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# Estrogen Receptor α, a Molecular Switch Converting Transforming Growth Factor-α-mediated Proliferation into Differentiation in Neuroblastoma Cells*

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**Transforming growth factor-α (TGF-α) is known to promote both proliferation and differentiation of neural cell progenitors. Using the human neuroblastoma cell line SK-N-BE that is induced to proliferate by TGF-α, we demonstrated that the expression of a single transcription factor, the estrogen receptor-α (ERα), can reroute the TGF-α mitogenic signaling toward a path leading to differentiation. With selected mutations in ERα and signal transducer and activator of transcription 3 (Stat3), we demonstrated that the blockade of TGF-α mitotic potential was not dependent on ERα DNA binding activity but required a transcriptionally active Stat3. In neuroblastoma cells, 17β-estradiol treatment induced a transient increase in the transcription of estrogen-responsive element-containing promoters including those regulating TGF-α and prothymosin α synthesis. Based on the data presented, we hypothesized that in the presence of prothymosin α, ERα activates its direct target genes and increases cell proliferation, whereas in the presence of high levels of TGF-α, ERα preferentially interacts with Stat3 and causes cell differentiation. Our results reveal a novel form of "end-product" regulation of an intracellular receptor that occurs through recruitment of membrane receptors and their signaling effector system. Cross-coupling between membrane and intracellular receptors has been described by several laboratories. This study proves the relevance of these interactions in cellular responses to growth factors.**

Transforming growth factor-α (TGF-α)[1] is a pleiotropic peptide, which belongs to the family of the epidermal growth factors and binds with high affinity to the epidermal growth factor receptor (EGFR). In the nervous system, TGF-α regulates the maturation of neural cell progenitors, promoting their proliferation and differentiation along the neuroepithelial lineage (1). TGF-α is synthesized as a 160 amino acid membrane-anchored precursor that can be proteolytically processed in different soluble forms. Both membrane-anchored and secreted TGF-α are able to bind and activate the EGFR through justacrine, paracrine, and autocrine mechanisms. In the recent years, the molecular paths conveying TGF-α signaling from the membrane into the cell nucleus have been identified. Ligand binding to the EGFR results in receptor dimerization and trans-phosphorylation. In ligand-activated receptor, phosphorylated tyrosine residues serve as docking sites for the recruitment of signaling molecules such as Shc, phosphatidylinositol 3-kinase, and Grb2, which in turn activate a cascade of effector molecules aimed at propagating intracellularly the TGF-α-mediated signal. Site-directed mutagenesis revealed that the activation of specific intracytoplasmic effectors is dependent on the tyrosine phosphorylated during receptor activation (2). However, still unclear is the mechanism enabling TGF-α to drive its target cells into differentiation or proliferation. Burrows *et al.* (3, 4) provided direct evidence that receptor and ligand concentration affects the decision of the neural progenitor cells to proliferate or to differentiate (3, 4). Indeed, during late embryonic and early postnatal development, the content of TGF-α and its receptor change significantly in the progenitors of neural cells (4, 5). The persistence of the signaling evoked at the receptor site may induce receptor homo/heterodimerization with consequent changes in the pattern of tyrosine phosphorylation necessary to select a specific cytoplasmic signaling pathway responsible for the final nuclear events decisive for the cell fate. Several reports have shown that estrogen receptors (ERs) are nuclear targets of growth factors. Because of the known effects of estrogens on proliferation and differentiation of reproductive tissues, these receptors may be good candidates as nuclear effectors completing the program originated by membrane receptor. Several studies, showing that estrogen has a key role in the differentiation of neurons localized in selected brain regions, support this view (6). Previous work from our laboratory demonstrated the existence of a cross-coupling between ERα and insulin growth factor in neural cells (7, 8) subsequently confirmed by several other groups (9–11). In addition, ERs co-localize with receptor tyrosine kinases (RTKs), such as insulin growth factor receptor or EGFR, in several areas of the developing and adult nervous system (5, 12, 13), and functional interactions between ERs and RTKs have been documented (7–10, 14).

This study stems from preliminary observations made in a neuroblastoma cell line we engineered to constitutively express the ERα (SK-ER3 cells) (15). In this cell line, TGF-α blocks cell

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[1] The abbreviations used are: TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor; ER, estrogen receptor; RTK, receptor tyrosine kinase; ProTα, prothymosin α; E2, 17β-estradiol; Stat, signal transducer and activator of transcription.

proliferation and induces phenotypic differentiation. This is in contrast with the marked mitotic effect of this hormone in the ER-negative parental cell line. We here investigate on the involvement of ERα in TGF-α signaling and demonstrate that ERα converts the mitotic potential of TGF-α into a stimulus to differentiate. Our results indicate that this effect does not require ERα DNA binding activity, is mediated by Stat3, and is blocked by prothymosin α (ProTα), a co-regulator of ERα transcriptional activity of which expression is positively associated with cell proliferation.

## EXPERIMENTAL PROCEDURES

*Cell Culture, Reagents, and Plasmids*—SK-N-BE and SK-ER3 cells were grown as previously described (15). Unless otherwise specified, chemicals were purchased from Merck KGaA (Darmstadt, Germany) and culture media and additives were from Invitrogen. 17β-estradiol (E2) and TGF-α were from Sigma, and ICI 182,780 was kindly donated by AstraZeneca (London, United Kingdom). TGF-α was used at 10 ng/ml, E2 was used at $10^{-9}$ M, and ICI 182,780 at $10^{-7}$ M was administered 1 h before hormone. pCMVProTα cDNA (16) and the variants obtained through mutagenesis were subcloned into the *Xho*I/*Xba*I sites of the expression vector pCDNA3.1A (Invitrogen). The internal control pTK-luciferase was generated in our laboratory (17). pCMV5hREA was kindly provided by B. Katzenellenbogen (University of Illinois, Urbana, IL), pBSTGF-α was provided by L. Beguinot (DIBIT Hospital San Raffaele, Milan, Italy), pGAS-luc was provided by E. Liboi (University of Verona, Verona, Italy), and RcCMVStat3, RcCMVStat3F, and RcCMVStat3D were provided by M. Greenberg (Harvard Medical School, Boston, MA).

