Estrogen receptors (ERα/ERβ) are expressed in neuronal cells and exhibit a variety of activities in the central nervous system. ER activity is regulated in a ligand-dependent manner and by co-regulatory factors. Caveolin-1 is a recently identified co-activator of ERα mediating the ligand-independent activation of this steroid receptor. Here, the influence of ERs on caveolin expression in human neuroblastoma SK-N-MC cells as well as in rodent brain was investigated. We found that ectopic expression of ERα in SK-N-MC cells (SK-ERα) leads to a ligand-independent transcriptional suppression of caveolin-1/-2 genes. This suppression is specifically mediated by ERα and not ERβ because ERβ counteracts the observed caveolin-silencing process. Interestingly, decreased caveolin expression in SK-ERα is accompanied by changes in the methylation pattern of caveolin promoters. The analysis of selected promoter regions of the human caveolin-1 gene showed that certain CpG dinucleotides were hypermethylated in SK-ERα cells, whereas the same sites were unmethylated in control, ERβ-, and ERα/β co-expressing SK-N-MC cells. Inhibition of DNA methylation or histone deacetylation led to partial re-expression of caveolin-1/-2 genes in SK-ERα. In vivo analysis revealed a down-regulation of caveolin-1 expression after long term estrogen exposure in certain regions of the mouse brain. In conclusion, we have shown for the first time that ERα and not ERβ silences caveolin-1/-2 expression in an epigenetic fashion in neuronal cells. The observed mechanism of gene silencing by ERα may have implications for the transcriptional regulation of further ERα target genes.

Estrogen receptors (ERs) belong to the family of steroid receptors that exert a great diversity of effects on mammalian, normal non-transformed cells as well as tumor cells. In addition to classical estrogen target tissues such as the female reproductive system (1) or bone tissue (2), ERs are also expressed in the central nervous system and influence a variety of functions including locomotor activity, mood, memory, and cognition (3, 4). To date, two structurally distinct subtypes of ERs, ERα (5) and ERβ (6), have been identified. These receptors show the capability to form homo- or heterodimers (7) ensuring a high level of complexity with respect to ER function. ERs exist as a protein complex assembled with a number of co-regulatory factors (8). The activity of ERs is regulated by the endogenous ligand estrogen and by interaction with co-repressors and co-activators. Co-repressors of ERs such as silencing mediator of retinoid and thyroid hormone receptor and nuclear receptor co-repressor have been shown to recruit histone deacetylases thereby mediating transcriptional repression. In contrast to co-repressors, co-activators enhance transcription of ER target genes in a ligand-dependent or -independent manner. Caveolin-1 has been recently identified as a co-activator exclusively of ERα (9). ERα binds to caveolin-1 directly via its AF-1 domain, which subsequently leads to a estrogen-independent potentiation of ERE-driven gene transcription (10).

Caveolin-1 is part of the caveolin gene family consisting of three members, caveolin-1, -2, and -3 (11). In general, caveolin proteins play an important role in the biogenesis and function of caveolae (12), vesicular invaginations of the plasma membrane. Caveolae are presumed to integrate different signaling pathways as they are enriched in certain signaling proteins such as epidermal growth factor receptor (13), H-Ras, Sre family tyrosine kinase, heterotrimeric G-protein (14), and endothelial nitric-oxide synthetase (15). Interaction of these proteins with caveolin suppresses their enzymatic activity by holding them in an inactive conformation. Recently, a putative membrane-bound form of ERs, which is thought to mediate the rapid non-genomic actions of ERs, was also found to be clustered in caveolae-like structures (16, 17). Moreover, caveolae are involved in processes of vesicular trafficking, and particularly in the transport of cholesterol (18, 19).

There are some differences in the expression pattern of the caveolin isoforms. Although caveolin-3 expression is mainly restricted to muscle cells (20), caveolin-1 and caveolin-2 were found to be abundant in other tissues, for example in adipoocytes, fibroblasts, and endothelial cells (21). For a long time, caveolin-1 and caveolin-2 proteins were considered to be absent from neuronal tissue (22), but there is a growing body of evidence that caveolins exert their function also in the central nervous system (23, 24). It has been demonstrated that caveolin modulates the signaling through p75NTR and TrkA in neuronal and glial cell populations (25) and that caveolin might be involved in the induction of synaptic potentiation in hippocam-
pal nerve cells (26). Interestingly, caveolae may also have a pivotal role for the processing of amyloid precursor protein and generation of β amyloid protein occurring in Alzheimer’s disease (27, 28) because amyloid precursor protein has been found in caveolae-like membrane domains (29).

From the evidence linking caveolae expression and ERα activation, ERα/estrogen may influence caveolae expression by means of an either synergistic or negative feedback regulation. Only a few studies have investigated changes in caveolae levels in response to ERα/estrogen in non-neuronal cells such as endothelial cells (30), uterine smooth muscle cells (31), and bovine aortic endothelial cells (32). In the present study we addressed the question whether ERα/estrogen affect caveolae expression in neuronal cells in vitro and in vivo focusing also on differences between ER subtypes and regulatory mechanisms.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human SK-N-MC cells (ATCC HTB-10) were obtained from the American Type Cell Collection and were propagated in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen). The medium for the SK-N-MC transfectants was supplemented with 2 μg/ml G418 (Calbiochem).

