HHS Public Access

Author manuscript

Endocr Relat Cancer. Author manuscript; available in PMC 2020 January 14.

Published in final edited form as:

Endocr Relat Cancer. 2011 ; 18(6): 731–742. doi:10.1530/ERC-11-0181.

CDC23 regulates cancer cell phenotype and is overexpressed in papillary thyroid cancer

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Abstract

Cancer gender disparities have been observed for a variety of human malignancies. Thyroid cancer is one such example where there is a dramatic difference in the incidence, aggressiveness, and death rate by gender. The molecular basis for gender disparity is poorly understood. To address this, we performed genome-wide gene expression profiling in matched papillary thyroid cancer (PTC) samples and identified nine candidate genes differentially expressed by gender. One of these genes was *CDC23* that was upregulated in PTC in men compared with women. Because the function and expression of *CDC23* is unknown in eukaryotic cells, we further characterized the expression of *CDC23* in normal, hyperplastic, and PTC tissue samples. We found *CDC23* was overexpressed in PTC and absent in normal and hyperplastic thyroid tissue. In thyroid cancer cells, functional knockdown of *CDC23* resulted in an increase in the number of cells in both the S and G_2M phases of the cell cycle, and an inhibition of cellular proliferation, tumor spheroid formation, and anchorage-independent growth. Cellular arrest in both S and G_2M phases was associated with significant cyclin B1 and securin protein accumulation after CDC23 knockdown. Moreover, the effect of *CDC23* on cellular proliferation and cell cycle progression was reversed on triple knockdown studies of CDC23, cyclin B1, and securin. Our data taken together suggests *CDC23* has important biologic effects on cell proliferation and cell cycle progression. The effect of *CDC23* on cellular proliferation and cell cycle progression is mediated, at least in part, by cyclin B1 and securin protein levels. Therefore, we propose that *CDC23* is a critical regulator of cell cycle and cell growth, and may be involved in thyroid cancer initiation and progression, and may explain the different tumor biology observed by gender.

Introduction

Gender-based differences have been observed in the incidence, aggressiveness, and mortality rate for many human malignancies (Naugler *et al*. 2007, Paggi *et al*. 2010, Rahbari *et al*. 2010, Yeh & Chen 2010, Fajkovic *et al*. 2011). Dietary, environmental, behavioral, and reproductive factors have been implicated in cancer gender disparity, but the molecular basis

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

for this disparity is poorly understood. Thyroid cancer is one such cancer with a dramatically different incidence, aggressiveness, and death rate by gender (Rahbari *et al.* 2010).

Thyroid cancer arises from follicular (papillary, follicular, hürthle cell cancer, and anaplastic) and parafollicular (medullary) cells. Papillary thyroid cancer (PTC) accounts for ~85% of all thyroid cancer cases. Although the overall incidence of cancer in the United States continues to decrease, the incidence of thyroid cancer has increased dramatically in the last three decades by about 4% annually (Jemal *et al.* 2004). The gender disparity in thyroid cancer incidence is striking. Incidence rates are particularly high in premenopausal women (four times higher than in men; Sakoda & Horn-Ross 2002, Jemal *et al.* 2004). On the other hand, thyroid cancer of follicular cell origin in men is more advanced at diagnosis and is associated with a worse outcome, even when confounding factors are accounted for. Furthermore, for the uniformly lethal anaplastic thyroid cancer, the gender distribution is even, unlike that for differentiated thyroid cancer of follicular cell origin (papillary and follicular; Kebebew *et al.* 2005).

The gender disparity in thyroid cancer incidence has prompted research into the role of sex hormone and reproductive factors in thyroid carcinogenesis. Several epidemiologic studies suggest that early or late menarche increases the risk of thyroid cancer by 50% (Horn-Ross *et al.* 2001, Iribarren *et al.* 2001, Sakoda & Horn-Ross 2002). Moreover, recent pregnancy increases the risk of thyroid cancer, whereas the consumption of cruciferous vegetables, antioxidant vitamins, and phytoestrogens protects against thyroid cancer (Horn-Ross *et al.* 2002). All these studies suggest that hormonal or reproductive, and dietary factors have a role in thyroid carcinogenesis and may account for the gender disparity in thyroid cancer.

Environmental, dietary and reproductive factors have important roles in cancer initiation, promotion and progression in a variety of human malignancies. There is growing evidence that these factors directly regulate gene expression in carcinogenesis (Kaput & Rodriguez 2004). Although epidemiologic and clinical studies suggest the gender disparity in thyroid cancer is influenced by dietary and reproductive factors, the molecular factors that may account for these differences are unknown. Therefore, we studied the expression profile of matched PTC samples by gender. Among several genes, we found *CDC23* was overexpressed in PTC in men compared with women, and in PTC compared with normal and hyperplastic thyroid tissue. Sex hormone had no effect on *CDC23* expression in thyroid cancer cell lines. Functional studies in thyroid cancer cell lines demonstrated *CDC23* had a dramatic effect on cell cycle and cellular proliferation, which were dependent on cyclin B1 and securin protein levels.

Materials and methods

Thyroid tissues

Six normal, ten hyperplastic human thyroid tissues, 96 PTCs, and 86 adrenocortical tumors were snap frozen and stored at −8°C on an Institutional Review Board approved tissue procurement protocol after written consent was obtained. Thirty-five conventional PTCs were used for genome-wide gene expression analysis, 34 separate-independent PTCs were used for validation of the genome-wide gene expression, and 34 PTC samples (27 separate-

independent samples and seven repeated samples used in quantitative RT-PCR (qRT-PCR) validation) were used for immunohistochemistry studies. The normal and hyperplastic thyroid tissue were used for immunohistochemistry and qRT-PCR. None of the PTC, normal, or hyperplastic tissue samples had any coexisting lymphocytic thyroiditis.

Cell culture

Human PTC cell line (TPC-1) and follicular thyroid cancer cell line (FTC-133) were maintained in DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (250 ng/ml), TSH (10 IU/l), and insulin (10 μg/ml) in a 5% CO_2 atmosphere at 37 °C. The human breast cancer cell line, MCF-7, was maintained in DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (250 ng/ml).

