[CANCER RESEARCH 63, 3899–3903, July 15, 2003]

Advances in Brief

Stimulation of Estrogen Receptor Signaling by γ Synuclein[1]

Yangfu Jiang, Yiliang Ellie Liu, AiPing Lu, Anu Gupta, Itzhak D. Goldberg, Jingwen Liu, and Y. Eric Shi[2]

Department of Radiation Oncology, Long Island Jewish Medical Center, The Long Island Campus for the Albert Einstein College of Medicine, New Hyde Park, New York 11040 [Y. J., Y. E. L., I. D. G., Y. E. S.], and Veterans Affairs Palo Alto Health Care System, Palo Alto, California 94304 [A. L., A. G., J. L.]

Abstract

Synucleins are emerging as central player in the fundamental neural processes and in the formation of pathologically insoluble deposits characteristic of Alzheimer's disease and Parkinson's disease. However, γ Synuclein (SNCG) is also highly associated with breast cancer and ovarian cancer progression. Whereas most studies of this group of proteins have been directed to the elucidation of their role in the formation of depositions in brain tissue, the normal cellular function of this highly conserved synuclein family remains largely unknown. A notable finding in this study is that SNCG, identified previously as a *breast cancer-specific gene 1*, strongly stimulated the ligand-dependent transcriptional activity of estrogen receptor-α (ER-α) in breast cancer cells. Augmentation of SNCG expression stimulated transcriptional activity of ER-α, whereas compromising endogenous SNCG expression suppressed ER-α signaling. The SNCG-stimulated ER-α signaling was demonstrated in three different cell systems including ER-α-positive and SNCG-negative MCF-7 cells, ER-α-positive and SNCG-positive T47D cells, and SNCG-negative and ER-α-negative MDA-MB-435 cells. The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth. Whereas overexpression of SNCG stimulated the ligand-dependent cell proliferation, suppression of endogenous SNCG expression significantly inhibited cell growth in response to estrogen. The stimulatory effect of SNCG on ERα-regulated gene expression and cell growth can be effectively inhibited by antiestrogens. These data indicate that SNCG is required for efficient ER-α signaling and, thus, stimulated hormone-responsive mammary tumors.

Introduction

Synucleins are a family of small proteins consisting of 3 known members, SNCA,[3] SNCB, and SNCG. Synucleins has been specifically implicated in neurodegenerative diseases such as AD and PD. Mutations in SNCA are genetically linked to several independent familial cases of PD (1). More importantly, wild-type SNCA is the major component of Lewy bodies in sporadic PD and in a subtype of AD known as Lewy body variant AD (2, 3). SNCA peptide known as nonamyloid component of plaques has been implicated in amyloidogenesis in AD (4, 5). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy body cases (6, 7). Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, synucleins have also been implicated in non-neural diseases, particularly in the hormone-responsive cancers of breast and ovary (8–13).

We have reported previously the isolation of differentially expressed genes in the cDNA libraries from normal breast and infiltrating breast cancer using differential cDNA sequencing approach (8, 14). Of many putative differentially expressed genes, a breast cancer-specific gene, *BCSG1,* was identified as a putative breast cancer-specific gene, which was highly expressed in a breast cancer cDNA library but scarcely in a normal breast cDNA library (8). Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather, BCSG1 revealed extensive sequence homology to neural protein synuclein, having 54% and 56% sequence identity with SNCA and SNCB, respectively. Subsequent to the isolation of *BCSG1*, *synuclein* γ (13) and *persyn* (15) were cloned independently from a brain genomic library and a brain cDNA library. In fact, BCSG1, SNCG, and persyn appear to be the same protein. Thus, the previously identified BCSG1, which is also highly expressed in brain, has been renamed as SNCG (16).

Although synucleins are abundant proteins expressed in presynaptic terminals and tightly associated with amyloid plaque in AD and Lewy body in PD, the normal cellular function of this highly conserved synuclein family remains largely unknown. Being identified as a breast cancer-specific gene, SNCG expression in breast follows a stage-specific manner: SNCG was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma *in situ*, but expressed at an extremely high level in advanced infiltrating breast cancer (8, 11). Overexpression of SNCG in cancer cells led to significant increase in cell motility and invasiveness *in vitro*, profound augmentation of metastasis *in vivo* (9), and resistance to chemotherapeutic drug-induced apoptosis (17). Overexpression of synucleins, especially SNCG and SNCB, also correlated with ovarian cancer development (11, 13). Whereas synuclein (α, β, and γ) expression was not detectable in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express either SNCG or SNCB, and 42% (19 of 45) expressed all three of the synucleins (α, β, and γ) simultaneously (11). The involvement of SNCG in hormone-responsive cancers of breast and ovary prompted us to explore the potential role of SNCG in cellular response to estrogen. In the present study, we evaluated the biological functions of SNCG on regulation of estrogen-receptor transcriptional activity in human breast cancer cells. The results suggest that one of the critical functions of SNCG on breast cancer pathogenesis is to stimulate ER-α transcriptional activity.