**Estrogen Receptor Constructs and Establishment of Stably Transfected SK-N-MC Cells**—Construction and characterization of SK-N-MC cells stably transfected with wild type human estrogen receptor α (ERα) were described previously (33). Briefly, the human cDNA of ERα (34) was ligated into the bicistronic eukaryotic expression vector pIRES (CLONTECH, BD Biosciences). For generation of the human estrogen receptor β (ERβ) expression vector, ERβ cDNA (7) was ligated into the EcoRI site of pBluescript II KS (Stratagene). This construct (ERβ/pBluescript II KS) was double-digested with XhoI/XbaI liberating an ~2-κb fragment that encodes the ERβ cDNA. Subsequently, the fragment was cloned into the XhoI/XbaI double-digested vector pIRES. In the case of co-expression of ERα and ERβ, cDNA of ERα was ligated into the SalI restriction site of the MCS B of pIRES. The XhoI/BesIII ERβ fragment derived from ERβ/pBluescript II KS was ligated into the XhoI/MulI opened MCS A of pIRES.

The orientation of insertions as well as sequences of ERα/β cDNAs were checked by sequencing (TopLab, Munich, Germany) in both directions before transfecting the cells. A mock transfection with the empty vector was performed as a control.

SK-N-MC cells were transfected with 2 μg of the ERα, ERβ, ERαβ expression vectors, using Superfect™ Transfection Reagent according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Twenty four hours after transfection, 2 μg/ml G418 was added to the medium. Clones were picked after 3 weeks and grown under selective conditions. The clones were analyzed by RT-PCR and luciferase reporter assay.

**Transient Transfection and Luciferase Reporter Assay**—Transient transfecion of SK-N-MC-cells with the reporter plasmid ERE-Luc was performed using the Superfect transfection reagent according to the manufacturer’s protocol (Qiagen). Briefly, cells were seeded in 24-well plates in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal/dextrans-treated FBS (HyClone, Perbio Science) 24 h before transfection. When cells reached 60% confluence, each well received 1 μg of reporter plasmid, and cells were stimulated with 10 nM 17β-estradiol (Sigma, diluted in 100% ethanol). After 48 h of estrogen treatment, cells were incubated for 5 min at room temperature with lysis buffer (K2HPO4, Triton), and lysates were then assayed for luciferase activity as described previously (35). The protein concentrations were determined by a standard BCA protein assay (Pierce), and luminescence readings were performed with an automatic counter (Wallac Victor, Wallac Inc.).

**Reverse Transcription-PCR**—Total RNA from the cells was prepared using the Absolutely RNA RT-PCR Miniprep Kit according to the manufacturer's instructions (Stratagene). Reverse transcription was performed on 2 μg of total RNA in a reaction volume of 20 μl containing 1 μl of oligo(dT)23 primer (Promega), 2 μl of 10 μM dNTPs (peqLAB), 0.5 μl of 10 μM oligo(dT)23 primer (Sigma), 10 units of RNasin (Promega), and 4 units of Omniscript Reverse Transcriptase (Qiagen). Synthesis of cDNA was carried out for 60 min at 37 °C. A mock control without reverse transcriptase was performed for each RT-PCR in order to exclude DNA contamination. PCR was carried out in a 25-μl reaction volume that contains 1–4 μl of cDNA, 2.5 μl of 10× PCR Buffer (Invitrogen), 0.75 μl of 50 mM MgCl2 (Invitrogen), 0.5 μl of 10 mM dNTPs (peqLAB), 0.5 μl of 10 μM sense and antisense primers, and 0.5 units of DNA Taq polymerase (Invitrogen). The sequences of PCR primers for the amplification of cavelin-1 cDNA were 5′-GACTTTGGA- GATCTGG-AACTCCG-3′ (sense) and 5′-GATTGGAATTGGTTGGCC-3′, this primer set amplified a 323-bp product within the ERα steroid binding domain. The primer pair 5′-GAGGCCCTCATGATGATGC-3′ (sense) and 5′-TTCCACGGAGGCTAC-3′ (antisense) was used for detection of ERβ mRNA and generated a 611-bp product corresponding to a part of the ERβ steroid binding domain. Primers for β-actin were used as control for the quantity of the RNA used. The β-actin specific primers amplified a 275-bp product and were composed of the following sequences: 5′-CTCAATGAGCTGGTGTGGC-3′ (sense) and 5′-CAGGTCCAGACGGAGTTGCC-3′ (antisense). All primers were purchased from Metabion (Martinsried, Germany). PCR cycle conditions were 94 °C for 30 s, 58 or 61 °C for 30 s, and 72 °C for 45 s for 25–30 cycles with an initial denaturation at 94 °C for 5 min and a final extension of 60 s at 72 °C. PCR products were fractionated by size on agarose gels and visualized with ethidium bromide.