*Reporter and Morphometric Assays*—The calcium phosphate precipitate procedure and reporter assays were described previously (8). Luciferase counts normalized *versus* protein content are expressed as fold induction with respect to untreated sample. For morphometric assays (18), cells were transfected in a 12-well plate with 0.2 μg/well pCMV-lacZ (Promega, Madison, WI) alone or with 0.4 μg/well of the expression vectors coding for transcription factors and/or Ras variants as specified in the figures or figure legends. Morphometric analysis was carried out 96 h after treatments, measuring the neurite length of β-galactosidase-stained cells (18). For each experimental group, a minimum of 50 cells/dish was evaluated.

*Cell Counting*—SK-N-BE and SK-ER3 were seeded in RPMI 1640 medium without Phenol Red + 0.5% dextran-coated charcoal in 24-well plates, and after the specified treatments, vital cells were counted in triplicate wells. For transfected SK-N-BE, β-galactosidase-positive cells were counted in 25 fields/well chosen at random for each experimental group. Transfection efficiency was normalized by including 0.2 μg of pTK-luciferase in three additional wells not subjected to β-galactosidase staining and transfected in parallel. The number of β-galactosidase-expressing cells was finally referred to $10^4$ luciferase counts.

*[$^3$H]Thymidine Incorporation Assay*—$10^4$ SK-ER3 cells/well (plated the day before the assay in a 6-well dish) were incubated for 3 h with 1 μCi/well [$^3$H]thymidine (Amersham Biosciences). Cells were washed twice with phosphate-buffered saline containing 2 mM cold thymidine and lysed, and the trichloroacetic acid precipitates were assayed in a scintillation counter (TRI-CARB 2100TR, Packard).

*Western Analysis and Immunoprecipitation*—Whole cell extracts were analyzed by Western blot or immunoprecipitation as previously reported (8) using as primary antibody anti-ERα ($H_{222}$, kindly provided by G. Greene, University of Chicago, IL), anti-ProTα (Calbiochem), anti-Stat3, or anti-Phospho-Tyr 705-Stat3 (New England Biolabs, Beverly, MA). For immunoprecipitation analysis, whole cell extracts from SK-N-BE cells expressing ERα were set at the final concentration of 150 mM NaCl.

*Northern Analysis*—20 μg of total RNA were loaded onto 1% denaturing agarose gel containing 2.2 M formaldehyde and blotted on nylon membrane (Hybond-N+, Amersham Biosciences). Pre-hybridization and hybridization were performed at 68 °C in Quick-Hyb solution (Stratagene, La Jolla, CA), and two washes were carried out in 0.1× saline/sodium phosphate/EDTA, 0.1% SDS at 50 °C for 30 min. Membranes were exposed to autoradiographic films (Hyperfilm, Amersham Biosciences) for 2 (ProTα) or 7 days (TGF-α).

*Nuclear Run-on Transcription Assay*—$50 \times 10^6$ cells were suspended in a lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and 10 mM NaCl), and nuclei were pelleted by Microfuge centrifugation. Nuclear run-on assays were performed as described previously (20). 4-h pre-hybridization and 24-h hybridization were carried on in

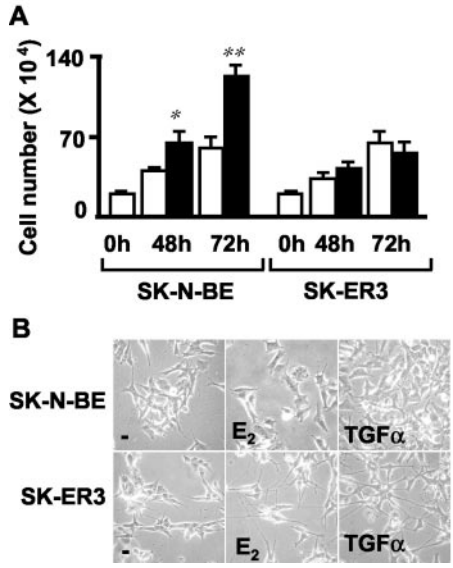


FIG. 1. **TGF-α action on proliferation and differentiation of neuroblastoma cells.** *A*, cells were grown in low serum (0.5% FBS) and counted with a Burker's chamber in the presence of a vital staining at the indicated time points. Data represent the mean ± S.E. of three separate experiments made in triplicates in the absence (*white bars*) or in the presence (*filled bars*) of TGF-α. *, $p < 0.05$, **, $p < 0.01$ *versus* vehicle calculated by two-way analysis of variance followed by Scheffé's test. *B*, microphotographs are representative of the morphology of cells treated for 96 h with vehicle (−), E2, or TGF-α (×200 magnification).

Church and Gilbert's solution at 72 °C. $2 \times 10^6$ cpm/ml were used in the hybridization reaction. After two washes of 45 min at 72 °C in a solution containing 40 mM $Na_2HP0_4$, 1% SDS, and 1 mM EDTA, filters were exposed to autoradiographic films with intensifying screens at −80 °C for 5 days.

*Reverse Transcription-PCR Analysis*—RNAs from transfected cells were reverse-transcribed using Moloney murine leukemia virus-reverse transcription (Promega), and the cDNAs of ProTα variants were amplified by PCR using the forward primer specific for the exogenous transcripts produced by the pCDNA3.1 vector (5′-TAATACGACTCACTATAGGG-3′) and the reverse primer specific for the 3′ region of the ProTα cDNA (5′-ctagtcatcctcgtcgg-3′). To assess the efficiency of the reverse transcriptase, cDNA encoding for the glyceraldehyde-3-phosphate dehydrogenase was also amplified using the following primers: forward 5′-atgacccctcattgacc-3′ and reverse 5′-tgcttcaccaccttcttg-3′. The number of cycles was chosen to remain in the logarithmic phase of the amplification curve.

