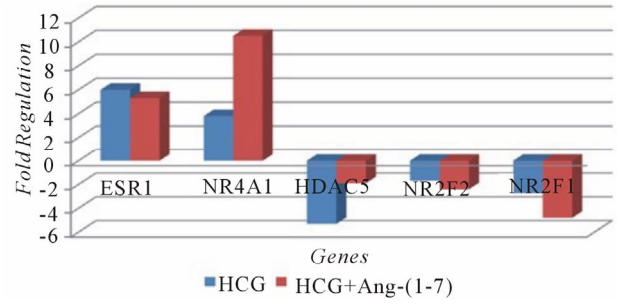

Figure 3. Most differentially expressed genes caused by both treatments in SKBR3 cells. Control cells were used as the calibrator sample.

activation of transcription. Estrogen receptors are also directly involved in different pathological processes including breast cancer, endometrial cancer, and osteoporosis [17].

Prolonged exposure to estrogens is a significant risk factor for the development of breast cancer. Estrogens exert carcinogenic effects by stimulating cell proliferation or through oxidative metabolism that forms DNA-damaging species. In SKBR3 cells, all of these estrogen-forming enzymes were expressed, although the lack of ESR1 and the low levels of ESR2 expression suggest that hCG and Ang-(1 - 7) modulate the expression of sexual hormone genes [18].

Nuclear Receptor Subfamily 2 (NR2F2) encodes a member of the steroid thyroid hormone superfamily of nuclear receptors. The encoded protein is a ligand inducible transcription factor involved in regulation of many different genes (pubmed). Members of this family inhibit cell differentiation and increase cell growth. Inhibition of COUP-TFII (Nr2f2) may offer a novel therapeutic approach to breast cancer [19]. In the present study hCG downregulated COUP-TFII, which might partially explain the breast cancer protection brought about by hCG. Besides that, hCG restores ESR1 gene expression, which

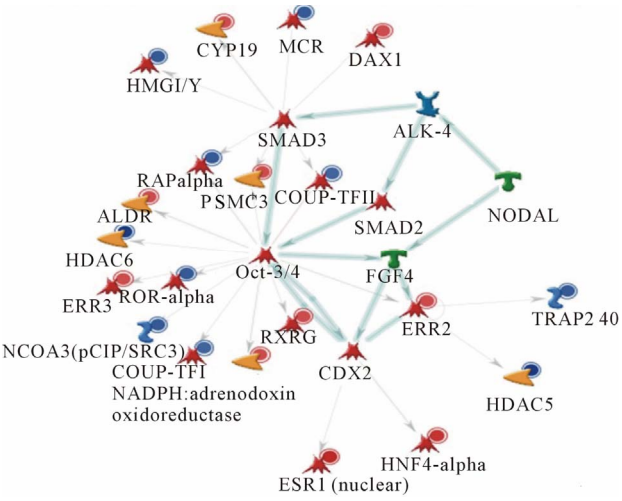

Figure 4. Top scored (by number of pathways) network generated by the active experiments. Thick cyan lines indicate the fragments of canonical pathways. Up-regulated genes are marked with red circles; down-regulated with blue circles. The checkerboard' color indicates mixed expression for the gene between files or between multiple tags for the same gene.

might be beneficial when considering the antineoplastic drugs available to treat breast cancer. At the same time, NR2F2 downregulation indicates that hCG seems to in duce cell differentiation in SKBR3 cells [19].

hCG also caused downregulation of the Nuclear Receptor Subfamily 2 (NR2F1), which may partially explain the anti-proliferative effects of this hormone [20].

Another important action of hCG was to increase the expression of the NR4A1 gene, which has an antimi-gration effect on normal cells.

Histone Deacetylase 5 (HDAC5) is an enzyme responsible for maintenance/assembly of the heterochromatin structure. As previously demonstrated its specific inhibition might contribute to increase the efficacy of DNA alteration-based cancer therapies in clinic [21]. hCG inhibits expression of HDAC5, reducing cancer progression and cell survival.

In summary, our results herein demonstrate that the modulation of sexual hormones and of other nuclear factor genes expression might underlie the tumorigenic protection effect and the induction of cell differentiation caused by the hormones hCG and Ang-(1 - 7) [22], especially in CSCs [15,23].

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