**Western Blot Analysis**—Cavelin-1, cavelin-2, phosphorylated and non-phosphorylated ERK1/2, as well as actin expression was examined by Western blot analysis. Cells from subconfluent 6- or 10-cm diameter dishes were washed with PBS and lysed in a buffer containing 62.5 mM Tris, 2% SDS, 10% sucrose, and protease inhibitors (phenylmethylsulfonyl fluoride, 5 μg/ml, and aprotinin, 1 μg/ml). Samples were briefly sonicated and boiled for 5 min at 95 °C. Prior to loading, the protein concentration of the samples was measured with the BCA method (Pierce) using bovine serum albumin as a standard. The samples were adjusted to loading buffer (10% SDS, 20% glycerin, 125 mM Tris, 1.0 mM EDTA, 0.02% bromophen blue, 10% β-mercaptoethanol), denatured by heating at 95 °C for 5 min, and subsequently separated by SDS-PAGE and transferred to nitrocellulose membranes. Protein bands were visualized with Ponceau S to verify equal loading of samples. Blots were placed in Tris-buffered saline, 0.05% Tween 20 (TBS/Tween) supplemented with 2.5% non-fat milk for 1.5 h at room temperature and then incubated with either anti-cavelin-1 (1:500, BD Transduction Laboratories), anti-cavelin-2 (1:250, BD Transduction Laboratories), anti-actin (1:1000, Sigma), or anti-phosphorylated and non-phosphorylated form; 1:1000, New England Biolabs) diluted in TBS/Tween, overnight at 4 °C. After washing the blots three times with TBS/Tween, membranes were incubated with horse-radish peroxidase-conjugated antibodies (1:3000, Amersham Biosciences) for 1.5 h and washed again as described previously. Membrane-bound secondary antibodies were detected using the ECL procedure developed by Amersham Biosciences. To ensure equal protein loading membranes were re-hybridized with an actin-specific antibody and developed.

**Cellular Immunofluorescence Microscopy**—Cells grown on glass coverslips were briefly washed twice with PBS and fixed at room temperature for 45 min with 4% formaldehyde in PBS. Cells were permeabilized by rinsing twice with 10 μM PBS containing 0.1% Tween 20. After permeabilization cells were rinsed in blocking solution (10% FBS in PBS) for 30 min. The coverslips were incubated for 2 h at room temperature with either cavelin-1 or cavelin-2 primary antibody (1:250 in PBS with 0.1% Tween 20 and 10% FBS). After three washes with PBS, 0.1% Tween 20, cells were incubated with the secondary antibody for 1 h at room temperature (anti-mouse IgG conjugated with Cy3, Dianova, diluted 1:200 in PBS with 0.1% Tween 20 and 10% FBS). Finally, cells were washed three times for 5 min in PBS, 0.01% Tween 20, and slides were mounted with Prolong Antifade® (Molecular Probes). Cells were visualized by confocal laser scanning microscopy (Leica confocal laser scanning microscope TCS NT) and digitally photographed.

5-AZA, TSA, and U0126 Treatment of SK-ERα Cells—Cells were grown to 75% confluence 24 h before treatment and then switched to culture medium supplemented with 10, 50, and 100 μM 5-aza-2-deoxycytidine (5-AZA; Sigma, diluted in acidic PBS), or cells were treated with PBS vehicle. The medium was replaced with fresh medium containing fresh 5-AZA every 24 h. In a separate experiment trichostatin A (TSA; Sigma, diluted in 100% ethanol) was added to the medium at a final concentration of 15 and 20 ng/ml, or cells were treated with the vehicle.
ER α-mediated Silencing of Caveolin Gene Expression

electron. Treatment of SK-ERα cells with 1, 5, and 10 μM U0126 (Calbiochem, diluted in MeSO) was performed in the presence of phenol-red free Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal/dextran-treated FBS. RNA was isolated using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene) after 4 days of 5-AZA, 2 days of TPA, or 3 days of U0126 treatment.

Methylation Analysis by Bisulfite Genomic Sequencing—Genomic DNA was isolated from cells using the Blood and Cell Culture DNA Mini Kit (Qiagen). The bisulfite reaction was performed using the CpG Genome Modification Kit according to the manufacturer’s protocol (In- tergen). In each bisulfite reaction, 1 μg of DNA was used initially. The DNA was incubated with bisulfite and eluted in 30 μl of TE. Each PCR contained 5 μl of bisulfite-modified DNA in a final volume of 50 μl of PCR mixture composed of 5 μl of 10× PCR Buffer (Invtrogen), 1.5 μl of 50 mM MgCl2 (Invtrogen), 1 μl of 10 mM of each dNTP (peqLAB), 1 μl of each 100-nmol primer, and 4 units of DNA Taq polymerase (Invtrogen). The first set of primers was designed after taking into account the bisulfite conversion reaction. Primer sequences were as follows: cav1prom1s (−877 → −848) 5′-GGATGGTGAATGATGGTGGAT-3′ (sense) and cav1prom1as (−547 → −525) 5′-CCATCTTCACTTAAACCACAT-3′ (antisense). The second pair of primer was cav1prom3s (−477 → −455) 5′-GGATGGTGAATGATGGTGGAT-3′ (sense) and cav1prom3as (−223 → −202) 5′-CATCTCCCAAATTACACTA-3′ (antisense) (36). The nucleotide positions are numbered relative to translation start codon (AUG) +1. PCR was performed at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 58 °C for 40 s, and 72 °C for 50 s and a final extension at 72 °C for 5 min. The amplified fragments were gel-purified and subcloned into the pGEM T-easy vector (Promega). Plasmids were purified using the QiAprep Spin MiniPrep Kit (Qiagen), and individual clones were sequenced by Genomed (Martinsried, Germany). In addition, amplified fragments derived from primer set cav1prom3s/3as were directly sequenced by TopLab (Martinsried, Germany). In addition, amplified fragments derived from primer set cav1prom3s/3as were directly sequenced by TopLab (Martinsried, Germany). In addition, amplified fragments derived from primer set cav1prom3s/3as were directly sequenced by TopLab (Martinsried, Germany).

