

γ Synuclein, a Novel Heat-Shock Protein-Associated Chaperone, Stimulates Ligand-Dependent Estrogen Receptor α Signaling and Mammary Tumorigenesis

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ABSTRACT

Synucleins are emerging as central players in the formation of pathologically insoluble deposits characteristic of neurodegenerative diseases. γ synuclein (SNCG), previously identified as a breast cancer-specific gene (BCSG1), is also highly associated with breast or ovarian cancer progression. However, the molecular targets of SNCG aberrant expression in breast cancer have not been identified. Here, we demonstrated a chaperone activity of SNCG in the heat-shock protein (Hsp)-based multiprotein chaperone complex for stimulation of estrogen receptor (ER)- α signaling. As an ER- α -associated chaperone, SNCG participated in Hsp-ER- α complex, enhanced the high-affinity ligand-binding capacity of ER- α , and stimulated ligand-dependent activation of ER- α . The SNCG-mediated stimulation of ER- α transcriptional activity is consistent with its stimulation of mammary tumorigenesis in response to estrogen. These data indicate that SNCG is a new chaperone protein in the Hsp-based multiprotein chaperone complex for stimulation of ligand-dependent ER- α signaling and thus stimulates hormone-responsive mammary tumorigenesis.

INTRODUCTION

Using the differential cDNA sequencing (1–3), we undertook a search for differentially expressed genes in the cDNA libraries from normal breast and breast carcinoma. Of many putative differentially expressed genes, a breast cancer-specific gene, BCSG1, was identified as a putative breast cancer marker (1). This gene was highly expressed in the advanced breast cancer cDNA library but scarce in a normal breast cDNA library. BCSG1 is not homologous to any other known growth factors or oncogenes. Rather, there is extensive sequence homology to the neural protein synuclein. Subsequent to the isolation of BCSG1, γ synuclein (SNCG; Ref. 4) and *persyn* (5) were independently cloned from a brain genomic library and a brain cDNA library. The sequences of these two brain proteins were found to be identical to BCSG1. Thus, the previously identified BCSG1 has also been named as SNCG and is considered to be the third member of the synuclein family (6).

Synucleins are a family of small proteins consisting of 3 known members, α synuclein (SNCA), β synuclein (SNCB), and γ synuclein (SNCG). Synucleins have been specifically implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Mutations in SNCA is genetically linked to several independent familial cases of PD (7). More importantly, wild type of SNCA is the major component of Lewy bodies in sporadic PD and in a subtype of AD known as Lewy body variant AD (8, 9). SNCA peptide known as nonamyloid component of plaques has been implicated in amyloidogenesis in AD (10, 11). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy

bodies cases (12, 13). Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, they have also been implicated in nonneural diseases, particularly in the hormone-responsive cancers of breast and ovary (1, 4, 14–22).

Being identified as a breast cancer-specific gene, SNCG expression in breast follows a stage-specific manner (1). Overall SNCG mRNA expression was detectable in 39% of breast cancers. However, 79% of stage III/IV breast cancers were positive for SNCG expression, whereas only 15% of stage I/II breast cancers were positive for SNCG expression. In contrast, the expression of SNCG was undetectable in all benign breast lesions (17). The expression of SNCG was strongly correlated with the stage of breast cancer. Overexpression of SNCG in breast cancer cells led to a significant increase in cell motility and invasiveness *in vitro* and a profound augmentation of metastasis *in vivo* (14). Overexpression of synucleins, especially SNCG and SNCB, also correlated with ovarian cancer development (4, 19). Although synucleins' (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 synucleins (α , β , and γ) simultaneously (19). The involvement of SNCG in hormone-responsive cancers of breast and ovary promoted us to explore the potential role of SNCG in cellular response to estrogen. Previously, we investigated the functions of SNCG in regulating estrogen receptor transcriptional activity and demonstrated that SNCG strongly stimulated the ligand-dependent transcriptional activity of estrogen receptor (ER)- α in breast cancer cells. Augmentation of SNCG expression stimulated the transcriptional activity of ER- α , whereas compromising endogenous SNCG expression suppressed ER- α signaling (16). In the present study, we evaluated the mechanism by which SNCG stimulated ER- α transcriptional activity and its biological relevance to estrogen-stimulated mammary tumorigenesis. The results indicated that SNCG stimulates ER- α signaling by acting as a chaperone in the heat-shock protein (Hsp)-based multiprotein chaperone complex of ER- α and enhances its high-affinity ligand binding.

MATERIALS AND METHODS

Reagents. Improved MEM, FCS, and charcoal-stripped FCS were obtained from Biosource International (Camarillo, CA). 17- β -Estradiol (E_2) and geldanamycin (GA) were purchased from Sigma Chemical Co. (St. Louis, MO). [3 H]Estradiol was from Perkin-Elmer Life Science, Inc. (Boston, MA). The mammalian expression plasmid pCI-neo was from Promega (Madison, WI).

Cell Culture. All cell lines used in this study (MCF-7, T47-D) were originally obtained from the American Type Culture Collection. Proliferating subconfluent human breast cancer cells were harvested and cultured in the phenol red-free improved MEM containing 5% charcoal-stripped FCS for 4 days before addition of indicated dose of E_2 . Cells in the absence or presence of E_2 were collected for ligand-binding assay or immunoprecipitation/Western blot analyses.

Gene Transfection. Subconfluent cells in 12-well plate were incubated with 2 μ g of expression vectors in 1 ml of serum-free improved MEM containing LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) 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. For stable transfection, transfected cells were routinely

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selected with G418 (600 μ g/ml). Individual colonies were picked to establish stable clones.

Immunoprecipitation. Cells were cultured in 100-mm cell culture dishes in ligand-free medium for 4 days as described in "Cell Culture." Cells were treated with or without E₂ for 3 days before the total cell lysates were prepared. Cells were lysed in solution containing 1 \times PBS, 1% Triton X-100, 10 mM sodium molybdate, 2 mg/ml aprotinin, 0.5 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cells then were disrupted by sonication and centrifuged for 10 min at 10,000 \times g. The protein concentrations of the supernatant were determined by BCA Protein Assay kit (Pierce). Cell lysates (1 mg of total cellular protein) were incubated with 2 μ g of indicated antibody at room temperature for 1.5 h followed by the addition of protein G-Sepharose. The beads were washed four times with the lysis buffer described above, and the bound proteins were eluted with 1 \times SDS gel-loading buffer followed by Western blotting.

