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Chenodeoxycholic acid (CDCA) through TGR5-dependent CREB-signaling activation enhances Cyclin D1 expression and promotes human endometrial cancer cell proliferation

Tutor

Dott. Ivan Casaburi

Dottoranda

Dott. Paola Avena

Coordinatore

Prof. Diego Sisci

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INDEX

Summary

Increasing epidemiologic data in humans as well as many in vitro investigative reports suggest that overweight and obesity are important risk factors for type-I EC which is the most common malignancy in women and accounts for 80% of all ECs. The strongest support for mechanisms to link obesity and cancer risk involves the metabolic and endocrine effects of obesity and the alterations that they induce in the production of peptide and steroid hormones. One way in which fat might exert its effect is stimulation of bile acids (BAs) synthesis and secretion.
Bile acids (BAs) are amphipathic detergents that are synthesized in the liver and stored in the gallbladder. An important physiological role of BAs is to facilitate the uptake of lipids together with the fat-soluble vitamins A, D, E and K from the intestine. BAs facilitate these absorptive processes through their detergent properties, which allow the emulsification of lipids. Moreover, BAs have evolved over the past few years from being considered as simple lipid solubilizers to complex metabolic integrators. Beyond the orchestration of bile acids, lipid and glucose metabolism by the nuclear receptor farnesoid X receptor (FXR), BAs also act as signaling molecules through the non-genomic pathway activated by a BA-dedicated G protein-coupled receptor (GPCR) TGR5 (also known as BG37 or MBAR). Despite the efficient enterohepatic recirculation of bile acids, a small amount of them can spill over into the systemic circulation particularly during high fat diet. Since in the obese women a strong correlation with endometrial cancer does exist, we investigated the biological effects of different concentrations of the primary bile acid CDCA in a human endometrial type-I cancer cell line, Ishikawa.
Ishikawa cell proliferation was determined by [3H]-thymidine incorporation assay after 72 hours of treatment with different concentrations of CDCA. The effects of CDCA on the expression of the cell cycle regulatory proteins were evaluated by RT-PCR and Western blotting assays. Transient transfection method and mutagenesis studies were performed to functionally characterize the Cyclin D1 promoter while ChIP assay was to highlight the direct involvement of the cis-elements in CDCA-dependent Cyclin D1 up-regulation. In addition, the effects of

impaired expression and function of the proteins involved in CDCA activated signaling were assessed by using small interfering RNAs (siRNAs) technology.

Our results indicate that low concentrations (< 30μM) of CDCA were able to stimulate Ishikawa cell growth as evidenced by [^{3}H]Thymidine incorporation and cell cycle analysis by inducing a significant increase in Cyclin D1(CD1) protein and mRNA expression. In contrast, according to previous findings, high doses (> 50μ) of CDCA showed cytotoxic effects concomitantly with an increase of CDK inhibitor $p21^{WAF1/CIP1}$ expression through a p53-indipendent pathway. Moreover, mutagenesis studies and ChIP analysis revealed that the CDCA-induced CD1 expression requires a cyclic AMP-responsive element (CRE) binding protein motif within the CD1 proximal promoter. Silencing of a cell surface bile acid sensor TGR5 and/or CREB gene expression by RNA interference reversed the CDCA-dependent induction of CD1 expression and proliferation in Ishikawa cells.

In conclusion, extrahepatic spillover of BAs following high fat diet particularly in overweight and obese women, could be involved in the onset and/or maintenance of endometrial cancer by stimulation of TGR5 that activates ERK signaling that in turn induces the recruitment of the transcription factor CREB to the Cyclin D1 gene promoter enhancing cell proliferation.

INTRODUCTION

Introduction

Worldwide, endometrial cancer (EC) is the seventh most common malignant disorder, but incidence varies among regions [1]. In less developed countries, risk factors are less common and endometrial cancer is rare, although specific mortality is higher [2] and [3]. The incidence is ten times higher in North America and Europe than in less developed countries; in these regions, this cancer is the commonest of the female genital tract and the fourth commonest site after breast, lung, and colorectal cancers. The most relevant environmental risk factors for endometrial cancer and its precursor, endometrial hyperplasia, include fat-rich diet, excess of body weight and physical inactivity.

Increasing epidemiologic data in humans as well as many in vitro investigative reports suggest that overweight and obesity are important risk factors for type-I EC which is the most common malignancy in women and accounts for 80% of all ECs [5–12].

Presently, the strongest support for mechanisms to link obesity and cancer risk involves the metabolic and endocrine effects of obesity and the alterations that they induce in the production of peptide and steroid hormones [13].

One way in which fat might exert its effect is stimulation of bile acids (BAs) synthesis and secretion [14,15]. Bile acids (BAs) are amphipathic detergents that are synthesized from cholesterol and actively secreted into bile by the liver and stored in the gallbladder. Usually bile is stored in the gallbladder, however, when a meal is ingested, it flows into the duodenum. The BAs are absorbed again by passive diffusion and active transport from the terminal ileum, and transported back to the liver via the portal vein, which completes their enterohepatic recirculation. The first pass extraction of BAs by the liver is remarkably efficient (70–90%). Thus after a meal, BA levels in the hepatocyte will increase. The hepatic extraction rate of BAs usually remains constant during the fasting state and during digestion [16]. Consequently, a significant amount of BAs can spill over into the systemic circulation. Therefore, after a meal, BAs levels will not only increase in the portal vein and the liver but also in the systemic circulation [17,18]. Indeed fasting serum BAs are usually below 5μM, whereas postprandial

levels rise up to 15 μM [19]. As a consequence of this phenomenon, serum BAs levels vary during the day following a rhythm dictated by the ingestion of meals that could be stressed out during a high fat diet which is one of the contributory causes of evolution from overweight to obesity.

Moreover, while the physiological and pathophysiological effects of bile acids have been well documented for the liver and intestine, where they play an essential role in dietary lipid absorption and cholesterol homeostasis [20], less is understood for BAs effects on tissues outside of the enterohepatic circulation. Recent studies have shown that bile acids can modulate intracellular signaling and gene expression which may in turn affect cell growth and tumor development.

Indeed, BAs can act as signaling molecules through the activation of specific receptors that control different systemic endocrine functions [21]. Specifically, BAs have been recognized as ligands for the nuclear farnesoid X receptor (FXR, also known as NR1H4) [22] that is now recognized as a master regulator of the pleiotropic actions of endogenous bile acids in the regulation of enterohepatic recycling of bile acids and in the feedback regulation of bile acid biosynthesis in the liver and intestine [21]. Activation of FXR protects against the toxic accumulation of bile acids through increased conjugation in the liver, followed by their excretion into bile canaliculi, thereby promoting bile flow [23-24]. FXR is also implicated in regulation of glucose metabolism through regulation of gluconeogenesis and glycogenolysis in the liver [25,26] and regulation of peripheral insulin sensitivity in the adipose tissue [27]. The discovery of FXR was followed by the pregnane X receptor (PXR; also known as NR1I2) and the vitamin D receptor (VDR; also known as NR1I1), which are other bile acid-activated receptors that play important roles in detoxification of bile acids, drugs and xenobiotics [28,29].

More recently, in addition to their genomic action, BAs have been also shown able to induce rapid non-genomic signaling pathways through the activation of TGR5, a novel plasma membrane G protein-coupled receptor, also known as M-BAR or BG37. This cell-surface BA receptor was discovered in 2002 [30], and first characterized in 2003 [31]. TGR5 is encoded by a single-exon gene, and its conservation among several vertebrate species underlines the relevance of this

GPCR in vertrebrate physiology [31]. Bile acids, including taurine-conjugated lithocholic acid (TLCA), lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and cholic acid (CA), are natural endogenous ligands for this G-protein coupled receptor. The main cellular responses following TGR5 stimulation include receptor internalization, activation of extracellular signal-regulated kinase (MAPK) and intracellular cAMP production with the following BAs rank order of potency: TLCA (0.33 μM) > LCA (0.53 μM) > DCA (1.01 μM) > CDCA (4.43 μM) > CA (7.72 μM) [31]. TGR5 is ubiquitously expressed in various human tissues, such as heart, spleen, skeletal muscle, kidney, liver, small intestine, placenta, lung, mammary gland, prostate, testis as well as uterus [30].

To date, the biological effects of bile acid on endometrial cancers cells has not been evaluated. The current study was designed to investigate the biological effects of the primary bile acid chenodeoxycholic on the human endometrial cancer type-I cell line Ishikawa.

The results obtained provide convincing evidence that low doses of CDCA are able to up-regulate Cyclin D1 expression through the activation of G-coupled membrane receptor, TGR5, leading to endometrial cancer cell growth.

Materials and Methods.

Materials

Modified Eagle's medium (MEM), L-glutamine, Eagle's non-essential amino acids, penicillin, streptomycin, fetal bovine serum (FBS), bovine serum albumin (BSA), phosphate-buffered saline, RNase A were purchased by Sigma (Milan, Italy). TRIzol and Lipofectamine 2,000 reagent by Invitrogen (Carlsbad, CA), FuGENE 6 by Roche Applied Science (Indianapolis, IN). TaqDNA polymerase, RETROscript kit, 100-bp DNA ladder, Dual Luciferase kit, and TK Renilla luciferase plasmid were provided by Promega (Madison, WI). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, and CDCA were purchased by Sigma (Milan, Italy). Antibodies against phospho p44/42 MAPK (Thr 202/Tyr 204) (#9101S), p44/42 MAPKinase (#9102), phospho-p38 MAPK (Thr180/Tyr182) (#9211S), p38 MAPK Antibody #9212, pCREB (Ser133) and CREB (48H2), were provided by Cell Signaling. Antibodies against cyclin D1 (M-20), GAPDH (FL-335), p21WAF1/Cip1 (H164), p53 (DO-1), and polymerase II (N20) by Santa Cruz Biotechnology (Santa Cruz, CA). An ECL system and Sephadex G-50 spin columns were purchased from Amersham Biosciences (Buckinghamshire, UK). [l32P]ATP and [3H]thymidine fromPerkinElmer Life Sciences (Wellesley, MA). H89, PD 98059 (Calbiochem, La Jolla, CA).

Plasmids

The plasmids containing the human cyclin D1 promoter or its deletions (p_2966/þ142, p_944/þ142, p_848/þ142, p_136/þ142 ,p_136/þ142 mut-creb) were kindly provided by Prof A. Weisz (University of Naples, Italy). These fragments were inserted into the luciferase vector pXP2.