Animal Treatment—Pregnant C57BL/6J mice were injected with vehicle or 1 μg/kg body weight of estradiol dipropionate daily from embryonic day 9.5–16.5. The pups were born, weaned, and sacrificed at 4 weeks of age. The brain was removed and frozen in isopentane on dry ice. Using a cryostat, 16-μm serial sections were taken and mounted directly onto Superfrost plus microscope slides and stored at −80 °C until processed.

Immunohistochemistry—Sections were removed from the freezer and allowed to defrost at room temperature for 5 min. The sections were then fixed in 4% (v/v) paraformaldehyde in PBS (pH 7.4) followed by permeabilization in 0.2% (v/v) Triton X-100 in PBS (PBST). Background staining was minimalized by incubating the sections in 50% (v/v) ethanol, 0.9% (v/v) hydrogen peroxide in PBS for 30 min to block endogenous peroxidase activity 0.9% followed by 4% (v/v) bovine serum albumin in PBS for 1 h at room temperature. A 1:100 dilution of the anti-caveolin-1 antibody was added to the slides and incubated overnight at 4 °C. After removing unbound antibody with PBST washes, immunoreactivity was detected with a biotinylated secondary antibody followed by an avidin-horseradish peroxidase complex (ABC kit, Vectastain). Immunoreactivity was visualized using a diaminobenzidine staining kit (Vectastain) for ~2 min. The reaction was terminated by dilution with water, and the sections were dehydrated, cleared in xylene, and coverslipped. Immunoreactivity was assessed in various brain regions by a person blind to the treatment of the mice.

Quantitation of Phospho-ERK1/2 Protein and Caveolin-1 Transcript Levels—Quantitation of U0126 inhibition of ERK1/2 phosphorylation as well as restoration of caveolin-1 transcription in SK-ERα cells was performed by measuring the optical density of bands using the scion imaging software program (Scion Corp.).

RESULTS

SK-N-MC Cells Stably Transfected with ERα and/or ERβ cDNA Respond to Estrogen—We examined the putative relationship between caveolin-1/2 and ERα/ERβ expression in neuroblastoma SK-N-MC cells in vitro. The SK-N-MC cell line has been demonstrated previously (33) to lack functional ERα and ERβ involved in ERE-mediated transcription. Therefore, expression vectors encoding wild type ERα and ERβ were stably transfected into SK-N-MC cells and subsequently characterized concerning the estrogen receptor status by RT-PCR analysis and luciferase assay. SK-N-MC cells overexpressing ERα cDNA, ERβ cDNA, or co-expressing ERα/β were named SK-ERα, SK-ERβ, or SK-ERα/β, respectively. Mock-transfected SK-N-MC cells were named SK01 and served as a control.

There were no ERα and ERβ mRNA transcripts detectable in SK01 cells after performing 25 PCR cycles as shown by RT-PCR (Fig. 1). RT-PCR analysis of SK-ERα cells and SK-ERα/β cells for ERα transcripts exhibited a specific band of the expected size using ERα-specific primers. Primer annealing sites are located within the steroid binding domain of the ER α cDNA. ERβ-mRNA transcripts were detected in ERβ-overexpressing cells (SK-ERβ) as well as in the SK-ERα/β double-transfectants using primers specific for a part of the steroid binding domain of ERβ cDNA. Taken together, we demonstrated that the corresponding receptor isoforms are transcriptionally expressed in the analyzed ER transfectants.

By employing an established luciferase-based reporter system (35), we tested the functional activity of the transfected ERα/β expression plasmids. The luciferase reporter plasmid contains 3 EREs linked to luciferase cDNA. When stimulating the cells with estrogen for 24 h, we observed a pronounced increase (~250–600-fold) in ERα and ERβ activity in the ER transfectants but not in the mock-transfected SK01 control cells. We next evaluated whether ER signaling is sensitive to the ER antagonist ICI 182 780. For this purpose we treated cells with ICI in the absence or presence of estrogen. The ER inhibitor ICI clearly abrogated the ERα/β activity in the ER transfectants when applied in combination with estrogen (data not shown).

Caveolin-1/2 Expression Is Negatively Regulated by ERα but not ERβ—In this study we demonstrated that SK-N-MC cells express high levels of caveolin-1/2 unlike most other previously analyzed neuroblastoma cell lines. Western blot analysis of cellular lysates derived from SK01 or ER-transfected SK-N-MC cells (SK-ERα, SK-ERβ, and SK-ERα/β) showed that the ectopic expression of ERα in SK-ERα cells led to a nearly complete abrogation of caveolin-1 and caveolin-2 expression (Fig. 2A). Unexpectedly, the observed effect occurred in a ligand-independent manner because there were no caveolin-1/2 isoforms detectable in estrogen-un-treated SK-N-MC cells. In contrast, ERβ-overexpressing cells (SK-ERα/β) exhibited high amounts of caveolin-1 and caveolin-2 proteins similar to caveolin-1/2 levels in SK01 cells. Estrogen treatment of SK01, SK-ERβ, and SK-ERα/β cells for 8–48 h did not markedly alter caveolin-1/2 expression.