Materials and Methods

Conditioned Cell Culture. All of the cell lines used in this study (MCF-7, T47-D, and MDA-MB-435) were originally obtained from the American Type Culture Collection. Proliferating subconfluent human breast cancer cells were harvested and cultured in the phenol red-free IMEM containing 5% charcoal-stripped FCS for 4 days before addition of indicated dose of E2. Cells in the absence or presence of E2 were collected 24 h after addition of E2 and were subjected to the assays for ER-α transcriptional activity.

Received 11/19/02; accepted 6/2/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported in part by Grant 99-028-04-CCE from American Cancer Society, Grants DAMD17-98-1-8118 and DAMD17-01-1-0352 from the United States Army Medical Research and Development Command, Grant from the Department of Veterans Affairs (Office of Research and Development, Medical Research Service), and by Grant 1RO1CA83648-01 from National Cancer Institute.

[2] To whom requests for reprints should be addressed, at Department of Radiation Oncology, Long Island Jewish Medical Center, New Hyde Park, NY 11040. Phone: (718) 470-3086; Fax: (718) 470-9756; E-mail: shi@lij.edu.

[3] The abbreviations used are: SNCA, α synuclein; AD, Alzheimer's disease; BCSG1, breast cancer specific gene 1; ER, estrogen receptor; Cat-D, cathepsin D; Hsp, heat shock protein; PD, Parkinson's disease; PR, progesterone receptor; SNCB, β synuclein; SNCG, γ synuclein; ERE, estrogen response element; RT-PCR, reverse transcription-PCR; E_2, 17β-estradiol; TGF, transforming growth factor; ERE4-Luc, ERE4-Luciferase; ICI, ICI 182,780.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/14/3899/2505538/3899.pdf by guest on 26 May 2022

Gene Transfection. Subconfluent proliferating cells in 12-well plate were incubated with 2 μg of expression vectors in 1 ml of serum-free IMEM containing LipofectAMINE for 5 h. Culture was washed to remove the excess vector and LipofecTAMINE, and then postincubated for 24 h in fresh culture medium to allow the expression of transfected gene.

Assays for the Transcriptional Activity of ER-α. Cells were transiently transfected with a firefly luciferase reporter construct (pERE4-Luc) containing four copies of the ERE (18). For the cotransfection experiments, the plasmid DNA ratio of pERE4Luc to expression vectors of ER-α or SNCG was 2:1. A renilla luciferase reporter, pRL-SV40-Luc, was used as an internal control for transfection efficiency. Luciferase activities in total cell lysate were measured using the Promega Dual Luciferase Assay System. Absolute ERE promoter firefly luciferase activity was normalized against renilla luciferase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition, and at least three independent transfection assays were performed.

RT-PCR Analysis. MCF-7-derived cells were cultured in ligand-free medium for at least 5 days, and treated with 10^{-9} M E_2 for 4 h as indicated. Total RNA from cells were isolated using RNeasy Kit (Qiagen Inc.). Approximately 4 μg of total RNA was subjected to semiquantitative RT-PCR analysis following a procedure described previously for estrogen-responsive genes (19, 20). The primer sequences (5′-3′) are as follows: TGF-α, sense CGCCCTGTTCGCTCTGGGTAT, antisense AGGAGGTCCGCATGCTCACAG (240-bp product); cathepsin-D, sense CCAGCCCCCAATCCCAACCCCACCTCCAG, antisense ACTGAAGCTGGGAGGCAAAGGCTACAAGC (842-bp product); and PS2, sense CATGGAGAACAAGGTGATCTG, antisense CAGAAGCGTGTCTGAGGTGTC (336-bp product).

Stable Expression of SNCG Antisense mRNA in T47D Cells. A 285-bp DNA fragment corresponding to the exon 1 region (−169 to +116) of SNCG gene was amplified from the plasmid pBS-SNCG759 and was cloned into the *EcoR*I site of the expression vector pcDNA3.1. The antisense or sense orientation of the exon 1 in the pcDNA3.1 vector was determined by restriction enzyme digestion and was verified by DNA sequencing. Vectors expressing SNCG antisense mRNA (pcDNA-SNCG-As) or SNCG sense mRNA (pcDNA-SNCG-S) were transfected separately into T47D cells by Effectin reagent. Isolated clones were picked up after G418 selection. The expression of SNCG antisense and sense mRNAs (285 bp) in the individual clones was confirmed by RT-PCR reaction. For antisense mRNA, the primer sets are: T7 as the forward primer, 5′ TAATACGACTCACTATAGGG 3′ and SNCG-Wf as the reverse primer, ACGCAGGGCTGGCTGGGCTCCA. The primer sets for detection of sense mRNA are: T7 as the forward primer, 5′ TAATACGACTCACTATAGGG 3′ and SNCG-Wr as the reverse primer, 5′ CCTGCTTGGTCTTTTCCACC 3′.