Site-directed mutagenesis

The cyclin D1 promoter plasmids bearing cyclic AMP-responsive element (CRE) mutated site (pCRE mut) were created by site-directed mutagenesis using QuickChange kit (Stratagene, La Jolla, CA).

Briefly, this was based on a PCR reaction with two complementary oligonucleotide primers containing the mutation. The PCR was performed with the Pfu DNA polymerase during 16 cycles (30 sec at 95 °C, 30 sec at 55 °C, and 8 min at 68 °C), using as template the human cyclin D1 promoter p_136/þ142 and the following mutagenic primers (mutations are shown as lowercase letters): 5'-GATCTTTGCTTAACAACAGTAACctcACACGGACTACAGGGGAG-3' and 5'-CTCCCCTGTAGTCCGTGTagaGTTACTGTTGTTAAGCAAAGATC-3' (CRE MUT).

The PCR products were then incubated with DpnI which only digests the parental methylated cDNA and the constructed mutated expression vectors were confirmed by DNA sequencing.

Cell culture

Ishikawa human endometrial cancer cells were obtained from D.Picard (University of Geneva, Geneva, Switzerland). Ishikawa cells were maintained in MEM without phenol red supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin and 1% non-essential amino acid. Cells were switched to medium without serum 24 h before each experiment.

DNA flow cytometry

Ishikawa cells were harvested, fixed, and stained with Propidium iodide (100 mg/ml) after treatment with RNase A (20 mg/ml). Stained cells were analyzed for DNA content by Flow Cytometry using FAC-Scan (Becton Dickingson and Co., Franklin Lakes, NJ).

Total RNA extraction and reverse transcription-PCR assay

Total cellular RNA was extracted from Ishikawa cells using Triazol reagent (Invitrogen, USA) as suggested by the manufacturer and then treated with DNase I (Ambion, Austin, TX, USA). The evaluation of genes expression was performed by the reverse transcription-PCR method using a RETROscript kit as suggested by the manufacturer.

The cDNAs obtained were amplified by PCR using the following primers:

GENE	SEQUENCE
Ciclina D1 forward	**5'-TCTAAGATGAAGGAGACCATC-3'**
Ciclina D1 reverse	**5'-GCGGTAGTAGGACAGGAAGTTGTT– 3'**
P21 forward	**5'-CTGTGCTCACTTCAGGGTCA-3'**
P21 reverse	**5'-CTCAACATCTCCCCCTTCTC-3'**
P53 forward	**5'-CCAGTGTGATGATGGTGAGG-3'**
P53 reverse	**5'-GCTTCATGCCAGCTACTTCC-3'**
TGR5 forward	**5'-TCGTCTACTTGGCTCCCAACTTCT-3'**
TGR5 reverse	**5'-AGCCCATAGACTTCGAGGTACAGGT-3'**
36B4 forward	**5'-CTCAACATCTCCCCCTTCTC-3'**
36B4 reverse	**5'-CAAATCCCATATCCTCGTCC-3**

The PCR was performed for 30 cycles for cyclin D1 (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min), 30 cycles for p21 (94°C for 1 min, 58°C for 1 min, and 72°C for 2 min), 28 cycles for p53 (95°C for 1 min, 56°C for 1 min, and 72°C for 2 min), 32 cycles for TGR-5 (95°C for 1 min, 68°C for 1 min, and 72°C for 2 min), and 15 cycles (94°C for 1 min, 58°C for 1 min, and 72°C for 2 min) to amplify 36B4 in the presence of 1 µl of first strand cDNA, 1 µM each of the primers mentioned above, 0.5 mM dNTP, Taq DNA polymerase (2 units/tube), and 2.2 mM magnesium chloride in a final volume of 25 µl. To check for the presence of DNA contamination, a reverse transcription-PCR was performed on 1 µg of total RNA without Moloney murineleukemia virus reverse transcriptase (the negative control).

DNA quantity in each lane was analyzed by scanning densitometry.

Immunoblotting

Ishikawa cells were grown in 10 cm dishes to 50–60% confluence and lysed in 500 µl of 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EdTA, 0.1% SDS, and a mixture of protease inhibitors (aprotinin, PMSF, and sodium ortho-vanadate). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories,

Hercules, CA USA). Equal amounts of total proteins were resolved on an 11% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the appropriated antibody. As internal control, all membranes were subsequently stripped (glycine 0.2 M, PH 2.6 for 30 min at room temperature) of the first antibody and reprobed with anti GADPH Ab. The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxidase-coupled goat anti-mouse or anti-rabbit IgG and revealed using the ECL System.

The blots were then exposed to film, and the bands of interest were quantified by Scion Image laser densitometry scanning program. The results obtained as optical density arbitrary values were transformed to percentages of the control (percent control) taking the samples from cells not treated as 100%.

Real-time reverse transcription–PCR

Total RNA was isolated as above mentioned and the cDNA obteined was diluted 1 : 3 in nuclease-free water and 5 µl were analysed in triplicates by real-time PCR in an iCycler iQ Detection System (Bio-Rad, USA) using SYBR Green Universal PCR Master Mix (Bio-Rad) with 0.1 µmol/l of each primer in a total volume of 30 µl reaction mixture.

The cDNAs obtained were amplified by PCR using the following primers:

GENE	SEQUENCE
TGR5 forward	**5′-TCAGCCAGGACACCAGACAT-3′**
TGR5 reverse	**5′-TGGGCCTTCCTGAGTGTCA-3′**
GAPDH forward	**5'-CCC ACT CCT CCA CCT TTG AC-3'**
GAPDH reverse	**5'-TGTTGC TGT AGC CAA ATT CGT-3'**

Negative controls contained water instead of first strand cDNA. Each sample was normalized on its GAPDH rRNA content. The GAPDH quantification was done using a TaqMan rRNA Reagent kit (Applied Biosystems, USA) following the manufacturer instructions. The relative gene expression levels were normalized to

a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as -fold differences in gene expression relative to GAPDH rRNA and calibrator, calculated using the $\Delta\Delta C_t$ method as follows: *n*-fold = $2^{-(\Delta Ctsample-\Delta Ctcalibrator)}$, where ΔC_t values of the sample and calibrator were determined by subtracting the average C_t value of the GAPDH rRNA reference gene from the average C_t value of the different genes analysed.

Transient transfection assay

Ishikawa cells were seeded into 24-well plates with 500 µl of regular growth medium/well the day before transfection. The medium was replaced with serum free medium on the day of trasfection which was performed using FuGENE 6 reagent with the mixture containing 0.25 µg of human cyclin D1 promoter constructs.

Twenty-four hours after transfection, the cells were untreated or treated with CDCA 5µM for 24 h. TK Renilla luciferase plasmid (20 ng per each well) was used. Firefly and Renilla luciferase activities were measured by Dual Luciferase kit (Promega). The firefly luciferase data for each sample were normalized based on the transfection efficiency measured by Renilla luciferase activity and data were reported as Relative Light Units (RLU) values. All the luciferase assays were carried out at least in triplicate, and the experiments were repeated thrice.

Electrophoretic mobility shift assay (EMSA)

Ishikawa cells plated into 10 cm dishes were grown to 70-80 % confluence shifted to SFM for 24 hours and then treated with 5µM of CDCA treatment for 2 hours. Thereafter, cells were scraped into 1.5 ml of cold PBS. Cells were pelleted for 10 seconds and resuspended in 400 µl cold buffer A (10 mM HEPES-KOH pH 7.9 at 4 °C, 1.5mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin). The cells were allowed to swell on ice for 10 minutes and then vortexed for 10 seconds. Samples were centrifuged for 10 seconds and the supernatant fraction discarded. The pellet was resuspended in 50 µl of cold Buffer B (20 mM HEPES-KOH pH 7.9, 25 % glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and

incubated in ice for 20 minutes for high-salt extraction. Cellular debris were removed by centrifugation for 2 minutes at 4 °C and the supernatant fraction (containing DNA binding proteins) was stored at - 80 °C.

The probe was generated by annealing single-stranded oligonucleotides, labeled with [λ32P] ATP and T4 polynucleotide kinase, and purified using Sephadex G50 spin columns.

The DNA sequences used as probe or as cold competitors are the following (the nucleotide motifs of interest are underlined, and mutations are shown as lowercase letters):

5'-TTAACAACAGTAACGTCACACGGACTA-3' and

5'-TAGTCCGTGTGACGTTACTGTTGTTAA-3' (CRE);

5'-C TTAACAACAGTAAttgCACACGGACTA-3' and

5'-TAGTCCGTGTGcaaTT ACTGTTGTTAAG-3' (CRE MUT);

In vitro transcribed and translated CREB protein was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system. The protein-binding reactions were carried out in 20 µl of buffer [20 mmol/L HEPES (pH 8), 1 mmol/L EDTA, 50 mmol/L KCl, 10 mmol/L DTT, 10% glycerol, 1 mg/ml BSA, 50 µg/ml poly(dI/dC)] with 50,000 cpm of labeled probe, 20 µg of Ishikawa nuclear protein or an appropriate amount of CREB protein, and 5 µg of poly (dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. For experiments involving CREB antibody, the reaction mixture was incubated with these antibody at 4 °C for 12 h before addition of labeled probe.

The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25 × Tris borate–EDTA for 3 h at 150 V.

Chromatin immunoprecipitation assay (ChIP)

For ChIP assay, Ishikawa cells were grown in 15 cm dishes to 50-60 % confluence, shifted to SFM for 24 hours and then treated with 5 µM CDCA for 2 hours.

Thereafter, cells were washed twice with PBS and crosslinked with 1 % formaldehyde at 37 °C for 10 minutes. Next, cells were washed twice with PBS at

4 °C, collected and resuspended in 200 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and left on ice for 10 minutes.

Then, cells were sonicated four times for 10 seconds at 30 % of maximal power (Sonics, Vibra Cell 500 W) and collected by centrifugation at 4 °C for 10 minutes at 14,000 rpm. The supernatants were diluted in 1.3 ml of IP 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) followed by immunoclearing with 80 µl of sonicated salmon sperm DNA/protein A agarose for 1 hour at 4 °C. The precleared chromatin was immunoprecipitated with specific anti-CREB and anti polymerase II antibodies or a normal mouse serum IgG as negative control. At this point, 60 µl salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 hours at 4 °C. After pelleting, precipitates were washed sequentially for 5 minutes with the following buffers: Wash A (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), Wash B (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1 % NP-40, 1 % sodium deoxycholate, 1 mM EDTA, 10 mM Tris- HCl pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immunocomplexes were eluted with elution buffer (1 % SDS, 0.1 M NaHCO3). The eluates were reverse crosslinked by heating at 65 °C and digested with proteinase K (0.5 mg/ml) at 45 °C for 1 hour. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. 2 µl of 10 mg/ml yeast tRNA (Sigma) were added to each sample and DNA was precipitated with 70 % ethanol for 24 hours at –20 °C, and then washed with 95 % ethanol and resuspended in 20 µl of TE buffer ; 3 µl of each sample were used for PCR amplification with the following primers flanking CRE sequence present in the cyclin D1 promoter region: 5'-TGCGCCCGCCCCCGCCCCCCTC-3' and 5'-TGTTCCATGGCTGGGGCTCTT-3'.

