Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 98–106
Journal of Nutritional Biochemistry

A potential proliferative gene, *NUDT6*, is down-regulated by green tea catechins at the posttranscriptional level

Mugdha Sukhthankar[a], Chang K. Choi[a], Anthony English[b], Jong-Sik Kim[c], Seung Joon Baek[a,*]

[a]*Laboratory of Environmental Carcinogenesis, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996, USA*

[b]*Department of Mechanical, Aerospace and Biomedical Engineering, College of Engineering, University of Tennessee, Knoxville, TN 37996, USA*

[c]*Department of Biology, Andong National University, Andong 760-749, South Korea*

Received 8 July 2008; received in revised form 23 October 2008; accepted 11 November 2008

Abstract

The main aims of this study were to elucidate the effect of green tea catechins on Nudix-type motif 6 (NUDT6) suppression and to characterize NUDT6's biological activity. Our microarray data showed that the green tea component epicatechin-3-gallate suppressed NUDT6 expression, and this was confirmed by RT-PCR. Subsequently, the use of different catechins showed that the effect of epigallocatechin-3-gallate (EGCG) was stronger than that of other catechins. At the posttranscriptional level, EGCG decreased the RNA stability of *NUDT6*, indicating it as a potential mechanism of NUDT6 suppression. Further cloning of the 3′ untranslated region of human *NUDT6* mRNA resulted in reduced luciferase activity by EGCG treatment. This effect was at least, in part, mediated by the extracellular-signal-regulated kinase and p38MAPK pathways. Finally, increased cell proliferation and cell growth in soft agar were observed in NUDT6-overexpressing cells. These findings provide a novel mechanism for the suppression of the proliferative gene *NUDT6* by green tea catechins in human colorectal cancer.

Keywords: NUDT6; GFG; EGCG; Catechin; mRNA stability

1. Introduction

Multiple components in fruits, vegetables, herbs and spices have been found to inhibit tumor formation in experimental animals, and many researchers have focused on polyphenolic compounds in this regard because of their potent biological properties [1]. Green tea (*Camellia sinensis*) is the second most commonly consumed beverage in the world (an estimated 18–20 billion cups daily) and represents a particularly important source of antitumorigenic polyphenols [2]. The most active constituents of green tea are catechins, including epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC). The antitumor effects of green tea catechins have been studied at the cell biological level, and the major cellular phenomena were found to be apoptosis and cell cycle arrest [3,4]. Potential mechanisms induced by catechins have been suggested to include induction of tumor-suppressor proteins [5,6], inhibition of angiogenesis [7], activation of p53 tumor-suppressor protein[8] and inhibition of telomerase activity [9]. While the preponderance of data strongly indicates significant antitumorigenic benefits of green tea, the molecular mechanisms by which catechins affect antitumorigenesis need to be elucidated in detail.

One of these mechanisms apparently involves Nudix-type motif 6 (NUDT6; also known as GFG), a member of the Nudix (*nu*cleoside *di*phosphate-linked moiety *X*) hydrolase superfamily. The Nudix superfamily is widespread among many species and consists mainly of pyrophosphohydrolases that act upon substrates of general structure nucleoside diphosphates linked to another moiety, X, to yield NMP plus P-X [10]. Interestingly, *NUDT6* is an antisense product of

Abbreviations: UTR, untranslated region; ARE, AU-rich element; DMSO, dimethyl sulfoxide; EGCG, epigallocatechin-3-gallate; EGC, epigallocatechin; ECG, epicatechin-3-gallate; EC, epicatechin; ERK, extracellular-signal-regulated kinase; FGF-2, fibroblast growth factor-2; NUDT6, Nudix-type motif 6.

* Corresponding author. Tel.: +1 865 974 8216; fax: +1 865 974 5616.
E-mail address: sbaek2@utk.edu (S.J. Baek).

0955-2863/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.11.002

the bidirectionally transcribed gene locus 4q26 whose sense product is basic fibroblast growth factor [bFGF/fibroblast growth factor-2 (FGF-2)] [11]. As a consequence, Li and Murphy [12] have shown that *NUDT6* regulates the expression of *FGF-2* in mammalian cells because it is the antisense of *FGF-2*. However, it has also been shown that *NUDT6* may have a role in modulating proliferation in response to growth signals via the protein itself [13].

In the present study, we found that *NUDT6* was one of the down-regulated genes affected by catechin treatment. EGCG modulates the RNA stability of the *NUDT6* transcript through the p38MAPK and extracellular-signal-regulated kinase (ERK) pathways. In addition, our data also suggest that NUDT6 is a cell proliferative protein, as assessed by cell growth, soft agar assay and cell impedance experiments.

2. Materials and methods

2.1. Cell culture, reagents and plasmid construction

Human colorectal cancer cells HCT-116, SW480, HT-29 and LoVo were purchased from the American Type Culture Collection (Manassas, VA). HCT-116 and HT-29 cells were grown in McCoy 5A, whereas SW-480 and LoVo cells were maintained in RPMI 1640 and Ham's F-12 media, respectively. Staurosporine was purchased from Biomol International (Plymouth Meeting, PA). All other chemicals were purchased from Fisher Scientific, unless otherwise specified. V5 and actin antibodies were obtained from Invitrogen (Carlsbad, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The luciferase-NUDT6 3′ untranslated region (3′UTR) hybrid construct was generated by PCR using two primers designed to contain the XbaI restriction enzyme site (underlined), as follows: 5′-gctctagattcacatttatatgtttag-3′ (forward) and 5′-gctctagacgaaaagaggcttttaaaat-3′ (reverse). A PCR product using cDNA from HCT-116 cells was digested with XbaI enzyme, followed by ligation into the pGL3 promoter vector digested with XbaI. The full-length human *NUDT6* cDNA (915 bp) was isolated by RT-PCR from HCT-116 cells using forward (5′-ggacgaattaagcggcgtggaga-3′) and reverse (5′-atcaattcctttcatagttttat-3′) primers obtained from the reported human *NUDT6* cDNA sequence (GenBank no. NM_007083). Amplified PCR products were then cloned into the *pcDNA3.1/V5-His-TOPO* vector (Invitrogen) and named *pcDNA 3.1/NUDT6* for correct orientation and *pcDNA 3.1/CONTROL* for reverse orientation.