Results derived from Western blot analysis were confirmed by cellular immunofluorescence using confocal laser scanning
microscopy. Caveolin immunostaining in estrogen-untreated SK01 and SK-ER\(\alpha\)/H9252 cells was characterized by the punctate distribution throughout the cytoplasm and the predominant location of the proteins at the cell membrane (Fig. 2B). Although SK01 control cells and ER\(\alpha\)/H9252-overexpressing cells (SK-ER\(\alpha\)/H9252) showed strong signals for both caveolin isoforms, ER\(\alpha\)/H9251-overexpressing cells (SK-ER\(\alpha\)/H9251) exhibited almost no detectable levels of caveolin-1/-2 proteins. The loss of caveolin-1 and caveolin-2 proteins might prevent the formation of caveolae in SK-ER\(\alpha\)/H9251 cells as it has been described for K562 cells lacking endogenous levels of caveolin-1 proteins (37).

We further examined by RT-PCR analysis whether the down-regulation of caveolin-1 and caveolin-2 expression is mediated at a transcriptional level. RT-PCR performed with 25 cycles produced a very faint amplification for caveolin-1 and caveolin-2 transcripts when using total RNA derived from estrogen-treated (8–48 h) and -untreated SK-ER\(\alpha\)/H9251 cells (Fig. 2C). On the other hand, caveolin-1- and caveolin-2-specific primers amplified a prominent band when applying total RNA of SK01, SK-ER\(\alpha\), or SK-ER\(\alpha\)/H9252 cells to RT-PCR. We observed no effect of estrogen treatment on the regulation of caveolin-1/-2 transcription in SK01, SK-ER\(\beta\), and SK-ER\(\alpha\)/H9252 cells. The results indicate an ER\(\alpha\)-mediated ligand-independent repression of caveolin-1 and caveolin-2 transcription in SK-ER\(\alpha\) cells, whereas ER\(\beta\) does not have any effect, or when co-expressed with ER\(\alpha\) (SK-ER\(\alpha\)/H9252) counteracts the caveolin-1/-2 silencing event. Treatment of SK-ER\(\alpha\) cells with the ER\(\alpha\) antagonist ICI 182 780 (10 \(\mu\)M) for 72 h did not restore caveolin-1 and caveolin-2 transcription in the cells (data not shown), pointing to a persistent change in the caveolin-1/-2 expression pattern in SK-ER\(\alpha\) cells.

The Demethylating Agent 5-Aza-2-deoxycytidine and the Histone Deacetylase Inhibitor Trichostatin A Partially Restore Caveolin Expression in SK-ER\(\alpha\) Cells—We further analyzed mechanisms that may be potentially involved in the caveolin-1/-2 silencing process in SK-ER\(\alpha\) cells. The 5'-regulatory regions of the caveolin genes have been demonstrated as TATA-less promoters containing CpG islands (38). Aberrant methylation of CpG loci within regulatory regions play a principal role in the tissue- and tumor-specific expression of genes by affecting the interaction of DNA with transcription factors (39). The role of CpG methylation and histone deacetylation in transcriptional silencing of the caveolin genes in our cellular model was investigated. SK-ER\(\alpha\) cells which originally do not express caveolin-1 and caveolin-2 were therefore incubated with different concentrations of the methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-AZA) and histone deacetylase inhibitor trichostatin A (TSA). Treatment of SK-ER\(\alpha\) cells with increasing doses of 5-AZA up to 4 days resulted in an up-regulation of caveolin-1 and caveolin-2 transcription as determined by RT-PCR analysis (Fig. 3). Inhibition of histone deacetylation by TSA did appear to re-activate the expression of the caveolin-1 gene but failed to re-activate caveolin-2 transcription even at the highest non-toxic dose (20 ng/ml).
5-AZA (μM) TSA (ng/ml)

**Fig. 3.** Induction of caveolin-1 and caveolin-2 expression in SK-ERα cells by treatment with the DNA methylation inhibitor 5-AZA and the histone deacetylase inhibitor TSA. SK-ERα cells were exposed for 4 days with the indicated concentrations of 5-AZA or 2 days with the indicated concentrations of TSA. Total RNA was extracted and submitted to RT-PCR (28 cycles) using caveolin-1- and caveolin-2-specific primers. Control SK01 cells expressing both caveolin members were used as a positive control. Actin RT-PCR provided a control for initial RNA amounts. M, DNA size marker.