## RESULTS

*In the Presence of ERα, TGF-α Ceases to Induce Proliferation of Neuroblastoma Cells*—The SK-ER3 neuroblastoma cell line used in this study was generated in our laboratory by stable transfection of the ERα in SK-N-BE neuroblastoma cells (15). E2 arrests SK-ER3 proliferation and induces differentiation toward a dopaminergic phenotype. Because of that, this cell line was proposed as a model that recapitulates estrogen activity in the maturing nervous system (21). Fig. 1*A* shows that TGF-α significantly increased the number of SK-N-BE cells (by 1.5- and 2-fold at days 2 and 3 of treatment, respectively) but that not of SK-ER3 cells. In addition, the treatment with TGF-α induced a marked morphological alteration of the ERα-positive neuroblastoma (Fig. 1*B*). Thus, the presence of ERα was sufficient to turn TGF-α from a proliferation into a differentiation stimulus. To ensure that this different response was not due to genetic alterations in the SK-ER3 subclone tested, ERα effect was investigated in SK-N-BE cells transiently co-transfected with a plasmid expressing ERα (pCMVERα) and pCMVLacZ encoding the transfection marker β-galactosidase. Cells were treated for 96 h with TGF-α or vehicle and then fixed and stained for the β-galactosidase activity. In control

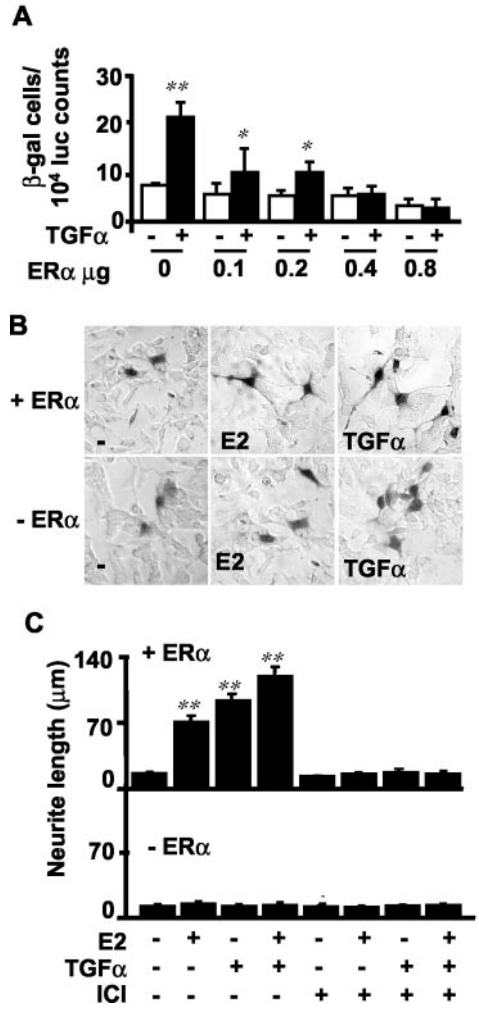


FIG. 2. **TGF-α promotes neurite sprouting in the presence of transfected ERα.** *A*, SK-NBE cells were transfected with plasmids encoding β-galactosidase (*β-gal*) (pCMVLacZ), ERα (pCMVERα), and with pTK-luciferase to allow normalization for transfection efficiency. β-gal-positive cells were counted 96 h after treatment with vehicle (*white bars*) or TGF-α (*filled bars*). Data represent the average of 20–30 fields chosen randomly and normalized for the transfection efficiency as described under "Experimental Procedures." *B*, morphology of cells after transfection and treatment for 96 h with vehicle (−), E2, or TGF-α. *C*, morphometric analysis of neurite lengths in samples of transfected SK-N-BE cells treated for 96 h with vehicle, E2, TGF-α, and the ERα pure antagonist ICI 182,780 (*ICI*). Results represent the mean ± S.E. values of three independent experiments done in triplicate. *, $p < 0.05$; **, $p < 0.01$ *versus* vehicle treated (analysis of variance followed by Scheffé's test).

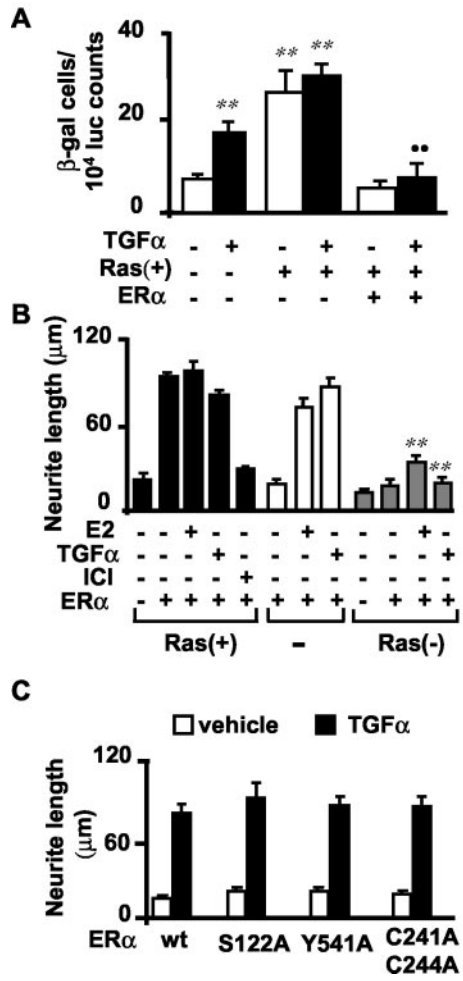


FIG. 3. **TGF-α-dependent neuroblastoma differentiation requires Ras and is independent from ERα DNA binding activity.** *A*, SK-N-BE were transfected with the expression vector encoding for the dominant positive Ras(+) with/without pCMVERα. Cells were counted after 96-h treatment with vehicle (*white bars*) or with TGF-α (*black bars*). Values are the mean ± S.E. of three independent experiments done on triplicate. **, $p < 0.01$ *versus* vehicle-treated cells in the absence of Ras(+) and ERα. ••, $p < 0.01$ *versus* cells treated with TGF-α in the absence of ERα (two-way analysis of variance followed by Scheffé's test). *B*, morphometric analysis was done in cells transfected with plasmids encoding β-galactosidase (*β-gal*), Ras(+), or Ras(−) in the presence or absence of pCMVERα and treated as indicated. Values are the mean ± S.E. of three independent experiments done on triplicate. **, $p < 0.01$ *versus* corresponding samples not transfected with Ras (two-way analysis of variance followed by Scheffé's test). *C*, in a similar set of experiments, the analyses were done in cells transfected with plasmids encoding ERα wild type (pMTMOR1–599) or ERα mutated in C241A/C244A (pMTMORC241A/C244A), S122A (pMTMORS122A), and Y541A (pMTMORY541A).