RNA isolation, microarray, and qRT-PCR

Total RNA was extracted from frozen tissue samples and thyroid cancer cell lines using TRIzol reagent (Invitrogen Life Technologies, Inc.) and the RNeasy Mini Kit (Qiagen).

Total RNA (1 μg) was amplified and labeled using the MessageAmp aRNA Kit (Ambion, Inc., Foster City, CA, USA). Twelve micrograms of labeled and fragmented complimentary RNA was hybridized to the Affymetrix Human Genome U133 plus 2.0 GeneChip (Santa Clara, CA, USA) for 16 h at 45 °C. The probe intensities were measured using argon laser confocal GeneArray Scanner (Hewlett-Packard, Palo Alto, CA, USA).

For qRT-PCR, 0.5–1 μg of total RNA was used for first-strand cDNA synthesis using the Superscript III First-strand Synthesis SuperMix for qRT-PCR (Invitrogen). TaqMan primer probes for *CDC23*, *GUSB*, and *GAPDH* were purchased from Applied Biosystems (Foster City, CA, USA), and relative expression was determined by the ΔC_t method described by the manufacturer (Applied Biosystems). All reactions were performed in triplicate.

Sex hormone treatment

TPC-1, FTC-133, and MCF-7 cells were plated in six-well plates (1×10^5, 4×10^4, and 5×10^4 cells/well respectively). After 24 h, cells were washed with PBS and cultured in phenol red-free medium. After 24 h of hormone deprivation (time 0) samples were collected. The cells were treated with four different conditions (1 nM estradiol, 1 nM testosterone, 1% DSMO vehicle, and medium only) in phenol red-free medium and harvested at 24 and 48 h after treatment. Total RNAs were extracted for qRT-PCR. All assays were conducted in biological and technical triplicates.

Small interfering RNA transfection, quantitative PCR, and immunoblotting

The small interfering (si)RNA for human CDC23 (siRNA ID- s16570 and s16572) and scrambled negative controls (Part #: 4390843) were purchased from Applied Biosystems. The siRNA for human cyclin B1 and PTTG (securin) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). FTC-133 and TPC-1 cells were reverse transfected with each individual siRNA at a concentration of 60 or 90 nmol/l using Lipofectamine RNAiMAX (Invitrogen). Total RNA was isolated and the level of *CDC23*

mRNA was determined by quantitative PCR as described earlier. Whole cell lysate was prepared with 1% SDS plus 10 mM Tris (pH 7.5), and was used for CDC23, cyclin B1, and securin protein detection by western blot (rabbit anti-CDC23, from Calbiochem (Darmstadt, Germany), mouse anti-cyclin B1 from Santa Cruz Biotechnology, Inc., and rabbit anti-securin from Abcam (Cambridge, MA, USA)).

Cell proliferation assay

Cell proliferation experiments were performed in 96-well in triplicates or quadruplicates. Cells were reverse transfected with individual siRNA in 96-well black plates at 2×10^3 cells/well and maintained in 200 μl serum-free media (DMEM/Ham's F-12 (1:1) supplemented with insulin (10 μg/ml), transferrin (5 μg/ml), somatostatin (10 ng/ml), and hydrocortisone (0.36 ng/ml)) in a humidified incubator. CyQuant proliferation assays were performed at each day after transfection according to the manufacturer's instructions (Invitrogen). The cell densities in the 96-well black plates were determined using a 96-well fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 485/538 nm.

Apoptosis assay

Cells (5×10^4 cells/well) were reverse transfected with different siRNA and maintained in serum-free medium as described earlier. Apoptosis was detected using ApoAlert Annexin-V-FITC Apoptosis kit (Clontech) following the manufacturer's instructions. Flow cytometric analysis for apoptosis was performed on a FACScan (BD Biosciences, Franklin Lakes, NJ, USA).

Cell cycle analysis

Cells (5×10^4 cells/well) were reverse transfected and maintained in serum-free medium as described earlier. Cells were harvested, washed, and resuspended with PBS, and fixed with ice-cold 70% ethanol at 4 °C. After washing with PBS, ribonuclease A was added to the cell suspension and incubated at 37 °C for 20 min. Then propidium iodide (PI; 50 μg/ml in PBS) was added, and samples were stored at 4 °C.

Flow cytometric analysis for cell cycle was performed on a FACScan using CellQuest software (BD Biosciences). Data files were generated for 10 000 events (cells) or more per sample gated on single cells. Doublets, cell clumps, and debris were excluded by PI fluorescence pulse width and pulse area measurements. Cell cycle analysis on the gated PI distribution was performed by Modfit software (Verity Software House, Inc., Topsham, ME, USA).

Soft agar assay for colony formation

Three days after siRNA transfection, FTC-133 cells were trypsinized, counted, and resuspended in culture media. Two-layered soft agar assays were performed in six-well plates. The bottom layer of agar (2 ml/well) contained 0.6% agar (Difco agar noble, Becton, Dickinson and Company, Sparks, MD, USA) in Ham's F-12 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (250 ng/ml).

Thirty thousand cells were mixed with 1 ml of upper agar solution (0.35% agar in culture media). Thirty minutes later, 1 ml of the culture media was added into each well. The plates were cultured at 37 °C in 5% CO_2, and the media was changed twice a week. After 18 days of culture, cell colonies were stained with Crystal Violet and examined by microscopy. Colony counting was performed in five different fields (5×10) per well.

Spheroid culture

Three days after siRNA transfection, FTC-133 cells were trypsinized, counted, and resuspended in culture media and plated in Ultra Low Cluster plate (Costar, Corning, NY, USA) at 3.5×10^4/well/24-well plates. The plates were cultured at 37 °C in 5% CO_2, and the medium was changed every 2–3 days. After 2 weeks of culture, cells were stained with Crystal Violet and photographed under microscope.

Immunohistochemistry

Paraffin-embedded tumor samples were cut into 5 mm thick sections and deparaffinized in xylene. The CDC23 protein expression was examined using a rabbit anti-*CDC23* antibody (Purified anti-APC8, BioLegend, San Diego, CA, USA). Staining was detected using vectastain ABC and DAB kits (Vector Laboratories, Inc., Burlingame, CA, USA).