### Differential Methylation Pattern of the Caveolin-1 Promoter Region in SK-ERα Cells and SK01, SK-ERβ, and SK-ERα/β Cells

Because numerous CpG loci constitute CpG islands in the 5′-flanking regions of caveolin genes, we further tested whether the transcriptional activity of the caveolin genes was related to the methylation status of certain CpG loci in caveolin-expressing SK01, SK-ERβ, and SK-ERα/β cells and caveolin-repressing SK-ERα cells. In our studies we focused on the analysis of the caveolin-1 promoter, because it has already been the subject of several investigations (36, 38). To analyze the methylation status high sensitivity mapping of methylated cytosines was carried out by sodium bisulfite modification before PCR amplification. PCR was performed for amplification of two distinct regions of the caveolin-1 promoter, designated A and B. Region A is spanning from position −877 to −525 relative to the translational start codon and contains 7 CpG dinucleotides, whereas region B is spanning from position −477 to −202 and contains 17 CpG dinucleotides (Fig. 4A). After modification and PCR, unmethylated cytosines appear as thymines, and methylcytosines appear as cytosines on the sequencing gel (Fig. 4C). To ensure an accurate methylation profile, we sequenced between 4 and 9 plasmid subclones from SK01, SK-ERα, SK-ERβ, and SK-ERα/β cells. For each of the sequenced clones, the presence or absence of a methylated cytosine at the analyzed CpG sites within the PCR fragments was scored (Fig. 4B). We found that almost all CpG dinucleotides of region A were unmethylated in the analyzed clones derived from SK01, SK-ERβ, and SK-ERα/β cells. One clone derived from SK-ERβ was found to have a single methylated CpG site at position 7. In two independent SK-ERα cell clones, which do not express caveolin-1, CpG sites 1–7 of region A showed a very high level of methylation. In these cells, 5-methylcytosine methylation frequency was in a range from 25 to 100%. For CpG positions 1–3 the mean values of methylation were 78–100%. All CpG dinucleotides of region B were unmethylated in SK01, SK-ERα, SK-ERβ, and SK-ERα/β clones (data not shown).

### Unaltered DNA Methyltransferase Transcription Levels in SK-ERα Cells

Next, we investigated a possible molecular basis underlying the aberrant methylation of the caveolin-1 promoter observed in the SK-ERα cells. A potential mechanism could be an up-regulation of methyltransferases in the ERα-overexpressing cells (SK-ERα) as it has been demonstrated for c-fos transformed fibroblasts (40). c-fos is a positively regulated target gene of ERα/estrogen (41). To clarify whether the hypermethylation of the caveolin promoter region is associated with an increased level of methyltransferase transcripts (42, 43) in SK-ERα cells, we performed RT-PCR to analyze the basal expression patterns of the three DNA methyltransferases DNMT1, DNMT3A, and DNMT3B (44, 45) in estrogen-un-treated SK01, SK-ERα, SK-ERβ, and SK-ERα/β cells. The mRNA levels of all three DNA methyltransferases appeared to be unaltered in the analyzed cells when normalized to the proliferation-associated gene histone H4 (46) (data not shown). These observations indicate that caveolin-1 promoter hypermethylation does not result from a simple overexpression of any of the three known DNA methyltransferase genes in SK-ERα cells.

### Participation of the MAP Kinase-Signaling Pathway in the Regulation of Caveolin-1 Expression in SK-ERα Cells

In the search for an upstream regulation of ERα-induced gene silencing, we analyzed the influence of MAP kinase-dependent signaling pathways on caveolin expression in SK-ERα cells. It was reported that constitutive activation of p42/44 MAP kinase results in a transcriptional down-regulation of caveolin-1 expression (47). We observed increased basal levels of the phosphorylated form of ERK1/2 kinase in SK-ERα cells compared with SK01 control cells and ERα/β-transfectants (Fig. 5). Treatment of the SK-ERα cells with the specific MAP kinase inhibitor U0126 for 3 days resulted in decreased levels of phosphorylated ERK1/2 as determined by Western blotting (Fig. 6A). Indeed, a partial up-regulation of caveolin-1 transcription could be observed by RT-PCR in the U0126-treated cells compared with the mock-treated controls (Fig. 6, B and C). Interestingly, U0126 treatment did not restore caveolin-2 expression in SK-ERα cells.

### Long Term Estrogen Exposure Leads to Down-regulation of Caveolin-1 Expression in Rodent Hypothalamic Brain Regions in Vivo

It has been shown in several studies that chronic estrogen exposure during development causes permanent changes in gene expression patterns accompanied by altered methylation of the corresponding gene promoter regions (48). To verify whether caveolin represents a target for ERs in the brain in vivo, we analyzed the long term effects of prenatal estrogen treatment on caveolin-1 expression in rodent hypothalamic brain regions. Therefore, pregnant mice were injected for 7 days with estradiol dipropionate, a synthetic estrogen. Brain sections were prepared from 4-week-old pups and stained using caveolin-1-specific antibodies. We focused on the caveolin-1 expression in hypothalamus because it represents a brain region highly responsive to estrogen, and it shows a great extent of ER expression (49). In control sections we observed an extensive caveolin-1 staining in the blood vessels throughout the brain (Fig. 7) as also shown by Ikezu et al. (24). Neuronal staining was particularly strong in the pre-optic area of the hypothalamus. After estrogen exposure, caveolin-1 expression was markedly down-regulated as seen both in the intensity of staining and the number of caveolin-1-positive cells. These findings suggest that prenatal estrogen treatment results in a persistent decrease in caveolin-1 protein levels in the hypothalamus during development.