2.2. RNA isolation and RT-PCR

RNA extraction was performed using Trizol (Invitrogen), and cDNA was prepared with an i-script synthesis kit (Bio-Rad Laboratories, Hercules CA). RT-PCR was carried out as previously described [6]. The PCR primers used were as follows: human *NUDT6*, 5′-catcctccaaagccgattta-3′ (forward) and 5′-aacttctcgaaccgctgtgt-3′ (reverse); human *NAG-1*, 5′-ctccagattccgagagttgc-3′ (forward) and 5′-agagatacgcaggtgcaggt-3′ (reverse); human *ATF3*, 5′-gtttgaggattttgctaacctgac-3′ (forward) and 5′-agctgcaatcttatttctttctcgt-3′ (reverse); human *NR4A1*, 5′-cacagcttgcttgtcgatgt-3′ (forward) and 5′-tcttgtcaatgatgggtgga-3′ (reverse); human *ID1*, 5′-cggatctgagggagaacaag-3′ (forward) and 5′-ctgagaagcaccaaacgtga-3′ (reverse); human *ID2*, 5′-cgtgaggtccgttaggaaaa-3′ (forward) and 5′-atagtgggatgcgagtccag-3′ (reverse). The *GAPDH* levels were used for the normalization of RNA using primers 5′-gggctgcttttaactctggt-3′ (forward) and 5′-tggcaggtttttctagacgc-3′ (reverse). The thermal cycle settings used on a Master Cycler Gradient (Eppendorf) were as follows: 94°C for 2 min as initial denaturation, 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. Amplification products (25–30 cycles) were analyzed on a 1.2% agarose gel, and bands were visualized using ethidium bromide as intercalating agent.

2.3. RNA stability and cycloheximide experiments

For the mRNA stability experiment, HCT-116 cells were grown in 6-cm plates and then treated with dimethyl sulfoxide (DMSO) and EGCG for 1 h. Actinomycin D (Fisher Bioreagents, Fairlawn, NJ) was added at a dose of 5 μM to terminate transcription. For the de novo protein synthesis experiment, HCT-116 cells were pretreated with 10 μg/ml cycloheximide or DMSO for 1 h in serum-free media, followed by treatment with EGCG (50 μM) for 24 h.

2.4. Transient transfection and luciferase reporter assays

HCT-116 cells were plated in 12-well plates at 2×10^5 cells/well and grown for 16 h. Plasmid mixtures containing 0.5 μg of reporter vector and 0.05 μg of pRL-null (Promega, Madison, WI) were transfected by LipofectAMINE (Invitrogen) in accordance with the manufacturer's protocol. After transfection, the media were replaced with serum-free media, and indicated reagents were added. The cells were harvested in 1× luciferase lysis buffer, and luciferase activity was determined and normalized to the pRL-null luciferase activity using a Dual-Glo luciferase assay system (Promega).

2.5. Stable cell lines

HCT-116 cells were plated in 6-cm plates and transfected with either NUDT6 expression vector (*pcDNA3.1/V5-His-TOPO/NUDT6*) or CONTROL vector (*pcDNA3.1/V5-His-TOPO/CONTROL*) using LipofectAMINE (Invitrogen), in accordance with the manufacturer's protocol. After 24 h, the cells were then transferred to a 10-cm plate with G418 (500 μg/ml) (Stratagene, Santa Clara, CA). Selection with G418 was carried out for 3 weeks, and then Western blot analysis was carried out to check the stable cell lines.

2.6. Western blot analysis

The stable cells were grown to 60–80% confluence in 6-cm plates followed by 24-h treatment in the presence of the indicated compounds. Total cell lysates were isolated

using RIPA buffer (1× phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate), and Western blot analysis was carried out as previously mentioned [6].

2.7. Cell proliferation analysis, soft agar cloning assay and impedance assay

Cell proliferation was carried out using *pcDNA3.1/NUDT6* and *pcDNA3.1/CONTROL* cell lines with or without staurosporine (5 μM), using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Briefly, cells were seeded at a concentration of 1000 cells/well in 96-well tissue culture plates in six replicates. The cells were then treated with 5 μM staurosporine for 1, 2, 3 and 4 days. At 0, 1, 2, 3 and 4 days, 20 μl of CellTiter96 Aqueous One solution was added to each well, and the plate was incubated for 1 h at 37°C. Absorbance at 490 nm was recorded in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments, Winooski, VT). Soft agar assays were performed to compare the clonogenic potential of the two stable cells in semisolid medium as previously described [14]. Cell colonies were visualized by staining with 0.5 ml of *p*-iodonitrotetrazolium violet (Sigma, St. Louis, MO) under the luminescent image analyzer (Fujifilm Co.). Colonies were counted using the Multi Gauge Program (Fujifilm Co.) in accordance with the instructions. For the impedance experiment, preliminary naked scans were performed to optimize sensitivity and to check for any electrode debris or defects. The electrodes were then inoculated with 400 μl of McCoy5A, seeding NUDT6 or CONTROL stable cancer cells at a concentration of 2.4×10^4 cells/well. During the cellular microimpedance scans, data were acquired at a rate of 32 Hz every 16 s using a 30-ms filter time constant and a 12-dB/decade rolloff for approximately 100 h. Averages and standard deviation estimates were obtained from 512 sampled data points over the 16-s time intervals. During the experiments, cell-inoculated electrodes were kept in a cell culture incubator at 37°C and 5% CO_2.

2.8. Statistical analysis

SAS for Windows (v. 9.1.3) (SAS Institute, Cary, NC) statistical analysis software was used. For quantitative analyses, analysis of variance with Tukey's multiple comparison test was used to compare mean values. Student's *t* test was also used to analyze the differences between samples. $P<.05$ was considered statistically significant (represented by an asterisk; $^{**}P<.01$, $^{***}P<.001$).