cells transfected with the pCMVLacZ alone, TGF-α treatment induced a 2.1-fold increase of the blue-labeled cells with respect to untreated cells. Co-transfection of increasing concentrations of pCMVERα progressively reduced the effect of TGF-α to reach control values (Fig. 2*A*). Morphological analysis indicates that treatment with TGF-α or E2 induced a differentiated morphology characterized by a decreased size of the soma and long neurites (Fig. 2*B*). Computer-assisted morphometric analysis, based on the measurement of neurite length, demonstrated that both E2 and TGF-α caused a significant increase in neurite outgrowth in the presence but not in the absence of ERα (Fig. 2*C*). This analysis is used to assess differentiation of cells of neuronal origin and has been previously shown as applicable to study the effects of ER ligands in the SK-N-BE cell system (21). Fig. 2*C* shows that TGF-α and E2 induce a similar increase in neurite length (3.5- and 4.0-fold, respectively). The observation that the pure ER antagonist, ICI 182,780 (*ICI*), blocked TGF-α-dependent neuritogenesis further demonstrated that ERα was indispensable to the differentiating activity of TGF-α.

*ERα-dependent Neurite Outgrowth Is Mediated by the Ras Pathway and Occurs through a DNA-binding Independent Mechanism*—Several studies reported that RTKs induce the transcriptional activation of unliganded ERα on ERE promoters via Ras/MAPKs enzymatic cascade, (8, 22–25). To investigate the role of Ras in the TGF-α effects above described, plasmids expressing the Ras dominant positive $p21(Leu61)^{Hras}$ (Ras+) or negative $p21(Asn17)^{Hras}$ (Ras−) mutants were co-transfected with pCMVERα and pCMVLacZ in SK-N-BE cells. The effects on cell number and neurite outgrowth were evaluated at 96 h after transfection. In the absence of exogenous E2, Ras(+) induced an increase in the number of β-galactosidase-