The PCR conditions were 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. The amplification products obtained in 35 cycles were analyzed in a 2% agarose gel and visualized by Ethidium bromide staining.

RNA interference (RNAi).

Cells were plated in 6-well dishes with regular growth medium the day before transfection to 60–70% confluence. On the second day the medium was changed with SFM without P/S and cells were transfected with an ON-TARGETplus SMARTpool L-005519-00-0005 of (25nM) siRNA targeted human TGR-5 Thermo Scientific Dharmacon, with RNA duplex of validate RNAi targeted human CREB mRNA sequence 5'- GGC UAA CAA UGG UACCGA Utt -3', with human FXR mRNA sequence, 5-CUGCAAGAUCUACCAGCCCGAGAA-3 or with a stealth RNAi control (Ambion) to a final concentration of 50nM using Lipofectamine 2000 as recommended by the manufacturer. After 5 h the transfection medium was changed with SFM in order to avoid Lipofectamine 2000 toxicity and cells were exposed to CDCA after 48h of transfection and for different times depending on the protocols performed (see results and figures legend).

[3H]thymidine incorporation.

Ishikawa cells were untreated or treated with 5μM CDCA for o72 h. For the last 6 h, [3H]thymidine (1 μCi/ml) was added to the culture medium. After rinsing with phosphate-buffered saline, the cells were washed once with 10% and three times with 5% trichloroacetic acid. The cells were lysed by adding 0.1 N NaOH and then incubated for 30 min at 37 8C. Thymidine incorporation was determined by scintillation counting.

Statistical analysis

Each datum point represents the mean ± SE of three different experiments. Statistical analysis was performed using ANOVA followed by Newman–Keuls testing to determine differences in means. $P<0.05$ was considered as statistically significant.

RESULTS

Results

Effects of chenodeoxicholyc acid on endometrial cancer cell growth

Divergent biological effects by bile acids, such as proliferation and apoptosis, have been reported by several authors in different experimental condition [33,34].

Thus, firs we examined, by thymidine incorporation assay, the effect of increasing doses of the primary bile acid (CDCA) on endometrial carcinoma Ishikawa cells growth. As shown in figure 1A, CDCA exerted divergent effects depending on its concentration. Indeed, low doses (2,5 and 5 μM) of CDCA strongly stimulated Ishikawa cell proliferation, while by contrast, the higher CDCA dose (100 μM) markedly suppressed Ishikawa cell growth. These data well correlated with cell cycle analysis showing a decrease of the percentage of cells in the G0/G1-phase and a concurrent increase in the S-phase, following 72h of 5μM CDCA treatment (Figure 1B and Table 1). As expected, 100 μM CDCA treatment instead inhibited cell cycle progression as pointed out by the significant decrease of cell population in S-phase (Figure 1B and Table 1).

Since Cyclin D1 is a critical modulator in the cell cycle G1/S transition and its overexpression is one of the most commonly observed alterations in human endometrial cancers [35,36], we aimed to evaluate the potential ability of CDCA to modulate Cyclin D1 mRNA and protein content in Ishikawa human endometrial cancer cells. In addition, the expression of p21WAF1/Cip1, the major cyclin-dependent-kinase inhibitor, was also analysed. As shown in figure 1C and D, Cyclin D1 mRNA and protein levels were significantly increased by low doses (2,5 or 5μM) of CDCA administration. In contrast, elevated concentrations of CDCA induced a down-regulation of Cyclin D1 expression in a dose dependent-manner concomitantly with an increase of p21WAF1/Cip1 expression (Figure 1C and D). The latter result appears to be mediated through a p53-indipendent pathway since the expression of the tumor suppressor p53 was unaffected by

CDCA exposure (Figure 1C and D). These findings indicate that CDCA exerted a biphasic effects on cell proliferation as well as cell cycle regulating proteins depending on its concentration. It is worth to underline that CDCA at low concentrations was able to stimulate cell proliferation and cell cycle progression.

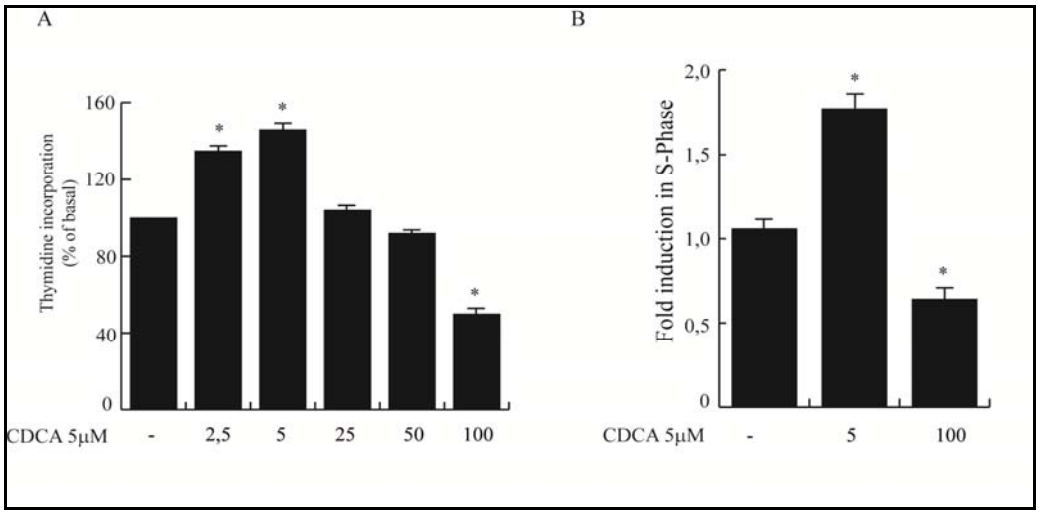


Table: 1

	G0/G1	G2/M	S-Phase
-	52.0 ± 1.7	19.6 ± 1.2	27.6 ± 1.2
CDCA 5 μM	29.3 ± 2	20.6 ± 1.2	49.6 ± 2.1
CDCA 100 μM	69.0 ± 2	14.0 ± 1.1	17.0 ± 1

Figure 1, A, *B. Divergent effects of CDCA treatment on Ishikawa cells*.

A) *Cell proliferation was determinated by [3H]-thymidine incorporation assay after 72 hours of treatment with different concentrations of CDCA as indicated in figure. 5x 10^4 cells per well were seeded on 12 wells/plate, then 24h later culture medium was changed to serum free medium before performing the proliferation experiments in MEM supplemented with 5% charcoal-treated FBS in absence (-) or presence of CDCA. On day 3 cells were incubated for the latest 5 hours with 1mCi/well [3H]-TdR . The results represent the mean ± SE of three independent experiments each performed in triplicate samples. *P< 0.01 compared with untreated cells.* ***B****) Ishikawa cells were synchronized*

in serum-free media for 24h and then exposed to 5 µM and 100 µM CDCA for 72 h or left untreated (–).

The distribution of Ishikawa cells in the cycle was determined by Flow Cytometry using Propidium-iodide stained nuclei. The results indicate the fold-increase of Ishikawa cells in S-phase after serum starvation or CDCA treatment. The ***table 1****shows the distribution of Ishikawa cells in the various phases of cell cycle. *P < 0.01 compared with untreated cells.*

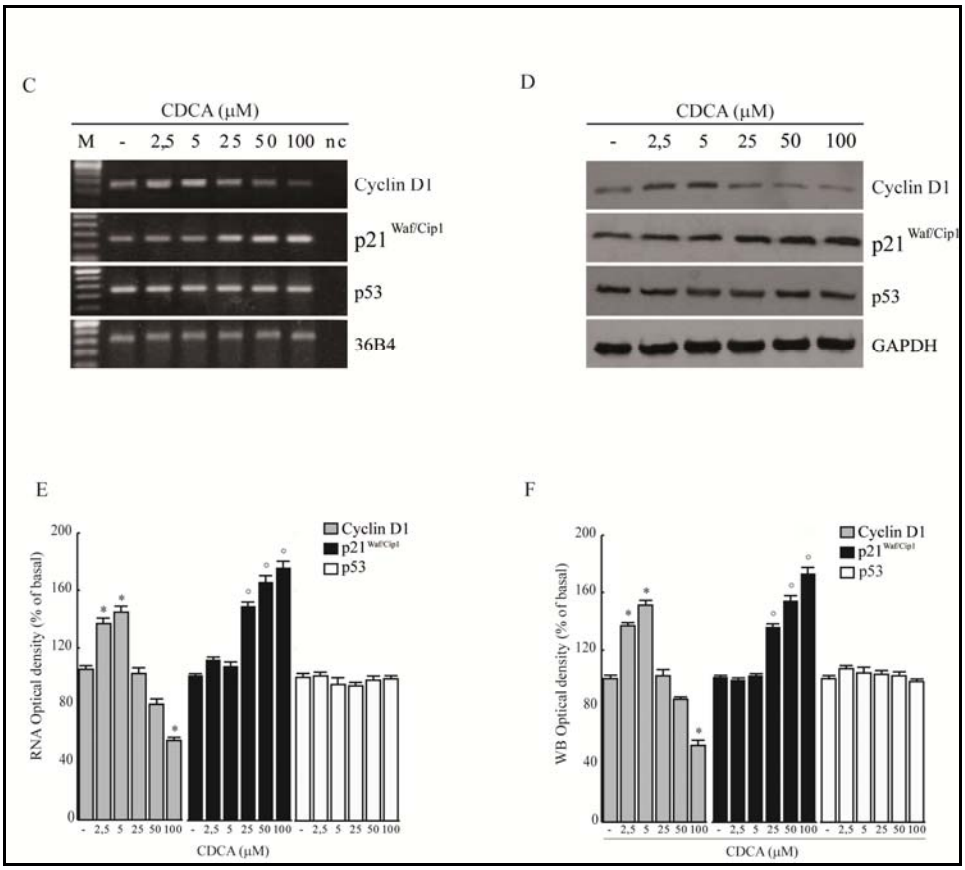


Figure 2 (*C-F *). ***Divergent effects of CDCA treatment on Ishikawa cells***. **C)** *Ishikawa cells were serum-starved for 24 h followed by treatment with 2.5 µM, 5 µM, 25 µM, 50 µM, 100 µM CDCA for 24 h or left untreated (–).Protein extracts obtained from Ishikawa cells were resolved by SDS–PAGE and subjected to immunoblot analysis with rabbit antiserum against human Cyclin D1 and p21WAF1/Cip1and with mouse antiserum against human p53. GAPDH served as loading control* **E)** *The histograms represent the mean ± SE experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. *P < 0.01 compared to untreated cells.* **D)** *Total RNA was isolated from Ishikawa cells*

and reverse transcribed cDNA was subjected to PCR using specific primers for Cyclin D1, p21WAF1/Cip1 or 36B4 (C). nc: negative control, RNA sample without the addition of reverse transcriptase. 36B4 mRNA levels were determined as control. **F***)The histograms represent the mean ± SE experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. *P < 0.01 compared to untreated cells.*

CDCA effects are specifically mediated by TGR5 receptor.