3. Results

3.1. Green tea catechins alter gene expression in human colorectal cancer cells

To elucidate the gene profiles affected by green tea catechins in human colorectal cancer cells, we performed a microarray analysis using HCT-116 cells treated with ECG. Among those genes altered by ECG, *NUDT6* was one of several down-regulated genes (data not shown). We selected three up-regulated genes (*ATF3*, *NAG-1* and *NR4A1*) and three down regulated genes (*NUDT6*, *ID2* and *ID1*). HCT-116 cells were treated with ECG (50 μM) for 24 h, and RNA was isolated. cDNA was made and subjected to RT-PCR. We found that ECG (50 μM) increased the expression of *ATF3*, *NAG-1* and *NR4A1* mRNA, and decreased the expression of *NUDT6*, *ID2* and *ID1* mRNA (Fig. 1A, left). Gel densitometry data from three independent experiments are shown in Fig. 1A (right). Since we have recently reported that a major green tea catechin, EGCG, suppresses bFGF at the posttranslational level [7] and that the *NUDT6* gene is an antisense of *bFGF* mRNA [11], we decided to further investigate NUDT6 in this study. NUDT6 belongs to the family of Nudix hydrolases, but its function is not well known. We carried out RT-PCR for *NUDT6* in other colorectal cancer cells, including HT-29, SW480 and LoVo. As shown in Fig. 1B, ECG down-regulated NUDT6 in these other colorectal cancer cells. We further investigated dose dependence and time dependence. HCT-116 cells were treated with ECG (50 μM) for 0, 6, 12 and 24 h (Fig. 1C, top), and with ECG (10, 50 and 100 μM) for 24 h (Fig. 1C, bottom). As shown in Fig. 1C, *NUDT6* mRNA expression was suppressed at 6 h at a dose of 10 μM, with almost complete suppression at 100 μM. Finally, we examined whether other catechins suppressed *NUDT6* by carrying out RT-PCR at a dose of 50 μM each. We found that among the major catechins of green tea, EGCG had a stronger effect on suppressing *NUDT6* mRNA expression, compared to other catechins (Fig. 1D). Therefore, we decided to use EGCG for further experiments.

3.2. Posttranscriptional regulation of NUDT6 by EGCG

To gain insight into the mechanisms responsible for inhibition of *NUDT6* mRNA by the green tea catechin EGCG, we examined the posttranscriptional regulation of *NUDT6*. Posttranscriptional control in eukaryotic gene expression can also provide a regulatory process by determining the abundance of a particular protein [15]. To determine the effects of EGCG on *NUDT6* mRNA stability, the half-life of the mRNAs was first determined by adding EGCG (50 μM) to HCT-116 cells for 1 h and then treating them with actinomycin D at different time points. After treatment, mRNA was isolated at 0, 1, 2, 4 and 8 h and subjected to RT-PCR (Fig. 2A, top). As a result, the half-life of *NUDT6* mRNA from vehicle- and EGCG-treated cells was estimated to be around 7 and 2 h, respectively (Fig. 2A, bottom). These results suggest that EGCG may affect *NUDT6* mRNA at the posttranscriptional level. Next, HCT-116 cells were pretreated with or without 10 μg/ml cycloheximide and vehicle (DMSO) for 1 h, followed by treatment with EGCG (50 μM) (Fig. 2B, top). The decreased mRNA expression of *NUDT6* was found to be insensitive to cycloheximide, suggesting that the mechanism of stabiliza-

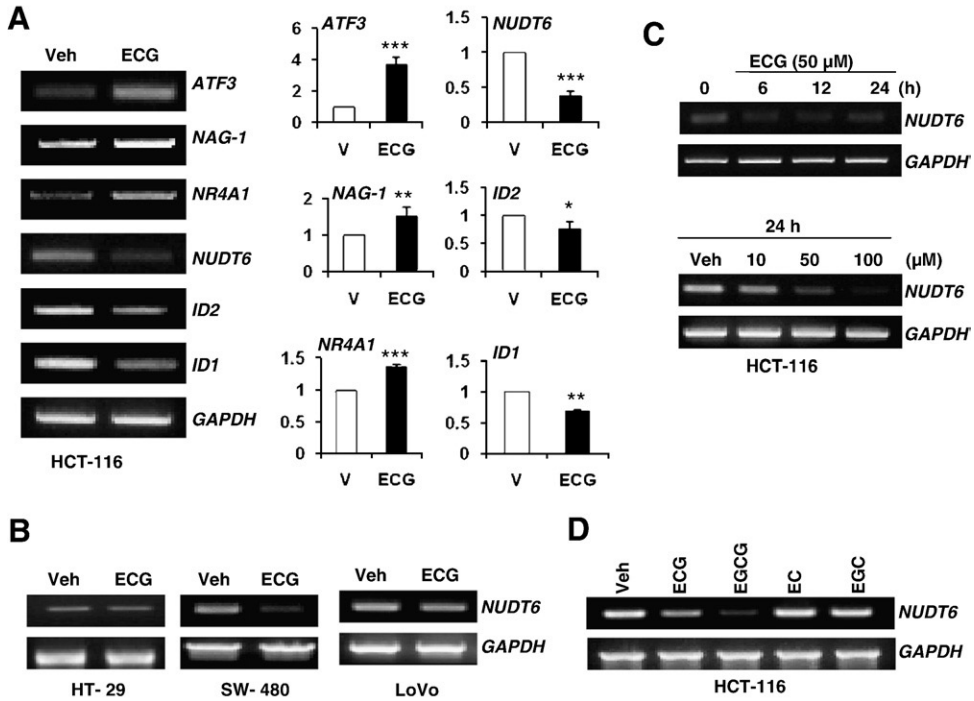

Fig. 1. *NUDT6* is suppressed in the presence of catechins. (A) Left: HCT-116 cells were treated with 50 μM ECG for 24 h, and RT-PCR was performed for *NUDT6*. *ATF3*, activating transcription factor 3; *NAG-1*, NSAID-activated gene-1; *NR4A1*, nuclear receptor subfamily 4, group A, member 1; *ID2*, inhibitor of DNA binding 2; *ID1*, inhibitor of DNA binding 1. Right: Gel densitometry results from three independent experiments for the respective genes from the left panel. Significance was set at $^{*}P$=.05, $^{**}P$=.01 and $^{***}P$=.001. (B) Different human colorectal cancer cells were treated with ECG (50 μM) for 24 h, and *NUDT6* RNA transcripts were measured by RT-PCR. (C) HCT-116 cells were treated with ECG at different time points and at different doses. (D) HCT-116 cells were treated with different catechins (50 μM each), and *NUDT6* gene expression was determined by RT-PCR.