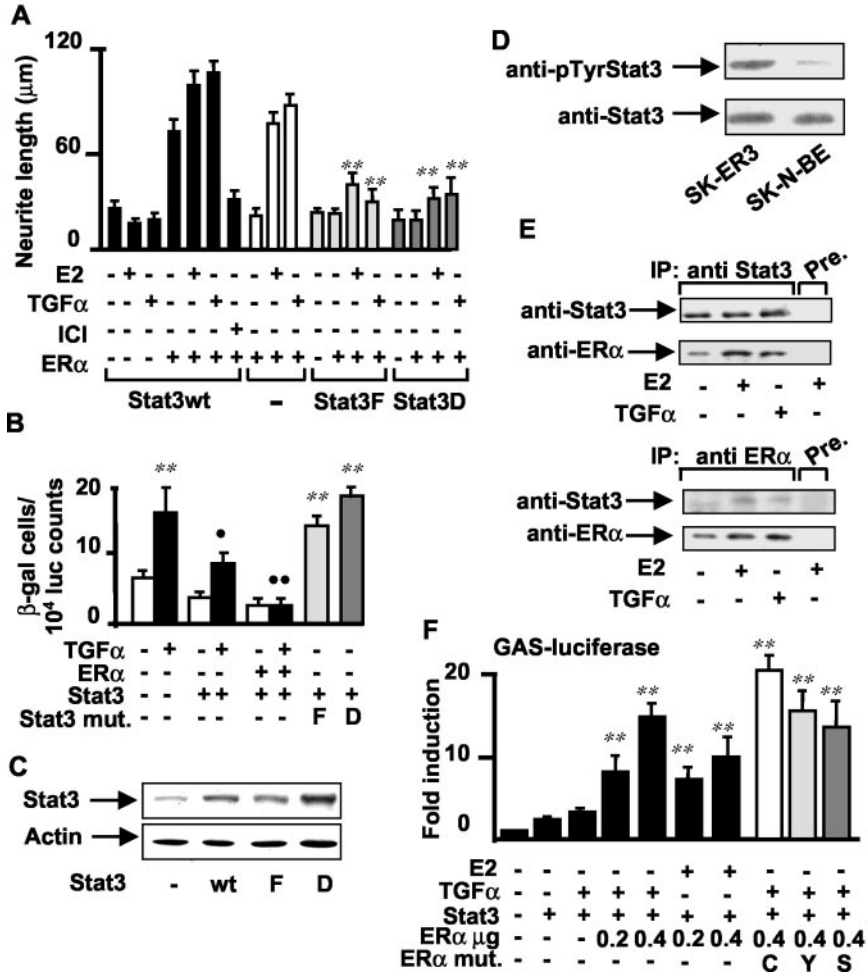


FIG. 4. **Stat3 is required for TGF-α- and ERα-dependent differentiation of neuroblastoma cells.** *A*, morphometric analysis of SK-N-BE cells transfected with plasmids encoding β-galactosidase (*β-gal*), ERα, and Stat3 wild type (*black bars*) or Stat3-negative mutants (*light gray bars*, Stat3F; *dark gray bars*, Stat3D). Cells were treated as indicated. Bars represent the mean ± S.E. of three independent experiments done in duplicate. **, $p < 0.01$ *versus* the corresponding samples without Stat3 wild type (*white bars*) (two-way analysis of variance followed by Scheffé's test). *B*, cells were transfected and treated as indicated. β-gal-positive cells were counted 96 h after treatment. Bars represent mean ± S.E. of three independent experiments done in duplicate. **, $p < 0.01$ vehicle-treated cells in the absence of Stat3 and ERα; •, $p < 0.05$; ••, $p < 0.01$ *versus* cells not transfected with pCMVERα and treated with TGF-α (two-way analysis of variance followed by Scheffé's test). *C*, representative Western blot of SK-N-BE whole cell extracts transiently transfected with the indicated Stat3 variants or with the control empty vector (−). *D*, representative Western blot of SK-N-BE and SK-ER3 whole cell extracts using anti-Stat3 and anti-phosphotyrosine-Stat3 (anti-pTyrStat3). *E*, physical interaction between ERα and Stat3. SK-N-BE cells expressing ERα were treated with E2 for 2 h or TGF-α for 15 min. Whole cell extracts were immunoprecipitated using anti-Stat3 antibody (*IP: anti-Stat3*), anti-ERα (*IP: anti-ERα*), or the pre-immune serum of the correspondent antibody (*Pre*). Immune complexes were separated by SDS-PAGE and blotted, and each probed with both antibodies were used for IPs. *F*, Stat3 transcriptional activity on the reporter pGAS-luciferase. SK-N-BE cells were co-transfected with pGAS-luciferase and with the plasmids encoding for Stat3 wild type, ERα (pMTMOR1–599) or its mutants, pMTMORC241A/C244A (*white bar*, Cys), pMTMORY541A (*light gray bar*, Tyr), and pMTMORS122A (*dark gray bar*, Ser). Luciferase activity was quantitated in cell extracts and normalized on the protein content. Bars are the mean ± S.E. of five independent experiments done in triplicate and represent the luciferase counts expressed as fold induction with respect to controls. **, $p < 0.01$ *versus* vehicle (two-way analysis of variance followed by Scheffé's test).

positive cells that was not further augmented by TGF-α. This effect was blocked by the presence of unliganded ERα (Fig. 3*A*). With regard to neuroblastoma differentiation, Ras(+) induced neurite outgrowth only in the presence of ERα. Its effect was not significantly augmented by estrogen or TGF-α and was blocked by ICI 182,780. On the contrary, the dominant negative Ras(−) completely prevented both TGF-α- and E2-induced differentiation (Fig. 3*B*). These data suggested that ERα is sufficient to redirect Ras pro-mitotic activity toward a program causing growth arrest and differentiation.

The current model on RTKs-ERα cross-coupling implies that selective phosphorylations of the receptor (26) and/or its co-activators (27, 28) trigger ERα activity on ERE promoters. However, ERα mutated in the sites shown to be indispensable for the cross-coupling to RTKs (Ser-122 in mouse corresponding to Ser-118 in human ERα) (8, 24, 25, 29, 30) or Src (Tyr-541 in mouse) (31, 32) could still interfere with TGF-α (Fig. 3*C*) or Ras(+)-proliferative action (data not shown). Most importantly, the use of C241A/C244A ERα variant impaired in its ability to bind DNA (Ref. 33 and data not shown) demonstrated that ERα-mediated neuroblastoma differentiation does not require binding to the canonical estrogen-responsive element and the transcriptional regulation of its primary target genes. This last observation led us to hypothesize that ERα-described activities in neuroblastoma were associated to its functional interactions with other nuclear transcription factors.

*Stat3 Is Indispensable to ERα-dependent Neuroblastoma Differentiation*—ERα is able to modulate the transcriptional activity of several transcription factors that are also downstream target of RTKs, including AP-1 (34), NF-kB (35), and STATs (36–39). This function does not require ERα direct binding to DNA. Therefore, we tested whether any of these factors were

able to induce SK-N-BE differentiation. Transfection of plasmids encoding NF-kB (NF-kB1/p50 and RelA/p65) and AP-1 (c-Jun and c-Fos) did not affect SK-N-BE morphology, nor did these factors alter TGF-α and E2 activity in the presence or absence of ERα (data not shown). Stat3 did not have any effect by itself, but when co-transfected with ERα, it induced a significant increase in neurite length, an activity that was completely prevented by the treatment with ICI 182,780 (Fig. 4*A*). The extent of Stat3 effect was only slightly modified by TGF-α and E2 treatments. The observation that the two Stat3 dominant negative mutants (40), Stat3F (Y705F) and Stat3D (E434A/E435A), blocked E2 and TGF-α activity suggested that Stat3 has a role in ERα-mediated differentiation of SK-N-BE. Dominant negative mutants of Stat3 also blocked the activity of ERα mutated in the DNA binding domain C241A/C244A (data not shown). On the other hand, cell count showed that Stat3 wild type opposed the proliferation of SK-N-BE cells, even in the absence of ERα. Conversely, the two Stat3 dominant negative mutants promoted cell proliferation. In the presence of ERα, Stat3 completely blocked TGF-α-induced proliferation (Fig. 4*B*). It is important to point out that transient transfection lead to the overexpression of the Stat3 variants in accordance with the dominant negative action hypothesized for Stat3F and Stat3D (Fig. 4*C*). These results therefore suggested a functional interaction between Stat3 and ERα. Interestingly, the two transcription factors appeared to interact also in the absence of E2 or TGF-α (Fig. 4*A*). Indeed the constitutive expression of ERα was sufficient to modify the basal levels of Stat3 tyrosine phosphorylation as shown by Western blot analysis of SK-ER3 and SK-N-BE cell extracts (Fig. 4*D*). Consistently with a functional interaction, immunoprecipitation experiments reported in Fig. 4*E* demonstrate that in neuroblastoma cells a physical interaction between Stat3 and ERα occurs. Interestingly, this interaction was better detected when the immunoprecipitation was performed with the anti-Stat3 antibody (Fig. 4*E*, *upper panel*) instead of anti-ERα ($H_{222}$) (Fig. 4*E*, *lower panel*), thus suggesting that the $H_{222}$ antibody might recognize an epitope in the vicinity of the interaction site. Finally, the interaction between ERα and Stat3 was also investigated at the level of Stat3 transcriptional activity by transient transfection assay using a luciferase reporter driven by the GAS sequence, a known Stat3-responsive element (pGAS-luciferase) (Fig. 4*F*). Treatment with TGF-α alone did not affect Stat3 transcriptional activity significantly; however, in the presence of increasing concentration of ERα, we observed a dose-dependent augmentation of luciferase transcription (7.9- and 14.4-fold stimulation *versus* Stat3 alone). Similar results were obtained when ERα was activated by E2 (6.7- and 9.4-fold stimulation). Consistent with our findings on neurite elongation, the ERα mutants C241A/C244A, Y541A, and S122A, had the same effect on the wild type receptor. The ability of activated ERα to physically interact with STATs family of transcription factors has been revealed also in other cell systems (36, 38, 39), and in accordance with our observation in neuroblastoma cells, this interaction has been shown to increase Stat3-dependent transcription (39). These experiments demonstrated the hypothesized transcriptional interaction between ERα and Stat3 and its relevance for TGF-α-induced differentiation of SK-N-BE cells.