Western Blot Analysis. Proteins were fractionated by electrophoresis through a SDS polyacrylamide gel, and the proteins were then transferred onto a nitrocellulose membrane. The membrane was blocked for 2 h in blocking buffer and then incubated with primary antibodies at room temperature for 2 h. After washing with Tris-buffered saline/0.2% Tween 20 buffer, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase, and the protein was detected using chemiluminescence method followed by autoradiography. Antibodies used for immunoprecipitations and Western blot analyses were as follows: anti- γ -synuclein antibody (goat polyclonal antibody E-20, 1:300 dilution); anti-ER- α antibody (rabbit polyclonal antibody HC-20, 1:300 dilution); anti-Hsp-70 antibody (goat polyclonal antibody sc-1060; 1:1000 dilution); anti-heat shock cognate 70 antibody (goat polyclonal antibody, 1:1000 dilution); anti-Hsp-90 antibody (rabbit polyclonal antibody sc-7947; 1:1000 dilution); normal goat IgG (sc-2028); normal rabbit IgG (sc-2027); and anti-actin antibody (goat polyclonal antibody sc-1615). These antibodies were from Santa Cruz Biotechnology. Anti-phospho-estrogen receptor α (Ser¹⁶⁷; 1:500 dilution), anti-phospho-Akt (Ser⁴⁷³; 1:500 dilution) and anti-Akt (1:1000 dilution) are from Cell Signaling Technology (Beverly, MA).

Ligand-Binding Assay. MCF-7 cells were plated into 24-well plate at 50,000 cells/well and cultured in estrogen-free medium for 3 days before the binding assay. E₂ binding by ER was assayed by measuring the bound [³H]estradiol following a 1.5-h incubation with different concentrations of [³H]estradiol as follows: 0, 0.0025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 nM. Cells were then washed three times with 0.5 ml of PBS and dissolved with 0.1 ml of 0.1 N NaOH. The cell lysate was transferred into a 1.5-ml Eppendorf tube and neutralized with 0.1 ml of 0.1 M NaAc. Aliquots of 150 μ l were counted in a liquid scintillation counter. Counts from samples, which were incubated with a 100-fold excess of unlabeled E₂ (nonspecific binding), were subtracted from the total counts to give the values for specific ligand binding. Triplicate wells were assayed for each condition. Each value was normalized against the protein concentration.

Assays for the Transcriptional Activity of ER- β . Cells were transiently transfected with a firefly luciferase reporter construct (pERE4-Luc) containing four copies of the estrogen response element (ERE). For the cotransfection experiments, the plasmid DNA ratio of pERE4Luc to expression vectors of ER- β 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.

Tumor Growth in Athymic Nude Mice. A nude mouse tumorigenesis assay was performed as we described previously (14). Briefly, estrogen pellets (0.72 mg/pellet; Innovative Research of America, Toledo, OH) were implanted s.c. in all athymic nude mice (experiment 1) and in some ovariectomized athymic nude mice (experiment 2; Frederick Cancer Research and Development Center, Frederick, MD). Approximately 5 \times 10⁶ cells (experiment 1) or 1.5 \times 10⁶ cells (experiment 2) were injected into a 6-week old female athymic nude mouse. Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined at weekly intervals by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor cell clone at each time point.

RESULTS

SNCG Participated in the Hsp-Based Multiprotein Chaperone Complex for ER- α .

It is well documented that the activation of steroid receptors is modulated by a heterocomplex with several molecular chaperones, particularly with Hsp90 and Hsp70, which associate with the unliganded steroid receptors and maintain them in a high-affinity hormone-binding conformation (23–26). The chaperone-like activity of synucleins has been demonstrated in the cell-free system by monitoring the aggregation of thermally denatured proteins (27). Because SNCG stimulated ligand-dependent transcriptional activity of ER- α , which can be blocked by antiestrogen (16), we reason that SNCG may have a chaperone activity and participate in Hsp-based multiprotein chaperone complex for ER- α . In this regard, we investigated if SNCG can physically and functionally interact with ER- α , Hsp70, and Hsp90 in SNCG-transfected MCF-7 cells by co-immunoprecipitation assays. Immunoprecipitation of ER- α coprecipitated SNCG, Hsp70, and Hsp90 in the absence of estrogen (Fig. 1A) and *vice versa* (Fig. 1B), indicating that SNCG participated in a heterocomplex with Hsp90, Hsp70, and ER- α in the absence of estrogen. However, SNCG dissociated from ER- α after cells were treated with E₂ (Fig. 1C). The binding pattern of SNCG to the unliganded ER- α is same to that of Hsp90 and Hsp70, which only bind to the unliganded ER- α (24). Similar to its binding pattern to ER- α , SNCG only bound to Hsp90 in the absence of estrogen (Fig. 1B). After cells were treated with E₂, the liganded ER- α dissociated from SNCG, Hsp70, and Hsp90 (Fig. 1C). However, in contrast to its binding pattern to ER- α and Hsp90, SNCG was found to bind to Hsp70 under the conditions both without (Fig. 1B) and with E₂ (Fig. 1D), indicating that SNCG binds to Hsp70 constitutively regardless of whether Hsp70 is associated with ER- α .