BAs are signaling molecules that can activate BA receptors to initiate signaling pathways and regulate gene expression. Two major receptors for BAs have been identified, the nuclear receptor, FXR [22], and the G protein-coupled receptor TGR5 [30,31]. To investigated through which receptor CDCA is able to induce its effects, we performed Thymidine incorporation assay in the presence of silencing TGR5 receptor and FXR receptor (Figure 2). Our results indicate that the effect of low doses of CDCA is specifically mediated by TGR5 since it was abrogated by silencing TGR5 gene expression but not in the presence of siRNA for FXR. Instead, the effect of high doses of CDCA was unaffected by silencing both receptors accounting for a general cytotoxic and/or apoptotic effect exerted by high doses of CDCA.

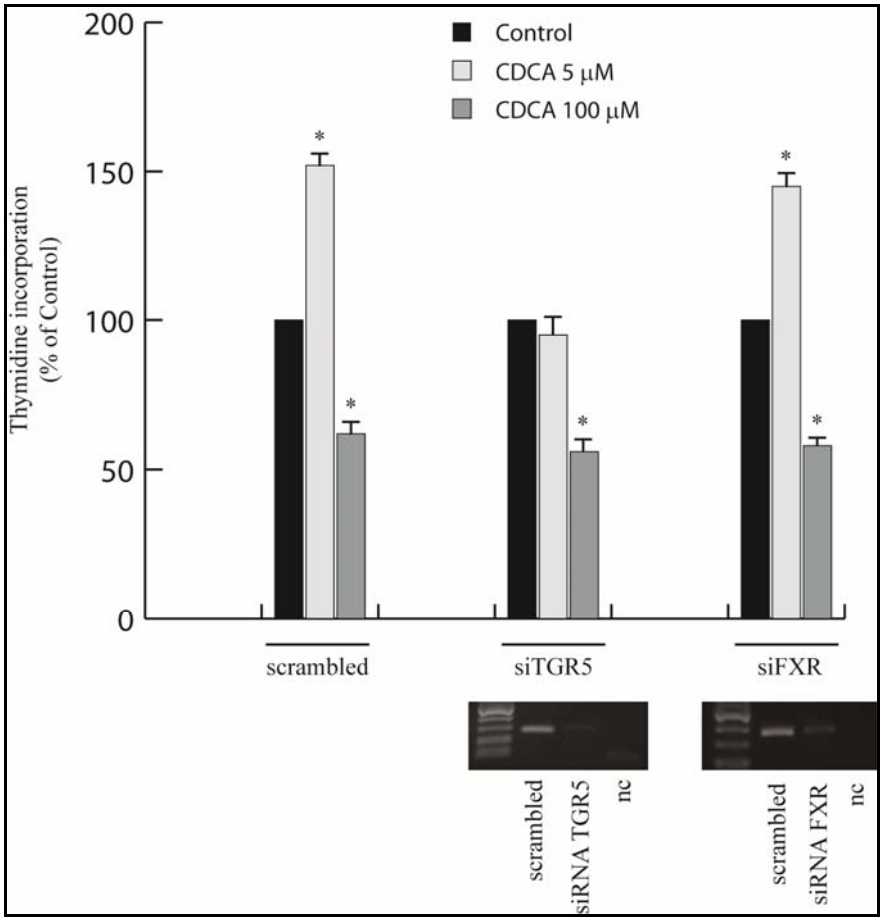


Figure 2. ***The effect of TGR5 and FXR silencing on CDCA-stimulated Ishikawa cell proliferation***. *Ishikawa cells were transfected with TGR5, FXR, controlsi RNA (scrambled) for 48h and untreated (control) or treated with 5 and 100 μM CDCA for 72h. RT-PCR analysis was performed to evaluate the expression of TGR5 and FXR in the presence of control siRNA (scrambled) and siRNA for TGR5 and FXR.he data represent the mean ± SE of three separate experiments.*

Chenodeoxicholic acid modulates Cyclin D1 expression through TGR5 mediated ERK activation

Next, since GPCRs signal transduction involves the activation of Extracellular signal-regulated kinase (ERK), we examined whether CDCA might promote ERK-42/44 activation. To this aim, serum starved Ishikawa cells were left untreated or treated, at different times, with the previously tested CDCA concentration that positively affected cell proliferation as well as Cyclin D1 expression. CDCA significantly induced phosphorylation of ERK 42/44 within 5 minutes of treatment while no changes were observed on the levels of total ERK (Figure 3A).

To explore the involvement of TGR5 in CDCA-dependent activation of ERK signaling, we knocked down TGR5 levels by using small interfering RNAs (siRNAs). TGR5 mRNA expression was effectively silenced as revealed by Real-Time-PCR after 48h of siRNA transfection (Figure 3B). Silencing of the TGR5 gene significantly affected CDCA-dependent ERK 42/44 phosphorylation, whereas the scrambled siRNA showed no effect (Figure 3C). Interestingly, silencing experimental approach also evidenced the involvement of the TGR5 in the positive regulation of Cyclin D1 expression by CDCA administration (Figure 3D).

To further evidence the involvement of activated ERK in CDCA dependent up-regulation of Cyclin D1 expression, specific chemical inhibitor of ERK 42/44 (PD 098059) was used (Figure 3E). Our results revealed that PD pretreatment effectively prevents CDCA induction of Cyclin D1 expression. These data

indicate that TGR5 may mediate CDCA-induced increase in Cyclin D1 expression by the involvement of rapid activation of ERK signaling.

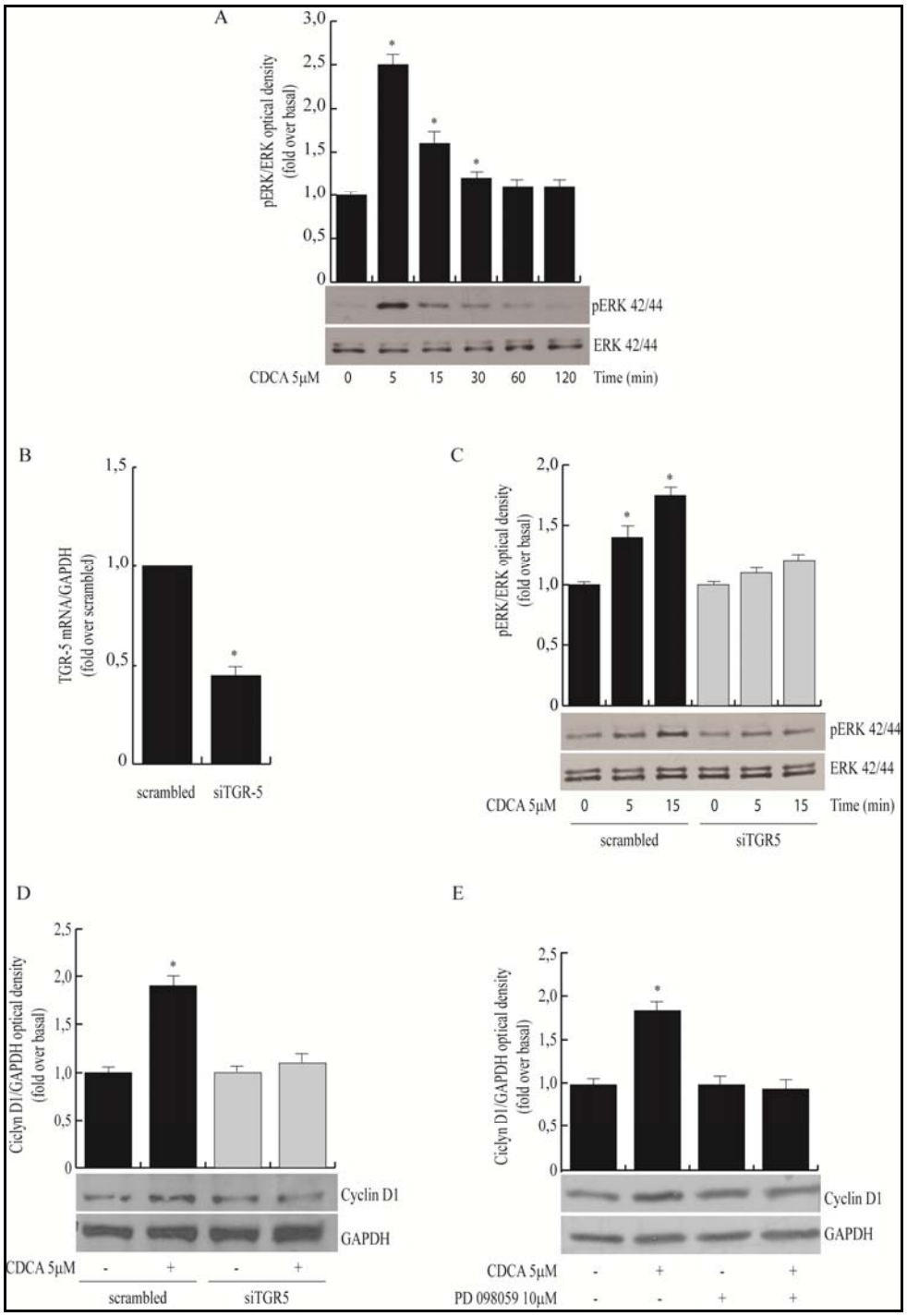


Figure 3

Figure 3. Chenodeoxicholic acid modulates Cyclin D1 expression through TGR5 mediated ERK activation.