*TGF-α- and E2-mediated Differentiation Is Blocked by the Constitutive Expression of ProTα*—We previously reported that ProTα and TGF-α are positively regulated by E2 in the SK-ER3 cell line (16). Other laboratories have shown that both genes are directly regulated by ERα through binding to EREs present in their promoters (41, 42). Run-on analysis using SK-ER3 nuclei demonstrated that the E2-mediated increase in ProTα and TGF-α mRNA is at least in part due to the activation of gene transcription (Fig. 5*A*). This effect was not detected in the ER-α-negative SK-N-BE cell line (data not shown). The prolonged hormonal effect observed on TGF-α and ProTα with respect to progesterone receptor gene transcription (Fig. 5*A*) suggested that factors other then ERα intervened to strengthen the effect triggered by the hormone. Northern blot analysis revealed that after E2 treatment, TGF-α and ProTα mRNAs slowly accumulated to reach a peak level at 48 h but with a different kinetics. ProTα reached the highest level of expression at 24 h while TGF-α reached at 48 h (Fig. 5*B*). The accumulation of TGF-α transcript was expected as a component of SK-ER3 differentiation. More puzzling was the prolonged high level of ProTα expression that generally is associated with proliferation rather then differentiation (43). Therefore, we investigated proliferative activity of SK-ER3 upon E2 treatment by studying [$^3$H]thymidine incorporation. In SK-ER3 cells, E2 induced a significant increase in the synthesis of nucleic acids up to 48 h (Fig. 5*C*). At 72 h, when cells visibly started to change their morphology, the [$^3$H]thymidine incorporation dropped below control levels. Thus, the decrease in ProTα mRNA content temporally correlated with the decreased

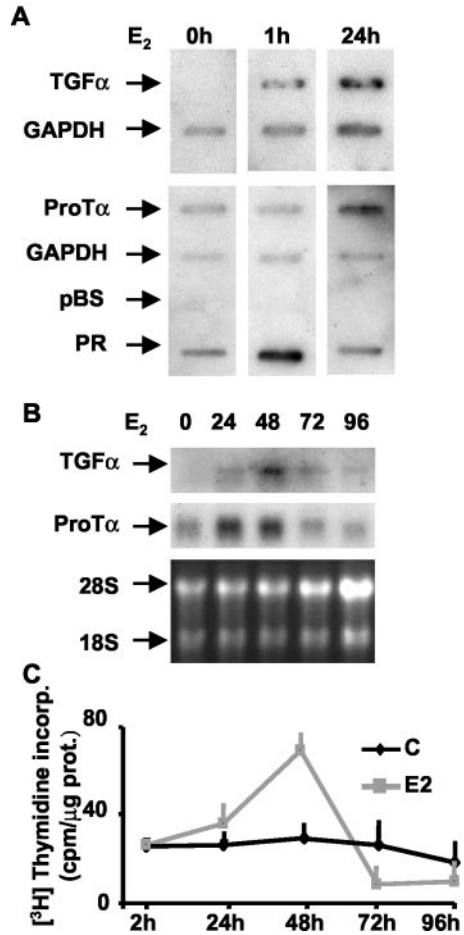


FIG. 5. **TGF-α and ProTα expression is estrogen-regulated in SK-ER3 cells.** *A*, run-on analysis performed on nuclei from SK-ER3 cells treated with E2 for the indicated times. Hybridized filters were exposed for autoradiography to x-ray films for 3 days at −70 °C. *B*, Northern blot analysis on total RNA from SK-ER3 treated with E2 for the indicated time. Hybridization was performed with $^{32}$P-labeled TGF-α and ProTα probe. Autoradiography of hybridized filters reveals, respectively, the ProTα 1.4-kb and TGF-α 4.5-kb transcripts. The ethidium bromide staining of the gel shows the 28 S and 18 S rRNA. *C*, effect of E2 treatment on SK-ER3 growth rate assayed by [$^3$H]thymidine incorporation. Data represent the mean ± S.E. of three separate determinations.