Cell Proliferation Assay. For [^{3}H]thymidine incorporation, cells were cultured and synchronized in the conditioned medium for 4 days as described in "Conditioned Cell Culture." Cells were treated with or without 1 nM of E_2 for 24 h. [^{3}H]Thymidine was added 12 h before harvesting. [^{3}H]Thymidine incorporation was determined by precipitation with 10% trichloroacetic acid followed by liquid scintillation counting. Triplicate wells were assayed for each cellular proliferation condition, and at least three independent assays were performed. Cell growth was also measured using a cell proliferation kit (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; Roche Molecular Biochemicals, Indianapolis, IN). Briefly, exponentially growing cells were seeded in quadruplicate at 1500 cells per well (96-well plate) in the conditioned medium. Cells were treated with indicated chemicals for 6 days before harvesting.

Soft Agar Colony Formation Assay. The anchorage-independent growth was carried out in 12-well plates as we described previously (10). The bottom layer consists of 0.5 ml of 5% charcoal-striped calf serum/IMEM containing 0.6% agar. The top layer consists of 0.25 ml of 5% charcoal-striped calf serum/IMEM containing 0.4% agar and ~2000 cells. In the E_2-treated groups, the top layer also contains 1 nM of E_2. Cells were cultured under high humidity condition. Cells were fed with 0.1 ml of culture medium with or without E_2 every 4 days. After 2 weeks, the number of colonies in each well was counted under a Nikon microscope at ×100 amplification. Triplicate wells were assayed for each condition.

Results

Overexpression of SNCG Stimulated Transcriptional Activity of ER-α. Estrogen response is mediated by two closely related members of the nuclear receptor family of transcription factors, ER-α and ER-β (21, 22). Because ER-α is the major ER in mammary epithelia, we measured the effect of SNCG on modulating the transcriptional activity of ER-α in human breast cancer cells. We first selected ERα-positive and SNCG-negative MCF-7 cells as recipients for SNCG transfection (Fig. 1, *A* and *B*). MCF-7 cells were transiently transfected with either the pCI-SNCG expression plasmid or control pCI-neo plasmid. Transfection of the *SNCG* gene into the SNCG-negative MCF-7 cells did not affect ER-α expression under the conditions both with and without E_2 (Fig. 1*A*). In the absence of E_2, the basal levels of ER-α on control and SNCG-transfected cells are the same. Although treatment of the control cells with E_2 resulted in a significant decrease in ER-α level, overexpression of SNCG did not affect E_2-mediated degradation of ER-α. Transfection of SNCG significantly stimulated E_2-mediated activation of ER-α (Fig. 1*B*). Treatment of wild-type and SNCG-transfected MCF-7 cells with E_2 resulted in a significantly differential increase in estrogen-responsive reporter ERE4-Luc activity relative to basal levels in untreated cells. Overexpression of the *SNCG* gene in MCF-7 cells increased E_2-stimulated reporter activity 3.2-fold over the SNCG-negative control cells. The SNCG-stimulated transcriptional activity of ER-α was ligand-dependent, because SNCG had no significant effect on the transcriptional activity of ER-α in the absence of E_2.

Consistent with the increased transcriptional activity of ER-α,

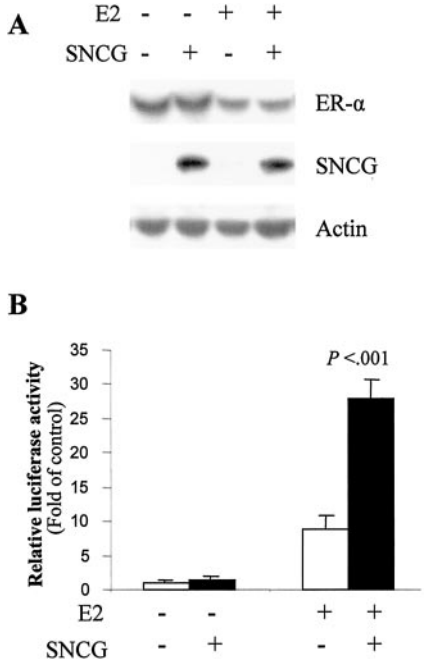

Fig. 1. SNCG stimulated ER-α transcriptional activity in MCF-7 human breast cancer cells. Cells were first transiently transfected with pCI-SNCG or the control vector pCI-neo. The transfected cells were selected with G418 and then transfected with pERE4-Luc, as well as control reporter pRL-SV40-Luc. After transfection, cells were cultured in the ligand-free medium for 4 days as described in the "Conditioned Cell Culture" of "Materials and Methods," treated with or without 1 nM E2 for 24 h before the promoter activities were determined by measuring the dual luciferase activity (*A*). Western analysis of ER-α and SNCG in MCF-7 cells transfected with pCI-SNCG or the control vector pCI-neo. Expression of SNCG did not affect the ER-α expression in the conditions both with and without 24-h E_2 treatment. SNCG stimulated ER-α signaling in MCF-7 cells (*B*). The ERE reporter luciferase activity was normalized against the control renilla luciferase activity to correct for transfection efficiency. All values were presented as the fold induction over the control luciferase activity in the nontreated SNCG-negative cells, which was taken as 1. The numbers represent means of three cultures; *bars,* ±SD.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/14/3899/2505538/3899.pdf by guest on 26 May 2022