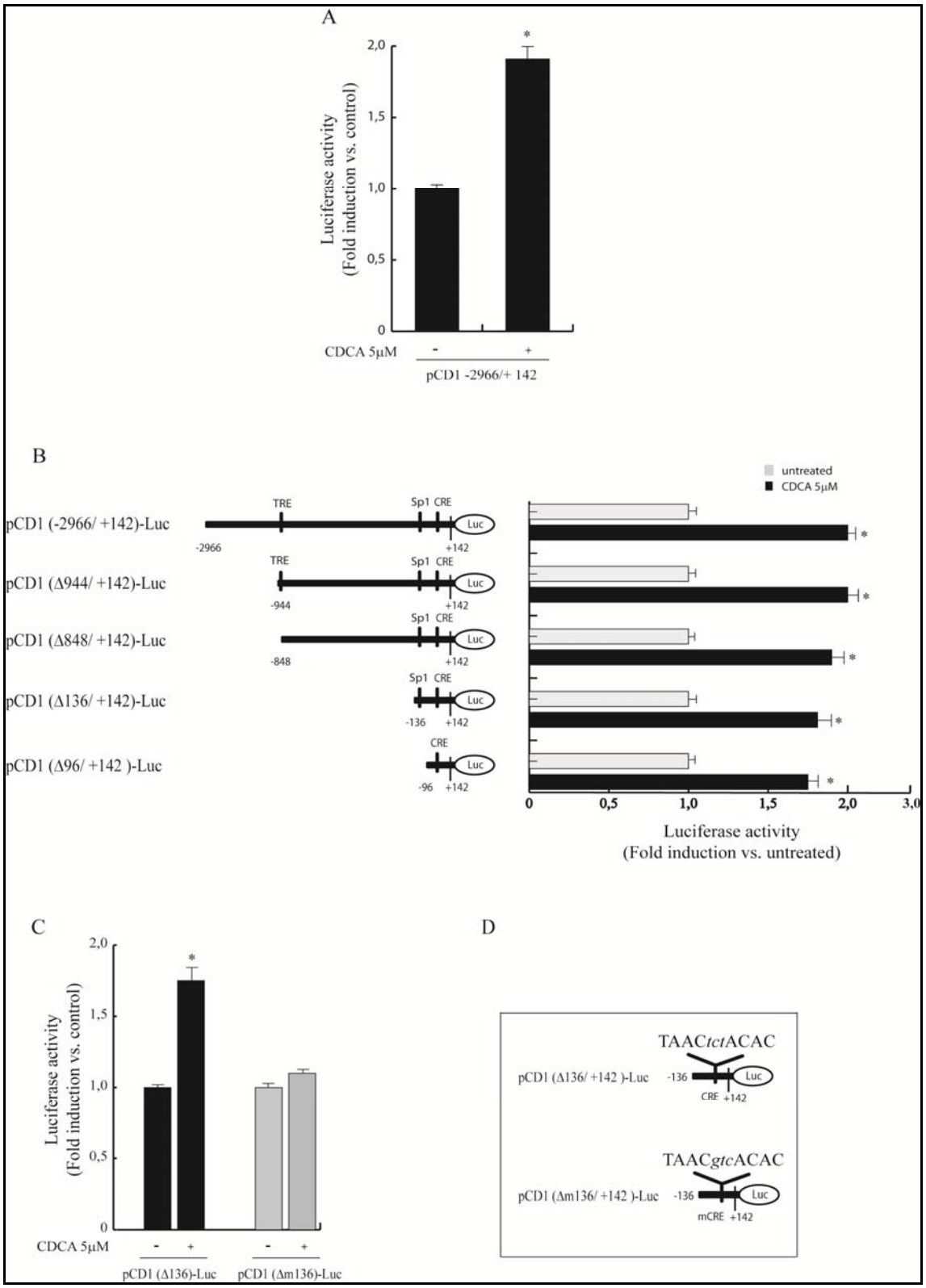
A*) Ishikawa cells were serum-starved for 24 h and then treated with 5 μM CDCA for various time intervals or left untreated (-). Protein extracts were resolved by SDS–PAGE and subjected to immunoblot analysis with specific antibodies against total or phosphorylated (p) forms of ERK. The upper histograms represent the mean ± SE experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. *P < 0.01 compared to untreated cells.* ***B)*** *Total RNA was isolated from Ishikawa cells and reverse transcribed cDNA was subjected to Real Time PCR using specific primers for TGR5. RT-PCR analysis showed that 48h of transfection using 25nM of TGR5-specific siRNA drastically reduced TGR5 mRNA expression.* ***C)*** *Ishikawa cells were transfected with siRNA for TGR-5, control siRNA (scrambled), untreated (-) or treated with 5 μM CDCA for various time intervals. Total proteins were extracted and Western blotting analysis was performed to evaluate the expression of total or phosphorylated (p) forms of ERK .* ***D****) Cells were transfected with siRNA for TGR-5, control siRNA (scrambled), untreated (-) or treated with 5 μM CDCA for 24 h. Total proteins were extracted and Western blotting analysis was performed to evaluate the expression of Cyclin D1. GAPDH was used as a loading control on the same stripped blot* **.** *The upper histograms represent the mean ± SE of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. *P < 0.01 compared to untreated cells.*

Characterization of functional CDCA responsive region(s) in the Cyclin D1 promoter

The aforementioned observations prompted us to evaluate whether CDCA signaling could affect the transcriptional activity of the Cyclin D1 gene promoter in Ishikawa cells.

To this aim, we transiently transfected Ishikawa cells with a luciferase reporter construct containing the upstream region of the Cyclin D1 gene spanning from -2.966 to + 142 (Figure 4A). A significant increase in promoter activity was observed in the transfected cells exposed to CDCA 5μM for 24h.

To delimit the cis-element involved in Cyclin D1 transcriptional activation by CDCA, a series of 5'-promoter-deleted mutants were used in transient transfection experiments. The constructs, pCD1Δ-944 D1Δ-848, pCD1Δ-136 and pCD Δ1-96 which include, 0.944 kb, 0.848 kb, 0.136 kb and 0.96 kb of the Cyclin D1 promoter fragments, respectively, showed an increased transcriptional activity upon CDCA treatment with respect to untreated cells. These results suggest that the region from -96 to + 142 was required for the transactivation of Cyclin D1 by CDCA. The nucleotide sequence analysis of this region evidenced at position -52 a CRE binding motif, a well-known down-stream target of ERK signaling that could represent a putative effector of CDCA treatment. Indeed, as shown in Figure 4C, mutation of CRE completely abolished the CDCA promoter responsiveness demonstrating its involvement in the up-regulation of Cyclin D1.


A
Luciferase activity
(Fold induction vs. control)
2,0
1,5
1,0
0,5
0
*
CDCA 5µM
-
+
pCD1 -2966/+ 142
B
pCD1 (-2966/ +142)-Luc
pCD1 (Δ944/ +142)-Luc
pCD1 (Δ848/ +142)-Luc
pCD1 (Δ136/ +142)-Luc
pCD1 (Δ96/ +142)-Luc
TRE
Sp1
CRE
Luc
-2966
-944
-848
-136
-96
+142
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Luciferase activity
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Luciferase activity
(Fold induction vs. control)
2,0
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CDCA 5µM
-
+
pCD1 (Δ136)-Luc
pCD1 (Δm136)-Luc
D
TAACtctACAC
pCD1 (Δ136/ +142)-Luc
-136
Luc
CRE
+142
TAACgtcACAC
pCD1 (Δm136/ +142)-Luc
-136
Luc
mCRE
+142

Figure 4

Figure 4 A-C. ***CDCA transactivates Cyclin D1gene promoter through CRE motif.***

A*) Ishikawa cells were serum-starved for 24 h, transfected with Cyclin D1 full-length promoter for 24 h and left untreated (-) or treated with 5 μM CDCA for 24 h.*

B, *left*) *Schematic representation of human Cyclin D1 promoter fragments used in this study. All the promoter constructs contain the same 3' boundary (+142). The 5' boundaries of the promoter fragments varied from -2966 to – 96).* **B, *right***) *Transcriptional activity of Ishikawa cells with promoter constructs is shown.* **C***). Ishikawa cells were serum-starved for 24 h, transfected for 24 h with mutated promoter constructs and left untreated or treated with 5 μM CDCA for 24 h.* **D).** *Schematic representation of the mutated constructs used in this study. The values represent the mean ± SE of three separate experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *P < 0.01 compared to untreated cells.*

CDCA administration increases CREB-DNA binding activity to Cyclin D1 promoter

To further investigate the specific role of CRE motif in the transcriptional activation of Cyclin D1 by CDCA, we performed EMSA experiments.

Using synthetic oligodeoxyribonucleotides corresponding to CRE motif, we observed the formation of a complex in nuclear extract from Ishikawa cells (Fig. 5A, lane 1), which was abrogated by 100-fold molar excess of unlabeled probe (Fig. 5A, lane 2) demonstrating the specificity of the DNA binding complex. This inhibition was not longer observed when mutated oligodeoxyribonucleotides were used as competitor (Fig. 5A, lane 3). CDCA-induced a strong increase in the DNA binding activity compared with untreated sample (Fig.5A, lane 4). Incubation with a specific anti-CREB antibody with the nuclear extracts appeared to deplete the band (Fig.5A, lane 5). Finally, using transcribed and translated in vitro CREB protein, we obtained a complex migrating at the same level as that of Ishikawa nuclear extracts (Fig. 5A, lane 6).

To better determine the physiological relevance of the CRE site in the modulation of Cyclin D1 promoter upon CDCA exposure, we investigated whether CREB interacts with Cyclin D1 gene promoter as it exists in native chromatin, performing ChIP assay. As shown in figure 5B, using anti-CREB antibody CREB occupancy of the CRE containing region of Cyclin D1 promoter was induced in a CDCA-dependent manner. The enhanced recruitment of CREB was concomitant with an increased association of polymerase II to this Cyclin D1 regulatory region consistent with the enhanced Cyclin D1 promoter activity previously demonstrated. Our results suggest that the molecular mechanism leading to CDCA mediated Cyclin D1 up-regulation in Ishikawa cells may involve recruitment of CREB transcription factor on its responsive region within the Cyclin D1 promoter.