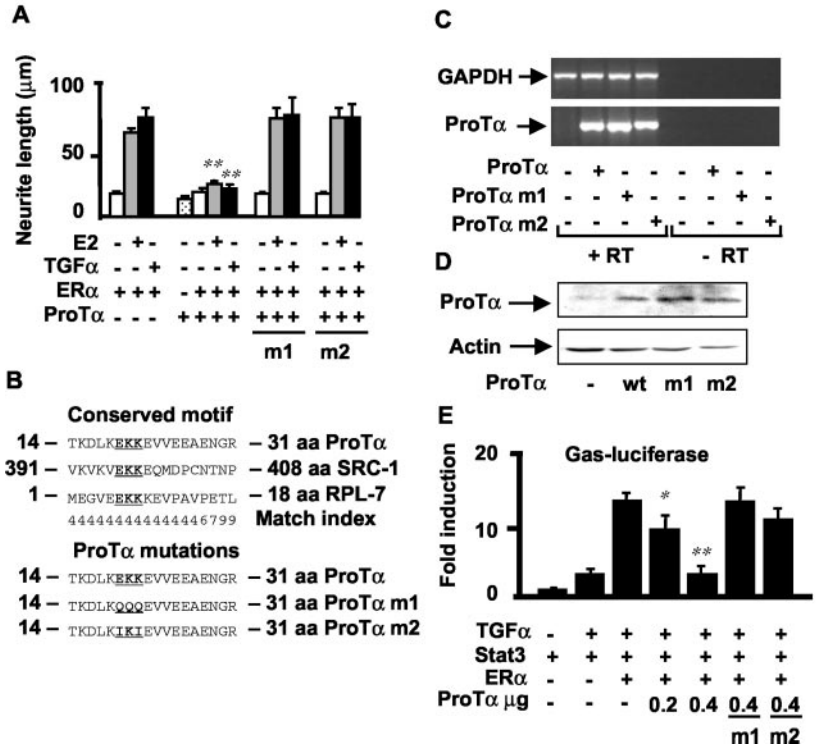


FIG. 6. **Constitutive expression of ProTα prevents ERα-mediated differentiation on SK-N-BE cells.** *A*, neurite lengths were determined in cells transfected with pCMVβ-galactosidase, pCMVERα, and plasmids encoding for ProTα wild type (pCMVProTα) or the mutated variants (pCMVProTαm1 or pCMVProT αm2). Transfected cells were treated with TGF-α or E2. Bars represent the average neurite length ± S.E. of three independent experiments done in triplicate. **, $p < 0.01$ *versus* the corresponding samples not transfected with ProTα as calculated by two-way analysis of variance followed by Scheffé's test. *B*, the EKK motif present in the cofactors of ERα activity (ProTα, steroid receptor coactivator-1 (*SRC-1*), and ribosomal protein large subunit-7 (*RPL-7*)). The match index is assigned by the program Match-Box and estimate the likelihood of correctly predicting the alignment (Match index = 4 corresponds to a range or likelihood of 73–100%). The mutated ProTα variants in the EKK motif are indicated. *C*, reverse transcription (*RT*)-PCR analysis performed on total RNAs extracted from SK-N-BE cells transfected with the expression vectors encoding for ProTα wild type or its mutant variants (m1 and m2). As negative controls, amplifications were also performed on reverse transcription reactions carried on without reverse transcriptase (−*RT*). *D*, representative Western blot of SK-N-BE whole cell extracts transiently transfected with the indicated ProTα variants or with the control empty vector (−). *E*, Stat3 transcriptional activity in SK-N-BE cells transfected with pGAS-luciferase together with RcCMVStat3, pCMVERα, pCMVProTα, or its mutated variant, pCMVProTαm1 or pCMVProTαm2. Extracts for luciferase activity were prepared at 48 h upon 24-h treatment with TGF-α. The enzyme activity was normalized on the protein contents. Bars represent the luciferase counts expressed as fold induction with respect to the control level and are an average ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* cells treated with TGF-α and transfected with RcCMVStat3 and pCMVERα as calculated by two-way analysis of variance followed by Scheffé's test.

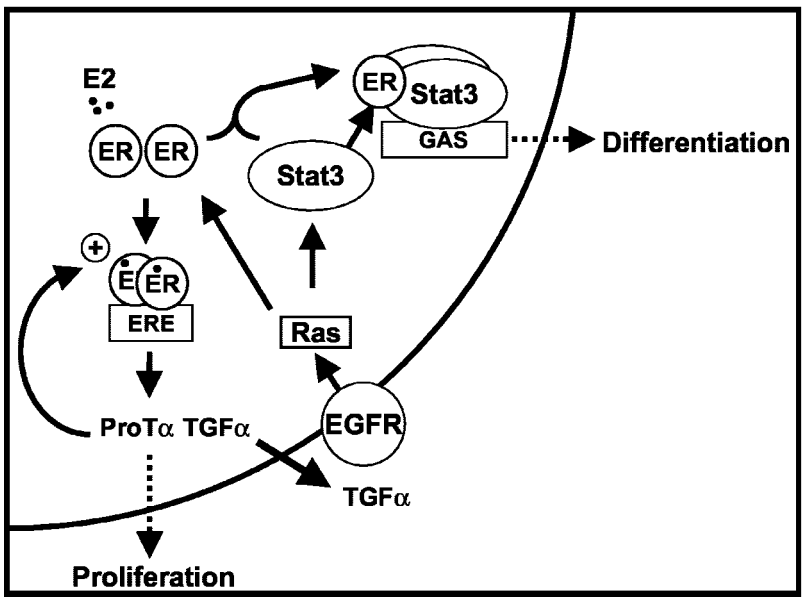


FIG. 7. **Proposed model for ERα role in neuroblastoma cell growth and differentiation.** In the presence of ERα, E2 induces synthesis of ProTα and TGF-α. ProTα initially strengthens ERα activity on ERE-driven promoters determining TGF-α accumulation. As a consequence, TGF-α increases signaling of Stat3 through interaction of ERα on GAS-driven promoters inducing neuroblastoma cell differentiation.