Statistical analysis

ANOVA *post hoc* tests (StatView) and *t*-test were used for statistical analysis. Significance for *P* values between 0.05 and 0.01 is indicated with one asterisk (*), values of *P* between 0.01 and 0.001 are indicated with two asterisks (**), and values of $P<0.001$ are indicated with three asterisks (***). All *in vitro* experiments were repeated two to five times.

Raw microarray data were analyzed using the affy package (R/Bioconductor). The robust multiarray average method was used to quantitate intensity values in log2 scale for each probe set (Bolstad *et al.* 2003, Irizarry *et al.* 2003). Hierarchical clustering was done using the Euclidean distance and complete linkage on the most variably expressed genes, as defined by the median absolute deviation (MAD, a robust measure of variance) across all arrays. For class comparison (female vs male), we used the limma package in R/Bioconductor to calculate the moderated *t*-statistics and the associated *P* values, as well as the log posterior odds ratio (B statistics) that a gene is differentially expressed vs not differentially expressed. *P* values were adjusted for multiple testing by controlling for false discovery rate using the Benjamini–Hochberg method. For the cross-validation of microarray data in PTC to that of the adrenocortical tumor samples, we examined the gender-specific expression of the probe sets for the genes (42 genes by B statistics >0) that showed significant differential expression in PTC by gender in the adrenocortical tumors as a function of gender. We classified the probe sets into three classes: those that show significant gender-specific adrenocortical tumor expression (Student's *t*-test *P* value <0.0002); those that show no evidence of gender-specific expression (*P* value >0.30); and those that could not be confidently assigned to either class ($0.0002<P<0.30$).

Results

Gene expression profile of PTC by gender

In 35 samples of PTC, we found a number of significantly differentially expressed genes by gender. By hierarchical cluster analysis, the tumor samples were segregated into two broad groups with tumor samples from women and men clustering together (Fig. 1). To eliminate gender-specific gene expression differences that occur in all cell types, such as Y- and X-linked gene expressions, we compared the differentially expressed genes in PTC to a genome-wide gene expression dataset of 86 adrenocortical tumors (56 women and 30 men). Adrenocortical tumors are endocrine tumors without significant gender disparity and thus are well suited for this type of analysis. We first examined 42 genes (B statistics >0) that showed significant PTC gender-specific expression in adrenocortical tumors as a function of gender. The majority of genes that showed increased expression in samples from male patients with thyroid or adrenocortical tumors were genes that were present on the Y chromosome. Conversely, many of the genes that showed increased expression in samples from female patients with either thyroid or adrenocortical tumors were genes that were involved in X-chromosome inactivation (Fig. 2A). There were, however, nine differentially expressed genes that showed gender-specific expression only in PTCs suggesting that these genes may contribute to gender-specific differences in thyroid biology and/or papillary thyroid tumorigenesis (Fig. 2B). Furthermore, we validated the differential expression of CDC23 by gender in 34 independent PTC samples using qRT-PCR (Fig. 2C). We found CDC23 was upregulated in PTC in men (1.4-fold) compared with PTC in women. We found no significant difference in CDC23 immunoreactivity in PTC samples by gender but most of the samples had strong staining. We also found no significant association between *CDC23* mRNA expression and other clinicopathologic variables or by common somatic mutation status (14 wild type, 15 BRAF V600E, and 5 RET/PTC3). Even though, we did not see a difference in the tissue samples, we also tested the idea of whether activating mutation in the MAPK pathways that are common in PTC may modulate *CDC23* mRNA expression. However, we found no significant change in *CDC23* mRNA expression when using a selective BRAF V600E and or MEK inhibitor in the 8505c BRAF V600E mutant cell line (Fig. 2D).

Sex hormones do not regulate CDC23 expression in thyroid and breast cancer cell lines

One of the genes, *CDC23*, was of particularly interest to us since the function of the gene in higher eukaryotic cells is unknown (Irniger & Nasmyth 1997, Zhao *et al.* 1998, Matyskiela & Morgan 2009). We first tested the hypothesis of whether the gender-specific difference in *CDC23* gene expression in PTC (which was upregulated in male tumor samples) was due to sex hormone gene expression regulation. To determine the effect of estrogen and testosterone on *CDC23* gene expression, we used thyroid cancer cell lines representing the common histologies of thyroid cancer (TPC-1 for PTC and FTC-133 for follicular thyroid cancer) and the breast cancer cell line MCF-7. Treatment of cells with either estradiol or testosterone had no significant effect on *CDC23* gene expression levels compared with their effect on a known sex hormone target gene (*GREB1*; Fig. 3A–D). This result suggests that the differential expression of *CDC23* in thyroid cancer we observed cannot be explained simply by differences in sex hormones in males vs females.

CDC23 protein expression in thyroid tissue

Immunohistochemistry staining was performed to examine the expression of CDC23 protein in normal thyroid tissue and PTC samples from 34 individual patient samples. In normal thyroid tissue, the anti-CDC23 antibody showed no staining in normal follicular cells (Fig. 4A and B). In contrast, PTC cells showed strong CDC23 immunoreactivity. Moreover, in cancer samples with adjacent normal thyroid follicles, strong staining was observed only in cancer cells, while the adjacent normal cells were negative for CDC23. Areas of hyperplastic thyroid tissue, follicular cells with increased proliferation and cell cycling, showed no CDC23 immunoreactivity. These findings suggest that CDC23 protein overexpression is specific to PTC cells.