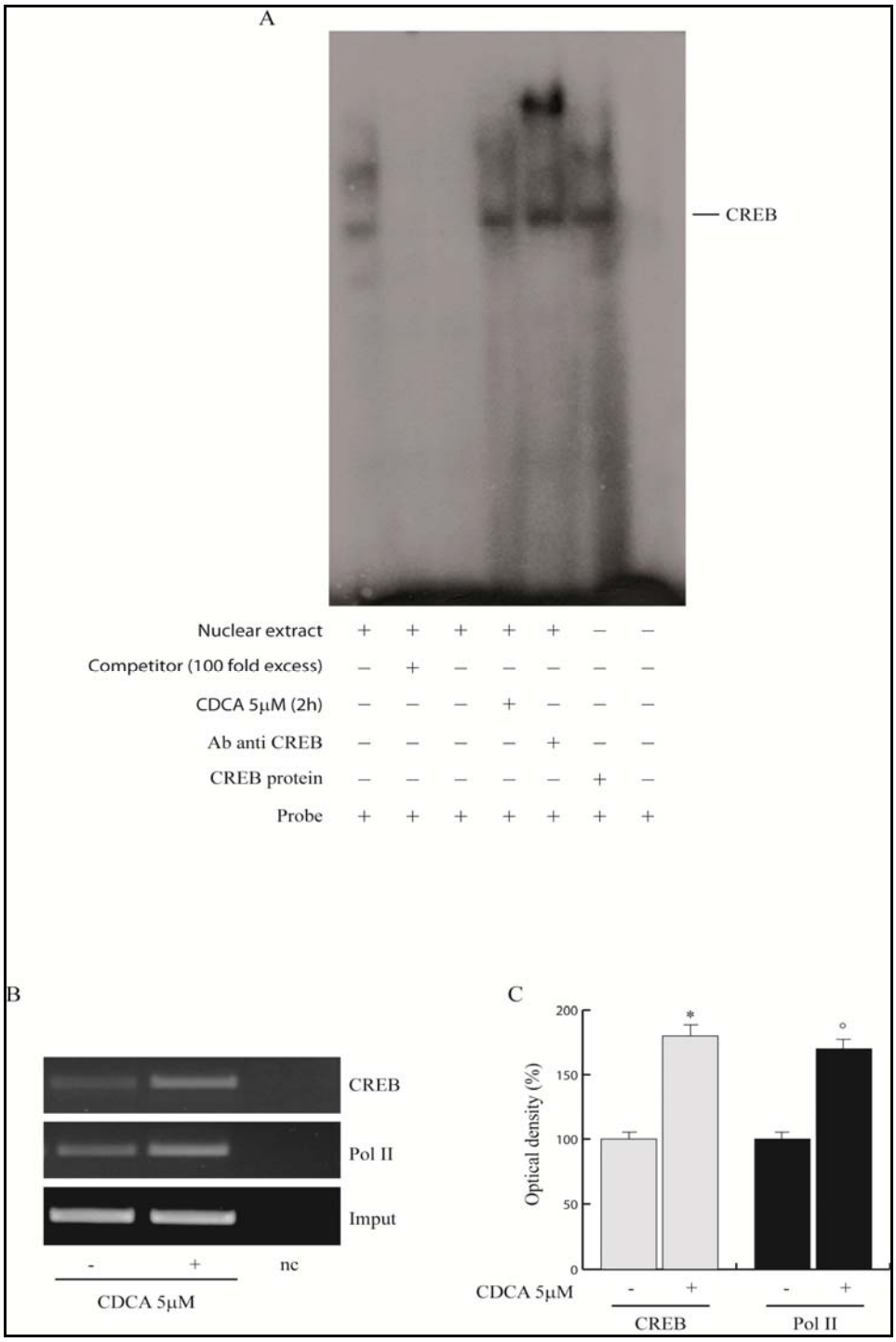

Figure 6 A-C. CDCA induces activation of CREB-DNA binding activity in Ishikawa cells.

A*) Nuclear extracts from Ishikawa cells were incubated with a CREB-specific consensus sequence probe labeled with [λ^{32}P] ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 1). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lane 2) Lane 3 contains a 100-fold molar*

excess of labeled mutated CRE oligonucleotide . Ishikawa nuclear extracts treated with 5μM CDCA for 2 h incubated with probe (lane 4).The specificity of the binding was tested by adding to the reaction mixture a CREB antibody (lane 5). We used as positive control a transcribed and translated in vitro CREB protein (lane 6). Lane 7 contains probe alone ***(A).***

B*) The cells were serum-starved for 24 h and left untreated (–) or treated with 5μM CDCA for 2 h. The preacleared chromatin was immunoprecipitated with specific antibody anti-CREB, Anti-polymerase II antibody, and with a normal mouse serum (NC) as negative control. Cyclin D1 promoter sequences containing CRE sites were detected by PCR with specific primers. To determine input DNA, the cyclin D1 promoter fragment was amplified from 30 μl, purified soluble chromatin before immunoprecipitation.* **C***) The histograms represent the mean ± SE of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary unit sand expressed as percentages of the control, which was assumed to be 100%.*P < 0.01compared to untreated cells.*

CREB siRNA prevent CDCA-induced Cyclin D1 expression and cell proliferation

To better define the contribution of CREB in CDCA-induced Cyclin D1 expression and cellular proliferation we knocked down CREB cellular levels by siRNA technology. CREB protein expression was effectively silenced as revealed by Western Blotting after 48h of siRNA transfection (Figure 7A). As shown in Figure 7A, CREB gene silencing significantly decreased Cyclin D1 expression induced by CDCA administration, while no changes were observed after transfection of cells with scrambled siRNA upon identical experimental conditions. Next, we determined the effect of CREB siRNA on growth stimulation by measuring changes in the rate of DNA synthesis. As previously

demonstrated treatment of Ishikawa cells with CDCA 5μM for 72h induces cell proliferation (Figure 7B). The growth stimulatory effect of CDCA was severely affected following CREB silencing (Figure 7B). These results confirmed the involvement of CREB in CDCA mediated up-regulation of Cyclin D1 and its biological significance for Ishikawa cells proliferation.

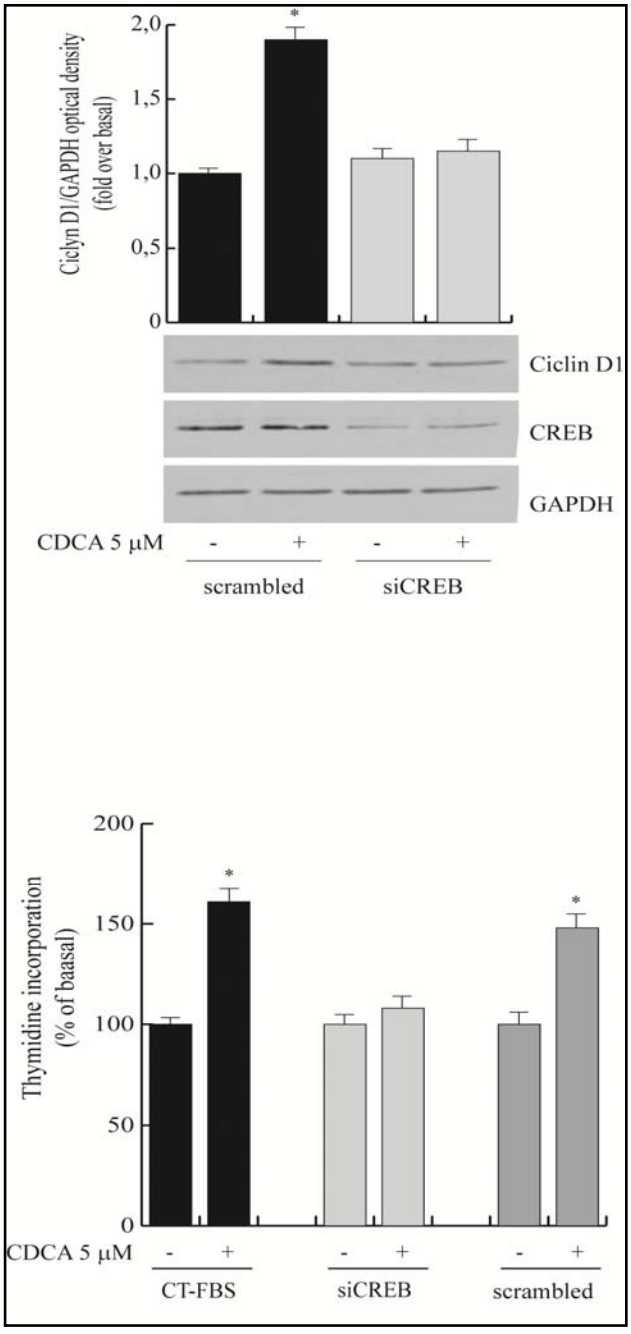

Figure 7

Figure 7. ***The effect of CREB silencing on CDCA stimulated cyclin D1 and Ishikawa cells proliferation.***

A) *Ishikawa cells were transfected with CREB, control si RNA, untreated (-) or treated with 5 μM CDCA for 24 h. Total proteins were extracted and Western blotting analysis was performed to evaluate the expression of cyclin D1 and CREB proteins. GAPDH was used as a loading control on the same stripped blot. The upper histograms represent the mean ± SE of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary unit sand expressed as percentages of the control, which was assumed to be 100%.*P < 0.01compared to untreated cells* ***B).*** *Ishikawa cells were transfected with CREB, control RNAi, untreated (-) or treated with 5 μM CDCA for 24 h. Thymidine incorporation assay was performed. The data represent the mean ± SE of three separate experiments. *P < 0.01 compared to untreated cells.*

DISCUSSION

Discussion

Increasing epidemiologic data in humans as well as many in vitro investigative reports suggest that overweight and obesity are important risk factors for type-I EC which is the most common malignancy in women and accounts for 80% of all ECs [5–12]. Given the epidemiological association between overweight, obesity and type-I EC, the search for a molecular link between these conditions is of great interest. Presently, the strongest support for mechanisms to link obesity and cancer risk involves the metabolic and endocrine effects of obesity and the alterations that they induce in the production of peptide and steroid hormones [13].

One way through which fat may exert its effect is stimulation of bile acids synthesis and secretion. In addition to the classic function of bile acids in facilitating hepatobiliary secretion of endogenous metabolites and xenobiotics and intestine absorption of lipophilic nutrients, bile acids also have also been recognized as signaling molecules with diverse endocrine and paracrine functions, including regulation of their own synthesis and lipids in the enterohepatic system, as well as glucose metabolism and energy expenditure in peripheral tissues [20,21].