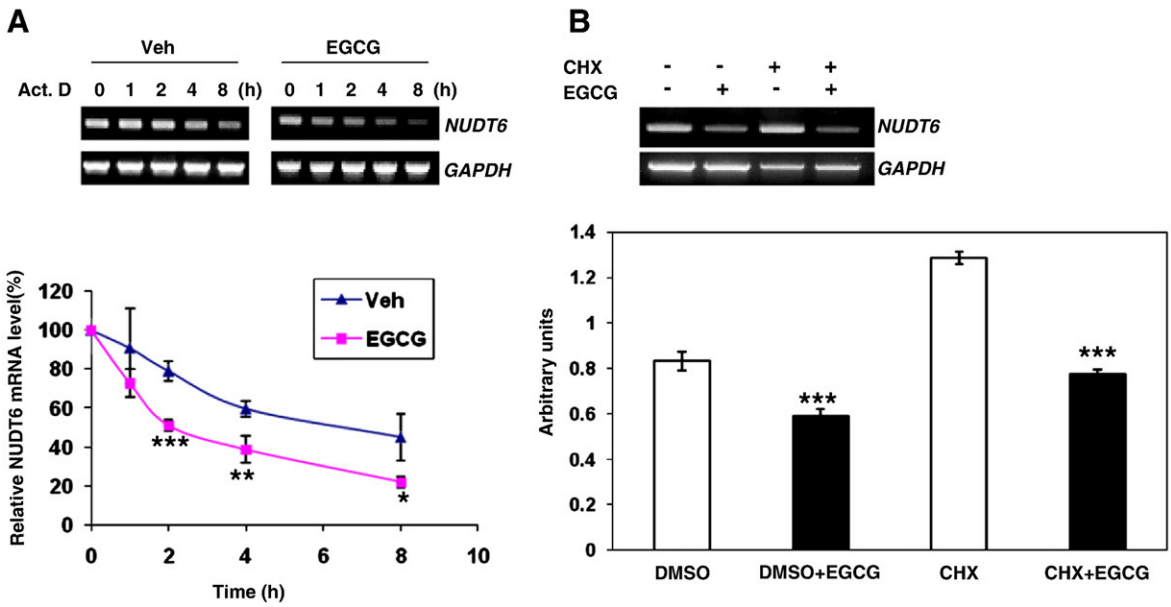

Fig. 2. Posttranscriptional regulation of *NUDT6*. (A) EGCG facilitates *NUDT6* mRNA degradation. HCT-116 cells were incubated for 24 h with vehicle or EGCG at a concentration of 50 μM and subsequently treated with actinomycin D (5 μM). At the indicated times, total RNA was isolated, and *NUDT6* transcripts were measured by RT-PCR. The relative level of *NUDT6* mRNA was calculated, and the results were plotted as the percentage of the mRNA level present at Time 0 of actinomycin D treatment (bottom). (B) HCT-116 cells were pretreated with 10 μg/ml cycloheximide and DMSO for 1 h in serum-free media, followed by treatment with EGCG (50 μM) for 24 h. The data are representative of three independent experiments. $^{***}P$<.001.

tion by EGCG is not involved in de novo protein synthesis (Fig. 2B, bottom).

3.3. Involvement of the 3′UTR sequence of NUDT6 in mRNA stability and MAPK pathways

The stability of mRNA is determined in many cases by interactions between specific RNA-binding proteins and *cis*-acting sequences located in the 3′UTR of the mRNA [15]. The AU-rich element (ARE), which targets mRNA for rapid degradation, is well known and characterized as a *cis*-acting sequence [16–18]. We found one ARE consensus sequence and seven ARE-like sequences [19] in the 3′UTR of NUDT6. The 3′UTR of the *NUDT6* gene was cloned into a pGL3 promoter vector downstream of the luciferase reporter gene (*pSV40-LUC-NUDT6 3′UTR*; Fig. 3A). After transfection, the cells were treated with different doses of EGCG (10, 50 and 100 μM), and luciferase activity was measured. As shown in Fig. 3B, luciferase activity decreased in a dose-dependent manner. This result clearly shows that the 3′UTR of *NUDT6* plays an important role in maintaining the stability of *NUDT6* and that EGCG affects the 3′UTR region in a dose-dependent manner.

We also checked the effect of different catechin treatments on the 3′UTR of *NUDT6* (Fig. 3C), and the result was similar to RT-PCR data shown in Fig. 1D. Although EC and EGC decreased the luciferase activity compared to vehicle, the effect was not statistically significant. Next, we examined whether kinase pathways might be involved in EGCG-induced NUDT6 suppression. ERK, p38MAPK and JNK have been shown to be involved in ARE-driven mRNA turnovers [20]. Thus, we examined