SNCG also stimulated E_2-regulated genes in MCF-7 cells (Fig. 2). Whereas SNCG had no effect on the transcription of Cat-D, PS2, and TGF-α in the absence of E_2, transcription of Cat-D, PS2, and TGF-α were increased 3.9-fold, 3.2-fold, and 4.2-fold in SNCG transfected cells *versus* control cells in the presence of E_2, respectively (Fig. 2*A*). To evaluate the effect of antiestrogen on SNCG-stimulated ER-α-regulated genes, we treated the cells with an antiestrogen ICI. As demonstrated in Fig. 2*B,* the basal levels of PR were very weak in both SNCG-transfected and control cells, but were increased significantly by E_2 treatment. Treatment of the cells with E_2 stimulated a 3.5-fold PR protein expression in SNCG-transfected cells compared with control cells. Although ICI slightly stimulated basal levels of PR, treatment of the SNCG-transfected MCF-7 cells with ICI significantly blocked E_2-stimulated PR expression, indicating that SNCG-stimulated gene expression in E_2-treated cells is mediated by ER-α.

We also investigated the effect of SNCG on the transcriptional activity of ER-α in ERα-negative and SNCG-negative MDA-MB-435 breast cancer cells (Fig. 3). Treatment of ER-α-transfected MDA-MB-435 cells with E_2 activated reporter activity, indicating the functional transcriptional activity of the transfected ER-α gene. A significant stimulation of ER-α signaling by SNCG was observed in MDA-MB-435 cells when the cells were cotransfected with ER-α and SNCG constructs. SNCG increased ligand-dependent transcriptional activity 3.7-fold over the control cells.

Reduced Levels of SNCG Compromised Transcriptional Activity of ER-α. The effect of SNCG expression on ER-α transactivation was additionally demonstrated by inhibiting endogenous SNCG expression with SNCG antisense mRNA in T47D cells that express high levels of SNCG (8). Stable transfection of the SNCG antisense construct into T47D cells significantly reduced SNCG expression to 25% of that in control T47D cells (Fig. 4*A*). Whereas E_2 significantly stimulated the reporter activity in the control T47D cells, inhibition of SNCG expression reduced E_2-responsive luciferase activity in two stable antisense-transfected T47D cell lines, AS-1 and AS-3 cells, to 21% and 13% of that in control T47D cells, respectively (Fig. 4*B*). Treatment of T47D cells with E_2 resulted in a 25-fold increase over the nontreated cells. However, only 3.3- and 5.2-fold increase was observed in the AS-3 and AS-1 cells, respectively. Taken together, the increased estrogen-responsive reporter activity in SNCG-transfected MCF-7 and MDA-MB-435 cells, as well as the increased estrogen-regulated gene transcription and the compromised transcriptional activity of ER-α in SNCG antisense-transfected T47D cells indicated that SNCG stimulated ligand-dependent transcriptional activity of ER-α.

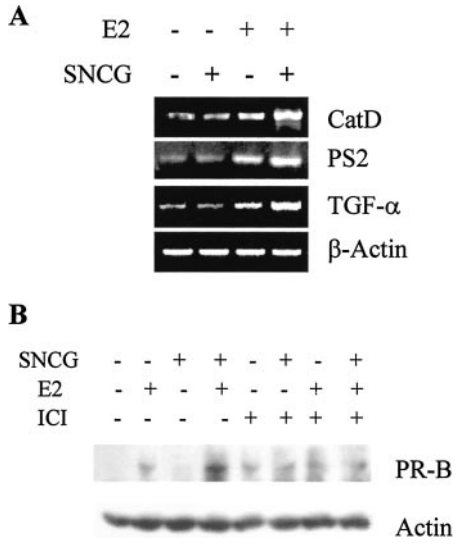

Fig. 2. SNCG stimulated estrogen-regulated gene transcription in MCF-7 cells. Cells were transiently transfected with pCI-SNCG or the control vector pCI-neo. After G418 selection, cells were cultured in the ligand-free medium for 4 days as described in the "Conditioned Cell Culture" of "Materials and Methods." *A,* RT-PCR. Cells were treated with or without 1 nM of E2 for 8 h before the isolation of total RNA. Expressions of mRNA of Cat-D, PS2, and TGF-α were studied in SNCG transiently transfected cells *versus* control cells by RT-PCR analyses. *A,* an 842-bp product of Cat-D, a 336-bp product of PS2, and a 240-bp product of TGF-α, were amplified by RT-PCR and normalized with actin. *B,* inhibition of SNCG-stimulated PR protein expression by antiestrogen ICI. Cells were treated with or without 1 nM of E_2 and 1 μM of ICI for 32 h. Total proteins were isolated, normalized, and subjected to Western analysis using anti-PR antibody.