Function of CDC23 in thyroid cancer cells

Human *CDC23* has a 30% homology with *Saccharomyces cerevisiae* Cdc23, a tetratricopeptide repeat protein component of anaphase-promoting complex (APC; Zhao *et al.* 1998). Since the function of *CDC23* in higher eukaryotic cells is unknown and CDC23 showed dramatic overexpression in PTC, we were interested in evaluating the role of *CDC23* in thyroid cancer cells. SiRNA directed *CDC23* knockdown was performed in thyroid cancer cell lines (TPC-1 and FTC-133) and qRT-PCR and immunoblot were used to confirm good CDC23 knockdown (Fig. 5). *CDC23* knockdown dramatically inhibited thyroid cancer cell growth (Fig. 6A and B). A significant difference in cellular proliferation between control and *CDC23* knockdown cells was observed after 4 or 5 days of transfection, which were later than the knockdown of CDC23 protein expression (Figs 5 and 6A and B). This delayed effect on cellular proliferation suggested that the effect of *CDC23* may depend on cell cycling and/or due to its regulation of downstream substrates that mediate its effect.

Given that *CDC23* was overexpressed in PTC and regulated thyroid cancer cell proliferation; we next examined the role of this gene in regulating other cancer cell phenotypes, including anchorage-independent growth. Compared with the negative control cells, *CDC23* knockdown significantly reduced the colony formation and size of FTC-133 cells (Fig. 6C and D). Specifically, in suspension cultures, cell aggregates were formed 16 h after plating and after 2 weeks a large solid spheroid with a diameter of 1 mm and a few separate small cell cluster balls were observed in the negative control cultures (Fig. 6E). The *CDC23* deficient cells also formed spheroids, however, their sizes were only about 1/3 (*CDC23* siRNA#1 transfected cells) or 1/10 (*CDC23* siRNA#2 transfected cells) of the control cells.

To investigate the possible mechanism of cell growth inhibition that occurs with *CDC23* knockdown, cell cycle and apoptosis analyses were performed. Compared with the negative control, *CDC23* deficiency resulted in an increase in the number of cells in both the S (4–19%) and G_2M (7–49%) phases of cell cycle (Fig. 7A). Annexin V staining was used to examine the effects of *CDC23* deficiency on thyroid cancer cell apoptosis. In TPC-1 cells, *CDC23* knockdown increased the percentage of Annexin V positive cells moderately (3–10%) after 5 days of transfection. Similarly, in FTC-133 cells, the deficiency in *CDC23* resulted in increased apoptosis (5–32%) 6–8 days after transfection (Fig. 7B). This time frame for increased apoptosis matched that of the reduction in cellular proliferation. In

addition, these phenotypic changes occurred later than CDC23 protein knockdown, further suggesting that a downstream target of *CDC23* may mediate this effect.

Given the profound effect of *CDC23* on thyroid cancer cell proliferation and cell cycle progression and the previous observation in yeast that mutations in APC genes lead to abnormal levels of cell cycle regulatory proteins (Peters 2006, Sullivan & Morgan 2007), especially the B-type cyclins, we explored the idea that cyclin B1 may be a substrate of CDC23 protein (Wasch & Cross 2002, Herbert *et al.* 2003, Thornton & Toczyski 2003, Soni *et al.* 2008). In both FTC-133 and TPC-1 cells, *CDC23* knockdown resulted in a dramatic buildup of cyclin B1 protein, starting 3 days after transfection (Fig. 7C). Of the two different *CDC23* siRNAs used to induce knockdown, siRNA#2 induced a more dramatic accumulation of cyclin B1 protein, which is consistent with this siRNA's more dramatic functional effects on thyroid cancer cell proliferation and cell cycle arrest.

Another important substrate of APC in yeast is securin, a protein involved in sister chromatids separation (Nasmyth 2005). The phosphorylation of securin by Cdk1 is critical for its binding with *separase* and also reduces the ubiquitination of itself by APC (Holt *et al.* 2008). In *Homo sapiens*, pituitary tumor-transforming 1 (PTTG1) is a homolog of yeast securin protein for which the function is unknown. Therefore, we examined the protein expression of *securin* following *CDC23* knockdown. Compared with control cells, *CDC23* deficiency resulted in the accumulation of *securin*. There was also a change in the migration pattern of the securin protein; a second slower migrating band was present (Fig. 7D). We speculate this may be due to increased phosphorylation of the securin protein. The time course of *securin* accumulation and the change in migration matched that of cyclin B1 protein accumulation and also the functional changes in these cells after *CDC23* knockdown. We next performed triple knockdown studies of CDC23, cyclin B1 and securin proteins to determine if the effect of CDC23 on cellular proliferation were dependent on these proteins. We found that the effect of CDC23 knockdown on cellular proliferation could be reversed by the simultaneous knockdown of *cyclin B1* and *securin* (Fig. 7E and F). These findings, taken together with the overexpression of *CDC23* in PTC provide a molecular basis by which the differential expression of *CDC23* in PTC by gender may result in differences in PTC progression.

Discussion

We studied the gene expression profile of PTC tumor samples by gender. Among several genes, we found *CDC23* was overexpressed in PTC in men compared with women. Furthermore, *CDC23* was overexpressed in PTC and was absent in normal and hyperplastic thyroid tissue. We found sex hormone treatment of thyroid and breast cancer cell lines had no effect on *CDC23* expression. Functional studies in thyroid cancer cell lines demonstrated *CDC23* had a dramatic effect on cell cycle, cellular proliferation, anchorage-independent growth, and tumor spheroid formation. The effect of *CDC23* on cellular proliferation and cell cycle was, at least in part, dependent on cyclin B1 and securin protein levels.

Although gender-based differences in the incidence, aggressiveness, and mortality rate for many human malignancies have been observed, the molecular basis for this is under studied

and poorly understood. Thyroid cancer is an excellent tumor type to study the molecular basis of cancer gender disparity as it has a dramatically different incidence, aggressiveness, and death rate by gender. Because environmental, dietary, and reproductive factors have important roles in cancer initiation, promotion, and progression and these factors may directly regulate gene expression, we used genome-wide gene expression profiling to identify candidate genes differentially expressed in PTC by gender (Mutter *et al*. 2001, Arimoto *et al*. 2003, Steinberg *et al*. 2008, Masotti *et al*. 2010). To minimize possible confounding factors and false-positive results, the samples were matched for extent of disease (TNM stage) and age (tumor samples from premenopausal women). We found nine candidate differentially expressed genes once we cross-validated the array data to exclude X- and Y-linked genes. This suggests that these candidate genes may account for the different epidemiologic and tumor behavior observed in thyroid cancer by gender (higher incidence in women but more aggressive tumors in men).