We also investigated the interactions among endogenous SNCG,

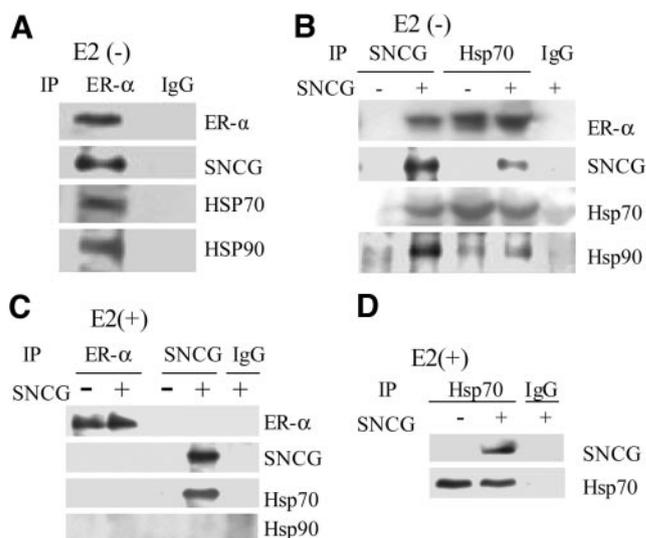


Fig. 1. Interaction between γ synuclein (SNCG) and estrogen receptor (ER)- α , heat-shock protein (Hsp70), and Hsp90 in SNCG-transfected MCF-7 cells. Cells were transiently transfected with pCI-SNCG or the control vector pCI-neo and then selected with G418 as we described previously (16). A and B, association of SNCG with endogenous ER- α , Hsp70, and Hsp90 in the absence of estradiol (E₂). Total cell lysates were isolated from the cells cultured in the E₂-free conditioned medium, and equal amounts of protein were subjected to immunoprecipitation (IP) with different antibodies. IP with anti-ER (A), anti-SNCG (B), anti-Hsp70 antibodies (B), and control rabbit (A) or goat IgG (B) followed by Western blotting for ER- α , SNCG, Hsp70, and Hsp90. C and D, association of SNCG with Hsp70 but not ER- α and Hsp90 in the presence of E₂. Total cell lysates were isolated from the cells cultured in the conditioned medium containing 10 nM E₂, and equal amounts of protein were subjected to IP with different antibodies. C, IP with anti-ER- α , anti-SNCG antibodies, and control goat IgG followed by Western blot for ER- α , SNCG, Hsp70, and Hsp90. D, IP with anti-Hsp70 antibody and control goat IgG followed by Western blotting for SNCG and Hsp70.

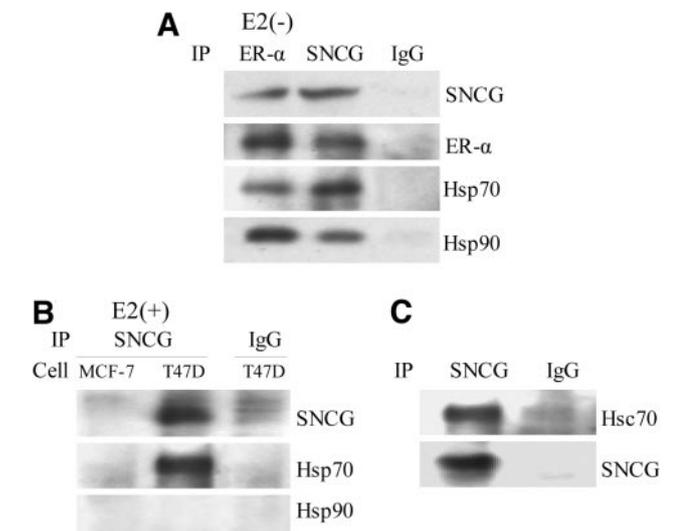


Fig. 2. Interaction between endogenous γ synuclein (SNCG), estrogen receptor (ER)- α , heat-shock protein (Hsp)70, heat-shock cognate (Hsc)70, and Hsp90 in T47D cells. **A**, association of endogenous SNCG with ER- α , Hsp70, and Hsp90 in the absence of estradiol (E_2). Total cell lysates were isolated from the cells cultured in the E_2 -free conditioned medium, and equal amounts of protein were subjected to immunoprecipitation (IP) with anti-ER- α , anti-SNCG antibodies, and normal IgG. The immunoprecipitates were analyzed for Hsp70, SNCG, Hsp90, and ER- α after Western blotting using anti-Hsp70, anti-SNCG, anti-Hsp90, and anti-ER- α antibodies. **B**, association of endogenous SNCG with Hsp70 in the presence of E_2 . Total cell lysates were isolated from the cells cultured in the conditioned medium containing 10 nM E_2 , and equal amounts of protein were subjected to IP with anti-SNCG antibody and normal goat IgG. The nontransfected MCF-7 cell lysates were also subjected to IP as a negative control for SNCG IP. The immunoprecipitates were subjected to Western blotting using antibodies against Hsp70, SNCG. **C**, association of endogenous SNCG with Hsc70. Lysates from T47-D cells were subjected to IP with anti-SNCG antibody and normal goat IgG. The immunoprecipitates were subjected to Western blotting using antibodies against Hsc70, SNCG.

ER- α and Hsp. T47D cells express both SNCG and ER- α and therefore were chosen to study the interaction between SNCG and ER- α and Hsp in the physiological situation. Same interaction pattern between endogenous SNCG and ER- α and Hsp was observed in T47D cells as that we demonstrated in SNCG transfected MCF-7 cells (Fig. 2). When T47D cells were cultured under the estrogen-free conditions, endogenous SNCG was coimmunoprecipitated with ER- α , Hsp70, and Hsp90 (Fig. 2A). However, after the treatment of E_2 , SNCG dissociated from ER- α and Hsp90 but still bound to Hsp70 (Fig. 2B), indicating that the interaction between the endogenous SNCG, ER- α , Hsp70, and Hsp90 proteins also occurs in the physiological situation. In addition, SNCG also bound to heat shock cognate 70, the cognate form of Hsp70 (Fig. 2C).

SNCG Enhances Ligand Binding by ER. It has been proposed that Hsp90-based chaperone complex inactivates the ER's transcriptional regulatory capabilities and maintains the ER in a conformation competent for steroid binding (24). We examined whether the SNCG-stimulated hormone-dependent ER- α transactivation results from increased E_2 binding by the receptor. This was investigated by detecting the hormone-binding activity of ER using a ligand-binding assay in MCF-7 cells. In this experiment, growing cell cultures were treated with [3 H]labeled E_2 over a range of hormone concentrations. As shown in Fig. 3, SNCG significantly enhanced ligand binding by ER. The biggest increases in the ligand binding were observed at the lower concentration range of 0.0025–1 nM ligand. At the concentration of 1 nM E_2 , the ligand binding was increased 160% in the SNCG-positive cells *versus* the SNCG-negative control cells. Although the levels of SNCG-enhanced ligand binding were gradually decreased with the increasing concentrations of ligand, the stimulated ligand binding was still considerably significant. At the concentration of 10 nM E_2 , SNCG overexpression led to a 50% increase in the ligand binding. However,

no difference in ligand binding was observed between MCF-7-neo and MCF-7-SNCG cells at 100 nM E_2 (data not shown). These data suggest that SNCG enhances ER's ability to respond to low levels of ligand.