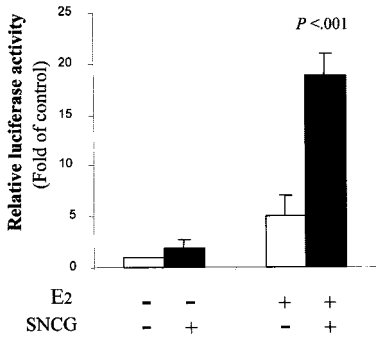

Fig. 3. SNCG stimulated ER-α transcriptional activity in MDA-MB-435 human breast cancer cells. SNCG-negative and ER-α-negative MDA-MB-435 cells were cotransfected with SNCG and ER-α constructs. Cells were cultured in the ligand-free medium for 4 days as described in the "Conditioned Cell Culture" of "Materials and Methods," treated with or without 1 nM E2 for 24 h before the promoter activities were determined by measuring the luciferase activity. The ERE reporter luciferase activity was normalized against the control renilla luciferase activity to correct for transfection efficiency. All values were presented as the fold induction over the control luciferase activity in the nontreated SNCG-negative cells, which was taken as 1. The SNCG-induced slight increase in the ligand-independent reporter activity over control was not statistically significant ($P > 0.05$); *bars,* ±SD.

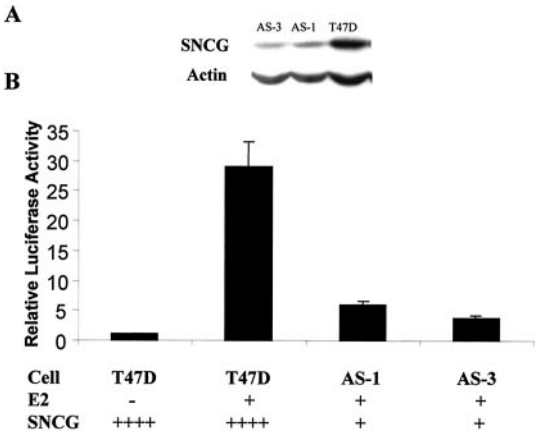

Fig. 4. Inhibition of SNCG expression reduced the transcriptional activity of ER-α. *A,* Western analysis of SNCG expression in control T47D and SNCG antisense transfected AS-1 and AS-3 cells. *B,* ERE-Luc reporter activity in control and antisense transfected T47D cells. Cells were cultured in the ligand-free conditioned medium for 4 days, treated with or without 10^{-11} M of E_2 for 24 h before harvesting. All values were normalized to the reporter activity of the nontreated T47D cells, which was set to 1. The numbers represent means of three cultures; *bars,* ±SD.

Stimulation of Cell Proliferation by SNCG. To determine the biological relevance of SNCG-stimulated ligand-dependent ER-α signaling, we analyzed the effect of SNCG overexpression on the growth of breast cancer cells. To determine whether SNCG overexpression affects ligand-dependent or ligand-independent cell growth, the cellular proliferation of the previously established two stable SNCG-transfected MCF-7 cell clones, MCF-SNCG2 and MCF-SNCG6, were compared with that of SNCG-negative cells, MCF-neo1 and MCF-neo2 (10). Data in Fig. 5*A* shows that whereas SNCG had no signif-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/14/3899/2505538/3899.pdf by guest on 26 May 2022

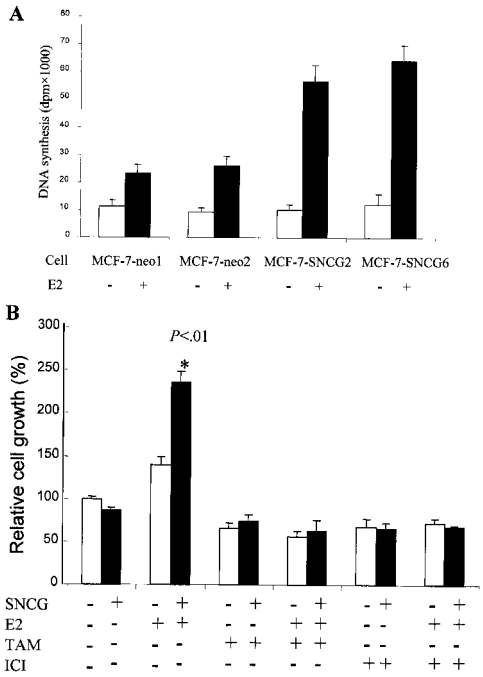

Fig. 5. SNCG stimulated ligand-dependent cell proliferation. For all experiments, cells were cultured and synchronized in the ligand-free conditioned cell culture medium for 4 days before the hormone treatments. *A,* stimulation of cell proliferation by SNCG overexpression. Cells were treated with or without 1 nM E_2 for 24 h. Cell proliferation was measured by [^{3}H]thymidine incorporation. Data are means of three cultures. *B,* effect of antiestrogens on SNCG-stimulated cell growth. Cells were treated with or without 1 nM of E_2, 1 μM of tamoxifen, or 1 μM of ICI for 6 days before harvesting. Media were changed every 2 days with fresh estrogen and antiestrogens. Cell growth was measured using a cell proliferation kit (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt). Data are the mean of quadruplicate cultures. □ represents MCF-neo1 cells; ■ represents MCF-SNCG6 cells; *bars,* ±SD.