CDC23 was one of the nine candidate genes differentially expressed by gender in PTC; 1.3-fold higher in tumor samples from men. We were interested in studying *CDC23* because its expression and function in cancer cells is unknown (Irniger & Nasmyth 1997, Zhao *et al*. 1998, Matyskiela & Morgan 2009). We first determined whether *CDC23* expression could be regulated by sex hormones as we found it to be differentially expressed by gender in PTC. In thyroid and breast cancer cell lines, treatment of cells with either estradiol or testosterone had no significant effect on *CDC23* gene expression levels. This suggests that the difference in *CDC23* expression level by gender is not likely to be due to sex hormone difference between women and men. Other possible reasons for the difference in *CDC23* expression in PTC by gender may be due to activating oncogenic changes which may occur at different frequencies by gender, differential effect of environmental and dietary factors by gender, and secondary changes that occur differently during cancer progression by gender (Nakagawa *et al*. 2009, Duma *et al*. 2010, Rahbari *et al*. 2010, Lista *et al*. 2011, Sighoko *et al*. 2011). The complex possible mechanisms that may regulate *CDC23* expression would need to be studied in the future to explain why this gene is differentially expressed in PTC by gender.

To determine if *CDC23* was specifically mis-expressed in cancer, we next characterized the expression of *CDC23* in normal and hyperplastic thyroid tissue samples, and PTC. We found *CDC23* to be overexpressed in PTC. CDC23 protein was absent in normal thyroid follicular cells and hyperplastic follicular thyroid tissue. This finding suggested that *CDC23* may be a positive regulator of PTC initiation and or progression. Thus, we tested the hypothesis if *CDC23* had any effect on cancer cell phenotype (cell proliferation, tumor spheroid formation, cell cycle, apoptosis, and anchorage-independent growth). We found dramatic inhibition of cell growth, anchorage-independent growth, and tumor spheroid formation with cell cycle arrest as a result of *CDC23* knockdown in thyroid cancer cells. Taken together, these findings all suggest that *CDC23* may promote thyroid cancer initiation and or progression.

Given the profound effect of *CDC23* on thyroid cancer cell proliferation and cell cycle progression, and the observation that alterations in APC genes lead to abnormal levels of cell cycle regulatory proteins, we explored the idea that *cyclin B1* and *securin* may be a substrate

of CDC23 protein and mediate its function (Wasch & Cross 2002, Herbert *et al*. 2003, Thornton & Toczyski 2003, Peters 2006, Sullivan & Morgan 2007, Soni *et al*. 2008). We show for the first time that the effect of *CDC23* knockdown on cellular proliferation could be reversed by the simultaneous knockdown of cyclin *B1* and *securin*. This finding suggests that the effect of *CDC23* is mediated, at least in part, by *cyclin B1* and *securin* which is consistent with the role of these two proteins in cancer progression (Bowers & Boylan 2004, Borlak *et al*. 2005, Winnepenninckx *et al*. 2006, Salehi *et al*. 2008).

In summary, our results demonstrate *CDC23* is differentially expressed by gender and is over-expressed in PTC. Although sex hormones do not regulate *CDC23* gene expression, our functional studies indicate that *CDC23* has important biologic effects on cell proliferation and cell cycle progression. The effect of *CDC23* on cancer cell phenotype is mediated, at least in part, by *cyclin B1* and *securin*. Therefore, we propose that *CDC23* is a critical regulator of cell cycle and cancer cell growth, and may be involved in thyroid cancer initiation and/or progression.

Funding

This study was supported by the intramural research program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health.

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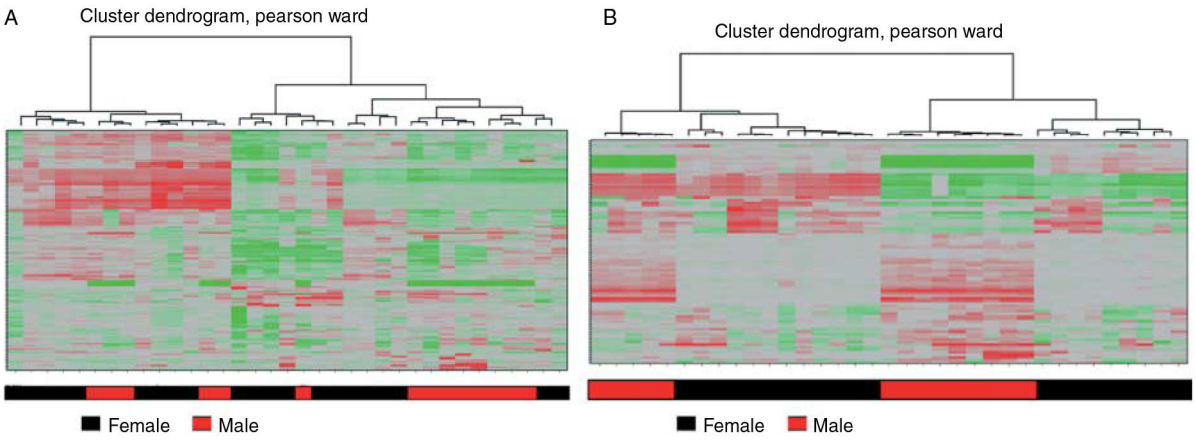

Figure 1.

Dendrograms of clustering from 35 papillary thyroid cancer samples. (A) Unsupervised hierarchical cluster analysis of the top 200 most variably expressed genes. (B) Supervised hierarchical cluster analysis showing differentially expressed genes by gender using the log posterior odds ratio (B statistics >0) statistical criterion. Each row represents the mean adjusted expression level of an individual gene and each column represents an individual tumor sample. Overexpressed genes are indicated in red and underexpressed genes are indicated in green.