### DISCUSSION

In our studies we have shown that ERα, and not ERβ, induces silencing of caveolin-1 and caveolin-2 gene transcription in the human SK-N-MC neuroblastoma cell line. This silencing process is accompanied by changes in the methylation pattern in the caveolin-1 promoter region, and it is partially reversible by inhibitors of methyltransferases and histone deacetylases, enzymes involved in the establishment of silenced chromatin structures. The steroid receptor subtype ERβ counteracts the ERα-mediated effects on caveolin expression. Moreover, we present strong evidence that in hypothalamic brain regions of mice, long term estrogen exposure led to marked decreases of caveolin-1 expression, indicating the presence of the caveolin gene.
gene silencing effect also in vivo.

Several lines of evidence indicate that estrogen and its receptors affect caveolin expression, and conversely, caveolin modulates the activity of ERs. However, there is a controversial discussion about the exact mode of caveolin regulation by estrogen. It has been demonstrated that estrogen administration leads to reduced levels of caveolin-1/-2 proteins in non-neuronal tissue such as uterine smooth muscle cells (31) and pial arteriols (30), whereas other investigators observed a slight up-regulation of caveolin-1 in aortic endothelial cells (32). Hence, the effect of estrogen on caveolin expression may be tissue-dependent. This difference could be due to the presence of different isoforms of ERs as well as the appropriate co-activators and co-repressors in the analyzed cellular systems. In recent years,

**FIG. 4.** Differential methylation pattern of the human caveolin-1 gene promoter region in SK01, SK-ERα, SK-ERβ, and SK-ERα/β cells. A, schematic illustration of the two analyzed regions (A and B) of the caveolin-1 promoter based on GenBank™ accession number AJ133269 is shown. Region A contains 7 CpG (1–7), and region B contains 17 CpG (8–24) dinucleotides that are numbered consecutively from 1 to 24. The translation start site is marked out with an arrow (+1). The nucleotide positions are numbered relative to translation start codon. The primers used for bisulfite genomic sequencing and their respective locations are indicated (s, sense primer; as, antisense primer). PCR products were subcloned, and individual clones were sequenced. B, each row of circles represents a single cloned allele of region A, whereas each circle depicts a single CpG site. Unmethylated or methylated cytosine residues within CpG dinucleotides are represented by open or closed circles, respectively. CpG sites of region B were all unmethylated in SK01 control cells and ER transfectants (data not shown). C, a chromatogram of representative sequences of the caveolin-1 promoter region A following bisulfite modification of DNA derived from SK01 and SK-ERα cells is shown. CpG dinucleotides (arrows) are corresponding to CpG loci in B.
an increasing number of co-activators and co-repressors have been identified which bind in a ligand-dependent or -independent fashion to ERs enhancing or suppressing activation of target gene transcription (8). Recently, caveolin-1 was identified as a co-activator exclusively for ERα (9). In addition to many other functions caveolin-1 mediates the ligand-independent activation of the steroid receptor ERα.

In the present study we evaluated the effects of the two ER isoforms ERα and ERβ on caveolin expression in neuronal cells in vitro and in vivo. The neuroblastoma cell line SK-N-MC used for in vitro studies was initially chosen because of its lack of functional ERs. We demonstrated that SK-N-MC cells express high levels of both caveolin isoforms 1 and 2. So far caveolin expression was considered not to be abundant in neuronal cells apart from a few exceptions (50, 51).

Interestingly, the ectopic expression of wild type ERα in SK-N-MC results in an almost complete abrogation of the caveolin gene transcription. The observed gene silencing mediated by ERα applies to both caveolin isoforms 1 and 2. In contrast, many other regulatory factors like Neu tyrosine kinase (52), MAP kinase, v-Src, v-Abl, or v-Raf (47) exert an influence on the regulation of only one caveolin isoform (caveolin-1). The remaining, very low basal transcription of the caveolin-1/-2 genes excludes the incident of a chromosomal deletion of the caveolin-1/-2 gene locus in SK-ERα cells, although loss of heterozygosity on 7q31.1 has been described for several breast or ovary carcinomas (53).

The silencing effect of ERα on caveolin expression occurs in a ligand-independent manner and is not reversible by the ER antagonist ICI 182 780. These observations point to a regulatory mechanism being involved in the permanent silencing of the caveolin-1 and caveolin-2 gene activity. A powerful mechanism for the constitutive suppression of gene activity comprises the concerted action of DNA methylation and histone deacetylation (39). There are several studies (54, 55) describing de novo methylation of CpG islands in normal and cancer cells, thereby silencing the expression of the associated genes. Indeed, the caveolin-1 and caveolin-2 promoter regions are embedded within CpG islands. Hypermethylation of the caveolin-1 promoter has already been reported to be associated with decreased caveolin-1 expression in various breast cancer cell lines such as MCF-7 and T-47D (38) as well as in prostate cancer (36). The analysis of two distinct regions of the caveolin-1 promoter revealed that certain CpG dinucleotides were highly methylated in caveolin-1 suppressing SK-ERα cells, whereas the same sites were unmethylated in SK01 control cells. Interestingly, a number of particular CpG sites remained unaffected from de novo methylation in all analyzed cells. Similar results were obtained for the determination of the methylation pattern of DNA derived from MCF-7 cells used as controls. Because MCF-7 and T-47D cells exhibit relatively high levels of endogenous ERα (56), we hypothesize an inverse correlation between ERα expression at high levels and decreased caveolin-1 gene expression through modulation of the caveolin-1 promoter methylation status.