To investigate the role of bile acid in extrahepatic tissues, we analyzed the effects of the primary bile acid chenodoxicholic acid on cell proliferation and cell cycle profile in Ishikawa cell line that is an excellent model for type-I endometrial cancer [32]. Our results showed that CDCA exerted divergent effects depending on its concentration. For low concentrations of CDCA (< 50 μM), cell proliferation of the Ishikawa cells was stimulated whereas concentrations greater than 100 μM induced cell death as the cells were detached from the culture flask, thus showing the two different effects (cell proliferation and cell death) that CDCA has, depending on the concentration. According to other research, the biphasic effects of bile acids were also found in entherohepatic tissues, as demonstrated in the Caco-2 intestinal cells [33], as well as in extrahepatic tissues as evidenced in human dermal fibroblast [34]. Low doses CDCA strongly stimulated Ishikawa cell proliferation, while by contrast, the higher CDCA dose

markedly suppressed Ishikawa cell growth. These data well correlated with cell cycle analysis showing a decrease of the percentage of cells in the G0/G1-phase and a concurrent increase in the S-phase, following 72h of low doses of CDCA exposure. As expected, 100 μM CDCA treatment instead, inhibited cell cycle progression leading to the accumulation of cells in G1 phase with the concomitant reduction of the S-phase.

The cell cycle is regulated by the coordinate action of cyclin-dependent kinases (cdk), specific cyclin proteins and cdk inhibitors. Alterations of the mechanisms controlling cell cycle progression play a relevant role in the pathogenesis of different human neoplasia. Among the molecules involved in cell cycle regulation cyclin D1 abnormalities may contribute to such malignant transformation [35-37]. Altered expression of cyclin D1 may result from rearrangement [38], translocation [39], amplification and/or overexpression in head and neck, breast, squamous cell carcinomas, non-small cell lung cancer, colon and urinary bladder cancer [40]. In addition, it has been reported that cyclin D1 overexpression in endometrial glands increases progressively in intensity and extent from normal endometrium to complex hyperplasia and carcinoma [41].

Of interest, we found that low doses of CDCA exposure up-regulates cyclin D1 mRNA and protein levels. By contrast, elevated concentrations of CDCA induced a down-regulation of cyclin D1 expression in a dose dependent-manner concomitantly with an increase of $p21^{WAF1/Cip1}$ expression without affecting the protein levels of the tumor suppressor p53.

It is now clear that, in addition to their important roles in nutritional absorption, BAs are signaling molecules that can activate BA receptors to initiate signaling pathways and regulate gene expression. Two major receptors for BAs have been identified, the nuclear receptor, FXR [22], and the G protein-coupled receptor TGR5 [30,31]. Our results indicate that the effect of low doses of CDCA is specifically mediated by TGR5 since it was abrogated by silencing of TGR5 gene expression but not in the presence of siRNA for FXR. Instead, the effect of high doses of CDCA was unaffected by silencing of both receptors accounting for a general cytotoxic and/or apoptotic effect exerted by high doses of CDCA.

Moreover, in our study, we demonstrated that CDCA trough the activation of a G-protein-coupled receptor, TGR5, induces the up-regulation of cyclin D1 gene expression as it emerges by the observation that silencing of TGR5 gene completely reversed the increase of cyclin D1 protein levels. By the same technical approaches we demonstrated the TGR5-dependent activation of MAPK signaling and its direct involvement in Cyclin D1 up-regulation as it was demonstrated by using a specific chemical inhibitor of the above mentioned pathways.

Therefore, in order to investigate the potential ability of low doses of CDCA to modulate the cyclin D1 promoter gene, we performed transient transfection experiments in Ishikawa cells using deleted constructs of the cyclin D1 promoter gene. The results indicated that CDCA up-regulates the full-length promoter activity of cyclin D1. Moreover, we documented that the region spanning from −96 to +142, which contains CRE site, was required for the responsiveness to CDCA. Mutation analyses of the CRE site on cyclin D1 promoter showed that this motif was the mediator of cyclin D1 regulation by CDCA since the mutated construct completely abolished CDCA-induced promoter activation. Other evidences strengthened our observation, since cyclin D1 has been reported to be transcriptionally regulated by CRE [42] in different cancer cells. Moreover, CREB is one of the major donwstream target of ERK1/2 signaling activation and this data well correlated with our findings [43,44].

The specific role of CREB was assess by EMSA experiments in which nuclear extracts from Ishikawa cells treated with CDCA showed an increased binding of the transcription factor to the CRE sequence located in the cyclin D1 promoter region. These findings were supported also by ChIP assay demonstrating the ability of CDCA to enhance the recruitment of CREB to the promoter of cyclin D1.

Finally, the relative contribution of this transcription factors emerges from our data showing that silencing of CREB gene expression is able to reverse the up-regulatory effect of CDCA on cyclin D1 expression and Ishikawa cells growth.

Taken together our results, for the first time, evidence that low doses of CDCA are able to induce a rapid non-genomic signaling through the activation of a G-

protein-coupled receptor, TGR5, that in turn activates ERK signaling that allows the transcription factor CREB to up-regulates Cyclin D1 gene expression and endometrial cancer cell proliferation.

This results indicate a new mechanism by which high-fat diet could potentially influence and clarify the role of bile acid dependent signaling in the progression of endometrial cancer, addressing it as a potential target of pharmacological therapy in obese women.

REFERENCES

References

1. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. *CA Cancer J Clin* 1999; 49: 33–64.
2. Hill HA, Eley JW, Harlan LC, Greenberg RS, Barrett RJ II, Chen VW. Racial differences in endometrial cancer survival: the black/white cancer survival study. *Obstet Gynecol* 1996; 88**:** 919–26.
3. Madison T, Schottenfeld D, James SA, Schwartz AG, Gruber SB. Endometrial cancer: socioeconomic status and racial/ethnic differences in stage at diagnosis, treatment, and survival. *Am J Public Health* 2004; 94: 2104–11.
4. Schouten LJ, Goldbohm RA, van den Brandt PA. Anthropometry, physical activity, and endometrial cancer risk: results from the Netherlands Cohort Study. J Natl Cancer Inst 2004; 96:1635–8.
5. Ryan AJ, Susil B, Jobling TW, Oehler MK. Endometrial. cancer. Cell Tissue Res 2005; 322: 53–61.
6. Sjöström L, Narbro K, Sjöström CD, Karason K, Larsson B, Wedel H, et al, Swedish Obese Subjects Study. Effects of bariatric surgery on mortality in Swedish obese subjects. N Engl J Med 2007; 357: 741–52.
7. Mokdad AH, Ford ES, Bowman BA, DietzWH, Vinicor F, Bales VS. Prevalence of obesity, diabetes, and obesity-related health risk factors,. JAMA 2003; 289: 76–9.
8. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer 2004; 4: 579–91.
9. Hossain P, Kawar B, el Nahas M. Obesity and diabetes in the developing world, a growing challenge. N Engl J Med 2007; 356: 213–5.
10. Abu-Abid S, Szold A, Klausner J. Obesity and cancer. J Med 2002; 33: 73–86.
11. Schouten LJ, Goldbohm RA, van den Brandt PA. Anthropometry, physical activity, and endometrial cancer risk: results from the Netherlands Cohort Study. J Natl Cancer Inst 2004; 96: 1635–8.

12. Soliman PT, Oh JC, Schmeler KM, Sun CC, Slomovitz BM, Gershenson DM. Risk factors for young premenopausal women with endometrial cancer. Obstet Gynecol 2005; 105: 575–80.
13. Eugenia E. Calle and Rudolf Kaaks.. Overweight, obesity and cancer:epidemiological evidence and proposed mechanism. Nature review Cancer 2004; 4: 579-591.
14. Reddy B.S.. Diet and excretion of bile acids, Cancer Res. 1981; 41: 3766–3768.
15. Cummings J.H., Wiggins H.S., Jenkins D.J.A., Houston H., Jivraj T., Drasar B.S., Hill M.J., Influence of diets high and low in animal fat on bowel habit, gastrointestinal transit time, fecal microflora, bile acid, and fat excretion, J. Clin. Invest 1978; 61: 953–963.
16. Cohen DE Pathogenesis of gallstones. In Hepatology: A Textbook of Liver Disease, Zakim D, Boyer TD (eds) 2003: 1713–1743. Philadelphia: Saunders.
17. Ho KJ. Circadian distribution of bile acid in the enterohepatic circulatory system in hamsters. J Lipid Res 1976; 17: 600–604.
18. Engelking LR, Dasher CA, Hirschowitz BI Within-day fluctuations in serum bile-acid concentrations among normal control subjects and patients with hepatic disease. Am J Clin Pathol 1980; 73: 196–201.
19. Everson GT Steady-state kinetics of serum bile acids in healthy human subjects: single and dual isotope techniques using stable isotopes and mass spectrometry. J Lipid Res 1987; 28: 238–252.
20. Russell DW. Fifty years of advances in bile acid synthesis and metabolism. J Lipid Res 2009; 50: S120-S125.
21. Houten, SM. Endocrine functions of bile acids. EMBO J. 2006; 25:1419–1425.
22. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. Science 1999 284: 1362–1365.

23. Houten, SM and Auwerx J. The enterohepatic nuclear receptors are major regulators of the enterohepatic circulation of bile acids. Ann. Med. 2004; 36: 482–491.
24. Moschetta A. Prevention of cholesterol gallstone disease by FXR agonists in a mouse model. Nat. Med. 2004; 10: 1352–1358.
25. Han, S.I. et al. Bile acids enhance the activity of the insulin receptor and glycogen synthase in primary rodent hepatocytes. Hepatology 2004; 39: 456–463.
26. Ma K. Farnesoid X receptor is essential for normal glucose homeostasis. J. Clin. Invest. 2006; 116: 1102–1109.
27. Cariou, B. The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice. J. Biol. Chem 2006; 281: 11039–11049.
28. Staudinger, JL. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc. Natl. Acad. Sci. U. S. A. 2001; 98: 3375–3380.
29. Makishima, M. Vitamin D receptor as an intestinal bile acid sensor. Science 2002; 296: 1313–1316.
30. Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Itadani H, Tanaka K. Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun 2002; 298: 714–719.
31. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M A G protein-coupled receptor responsive to bile acids. J Biol Chem 2003; 278: 9435–9440.
32. Albitar L, Pickett G, Morgan M, Davies S, Leslie KK. Models representing type I and type II human endometrial cancers: Ishikawa H and Hec50co cells. Gynecologic Oncology 2007; 106**:** 52–64
33. Milovic V, Teller IC, Faust D, Caspary WF, and Stein J: Effects of deoxycholate on human colon cancer cells: apoptosis or proliferation. Eur J Clin Invest 2002; 32: 29–34.
34. Bouscarel B., Meng J.P., Ceryak S., Aratsu Z., Jones L., Epstein L.. Biphasic regulation by bile acids of dermal fibroblast proliferation

through regulation of cAMP production and COX-2 expression level. Am J Physiol Cell Physiol 2006; 291: C546–C554.