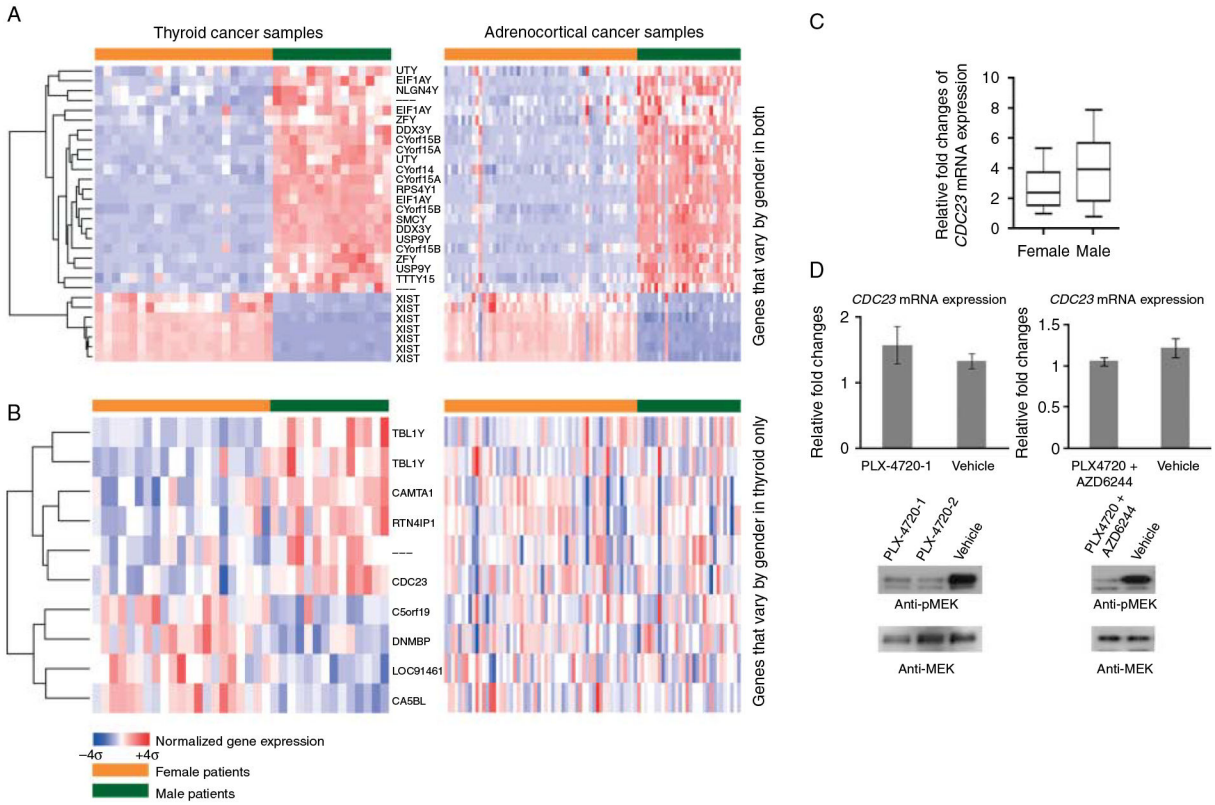

Figure 2.

CDC23 was differentially expressed by gender in thyroid cancer. (A) The gender-specific genes identified by microarray analysis in both thyroid and adrenocortical tumors. (B) Genes with gender-specific expression in papillary thyroid cancer (PTC) only. (C) Quantitative RT-PCR (qRT-PCR) validation of *CDC23* mRNA expression in independent PTC samples. Seventeen paired PTC samples (matched for age at diagnosis (21–45 years, same age or 1 year difference in each pair) and tumor size) were analyzed. *CDC23* mRNA expression was normalized to β-glucuronidase. Box plot is for mean±95% interval. $P<0.01$. (D) Inhibiting the MAPK pathway does not significantly alter *CDC23* mRNA expression. 8505c cells were treated with 3000 nM of PLX 4720 (Braf inhibitor) (left panels), 3000 nM of PLX 4720 plus 80 nM of AZD6244 (MEK inhibitor) (right panels), or vehicle for 3.5 h. CDC23 expression was determined by qRT-PCR using β-glucuronidase as a reference gene (mean±S.E.M.). By *t*-test *P* value was >0.05. Effective inhibition of the MAPK pathway was detected by immunoblotting with anti-phospho-MEK or anti-MEK antibodies.

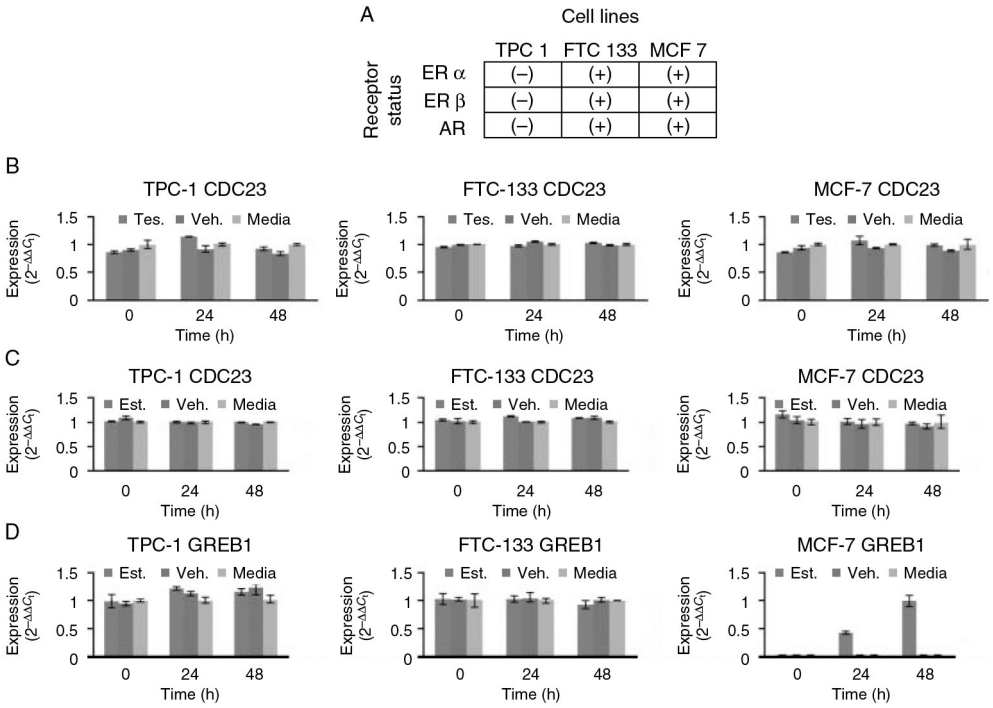

Figure 3.