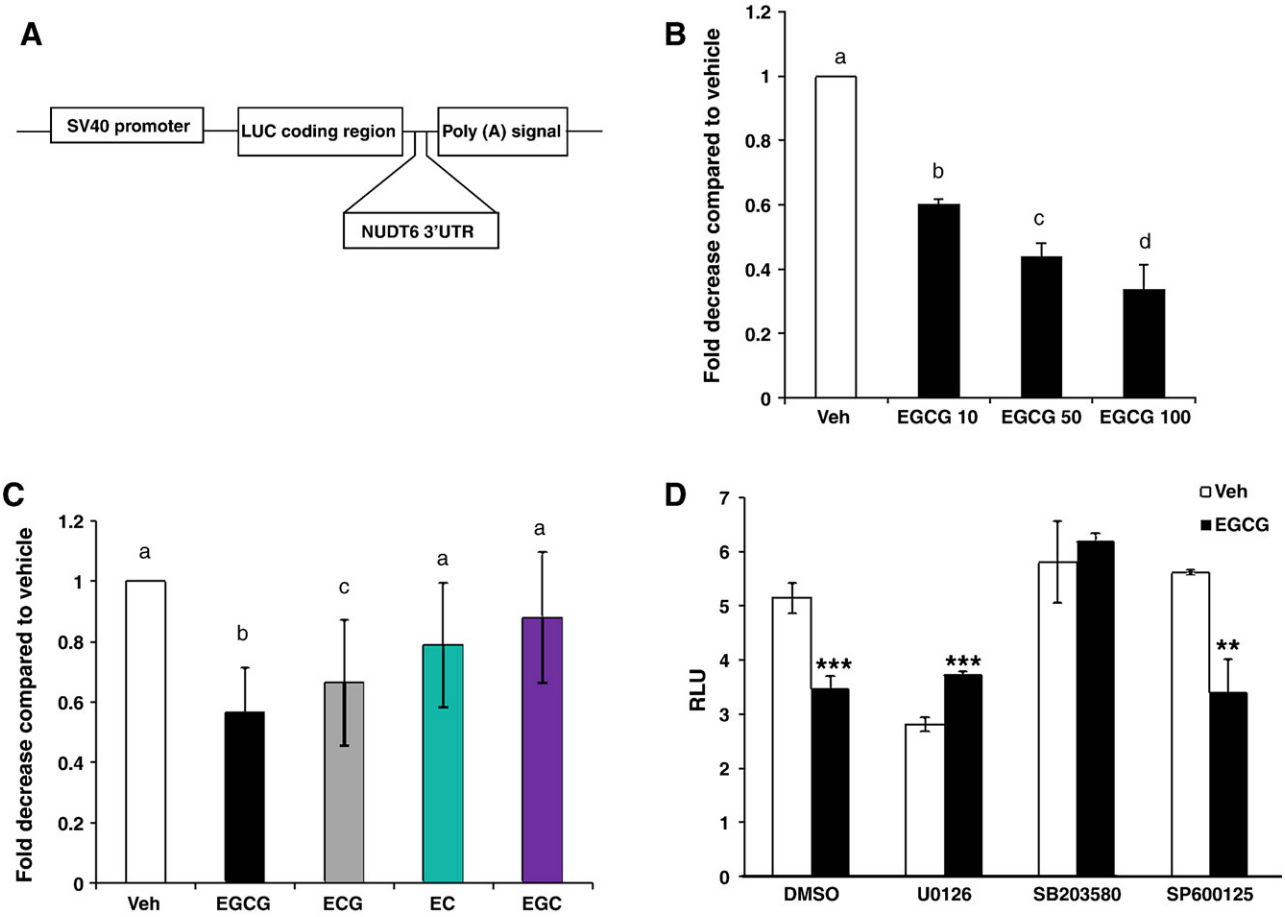

Fig. 3. Involvement of 3′UTR sequences in EGCG-induced *NUDT6* mRNA suppression. (A) Schematic diagram of the pSV40-LUC-*NUDT6* 3′UTR construct. (B) The construct was transfected (0.5 μg) along with pRL-null vector (0.05 μg) into HCT-116 cells, and the cells were treated with different doses of EGCG (10, 50 and 100 μM). After 24-h treatment, luciferase activity was measured. All other values were normalized to the control (Veh) value set as 1.0. Tukey's multiple comparison test was used to express the results as the mean±SD of three independent transfections, and different letters indicate significant difference ($P<.05$). (C) After transfection, HCT-116 cells were treated with four different catechins (EGCG, ECG, EC and EGC, at a dose of 50 μM), and luciferase activity was measured. The values obtained from vehicle-treated samples (Veh) were defined as 1.0, and data were analyzed using Tukey's multiple comparison test. (D) HCT-116 cells were plated in a 12-well plate; transfected with the construct mentioned in (A); pretreated with inhibitors for the ERK (U0126; 5 μM), p38MAPK (SB203580; 15 μM) and JNK pathways (SP600125; 30 μM) for 30 min; and then treated with EGCG (50 μM) for 24 h. The *y*-axis represents luciferase activity measured by relative luciferase unit (RLU). $^{**}P<.01$ and $^{***}P<.001$, based on Student's *t* test. All experiments were performed in triplicate, with columns representing means and with bars representing S.D.

the effects of MAPK pathway inhibitors on EGCG-affected *NUDT6* mRNA stability. HCT-116 cells were transfected with pSV40-LUC-*NUDT6* 3′UTR construct for 24 h; pretreated with U0126 (ERK inhibitor; 5 μM), SB203580 (p38 inhibitor; 15 μM) and SP600125 (JNK inhibitor; 30 μM) for 30 min; and treated with EGCG (50 μM) for 24 h in serum-free media. The treatment of U0126 and SB203580 with EGCG did not decrease luciferase activity compared to vehicle-treated samples (Fig. 3D), suggesting the potential role of ERK and, to some extent, p38MAPK in the posttranscriptional regulation of *NUDT6* by EGCG.

3.4. Biological activity of NUDT6

The biological activity of NUDT6 has not been elucidated in detail, although there are some reports indicating that NUDT6 functions as tumor suppressor and tumor promoter [12,13]. Commercially available NUDT6 antibody did not work properly in Western blot analysis (data not shown). Therefore, we amplified the full-length *NUDT6* gene and were successful in getting the forward and reverse clones of *NUDT6* in the expression vector pCDN3.1/V5-His-TOPO (Fig. 4A). We then made stable cell lines for NUDT6 in both forward and reverse orientations. The stable cell lines pcDNA 3.1/NUDT6 and pcDNA 3.1/CONTROL were plated in 6-cm plates, harvested and subjected to Western blot analysis. First, NUDT6 expression was confirmed using antibody against the V5 tag (Fig. 4A). Cell proliferation was carried out to determine whether NUDT6 expression affects cell growth. Staurosporine was also added to induce apoptosis, and absorbance was measured on Days 0, 1, 2, 3 and 4. NUDT6-overexpressing cells significantly increased cell proliferation in the presence of staurosporine at 2, 3 and 4 days; however, a significant increase was also seen at 0 and 1 day without staurosporine (Fig. 4B). The protumorigenic activity of NUDT6 was evaluated by determining whether