The binding data are complex because there are two binding states that differed in their affinity for the hormone. As revealed by Scatchard analysis (Fig. 4), the cutoff value for estrogen concentration to differentiate high- from low-affinity binding is 1 nM. The high-affinity state was apparent in a linear plot between 0.0025 and 1 nM E_2 , and the low-affinity state between 1 and 10 nM E_2 . The existence of both high- and low-affinity states of steroid receptors have been observed previously (28). To more closely examine the effect of SNCG on high-affinity ligand binding, data in Fig. 4C shows the high-affinity binding state in MCF-7-neo and MCF-7-SNCG cells. SNCG overexpression significantly enhanced the high-affinity binding capacity. Although the high-affinity binding capacity in MCF-7-neo cells was saturated when the bound E_2 reached 0.06 pmol/mg protein, the high-affinity binding capacity in MCF-7-SNCG cells was saturated when the bound E_2 reached 0.14 pmol/mg protein. These data indicate that SNCG affects ER- α signal transduction pathway at the step of ligand binding by increasing the number of high-affinity ligand-binding sites.

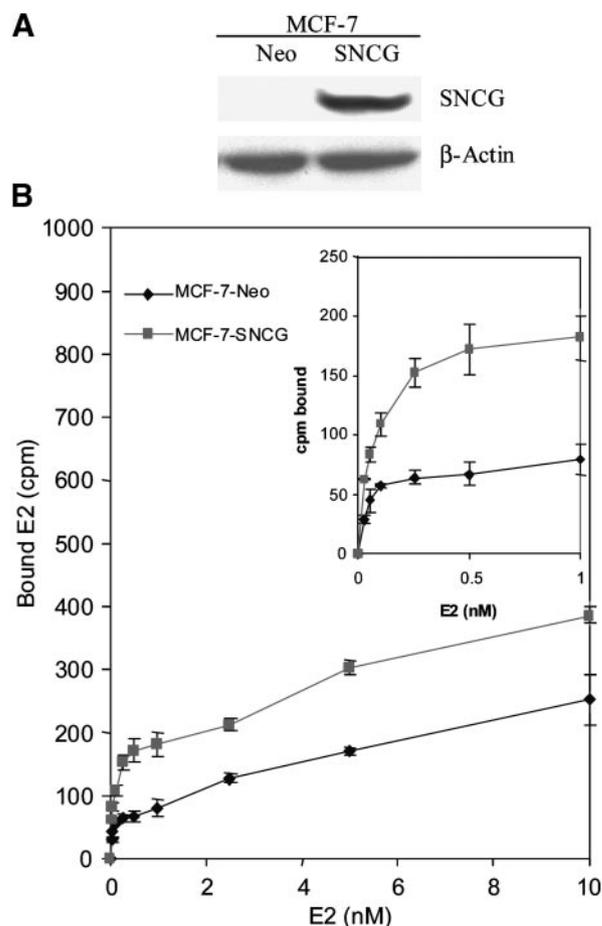


Fig. 3. Estrogen-binding capability for estrogen receptor in γ synuclein (SNCG)-transfected MCF-7 cells (MCF-7-SNCG) and control neo-transfected cells (MCF-7-Neo). Cells were transiently transfected with pCI-SNCG or pCI-neo plasmids. The transfected cells were enriched with Neomycin selection for 12 days before the hormone-binding assay. **A**, Western blot analysis of SNCG expression in pooled SNCG-transfected MCF-7 cells after selection with G418. **B**, titration of [3 H]estradiol (E_2) in MCF-7-Neo and MCF-7-SNCG cells. *Inset*, enlarged view of [3 H] E_2 titration from 0 to 1 nM of ligand.

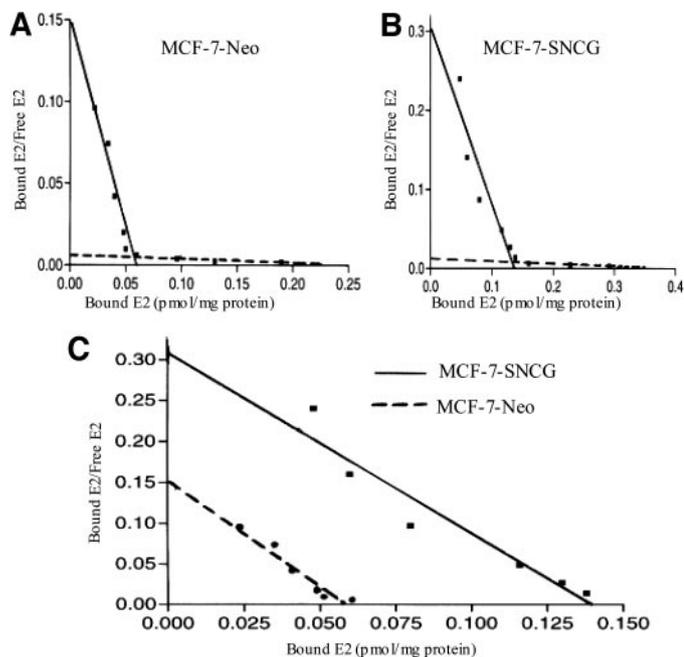


Fig. 4. Scatchard analysis of the ligand binding by estrogen receptor from MCF-7-Neo cells (A) and MCF-7-SNCG cells (B). Solid line, high-affinity sites; dashed line, low-affinity sites. C, the high-affinity sites of MCF-7-Neo and MCF-7-SNCG cells. Specific binding was determined by subtracting the nonspecific binding from samples incubated with 100-fold excess of nonlabeled estradiol (E_2). Each data point is the mean \pm SD of triplicate samples.