Sex hormone effect on *CDC23* mRNA expression. Estradiol-17β and testosterone have no significant effect on *CDC23* mRNA expression in TPC-1, FTC-133, and MCF-7 cell lines. (A) Estrogen receptor and androgen receptor expression status in cell lines. Estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and androgen receptor (AR). (B) Relative expression levels of *CDC23* compared with control in three cell lines (FTC-133, TPC-1, and MCF-7) after exposure to 1 nM testosterone (blue), vehicle (1% DSMO) (red), and medium only (green). (C) Relative expression levels of *CDC23* compared with control in three cell lines after exposure to 1 nM 17β-estradiol, vehicle, and medium only. (D) Relative expression levels of *GREB1* compared with control in three cell lines after exposure to 1 nM 17β-estradiol, vehicle, and medium only. *GREB1* was used as a positive control target gene for 17β-estradiol.

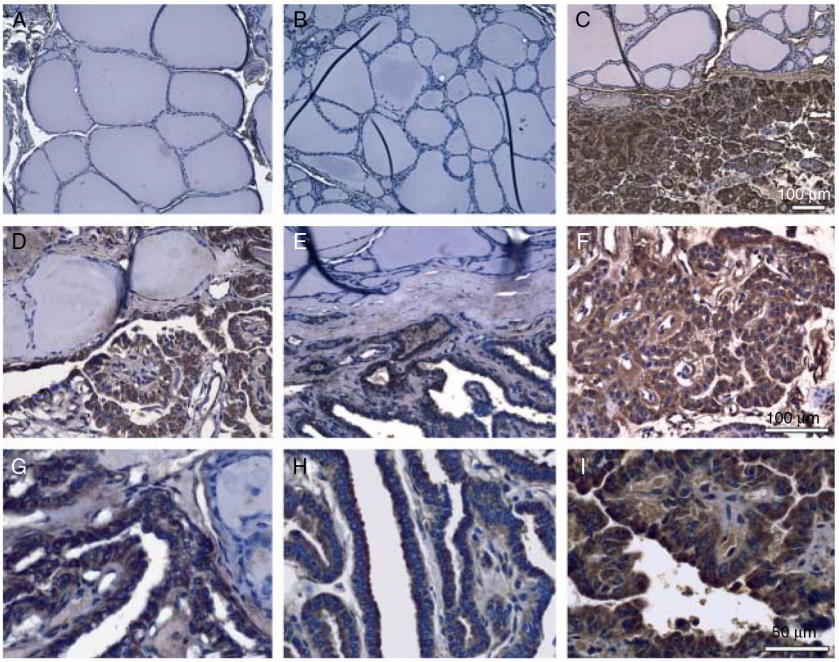

Figure 4.

CDC23 was overexpressed in papillary thyroid cancer (PTC). Representative immunohistochemistry of (A and B) normal thyroid tissues from two different patients with PTC (magnification 10×) and (C–I) PTC samples from six individual patients (magnification: 10× for C; 20× for D and E, F; 40× for G–I).

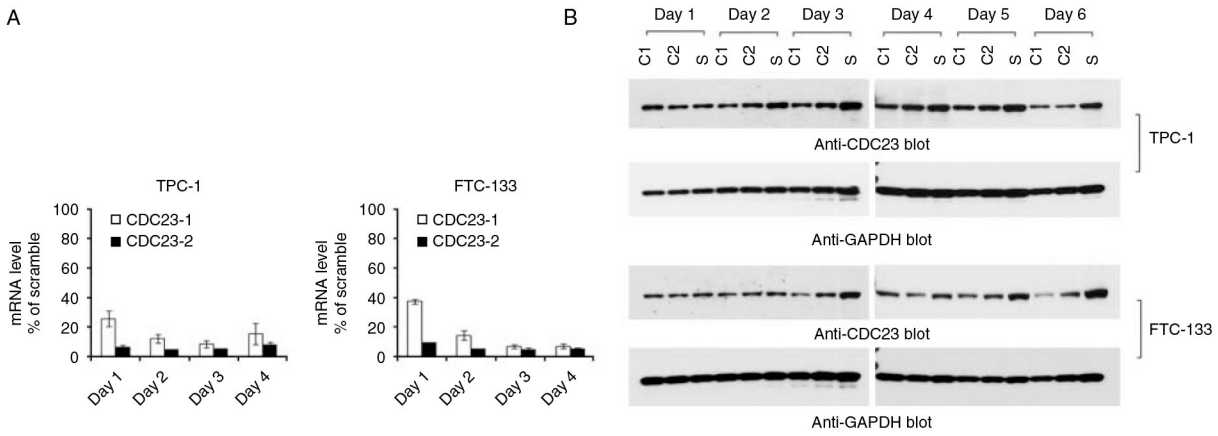

Figure 5.

SiRNA-induced *CDC23* knockdown in thyroid cancer cells. The transfection of *CDC23* specific siRNA reduced *CDC23* mRNA (A) and protein (B) expression in both TPC-1 and FTC-133 cells. After 24 h of transfection, the *CDC23* mRNA was reduced more than 60% with *CDC23*-siRNA1 and more than 90% with *CDC23*-siRNA2 in both cell lines, while the level of CDC23 protein was reduced after 48 h (TPC-1 cells) or 72 h (FTC-133 cells) suggesting that this protein had a relatively long half-life.