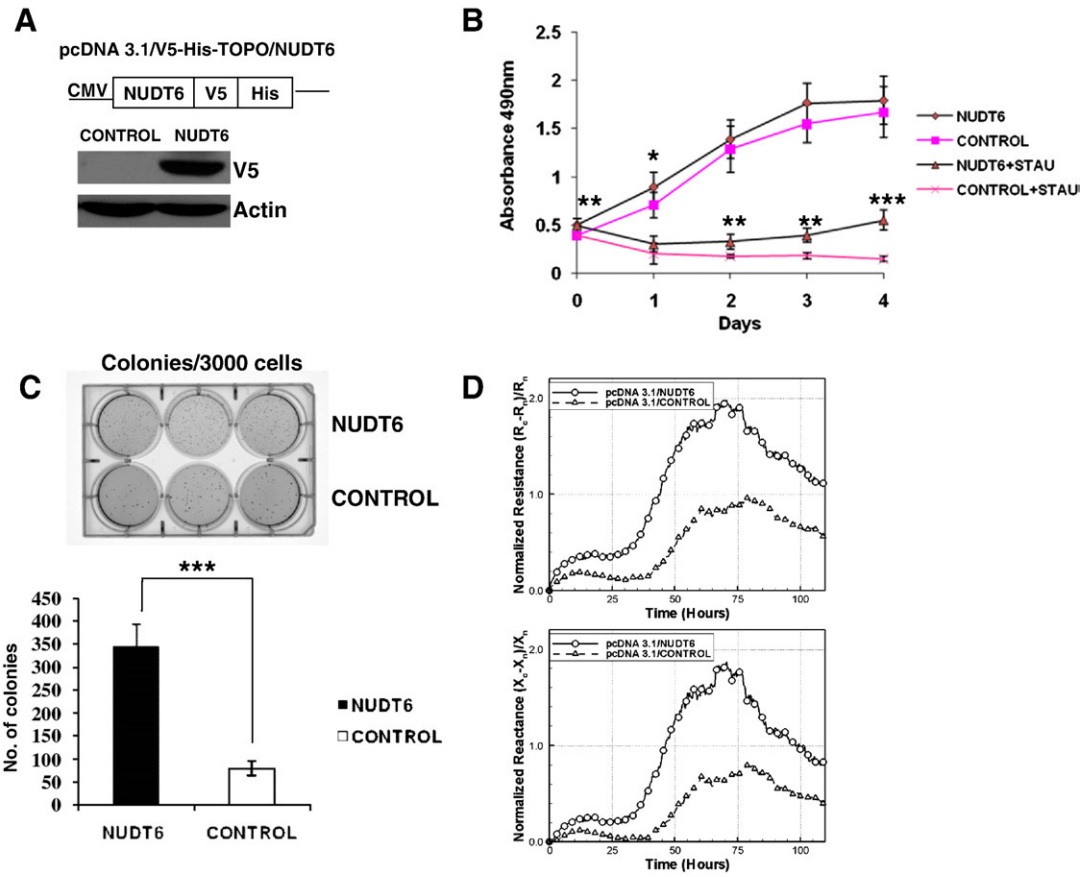

Fig. 4. Characterization of the biological activity of NUDT6 in human colorectal cancer cells. (A) The stable cell lines pcDNA3.1/NUDT6 and pcDNA3.1/CONTROL were made as described in Materials and Methods. These clones contained tag proteins, V5 and histidine. Western blot analysis was carried out using anti-V5 antibody to confirm the integration of plasmid. Actin served as loading control. (B) Cell growth curve for CONTROL and NUDT6 cells. CONTROL and NUDT6 cells were plated onto a 96-well plate (1 day before treatment), and either vehicle or 5 μM staurosporine was added at 0 day. Cell growth was measured using the CellTiter96 Aqueous One Solution Cell Proliferation Assay. Values are expressed as the mean±S.D. of six replicates. $^{*}P<.05$, $^{**}P<.01$ and $^{***}P<.001$ versus control cells. (C) Soft agar assay. The stable cell lines were grown for 2–3 weeks in 0.4% soft agar and stained with *p*-iodonitrotetrazolium violet solution. The results are representative of three different experiments. The data represent mean±SD. (D) Microimpedance measurement of HCT-116 cells with stable cell lines. The corresponding normalized resistance (R) and reactance (X) were measured in HCT-116 cells over a 3.5-day period. The subscripts "c" and "n" indicate cell-covered and naked scans, respectively. For the sake of clarity, symbols represent 20 data intervals. The starting cell concentration used here was 2.4×10^4 cells/ml.

NUDT6 expression would affect cell growth in agarose because the ability to form colonies in soft agar is reflective of tumorigenesis [14]. The NUDT6 stable cell line resulted in a dramatic increase in the clonogenic capacity of the cells (Fig. 4C). To further confirm the biological activity of NUDT6, an electrical impedance assay was carried out, and resistance and reactance were recorded as described in Materials and Methods. The pcDNA 3.1/NUDT6 cells showed increased resistance and reactance compared to pcDNA 3.1/CONTROL cells (Fig. 4D, top and bottom, respectively). These data also suggest that NUDT6 may have oncogenic activity. Overall, our data suggest that NUDT6 may act like a cell-proliferating factor in human colorectal cancer cells, and green tea catechins suppress its expression at the posttranscriptional level.