Hsp90-Specific Compound GA Abolished the Stimulatory Effect of SNCG on ER- α Activity. To address whether the stimulatory effect of SNCG on ER- α ligand-binding activity is mediated by Hsp chaperone activity, we investigated the effect of the Hsp90 inhibitor GA on the ligand-binding activity of ER- α in MCF-7-neo cells and MCF-7-SNCG cells. GA inhibits the ATPase activity of Hsp90. We first analyzed the effect of GA treatment on ER- α levels. As demonstrated in Fig. 5A, transfection of SNCG gene into the SNCG-negative MCF-7 cells did not affect ER- α expression under the conditions with or without GA treatment. In the absence of GA, the basal levels of ER- α in control and SNCG-transfected cells are the same. Although treatment of the control cells with GA resulted in a decrease in ER- α levels, overexpression of SNCG did not affect GA-mediated degradation of ER- α . We next investigated the effect of GA on the ER- α ligand-binding activity in MCF-7-neo and MCF-7-SNCG cells. In the absence of GA, SNCG increased the ligand-binding capability of ER- α by 91%. Upon the treatment with GA, although considerable amounts of ER- α protein were still present in both MCF-7-neo and MCF-7-SNCG cells, the ligand-binding activity of ER- α in both cells was abolished (Fig. 5B). This data indicates that the stimulatory effect of SNCG on ER- α activity is Hsp dependent.

SNCG Did Not Affect Phosphorylation of ER- α . To additionally determine the molecular mechanisms for SNCG-stimulated ER- α transcriptional activity, we also studied if SNCG regulates ligand-dependent or ligand-independent phosphorylation of ER- α . Although E_2 directly binds to and activates ER, thereby enhancing estrogen-responsive genes transcription, ER- α can also be activated by ligand-independent phosphorylation, which is mediated by cytoplasmic proteins and signaling pathways such as mitogen-activated protein kinase- and Akt-mediated phosphorylation (29). Many growth factors such as epidermal growth factor can also activate ER- α by such cytoplasmic signaling pathways. As expected, treatment of MCF-7 cells with E_2 resulted in decreased levels of ER- α and stimulated Ser¹⁶⁷ phosphorylation of ER- α . However, transfection of SNCG to

MCF-7 cells didn't enhance Ser¹⁶⁷ phosphorylation of ER- α in response to E_2 compared with mock-transfected cells (Fig. 6A). There was also no significant difference in Ser¹⁰⁴ or Ser¹¹⁸ phosphorylation of ER- α (data not shown). In addition, SNCG overexpression in MCF-7 cells didn't enhance the phosphorylation of Akt in response to both E_2 and epidermal growth factor (Fig. 6B).

Effect of SNCG on Transcriptional Activity of ER- β . In an effort to address whether SNCG has general effect on other steroid hormone receptors relevant in breast cancer, we investigated the effect of SNCG on the transcriptional activity of ER- β . Because both ER- α and ER- β bind to the same EREs, we cotransfected ER- α -negative MDA-MB-435 cells with ER- β and ER reporter ERE4-luciferase (Fig. 7). In contrast to the previously demonstrated stimulation of ER- α signaling (16), SNCG did not stimulate the transactivation of ER- β . When cells were treated with E_2 , a 15.7- and 13.5-fold increase relative to basal levels in ER- β reporter activity was observed in SNCG-negative and SNCG-positive cells, respectively. Therefore, expression of SNCG in breast cancer cells stimulates the transcriptional activity of ER- α but not ER- β , indicating that SNCG may not have a general effect on stimulation of transcriptional activity of steroid receptors.

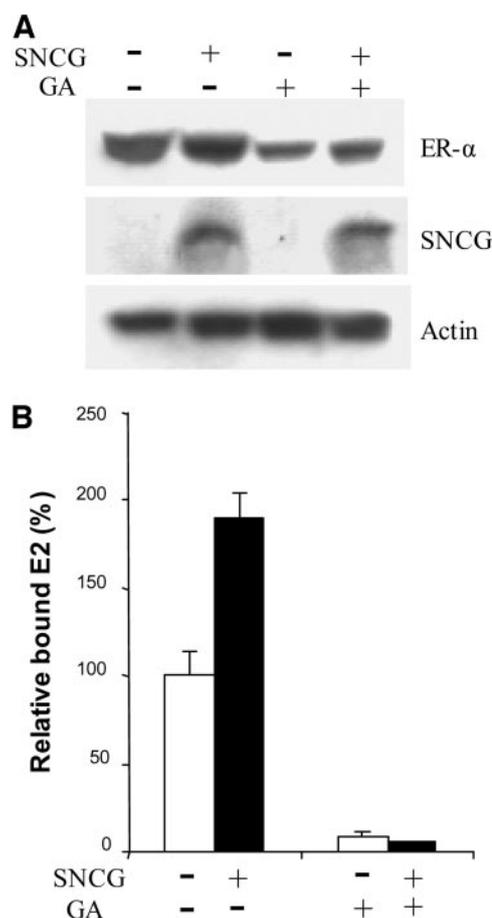


Fig. 5. Effect of heat-shock protein (Hsp90) inhibitor geldanamycin (GA) on estrogen-binding capability of estrogen receptor (ER) in MCF-7 cells. A, Western blot analysis of ER and γ synuclein (SNCG) levels in pooled MCF-7-Neo and MCF-7-SNCG cells treated with or without GA. The pooled MCF-7-Neo and MCF-7-SNCG cells were cultured in the presence of 0.1 nM estradiol (E_2) and treated with or without 0.25 μ M GA for 24 h. Cell lysates were normalized and subjected to Western blot analysis using the antibody against ER- α , SNCG, and actin, respectively. B, GA abolished the stimulatory effect of SNCG on estrogen-binding capability in MCF-7 cells. MCF-7-Neo and MCF-7-SNCG cells were treated with or without 0.25 μ M GA for 24 h followed by the ligand binding assay with 0.1 nM [³H] E_2 . The data were presented as the percentage of the nontreated MCF-7-Neo controls, which was taken as 100%. Each data represent the mean \pm SD of triplicate cultures.