Treatment of SK-ERα cells with the methyltransferase inhibitor 5-AZA leads to a restoration of caveolin-1 and caveolin-2 mRNA, indicating that CpG island hypermethylation is a crucial event in the inactivation of the caveolin isoforms. Moreover, inhibition of histone deacetylases by TSA re-activated caveolin-1 but failed to re-activate caveolin-2 transcription. Thus, histone deacetylation and subsequent chromatin condensation appears to be playing a role for caveolin-1 silencing in SK-ERα cells.

The molecular mechanisms responsible for a region-specific de novo methylation are not well understood. As caveolins have
been reported to be down-regulated by a number of activated oncogenes, it might be possible that ectopic ERs expression leads to either a directly ERα-targeted or indirect suppression of caveolin gene expression. The resulting constitutive suppression of caveolin genes caused by the permanent presence of ERs could subsequently lead to hypermethylation as a secondary event, which stabilizes the transcriptionally inactive status of caveolin promoters.

We assume that stable down-regulation of caveolin in SK-ERα cells prevents the formation of caveolae in the cell membrane, which might have various consequences for the cells. Caveolae are proposed to be important integrators for the cross-talk of different signal transduction pathways. For example, targeted down-regulation of caveolin-1 transcription by an antisense approach results in an hyperactivation of the p42/p44 MAP kinase cascade in NIH3T3 cells (57).

We investigated the MAP kinase cascade in the search for an upstream modulation of the caveolin gene silencing. We found high basal levels of activated ERK1/2 in SK-ERα cells. Treatment of the cells with the potent MEK inhibitor U0126 slightly increased caveolin-1 expression at a transcriptional level, suggesting a participation of MAP kinase pathways in suppression of caveolin-1 in SK-ERα cells. Similar results were obtained by Wiechen et al. (58), showing that in the human ovarian carcinoma cell line OAW42, which is presumed to suppress caveolin-1 transcription through DNA methylation, caveolin-1 expression was up-regulated after inhibition of MEK1/2 by PD98059.

A second isoform of the estrogen receptor, ERβ, was discovered a few years ago (6). Comparison of amino acid sequences of ERα and ERβ reveals a 95% homology in the DNA binding domain, 58% homology in the ligand binding domain, and a high sequence diversity in the N-terminal domain, the hinge domain, and the C-terminal domain (6). The ERβ protein exhibits similar affinity to estrogen and consensus ERE as ERα (59, 60). When co-expressed with ERα, the steroid receptor subtypes form heterodimeric complexes within target cells (7). There is some evidence that ERβ modulates the action of ERα, showing that heterodimers of ER subtypes result in receptor activity which is distinct from that of homodimers (61, 62).

In our studies we demonstrated that ectopic expression of wild type ERβ in SK-N-MC cells does not alter caveolin-1 and caveolin-2 expression at a transcriptional and translational level compared with SK01 control cells. When co-expressing both receptor subtypes at equal levels, the silencing effect of ERα on caveolin-1/2 expression is abolished by the presence of ERβ. In addition, we found that the same caveolin-1 promoter region, which has been shown to be strongly methylated in ERα-overexpressing cells, was non-methylated in SK-ERβ and SK-ERα/β caveolin-1-expressing cells. These observations are in line with the previous results illustrating that the methylation status of the caveolin-1 gene promoter region correlates with the silenced stage of caveolin-1 gene transcription. In summary, ERβ counteracts the ERα-mediated down-regulation of caveolin expression in SK-N-MC cells.

Furthermore, we have shown that caveolin-1 expression is targeted by ERs/estrogen in vitro in a model of prenatal estrogen long term treatment of mouse embryos. As already reported by other investigators (23, 24), we found a strong caveolin-1 expression in endothelial cells in the brain, where caveolin-1 has been supposed to be involved in transcytosis of certain proteins and in regulation of endothelial nitric-oxide synthase activity. In addition to blood vessels, cells of neuronal origin also exhibit caveolin-1 proteins. Hypothalamic neural cells, which partially appear to express high levels of caveolin-1, show a marked and persisting down-regulation of caveolin-1 after estrogen treatment. The hypothalamic brain region is highly susceptible to effects of the steroid hormone estrogen, because it is one prime target site of estrogen action in the brain (49). The transfer from in vitro to in vivo data has intrinsic limitations because levels of ectopically expressed ERs might have been higher in SK-N-MC cells than those expressed endogenously in the brain. This might explain the observation that ERs causes ligand-independent down-regulation of caveolin in vitro, whereas in vivo, long term estrogen treatment leads to similar effects in ER-rich brain regions. Nevertheless, results derived from both models (in vitro and in vivo) indicate a negative reciprocal relationship between ERs/estrogen and
the ER co-activator caveolin in neuronal cells. Moreover, we have demonstrated the establishment of a stable phenotype, which is characterized by the maintained suppression of caveolin expression induced by ERs/estrogen. In summary, our findings may have important implications for the following: (i) a better understanding of the functional interaction between caveolin and ERs in tumor cells and of the consequences for tumorigenesis as ERs are reported to play a critical role in the pathogenesis and prognosis of certain types of cancer; (ii) the discovery of new mechanisms leading to epigenetic alterations in ER target gene expression in neuronal and non-neuronal tissue.

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