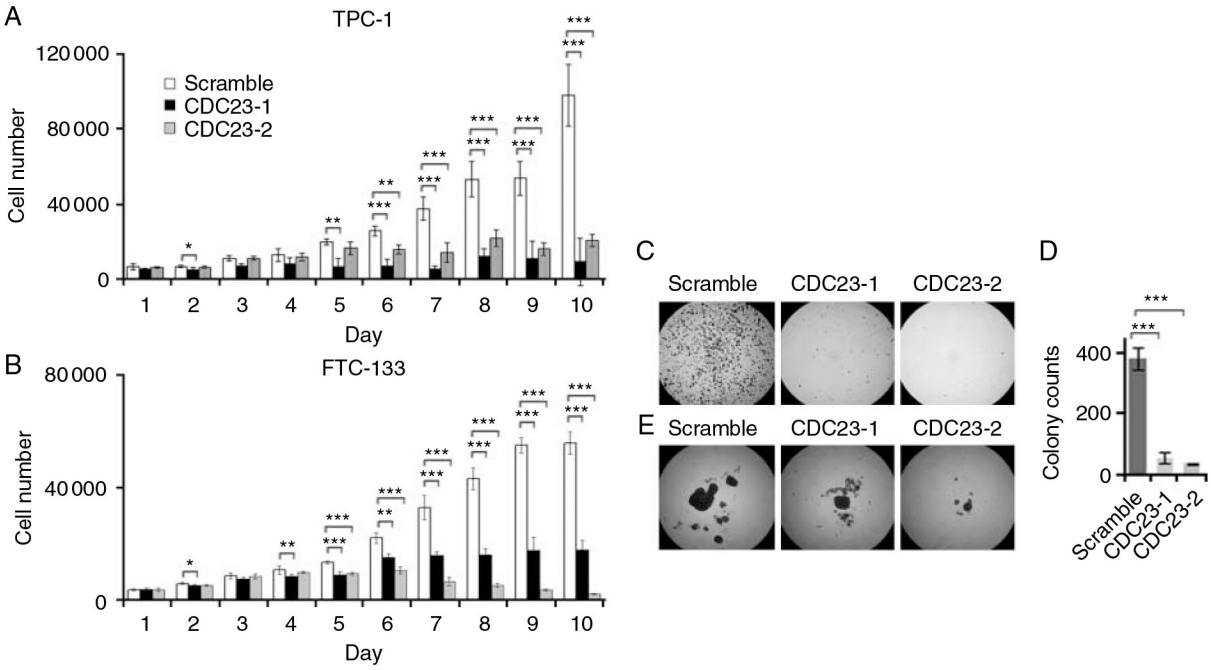

Figure 6.

CDC23 knockdown decreases thyroid cancer cell proliferation and anchorage-independent growth. Cellular proliferation of TPC-1 cells (A) and FTC-133 cells (B) transfected by either scrambled or two different CDC23 specific siRNAs (CDC23–1 and CDC23–2). Data are presented as the mean±S.D. (C) Soft agar colony formations of FTC-133 cells following transfection with the indicated siRNA. (D) Quantitative measures of soft agar assay in four different transfections. Data are the mean±S.D. (E) Spheroid formation of FTC-133 cells after transfection with the indicated siRNA. FTC-133 cells, but not TPC-1 cells, formed colonies in soft agar and grew as spheroids in suspension culture. *, $0.01<P<0.05$; **, $0.001<P<0.01$; ***, $P<0.001$.

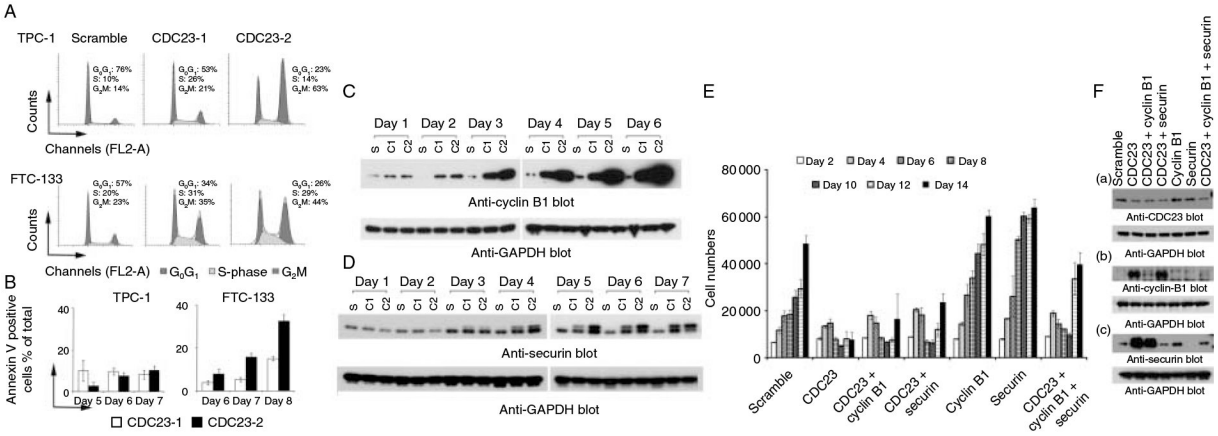

Figure 7.

CDC23 deficiency induced cell cycle arrest, cell apoptosis, and the accumulation of cyclin B1 and securin proteins. (A) Cell cycle analysis after 5 days (TPC-1 cells) or 7 days (FTC-133 cells) of transfection with the indicated siRNAs. (B) The percentages of Annexin V positive cells in the *CDC23* siRNA transfected cells compared with control cells. Data are presented as the mean±S.E.M. (C) *Cyclin B1* expression assessed by immunoblotting of whole cell lysates prepared from FTC-133 cells transfected with the indicated siRNAs. S, Scramble; C1, *CDC23*-1; C2, *CDC23*-2. (D) Securin protein expression assessed by immunoblotting of whole cell lysates prepared from FTC-133 cells transfected with the indicated siRNAs. (E) Effect of CDC23, cyclin B1, and/or securin knockdown on cell proliferation. Simultaneous cyclin B1 and/or securin knockdown reversed the cell growth inhibition effect of CDC23 deficiency as expected. (F) Western blot analysis of CDC23, cyclin B1, and/or securin knockdown.