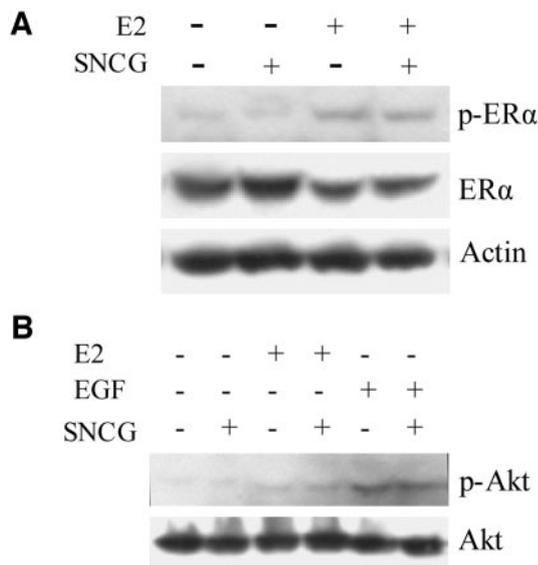


Fig. 6. Effect of γ synuclein (SNCG) overexpression on ER- α and AKT phosphorylation. **A**, MCF-7 cells were transiently transfected with pCI-SNCG or pCI-neo plasmids. After G418 selection for 12 days, the pooled population of transfected cells were treated with or without 10^{-9} M estradiol (E_2) or 50 ng/ml epidermal growth factor (EGF). Cell lysates were subjected to Western blot analysis using the antibodies against human Ser¹⁶⁷-phosphorylated ER- α , ER- α , and actin, respectively. SNCG overexpression didn't affect E_2 and EGF-induced ER- α phosphorylation. **B**, the pooled population of SNCG-transfected cells and mock-transfected cells were treated with or without 10^{-9} M E_2 or 50 ng/ml EGF for 15 min. Cell lysates were subjected to Western blot analysis using the antibodies against human AKT and Ser⁴⁷³-phosphorylated AKT, respectively. SNCG overexpression didn't affect E_2 and EGF-induced AKT phosphorylation.

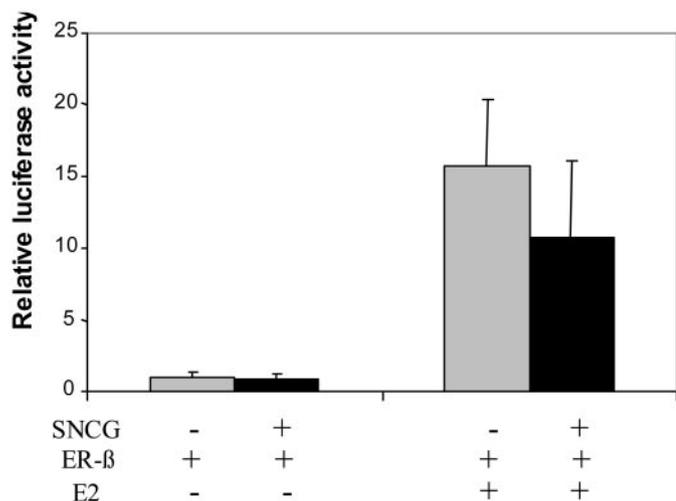


Fig. 7. Effects on transcriptional activity of ER- β . MCF-7 cells were transiently cotransfected with pSG5-hER β and pCI-SNCG or the control vector pCI-neo. After selection with G418, the transfected cells were transfected with pERE4-Luc, as well as control reporter pRL-SV40-Luc, cultured in the ligand-free medium for 4 days, treated with or without 1 nM estradiol (E_2) for 24 h before harvesting. The promoter activities were determined by measuring the dual luciferase activity. 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 \pm SD of three cultures.

Stimulation of Estrogen-Dependent Mammary Tumorigenesis by SNCG. An orthotopic nude mouse model was used to study the effects of SNCG on tumor growth. Two independent experiments under different conditions were done to determine the effects of SNCG on mammary tumorigenesis. We first analyzed the tumorigenesis in response to E_2 in nonovariectomized mice. It was previously demonstrated that the circulating E_2 level in the nonovariectomized mice is about 26 pg/ml (30), which compares to the low levels found in postmenopausal women (31). To override the endogenous levels of

estrogen, all of the mice were supplemented with E_2 (0.72 mg/pellet) 1 day before injection of 5×10^6 cells. After a lag phase of 8–10 days, 29 of 32 (91%) injections in the mice given implants of SNCG-positive MCF-SNCG2 and MCF-SNCG6 cells developed tumors. In contrast, only 21 of 32 (66%) injections in the mice given implants of SNCG-negative MCF-neo1 and MCF-neo2 cells developed tumors (Table 1). The tumor growths in MCF-SNCG clones were significantly stimulated. At 35 days after tumor cell injection, the size of MCF-SNCG6 tumors, which expressed a relative high level of SNCG mRNA, was 4.8-fold of that in parental MCF-neo2 tumors and 3.7-fold of that in MCF-neo1 tumors. In addition, the tumor incidence was also increased. With 16 injections, whereas 15 implants in MCF-SNCG6 cells developed tumors, only 10 implants from MCF-neo1 and 11 implants from MCF-neo2 developed tumors, respectively. The tumor growth of MCF-SNCG2 cells was also significantly stimulated, with 3.5- and 2.7-fold increase in tumor size as compared with MCF-neo2 and MCF-neo1 tumors, respectively. Fig. 8 shows growth kinetics. After a slow growth phase of 14 days, tumor growth of MCF-SNCG2 and MCF-SNCG6 clones were significantly enhanced as compared with that of MCF-neo1 and MCF-neo2 clones. Thus, the tumorigenesis of MCF-7 cells in response to E_2 was significantly stimulated by SNCG overexpression.

We then analyzed the requirement of E_2 for SNCG-mediated tumor

Table 1 Effects of SNCG expression on tumor incidence and tumor growth of MCF-7 cells^{a,b}

Experiment group	E_2	Tumor incidence		Tumor volume (mm ³)	
		Tumor/Total(%)	Day 21	Day 35	
MCF-SNCG2	+	14/16 (88)	234 \pm 69	1850 \pm 280	
MCF-SNCG6	+	15/16 (94)	351 \pm 78	2521 \pm 390	
MCF-neo1	+	10/16 (63)	101 \pm 39	491 \pm 92	
MCF-neo2	+	11/16 (69)	123 \pm 25	625 \pm 130	

^a Cells were injected into the mammary fat pads, and tumor volumes and tumor incidence were determined as described in "Materials and Methods." Each mouse received two injections. Tumor volumes were measured at 21 and 35 days after cell injection and are expressed as means \pm SEs (number of tumors assayed). All the nonovariectomized mice received an estrogen implantation 1 day before the cell injection. There were total 16 injections for 8 mice in each group, and each injection had 5×10^6 cells. Statistical comparisons for SNCG-positive clones relative to SNCG-negative clones indicated $P < 0.01$ for the mean tumor sizes and $P < 0.05$ for the tumor incidence. Statistical comparison for primary tumors was analyzed by Student's t test. A χ^2 test was used for statistical analysis of tumor incidence.