[$^3$H]thymidine incorporation and the onset of E2-dependent differentiation. We then tested the effect of constitutive expression of ProTα by transfection of pCMVProTα in SK-N-BE cells. Fig. 6*A* shows that maintaining high levels of ProTα expression the morphological differentiation mediated by ERα was completely prevented. To identify motifs relevant to ProTα function, we compared its amino acid sequence with other proteins implicated in ERα signaling. ProTα does not contain the se-

quence motif L*XX*L (44); however, a computational analysis using the Match-Box website server (45) identified a conserved motif (EKK) present in ProTα, steroid receptor coactivator-1, and ribosomal protein large subunit-7 (Fig. 6*B*). The program assigned a high score (=4) in terms of calculated match index corresponding to a 73–100% likelihood of correctly predicting the residue alignment. Searching the ExPASY protein databases, SWISS-PROT and TrEMBL, for the identified box by means of PATTINPROT program at NPS@ server (46), we obtained only the three above mentioned co-regulators. On the basis of this analysis we devised two different mutated sequences to replace the EKK pattern: QQQ and IKI (Fig. 6*B*). The former was chosen to completely remove the positive charge present in the wild type sequence without gross alteration of hydrophilicity, the latter to drastically reduce this parameter. Both ProTα mutations were unable to prevent ERα-mediated neurite elongation (Fig. 6*A*), even though the mutated variants were correctly expressed at a concentration comparable to wild type ProTα as demonstrated by reverse transcription-PCR assays on total RNA samples from transfected cells (Fig. 6*C*) and by Western blot on whole cell extracts shown in Fig. 6*D*. These experiments suggest that the effect of constitutive expression of ProTα on neuroblastoma differentiation is mediated by the EKK motif. The experiments reported in Fig. 4 led us to hypothesize that TGF-α induces neuroblastoma differentiation via ERα/Stat3 interaction. Therefore, we tested whether ProTα effect on differentiation correlated to a blockade of ERα activity on a GAS-containing promoter. ProTα wild type, but not the EKK-mutated variants, abolished the transcriptional response of the GAS promoter to Stat3 and ERα in a concentration-dependent manner (Fig. 6*E*). Co-transfection of the co-regulator activity-modulating protein REA (47) with ERα had no effect on GAS-luc transcription and on neuroblastoma differentiation induced by Stat3/ERα (data not shown); this suggest that REA does not play a role in the interaction between Stat3 and ERα, leading to neuroblastoma differentiation.

## DISCUSSION

The molecular events inducing a proliferating cell to stop dividing and undertaking a differentiation program are still largely unknown. Using a neuroblastoma model, we studied how TGF-α may induce the same cell type to proliferate or differentiate. We here demonstrate that the molecular switch between the SK-N-BE proliferation and differentiation programs may be a single transcription factor, ERα. TGF-α induces SK-N-BE to proliferate or differentiate depending on the absence or presence of ERα. Because E2 increases the transcription of *TGF-α* gene in SK-ER3 cells, it might be argued that E2-dependent differentiation of SK-ER3 cells is due to a mechanism of signal amplification (48, 49). We believe that this is not the case because exogenous administration of TGF-α (thus the persistent stimulation of its receptor) or the constitutive expression of Ras(+) into the ERα-negative SK-N-BE cells causes an increase in cell growth but is not associated with any morphological alteration (Fig. 1 and 3, respectively).

The classical view of ERα activity involves the binding of the hormone receptor complex to specific sequences in the promoter of responsive genes, thereby favoring their transcription (50). More recent studies have shown that the unliganded receptor may also be transcriptionally activated by molecules of the RTK signaling pathways (8, 22, 24, 25, 50). This study shows that mutations of ERα impairing its ability to bind ERE or to be the target of RTK-dependent kinases still allow neuroblastoma differentiation induced by TGF-α. It is well known that in the absence of DNA binding function through protein/protein interaction, ERα can still modulate the activity of other transcription factors including AP-1, NF-kB, or Stats (34–36). Little is known with regard to the functional relevance of these interactions. We here demonstrate that ERα/Stat3 functionally interacts to regulate a GAS-containing promoter and that this interaction is necessary and sufficient to prevent TGF-α-induced proliferation. This study underscores a novel mechanism of cross-coupling between growth factors and intracellular receptors that does not require the phosphorylation of the AF-1 domain and that does not result in ERα activation on ERE promoters but facilitates its functional interaction with other transcription factors, namely Stat3.

Several lines of evidence demonstrate that ProTα is associated to cell proliferation (43), and its transcription is positively regulated by estrogen (16, 42). Indeed, E2 treatment of SK-ER3 cells induces an increase in ProTα mRNA and thymidine incorporation (Fig. 5). Furthermore, ProTα has been shown to facilitate ERα-mediated transcription on its responsive element ERE by removing REA (47). We speculate that the initial synthesis of ProTα helps to release REA from ERα, thus increasing TGF-α synthesis. Indeed, here we show that the initial response of SK-ER3 to E2 is increased proliferation. However, after 48 h, ProTα mRNA levels decline and cells strongly decrease their proliferation rate and start differentiating. This raises the question regarding the mechanism interrupting the receptor activity on ERE promoters and initiating the regulation of genes associated with cell differentiation that are not primary targets for ER. On the basis of our data, it might be speculated that the initial production of TGF-α triggers, through the EGFR signaling cascade, ligand-independent activation of ERα, resulting in its association with intranuclear factors abundant in SK-ER3 such as Stat3. This protein/protein interaction subtracts ER from the binding to the ERE-containing promoters, shifting the cell program toward differentiation (Fig. 5). The fact that REA cannot modify cell differentiation suggests that this factor is not implicated in Stat3/ERα interaction.

We here propose that ERα might have proliferative or antiproliferative action depending on the relative content of the nuclear factors capable of interaction with the receptor. Our model predicts that ProTα strengthens the action of ERα on the ERE promoters. In its absence, ERα is free to interact with other transcription factors, such as Stat3, with opposite physiological consequences. Indeed, we here show that ProTα prevents ER-α-dependent transcriptional activation of Stat3 on its responsive element and that, conversely, high expression of Stat3 induces ERα-dependent differentiation of SK-N-BE cells (Fig. 7). Supporting this view is the fact that in SK-ER3 neuroblastoma cells estrogen acts as a differentiation factor, whereas in other cell types, E2 induces proliferation. Interestingly, high levels of ProTα are associated with high proliferative ER-positive neoplasia (19). The results of this study might be of relevance also for the understanding of the activity of ER in other tissues and might provide novel target for limiting ERα-dependent proliferation in ER-positive neoplasia.

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