4. Discussion

Colorectal cancer is the third leading cause of cancer-related deaths in the United States [21]. Prevention of colorectal cancer is gaining more attention than its treatment because therapeutic drugs generate many side effects and discomforts. Dietary compounds have been known to have fewer side effects and to play an important role in the pathogenesis and prevention of cancer [22]. Green tea catechins are among such dietary compounds, and they are well established as anticancer agents [23,24]. Green tea catechins are known to inhibit cell growth, angiogenesis and metastasis, and to promote apoptosis accompanied by alterations in the gene expression of the tumor-suppressor proteins NAG-1 and EGR-1 and of oncogenic proteins such as bFGF [5,7,25]. In this work, we have provided a new mechanism by which EGCG/ECG acts as an anticancer agent. EGCG affects the posttranscriptional regulation of the cell proliferative gene *NUDT6* through the ERK and p38MAPK pathways.

Regulation of gene expression by posttranscriptional modification of mRNA stability is an important mechanism used in the control of cell growth. Namely, the rate of mRNA degradation determines the extent and duration of gene expression. The 3′UTR of some mRNAs contains AREs, which regulate degradation of mRNAs and provide an effective way to control protein expression by regulating mRNA half-life and translation [26]. In particular, cellular events such as differentiation, proliferation, apoptosis and inflammation are known to be related to modifications in the rate of RNA degradation mediated by the ARE motif [16,26]. Based on their sequences and their effects on mRNA stability, three classes of AREs have been defined [27]. Class I AREs contain scattered copies of the AUUUA sequence within a U-rich region. Class II AREs contain overlapping AUUUA motifs within a U-rich region. Class III AREs contain U-rich sequences rather than the AUUUA motif. Emerging evidence suggests that there is another novel ARE that does not fit into any of the three classes. Examination of sequences revealed that the 3′UTR of *NUDT6* mRNA contains a highly conserved ARE (Fig. 3A) and seven ARE-like sequences, and these AREs should indicate the rapid degradation of mRNA. Indeed, insertion of the 3′UTR of *NUDT6* into the reporter vector caused a dramatic reduction in luciferase activity in the presence of EGCG (Fig. 3B). In addition, EGCG affected the mRNA stability of *NUDT6* and degraded the mRNA as quickly as 2 h compared to 7 h in vehicle (DMSO) (Fig. 2A, top and bottom). There was, however, no involvement of any de novo protein synthesis as depicted in the cycloheximide study (Fig. 2B, top and bottom), indicating the involvement of signaling pathways that do not require new protein synthesis.

The MAPK pathway could play a role in the posttranscriptional regulation of genes because a number of ARE-binding proteins require phosphorylation by MAPK for activation [28]. We have shown that treatment with JNK inhibitor and EGCG did not affect the luciferase activity of the pSV40-LUC-*NUDT6* 3′UTR construct (Fig. 3D); however, luciferase activity was even increased and restored when U0126 and SB203580 were added, respectively. Interestingly, U0126 treatment with EGCG increased luciferase activity (Fig. 3D), indicating that the ERK pathway may affect not only the 3′UTR of NUDT6 but also SV40 promoter activity located in the promoter of this reporter vector. Luciferase activity was decreased in the presence of U0126 compared to the vehicle-treated samples, clearly suggesting that the ERK pathway is involved in stabilizing the mRNA of *NUDT6.* Overall, our data suggest that EGCG modulates the ERK and p38MAPK pathways and leads to degradation of *NUDT6* mRNA.

NUDT6 belongs to the Nudix hydrolase family and is hypothesized to have both proliferative and antiproliferative effects [10]. There was a 4.44-fold decrease in *NUDT6* gene expression in our microarray data treated with ECG. To confirm the microarray data, we selected three up-regulated genes (*ATF3*, *NAG-1* and *NR4A1*) and three down-regulated genes (*NUDT6*, *ID2* and *ID1*). We previously reported that *NAG-1* and *ATF3* are induced by green tea catechins and that *NR4A1*, *ID1* and *ID2* are known to play a role in tumorigenesis [29–31]. In contrast, the biological function of NUDT6 and its role in tumorigenesis have not been well elucidated [11]. To elucidate the biological function of *NUDT6* in human colorectal cancer cells, we generated stable cell lines with V5/His tags. The cell proliferation assay showed that the NUDT6 cells had an increased rate of cell growth compared to controls at the early time points (Fig. 4B, 0 and 1 day); however, cell proliferation was seen at later time points (Fig. 4B, 2, 3 and 4 days) when an apoptosis inducer was added to the cells. This suggests that NUDT6 may contribute to the initiation of cell proliferation and that its expression is less sensitive to the apoptosis inducer. The cell proliferative properties of NUDT6 are more evident by soft agar cloning and impedance assays. The NUDT6 stable cell line had more clonogenic properties compared to control

cells, as shown in Fig. 4C. The impedance technique has been applied to a number of biological studies that deal with cellular barrier function, attachment, spreading and adhesion [32]. In addition, frequency-dependent electrical impedance measurements have been used to evaluate the model parameters associated with cell–cell and cell–matrix junction formation [33]. In the present study, microimpedance measurements were used to quantitatively examine proliferation and morphological changes such as cell–cell adhesion and cell–substrate adhesion under NUDT6 expression. The electrical impedance assay confirmed that NUDT6 expression resulted in cell proliferation with enhanced cell motility (Fig. 4D, top and bottom). All these results show that EGCG decreased the expression of protumorigenic *NUDT6* by modulating the ERK and p38MAPK pathways at the posttranscriptional level. Further studies are required to determine the ERK-pathway- and p38MAPK-pathway-related ARE-binding proteins.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R21CA 109423 and R01CA 108975) and by the Center of Excellence in Livestock Diseases and Human Health from the University of Tennessee (S.J.B.). This work was also supported, in part, by NSF CAREER Award BES-0238905 (A.E.).

The authors thank Misty Bailey and Nichelle C. Whitlock for their critical review.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2008.11.002.

References

[1] Wenzel U, Kuntz S, Brendel MD, Daniel H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. Cancer Res 2000; 60:3823–31.

[2] Azam S, Hadi N, Khan NU, Hadi SM. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. Toxicol In Vitro 2004;18:555–61.

[3] Ahmad N, Cheng P, Mukhtar H. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. Biochem Biophys Res Commun 2000;275:328–34.

[4] Gupta S, Ahmad N, Nieminen AL, Mukhtar H. Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent(-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. Toxicol Appl Pharmacol 2000;164:82–90.