icant effect on the proliferation of SNCG-transfected cells compared with MCF-neo cells in the absence of E_2, overexpression of SNCG significantly stimulated the ligand-dependent proliferation. Treatment of neo clones with E_2 stimulated an average cell proliferation 2.4-fold over controls. However, E_2 treatment of SNCG clones resulted in an average of 5.4-fold increase in the proliferation *versus* controls, suggesting that SNCG expression renders the cells more responsive to E_2-stimulated cell proliferation. To address whether the stimulatory effect of SNCG on cell growth is mediated by ER-α, we investigated the effect of the antiestrogen tamoxifen and ICI. As shown in Fig. 5*B,* E_2-stimulated growth in both MCF-neo1 and SNCG-MCF6 cells was significantly blocked by tamoxifen and ICI. These data indicate that SNCG-stimulated cell growth is mediated by ER-α.

The effect of SNCG expression on cell growth was also demonstrated in the SNCG antisense construct-transfected T47D cells. SNCG antisense mRNA expression reduced SNCG protein expression to the level of 25% of that in control T47D cells (Fig. 4*A*). Soft agar colony assays demonstrated that the anchorage-independent growth of T47D cells expressing SNCG antisense mRNA was suppressed significantly. When cells were cultured in soft agar without E_2, there were very few colonies formed in both the T47D group and T47D-SNCG antisense group. Treatment of T47D cells with E_2 resulted in a 19-fold increase of colonies over the nontreated cells. However, treatment of T47D cells expressing SNCG antisense mRNA with E_2 resulted in only 3-fold increase over the nontreated cells (Fig. 6). These data demonstrated that inhibition of endogenous SNCG expression dramatically diminished the cell growth in response to estrogen. Consistent with its stimulatory effect on ligand-dependent cell proliferation, overexpression of SNCG did not affect the proliferation of ER-α-negative MDA-MB-435 cells (9).

Discussion

Synucleins are small proteins expressed predominately in neurons, and have been specifically implicated in the neurodegenerative disorders such as AD and PD. Most studies of this group of proteins have been directed to the elucidation of their role in the formation of depositions in brain tissue. However, studies also indicated the potential role of synucleins, particularly SNCG, in the pathogenesis of steroid-responsive tumors of breast and ovary. SNCG was first identified and cloned as a breast cancer-specific gene, which is highly expressed in advanced infiltrating breast carcinomas but not in normal or benign breast tissues (8). Aberrant expression of SNCG was also associated with ovary cancer progression (11). What role SNCG has in breast and ovary, and how it is implicated in breast and ovary cancer remains a mystery. The association between SNCG expression and the progression of steroid-dependent cancers of breast and ovary led us to investigate the role of SNCG in the regulation of ER-α. Here we reported that SNCG strongly stimulated the ligand-dependent transcriptional activity of ER-α. Whereas SNCG overexpression stimulated transcriptional activity of ER-α, compromising SNCG expression suppressed ER-α signaling. The SNCG-stimulated ER-α signaling was demonstrated in three different cell systems including: (*a*) overexpression of SNCG in ER-α-positive and SNCG-negative MCF-7 cells; (*b*) antisense blocking SNCG expression in ER-α-positive and SNCG-positive T47D cells; and (*c*) cotransfection of SNCG and ER-α into SNCG-negative and ER-α-negative MDA-MB-435 cells. The results shown in this report demonstrated that human ER-α requires SNCG for efficient transcriptional activity.

The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth. It has been demonstrated in different systems that the interaction between SNCG and ER-α stimulated cell growth. First, whereas expression of SNCG in MCF-7 cells had no effect on the cell growth in the absence of E_2, SNCG significantly stimulated the ligand-dependent cell growth, which can be blocked by antiestrogens. This growth stimulation was also demonstrated previously in the anchorage-independent growth assay (10). Second, when endogenous SNCG expression in T47D cells was blocked by expressing SNCG antisense mRNA, the anchorage-independent growth in response to E_2 was significantly suppressed in the cells expressing antisense SNCG. Third, although the alternation of SNCG expression affected the cell growth of ER-α-positive MCF-7 and T47D cells, it had no effect on the cell growth of ER-α-negative MDA-MB-435 cells (9). Consistent with the requirement of E_2 for SNCG-stimulated cell growth, we also demonstrated previously that SNCG has no significant effect on tumor growth of ER-α-negative MDA-MB-435 cells (9).