35. Cordon-Cardo C. Mutation of cell cycle regulators. Am J Pathol 1995; 147: 545–560.
36. Fu M, Wang C, Li Z, Sakamaki T, Pestell RG.. Cyclin D1: Normal and abnormal functions. Endocrinology 2004; 145: 5439–5447.
37. Knudsen KE, Diehl JA, Haiman CA, Knudsen ES.. Cyclin D1: Polymorphism, aberrant splicing and cancer risk. Oncogene 2006; 25: 1620–1628.
38. Arnold A, Kim HG, Gaz RD, Eddy RL, Fukurushima Y, Byers MG, Shows TB, Kronemberg HM.. Molecular cloning and chromosomal mapping of DNA rearranged with the parathyroid hormone gene in parathyroid adenoma. J Clin Invest 1989; 83: 2034–2040.
39. Withers DA, Harvey RC, Faust JB, Melnyk O, CareyK, Meeker TC.. Characterization of a candidate bcl-1 gene. Mol Cell Biol 1991; 11: 4846–4853.
40. Hall M, Peters G.. Genetic alteration of cyclins, cyclin-dependent kinases, and CDK inhibitors in human cancer. Adv Cancer Res 1996; 68:67–108.
41. Ruhul-Quddus M, Latkovich P, Castellani WJ, James-Sung C, Steinhoff MM, Briggs RC Miranda RN.. Expression of cyclin D1 in normal, metaplastic, hyperplastic endometrium and endometrioid carcinoma suggests a role in endometrial carcinogenesis. Arch Pathol Lab Med 2002; 126: 459–463.
42. Sabbah M, Courilleau D, Mester J, Redeuilh G. Estrogen induction of the cyclin D1 promoter: Involvement of a cAMP response-like element. Proc Natl Acad Sci USA 1999; 96: 11217–11222.
43. 10. Bonni A, Brunet A, West AE. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 1999; 286: 1358–1362.
44. Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 1996; 273: 959–963.

Scientific Publications Performed during the Program

1. Guido C., Santoro M., Vizza D., **Avena P**., Carpino A. and Aquila S. Peroxisome Proliferator-Activated Receptor (PPAR) γ modulates sperm metabolism in pig sperm.*(submitted Journal of Molecular Endocrinology).*

2. Perrotta I., Santoro M., Guido C., **Avena P**., Tripepi S., Andò S. and Aquila S. Different expression of the Cyclooxygenase (COX)1 and COX2 in human male gamete from normal, varicocele and diabetes patients. (Submitted Histopatology).

3. Guido Carmela, Perrotta Ida, Panza Salvatore, Middea Emilia, **Avena Paola**, Santoro Marta, Marsico Stefania, Imbrogno Pietro, Andò Sebastiano and Aquila Saveria. Human sperm physiology: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) influence sperm metabolism and may be involved in the pathophysiology of varicocele-associated male infertility. **J. Cell. Physiol**. 2011 Feb 22.

4. Guido Carmela**,** De Amicis Francesca, Perrotta Ida, **Avena Paola,** Panza Salvatore, Andò Sebastiano, Aquila Saveria. Conventional progesterone receptors PR-B and PR-A are expressed in human spermatozoa and may be involved in the pathophysiology of varicocele: a role for progesterone in metabolism. **Int. J. Androl**.

Comunications in National and International Conferences.

1. **Paola Avena**, Ivan Casaburi , Marilena Lanzino , Catia Morelli, Carmela Guido, Pamela Maris, Diego Sisci, Saveria Aquila, Sebastiano Andò. Chenodeoxycholic acid (CDCA) through TGR5-dependent CREB-signaling activation enhances Cyclin D1 expression and promotes human endometrial cancer cell proliferation. 31esimo ***Congresso Nazionale della Società Italiana di Patologia*** in collaborazione con l'American Society of Investigative Pathology (ASIP) 2011, Bologna. (Comunicazione orale).

2. Carmela Guido, Salvatore Panza, Marialuisa Panno, **Paola Avena**, Francesca Giordano, Ivan Casaburi, Stefania Catalano, Ida Perrotta, Marta Santoro, Saveria Aquila and Sebastiano Andò. Estrogen Receptor beta produces cell death in TCAM2 human seminoma cell line binding through Sp1 on the phosphatase and tensin homologue deleted from chromosome 10 (*PTEN*) promoter gene. 31esimo *Congresso Nazionale della Società Italiana di Patologia* in collaborazione con l'American Society of Investigative Pathology (ASIP) 2011, Bologna

3. M. Lanzino, C. Morelli, D. Sisci, I. Casaburi, P. Maris, C. Capparelli, **P. Avena**, S. Andò. University of Calabria, Arcavacata di Rende, Italy. First Evidence of the up-Regulatory Effect of Androgens on DAX-1 Gene Expression: Is This a Novel Mechanism for Inhibiting Breast Cancer Cell Proliferation? ENDO The Endocrine Society's 93th Annual meeting & EXPO June 4-7, 2011- Boston, Massachussets.

4. Carmela Guido, Salvatore Panza, Marialuisa Panno, **Paola Avena** , Francesca Giordano, Ivan Casaburi, Stefania Catalano, Ida Perrotta, Marta Santoro, SaveriaAquila and Sebastiano Andò. Estrogen Receptor beta produces cell death in TCAM2 human seminoma cell line binding through Sp1on the phosphatase and tensin homologue deleted from chromosome10 (*PTEN*) promoter gene. ASIP Annual Meeting at Experimental Biology 2011 April 9-13, 2011- Washington DC- USA.

5. **Avena Paola.** , Lanzino Marilena, Morelli Catia, Capparelli Claudia, Guido Carmela, Maris Pamela, Sisci Diego, Aquila Saveria, Casaburi Ivan. Chenodeoxycholic acid (CDCA) through TGR5-dependent CREB-signaling activation enhances Cyclin D1 expression and promotes human endometrial cancer cell proliferation. XXX National Congress, Italian Society of Pathology in collaboration with American Society for Investigative Pathology, Salerno, 14-17 Ottobre, 2010. (Italy).

6. Guido Carmela, Panza Salvatore, **Avena Paola**, Casaburi Ivan, Giordano Francesca, Bifulco Maurizio, Ando' Sebastiano, Aquila Saveria. Estrogen Receptor beta induces apoptosis in TCAM2 human seminoma cell line. XXX National Congress, Italian Society of Pathology in collaboration with American Society for Investigative Pathology, Salerno, 14-17 Ottobre, 2010. (Italy).

7. Sisci Diego, Morelli Catia, Maris Pamela, Capparelli Claudia, **Avena Paola**, Lanzino Marilena, Casaburi Ivan, Ando' Sebastiano. Akt inhibition enables the Forkhead Factor Foxo3a to a repressive role for ER alpha transcriptional activity in breast cancer cells. XXX National Congress, Italian Society of Pathology in collaboration with American Society for Investigative Pathology, Salerno, 14-17 Ottobre, 2010. (Italy).

8. Santoro Antonietta, Guido Carmela, **Avena Paola**, Aquila Saveria, Andò Sebastiano and Bifulco Maurizio**.** Cannabinoid type-1 receptor (CB1-R) signaling modulates human sperm physiology: implications for fertility. XXX National Congress, Italian Society of Pathology in collaboration with American Society for Investigative Pathology, Salerno, 14-17 Ottobre, 2010. (Italy).

9. Marilena Lanzino, Diego Sisci, Catia Morelli, Stefania Catalano, Ivan Casaburi, Claudia Capparelli, **Paola Avena** and Sebastiano Andò. Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells: identification of a novel androgen response element. Asip, Anahim, CA, April 22-28, 2010.

10. De Amicis Francesca, Guido Carmela, **Avena Paola**, Perrotta Ida, Carpino Amalia, Andò Sebastiano and Aquila Saveria. Conventional Progesterone Receptor (PR) B and PRA are espressed by human sperm and may be involved in the pathophysiology of the varicocele condition. ISCAA 2010, 2nd International Symposium of Clinical and Applied Anatomy, Praga, 2010.

11. Saveria Aquila, Carmela Guido, **Paola Avena**, Ivan Casaburi and Amalia Carpino. Expression of NOS enzymes in pig sperm: evidence of intracellular NO increase in leptin treated sperm. ISCAA 2010, 2nd International Symposium of Clinical and Applied Anatomy, Praga, 2010.

12. Guido Carmela, Panza Salvatore, **Avena Paola**, Casaburi Ivan, Andò Sebastiano and Aquila Saveria. Estrogen Receptor beta induces both authofagy and apoptosis in TCAM2 human seminoma cell line. ISCAA 2010, 2nd International Symposium of Clinical and Applied Anatomy.

13. Morelli Catia, Lanzino Marilena, Maris Pamela, Garofalo Cecilia, Casaburi Ivan , **Avena Paola**, Capparelli Claudia, Sisci Diego, Ando' Sebastiano. Regulation of ER mediated transcription by akt2/foxo3a signalling in breast cancer cells. The EMBO Conference on Nuclear Receptors, Cavtat/Dubrovnik, Croatia, September 25-29, 2009.

14. Lanzino Marilena, Sisci Diego, Morelli Catia, Catalano Stefania, Casaburi Ivan, Capparelli Claudia, Marsico Stefania, Bruno Rosalinda, **Avena Paola**, Ando' Sebastiano. Androgen receptor regulation of the cyclin D1 gene through an androgen response element in the proximal promoter. The EMBO Conference on Nuclear Receptors, Cavtat/Dubrovnik, Croatia, September 25-29, 2009.

15. **Avena Paola**, Morelli Catia, Lanzino Marilena, Casaburi Ivan, Catalano Stefania, Bruno Rosalinda, Andò Sebastiano and Sisci Diego. AKT2/FOXO3A signalling regulates ERα expression and transcriptional activity in MCF-7 Breast Cancer Cells. XII Workshop on Apoptosis in Biology and Medicine. Parghelia (VV), Calabria, Italy 22 May, 2009.

16. Casaburi Ivan, Lanzino Marilena, Catalano Stefania, Sisci Diego, Aquila Saveria, Vivacqua Adele, **Avena Paola**, Maggiolini Marcello, Andò Sebastiano. Different Biological Effects of Chenodeoxycholic Acid (CDCA) in Endometrial Cancer Cells. XXIX National Congress, Italian Society of Pathology in collaboration with American Society for Investigative Pathology, University of Calabria, Rende-Cosenza (Italy), September 10-13, 2008