^b SNCG, γ synuclein; E_2 , estradiol.

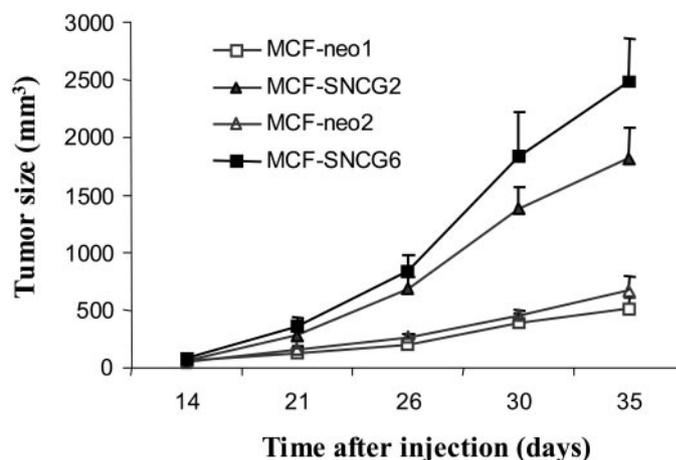


Fig. 8. Stimulation of MCF-7 tumor growth by γ synuclein (SNCG). Each of the eight estradiol-supplemented nonovariectomized mice in each group received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each clone at each time point. Each point represents the mean of tumors \pm SE (bars).

Table 2 Stimulation of estrogen-mediated tumorigenesis by SNCG^{a,b}

Group	E ₂	Tumor incidence (%)							Tumor volume (mm ³)
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 7
MCF-neo1	-	0	0	0	0	0	0	0	44 ± 10
MCF-neo1	+	0	0	0	0	20	30	30	
MCF-SNCG6	-	0	0	0	0	0	0	0	194 ± 35
MCF-SNCG6	+	40	80	90	90	90	90	90	

^a Ovariectomized mice were treated with or without E₂ pellet. There were total 10 injections for 5 mice in each group, and each injection had 1.5×10^6 cells. Only measurable tumors were used to calculate the mean tumor volume. Statistical comparisons for SNCG-positive clones relative to SNCG-negative clones indicated $P < 0.001$ for both tumor incidence and mean tumor sizes in the presence of E₂.

^b SNCG, γ synuclein; E₂, estradiol.

stimulation under more stringent conditions, including use of ovariectomized mice and reduction of injected tumor cells from 5×10^6 to 1.5×10^6 . As shown in Table 2, when the number of injected MCF-neo1 cells was reduced, the tumor incidence was greatly reduced from 63% to 0%, both in the absence and presence of E₂ supplement for up to 4 weeks, indicating a nontumorigenic condition for SNCG-negative MCF-7 cells even with estrogen stimulation for 4 weeks. At week 7, a 30% of tumor incidence with small tumor size (44 mm³) was observed. However, for MCF-SNCG6 cells, although the same nontumorigenic phenotype was observed under the conditions with reduced cell number and in the absence of E₂ when E₂ was supplemented, the tumor incidence reached 90% 3 weeks after cell inoculation, which is similar to 94% of tumor incidence in the experiment with higher injected cell numbers (Table 1). Furthermore, the tumor size of SNCG-positive cells is 4.4-fold of control. These data indicate that estrogen is necessary for SNCG-mediated tumor stimulation in this xenograft model.

DISCUSSION

Synucleins are small proteins expressed predominately in neurons and have been specifically implicated in neurodegenerative disorders such as AD and PD. However, 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 (1). Aberrant expression of SNCG was also associated with ovary cancer progression (4, 19). The association between SNCG expression and the progression of steroid-dependent cancers of breast and ovary suggest a potential role of SNCG in regulation of ER- α . Previously, we demonstrated a SNCG-stimulated ER- α signaling in three different cell systems, including (a) overexpression of SNCG in ER- α -positive and SNCG-negative MCF-7 cells, (b) antisense blockage of 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 (16). The results shown in this study demonstrated that SNCG stimulated mammary tumorigenesis in response to estrogen, which is mediated by its participation in Hsp-based chaperone complex for regulation of ER- α transcriptional activity.

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, p23, and others (23, 24). Hsp70 and Hsp90 associate with the unliganded steroid hormone receptors and maintain the conformational state for efficient ligand binding and receptor activation. Consistent with the previous report on chaperone-like activity of synucleins (27), here, we provided evidences suggesting that SNCG is a new member of molecular chaperone proteins that participates in Hsp-based chaperone complex for regulating ER- α

activity. These evidences include that (a) SNCG bound to the unliganded form of ER- α , Hsp90, and Hsp70, (b) SNCG enhanced the high-affinity ligand-binding state of ER, and (c) SNCG significantly stimulated the transcriptional activity of ER- α and ligand-dependent mammary tumorigenesis. The binding of SNCG to ER- α and Hsp90 only occurs in the absence of ligand, which is same to the binding of Hsp90 and Hsp70 to the unliganded ER- α . However, the binding between SNCG and Hsp70 was observed under the conditions both with and without the ligand, suggesting that SNCG is an Hsp70-binding protein.

It has been previously demonstrated in the cell-free system that Hsp70-free reticulocyte lysate is inactive at glucocorticoid receptor heterocomplex formation with Hsp90 and that the activity is restored by readdition of purified Hsp70 (32, 33), indicating that the binding of Hsp70 to the unliganded steroid receptor is necessary for the efficient Hsp90 binding for maintaining the high transcriptional activity of steroid receptors. Hsp90 is absolutely essential for hormone binding to glucocorticoid receptor under all conditions (34). Recent studies from *in vivo* yeast system and *in vitro* mammalian cell-free system also indicate that ER requires the molecular chaperone Hsp90 for efficient hormone binding (35). There were two pools of ER, one with high hormone affinity and one with low affinity (Fig. 4). Although the nature of the low-affinity state is unclear, its existence in cells may reflect an equilibrium between the receptors with high hormone affinity and poised for activation and those with low affinity. The low-affinity hormone-binding state may reflect a kinetically trapped folding intermediate that requires the action of SNCG, Hsp90, and other proteins for the conversion into the high-affinity state. A working model for the role of SNCG on ER- α transcriptional activation is presented in Fig. 9. In this model, SNCG binds to Hsp70 and forms a complex including Hsp90, Hsp70, SNCG, ER- α , and others. This