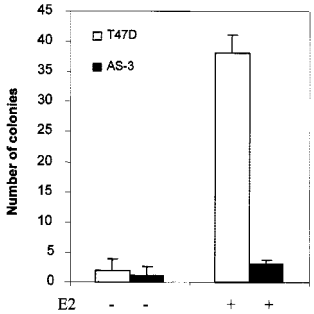

Fig. 6. Effect of inhibiting endogenous SNCG expression on soft agar colonies formation capability of T47D cells. T47D and SNCG antisense stably transfected AS-3 clone were cultured into the top layer soft agar and treated with or without 1 nM of E_2 as described in "Materials and Methods." The number of colonies was counted after 2 weeks of plating using a Nikon microscope at ×100 amplification. Triplicate wells were assayed for each condition; *bars,* ±SD.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/14/3899/2505538/3899.pdf by guest on 26 May 2022

To acquire the ability to bind hormone, steroid hormone receptors undergo a series of transformation steps in which they are brought into the correct conformation by molecular chaperones and cochaperones. The most extensively studied chaperones for steroid receptors are a multiprotein Hsp70- and Hsp90-based chaperone system, which includes Hsp90, Hsp70, Hop, Hsp40, and p23 (21–23). Hsp70 and Hsp90 associate with the unliganded steroid hormone receptors, and maintain the conformational state for efficient ligand binding and receptor activation (21, 23). Interestingly, the chaperone-like activity has been suggested for synucleins based on the cell-free system (24). However, the molecular targets for synuclein-mediated chaperone activity remain to be identified. It is likely that SNCG is a new member of molecular chaperone proteins that participate in Hsp-based chaperone complex for regulating ER-α activity. Studies are under way to investigate the mechanism by which SNCG regulates ER-α signaling.

Like SNCA of which the mutations have been detected in several cases of familial PD (1), mutations of SNCG could be linked to the development of breast carcinomas. However, after analysis of a number of breast tumors and breast cancer cell lines, it was found that the malignant phenotype correlated with the high level expression of wild-type SNCG protein (8, 11, 15). Moreover, in addition to the absence of mutation, SNCG gene amplification was also not detected in breast tumors (15). To elucidate the molecular mechanisms underlying the abnormal transcription of SNCG in breast cancer cells, we isolated a 2195-bp promoter fragment of human *SNCG* gene and demonstrated that demethylation of exon 1 region of *SNCG* gene is an important factor responsible for the aberrant expression of *SNCG* in breast carcinomas (12). However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. Here we demonstrated ER-α as one of the critical target molecules for the action of SNCG in breast cancer pathogenesis. Thus, aberrant expression of SNCG stimulates breast cancer growth and progression, at least in part, by enhancing the transcriptional activity of ER-α. The role of SNCG in breast cancer progression may also be involved in non-ER-mediated functions such as stimulation of tumor motility and metastasis as we described previously in hormone-independent breast cancer cells (9).

The preventive effect of estrogen on AD has become clear with epidemiological data, suggesting that estrogen may act as a neuroprotectant against the neurodegenerative diseases (25–28). The cellular functions of synucleins remain elusive. The demonstration of ER-α as the critical target for SNCG may indicate a new direction of normal cellular function of synucleins. In this regard, SNCG-mediated stimulation of ER-α signaling not only supports its pathological role in the growth of steroid-responsive tumors, but may also shed some light on the cellular functions of synucleins in brain cells and their complex roles in the development of neurodegenerative disorders.