[5] Baek SJ, Kim JS, Jackson FR, Eling TE, McEntee MF, Lee SH. Epicatechin gallate-induced expression of NAG-1 is associated with growth inhibition and apoptosis in colon cancer cells. Carcinogenesis 2004;25:2425–32.

[6] Cho KN, Sukhthankar M, Lee SH, Yoon JH, Baek SJ. Green tea catechin (-)-epicatechin gallate induces tumour suppressor protein ATF3 via EGR-1 activation. Eur J Cancer 2007;43:2404–12.

[7] Sukhthankar M, Yamaguchi K, Lee SH, et al. A green tea component suppresses posttranslational expression of basic fibroblast growth factor in colorectal cancer. Gastroenterology 2008;134:1972–80.

[8] Hofmann CS, Sonenshein GE. Green tea polyphenol epigallocatechin-3 gallate induces apoptosis of proliferating vascular smooth muscle cells via activation of p53. FASEB J 2003;17:702–4.

[9] Naasani I, Oh-Hashi F, Oh-Hara T, et al. Blocking telomerase by dietary polyphenols is a major mechanism for limiting the growth of human cancer cells in vitro and in vivo. Cancer Res 2003;63: 824–30.

[10] McLennan AG. The Nudix hydrolase superfamily. Cell Mol Life Sci 2006;63:123–43.

[11] Zhang SC, Barclay C, Alexander LA, et al. Alternative splicing of the FGF antisense gene: differential subcellular localization in human tissues and esophageal adenocarcinoma. J Mol Med 2007;85: 1215–28.

[12] Li AW, Murphy PR. Expression of alternatively spliced FGF-2 antisense RNA transcripts in the central nervous system: regulation of FGF-2 mRNA translation. Mol Cell Endocrinol 2000;162:69–78.

[13] Asa SL, Ramyar L, Murphy PR, Li AW, Ezzat S. The endogenous fibroblast growth factor-2 antisense gene product regulates pituitary cell growth and hormone production. Mol Endocrinol 2001;15: 589–99.

[14] Baek SJ, Kim KS, Nixon JB, Wilson LC, Eling TE. Cyclooxygenase inhibitors regulate the expression of a TGF-{beta} superfamily member that has proapoptotic and antitumorigenic activities. Mol Pharmacol 2001;59:901–8.

[15] Yamaguchi K, Lee SH, Eling TE, Baek SJ. A novel peroxisome proliferator-activated receptor gamma ligand, MCC-555, induces apoptosis via posttranscriptional regulation of NAG-1 in colorectal cancer cells. Mol Cancer Ther 2006;5:1352–61.

[16] Bevilacqua A, Ceriani MC, Capaccioli S, Nicolin A. Post-transcriptional regulation of gene expression by degradation of messenger RNAs. J Cell Physiol 2003;195:356–72.

[17] Dixon DA. Dysregulated post-transcriptional control of COX-2 gene expression in cancer. Curr Pharm Des 2004;10:635–46.

[18] Shaw G, Kamen R. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 1986;46:659–67.

[19] King PH, Levine TD, Fremeau Jr RT, Keene JD. Mammalian homologs of *Drosophila* ELAV localized to a neuronal subset can bind in vitro to the 3′UTR of mRNA encoding the Id transcriptional repressor. J Neurosci 1994;14:1943–52.

[20] Eberhardt W, Doller A, Akool ES, Pfeilschifter J. Modulation of mRNA stability as a novel therapeutic approach. Pharmacol Ther 2007;114:56–73.

[21] Vincenzi B, Santini D, Rabitti C, et al. Cetuximab and irinotecan as third-line therapy in advanced colorectal cancer patients: a single centre phase II trial. Br J Cancer 2006;94:792–7.

[22] Singh PN, Fraser GE. Dietary risk factors for colon cancer in a low-risk population. Am J Epidemiol 1998;148:761–74.

[23] Bigelow RL, Cardelli JA. The green tea catechins, (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin-3-gallate (ECG), inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells. Oncogene 2006;25:1922–30.

[24] Chung FL, Schwartz J, Herzog CR, Yang YM. Tea and cancer prevention: studies in animals and humans. J Nutr 2003;133: 3268S–74S.

[25] Issa AY, Volate SR, Wargovich MJ. The role of phytochemicals in inhibition of cancer and inflammation: new directions and perspectives. J Food Comp Anal 2006;19:405–19.

[26] Winzen R, Gowrishankar G, Bollig F, Redich N, Resch K, Holtmann H. Distinct domains of AU-rich elements exert different functions in mRNA destabilization and stabilization by p38 mitogen-activated protein kinase or HuR. Mol Cell Biol 2004;24:4835–47.

[27] Brennan CM, Steitz JA. HuR and mRNA stability. Cell Mol Life Sci 2001;58:266–77.

[28] Dean JL, Sully G, Clark AR, Saklatvala J. The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation. Cell Signal 2004;16:1113–21.
[29] Jang KS, Han HX, Paik SS, Brown PH, Kong G. Id-1 overexpression in invasive ductal carcinoma cells is significantly associated with intratumoral microvessel density in ER-negative/node-positive breast cancer. Cancer Lett 2006;244:203–10.
[30] Nilsson JA, Nilsson LM, Keller U, Yokota Y, Boyd K, Cleveland JL. Id2 is dispensable for myc-induced lymphomagenesis. Cancer Res 2004;64:7296–301.
[31] Rajpal A, Cho YA, Yelent B, et al. Transcriptional activation of known and novel apoptotic pathways by Nur77 orphan steroid receptor. EMBO J 2003;22:6526–36.
[32] Choi CK, English AE, Jun SI, Kihm KD, Rack PD. An endothelial cell compatible biosensor fabricated using optically thin indium tin oxide silicon nitride electrodes. Biosens Bioelectron 2007;22:2585–90.
[33] English AE, Squire JC, Bodmer JE, Moy AB. Endothelial cell electrical impedance parameter artifacts produced by a gold electrode and phase sensitive detection. IEEE Trans Biomed Eng 2007;54: 863–73.