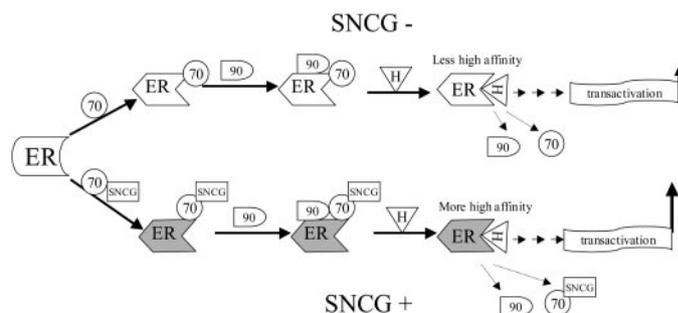


Fig. 9. A model for γ synuclein (SNCG)-regulated estrogen receptor (ER)- α activation. In the model, 90 refers to heat-shock protein (Hsp)90, 70 refers to Hsp70, H refers to hormone. According to the model, SNCG and Hsp70 preassociate to form SNCG-Hsp70 chaperone complex. This complex binds to the unstable hormone-binding domain of the native receptor. The recruitment of Hsp90 to the ER- α would occur once the receptor was bound with the SNCG-Hsp70 complex. At this stage, the receptor undergoes structure changes and assumes a conformation with high affinity for estradiol. Upon the binding to estradiol, the receptor disassociates with SNCG-Hsp70 complex and Hsp90, which leads to receptor dimerization, interaction with coactivators, and transactivation.

chaperone complex pushes the equilibrium toward the high-affinity hormone-binding conformation. Although the nature of the conformational changes in ER- α is unknown, SNCG overexpression would be expected to result in a greater number of mature ER complexes with high-affinity ligand-binding capability. As a result, the physiological levels of E₂ binding will increase, which is consistent with the E₂-binding assays (Figs. 3 and 4). Thus, overexpression of SNCG, by increasing the number of mature receptor complexes with high-affinity ligand-binding capability, will manifest itself as an increase in transcriptional activation by ER at a given hormone concentration.

One of the critical questions need to be addressed is whether SNCG-stimulated ER- α transactivation is mediated by a Hsp-based chaperone complex, particularly Hsp70. Using Hsp90 inhibitor GA, our data demonstrated that treatment of cells with GA completely abolished SNCG-stimulated receptor ligand binding, indicating that the SNCG-mediated stimulation of ER- α is Hsp dependent. However, this study needs to be additionally confirmed with more specific approaches such as small interfering RNAs to knock out Hsp70 or Hsp90. We realize that Hsp70 and Hsp90 play a critical role in maintaining ER- α in a right conformation for ligand binding. Without chaperone activity of Hsp70 and Hsp90, the transcriptional activity of ER- α might not be efficiently activated. In this regard, inhibition of endogenous levels of Hsp70 or Hsp90 by small interfering RNA may greatly affect ER- α transactivation, and thus, the SNCG-mediated stimulation of ER- α transactivation may be jeopardized. SNCG may also regulate ER- α signaling by Hsp-independent pathways such as direct binding to and chaperoning ER- α . However, our *in vitro* translation study indicates that SNCG did not physically interact with ER- α directly (data not shown). Nevertheless, our model suggests that SNCG-stimulated transcriptional activity of ER- α is mediated, at least in part, by participating in Hsp-based multiprotein chaperone complex and maintaining the ER- α in a high-affinity hormone-binding conformation. Thus, SNCG and Hsps act cooperatively in ER- α signaling.

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 in response to estrogen. First, although expression of SNCG in MCF-7 cells had no effect on the cell growth in the absence of E₂, SNCG significantly stimulated the ligand-dependent cell growth, which can be blocked by antiestrogens (16). This growth stimulation was also previously demonstrated in the anchorage-independent growth assay (15). Second, when endogenous SNCG expression in T47D cells was blocked by expressing SNCG antisense mRNA, the anchorage-independent growth in response to E₂ was significantly suppressed in the cells expressing antisense SNCG (16). Third, although the alteration 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 (14). Finally, SNCG overexpression significantly stimulated the tumorigenesis of MCF-7 cells in response to estrogen, whereas it has no effect on tumor growth in the absence of estrogen. Consistent with the requirement of E₂ for SNCG-stimulated tumor growth, it was demonstrated that SNCG had no significant effect on tumor growth of ER- α -negative MDA-MB-435 cells (14).

We isolated a 2195-bp promoter fragment of a 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 (21, 22). However, the molecular targets of SNCG aberrant expression in breast cancer have not been identified. Our findings suggest that SNCG functions as a chaperone and participates in Hsp-based multiprotein chaperone system for efficient activation of ER- α . 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 involve other non-ER-mediated functions such as stimulation of tumor motility and metastasis as we previously described in hormone-independent breast cancer cells (14).

The cellular functions of synucleins remain elusive. Although the chaperone-like activity has been suggested for synucleins based on the cell-free system (27), the molecular targets for chaperone activity remain to be identified. Recently, the protective effect of molecular chaperone Hsp70 on SNCA-induced dopaminergic neuronal loss in *Drosophila* has been reported (36), indicating that chaperone activity of Hsp70 helps to protect neurons against the neurotoxic consequences of SNCA expression. Interestingly, although filamentous SNCA is the major deposit in intracellular inclusions in neurons, SNCG and SNCB inhibit SNCA fibril formation, suggesting a protective effect of SNCG and SNCB on SNCA aggregation (37). SNCG-mediated chaperone activity on ER- α may indicate a new direction of normal cellular function of synucleins. In this regard, SNCG may be involved in regulating Hsp70 and mediating the activation of ER- α in neuronal cells; thus, the down-regulation of SNCG expression may lower the beneficial effects of estrogen on protecting neurons against PD and AD. The potential role of SNCG as a neuroprotectant warrants additional investigation. Demonstration of direct interaction with ER- α chaperone complex and stimulation of ER- α signaling as one of the cellular functions of SNCG not only support 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.

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γ Synuclein, a Novel Heat-Shock Protein-Associated Chaperone, Stimulates Ligand-Dependent Estrogen Receptor α Signaling and Mammary Tumorigenesis

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