References

1. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science (Wash. DC), *276:* 2045–2047, 1997.
2. Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. α-Synuclein in Lewy bodies. Nature (Lond.), *388:* 839–840, 1997.
3. Takeda, A., Mallory, M., Sundsmo, M., Honer, W., Hansen, L., and Masliah, E. Abnormal accumulation of NACP/alpha-synuclein in neurodegenerative disorders. Am. J. Pathol., *152:* 367–372, 1998.
4. Ueda, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D. A., Kondo, J., Ihara, Y., and Saitoh, T. Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. Proc. Natl. Acad. Sci. USA, *90:* 11282–11286, 1993.
5. Yoshimoto, M., Iwai, A., Kang, D., Otero, D. A., Xia, Y., and Saitoh, T. NACP, the precursor protein of the non-amyloid beta/A4 protein (A beta) component of Alzheimer disease amyloid, binds A beta and stimulates A beta aggregation. Proc. Natl. Acad. Sci. USA, *92:* 9141–9145, 1995.
6. Duda, J. E., Lee, V. M., and Trojanowski, J. Q. Neuropathology of synuclein aggregates. J. Neurosci. Res., *61:* 121–127, 2000.
7. Galvin, J. E., Uryu, K., Lee, V. M., and Trojanowski, J. Q. Axon pathology in Parkinson's disease and Lewy body dementia hippocampus contains alpha-, beta-, and gamma-synuclein. Proc. Natl. Acad. Sci. USA, *96:* 13450–13455, 1999.
8. Ji, H., Liu, Y. E., Jia, T., Wang, M., Liu, J., Xiao, G., Joseph, B. K., Rosen, C., and Shi, Y. E. Identification of a breast cancer-specific gene. BCSG1, by direct differential cDNA sequencing. Cancer Res., *57:* 759–764, 1997.
9. Jia, T., Liu, Y. E., Liu, J., and Shi, Y. E. Stimulation of breast cancer invasion and metastasis by synuclein gamma. Cancer Res., *59:* 742–747, 1999.
10. Liu, J., Spence, M. J., Zhang, Y. L., Jiang, Y., Liu, Y. E., and Shi, Y. E. Transcriptional suppression of synuclein gamma (SNCG) expression in human breast cancer cells by the growth inhibitory cytokine oncostatin M. Breast Cancer Res. Treat., *62:* 99–107, 2000.
11. Bruening, W., Giasson, B. I., Klein-Szanto, A. J., Lee, V. M., Trojanowski, J. Q., and Godwin, A. K. Synucleins are expressed in the majority of breast and ovarian carcinomas and in preneoplastic lesions of the ovary. Cancer (Phila.), *88:* 2154–2163, 2000.
12. Lu, A., Gupta, A., Li, C., Ahlborn, T. E., Ma, Y., Shi, E. Y., and Liu, J. Molecular mechanisms for aberrant expression of the human breast cancer specific gene 1 in breast cancer cells: control of transcription by DNA methylation and intronic sequences. Oncogene, *20:* 5173–5185, 2001.
13. Lavedan, C., Leroy, E., Dehejia, A., Buchholtz, S., Dutra, A., Nussbaum, R. L., and Polymeropoulos, M. H. Identification, localization and characterization of the human gamma- synuclein gene. Hum. Genet., *103:* 106–112, 1998.
14. Xiao, G., Liu, Y. E., Gentz, R., Sang, Q. A., Ni, J., Goldberg, I. D., and Shi, Y. E. Suppression of breast cancer growth and metastasis by a serpin myoepithelium-derived serine proteinase inhibitor expressed in the mammary myoepithelial cells. Proc. Natl. Acad. Sci. USA, *96:* 3700–3705, 1999.
15. Ninkina N. N., Alimova-Kost M. V., Paterson J. W., Delaney L., Cohen B. B., Imreh S., Gnuchev N. V., Davies A. M., and Buchman V. L. Organization, expression and polymorphism of the human persyn gene. Hum. Mol. Genet., *7:* 1417–1424, 1998.
16. Clayton, D. F., and George, J. M. The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. Trends Neurosci., *21:* 249–254, 1998.
17. Pan, Z. Z., Bruening, W., Giasson, B. I., Lee, V. M., and Godwin, A. K. gamma-Synuclein promotes cancer cell survival and inhibits stress- and chemotherapeutic drug-induced apoptosis by modulating MAPK pathways. J. Biol. Chem., *277:* 35050–35060, 2002.
18. Li, C., Briggs, M. R., Ahlborn, T. E., Kraemer, F. B., and Liu, J. Requirement of Sp1 and estrogen receptor alpha interaction in 17beta- estradiol-mediated transcriptional activation of the low density lipoprotein receptor gene expression. Endocrinology, *142:* 1546–1553, 2001.
19. Knowlden, J. M., Gee, J. M., Bryant, S., McClelland, R. A., Manning, D. L., Mansel, R., Ellis, I. O., Blamey, R. W., Robertson, J. F., and Nicholson, R. I. Use of reverse transcription-polymerase chain reaction methodology to detect estrogen-regulated gene expression in small breast cancer specimens. Clin. Cancer Res., *3:* 2165–2172, 1997.
20. Ferguson, A. T., Lapidus, R. G., Baylin, S. B., and Davidson, N. E. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res., *55:* 2279–2283, 1995.
21. Perlmann, T., and Evans, R. M. Nuclear receptors in Sicily: all in the famiglia. Cell, *90:* 391–397, 1997.
22. Katzenellenbogen, B. S. Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biol. Reprod., *54:* 287–293, 1996.
23. Pratt, W. B., and Toft, D. O. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr. Rev., *18:* 306–360, 1997.
24. Souza, J. M., Giasson, B. I., Lee, V. M., and Ischiropoulos, H. Chaperone-like activity of synucleins. FEBS Lett., *474:* 116–119, 2000.
25. Dluzen, D. E., and McDermott, J. L. Gender differences in neurotoxicity of the nigrostriatal dopaminergic system: implications for Parkinson's disease. J. Gend. Specif. Med., *3:* 36–42, 2000.
26. Shepherd, J. E. Effects of estrogen on congnition mood, and degenerative brain diseases. J. Am. Pharm. Assoc. (Wash), *41:* 221–228, 2001.
27. Tang, M. X., Jacobs, D., Stern, Y., Marder, K., Schofield, P., Gurland, B., Andrews, H., and Mayeux, R. Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. Lancet, *348:* 429–432, 1996.
28. Chae, H. S., Bach, J. H., Lee, M. W., Kim, H. S., Kim, Y. S., Kim, K. Y., Choo, K. Y., Choi, S. H., Park, C. H., Lee, S. H., Suh, Y. H., Kim, S. S., and Lee, W. B. Estrogen attenuates cell death induced by carboxy-terminal fragment of amyloid precursor protein in PC12 through a receptor-dependent pathway. J. Neurosci. Res., *65:* 403–407, 2001.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/14/3899/2505538/3899.pdf by guest on 26 May 2022