p38 affect the apoptosis process at the premitochondrial or mitochondrial stage.

The effect of U0126 on the several steps of apoptosis described above was also investigated in CC3 cells. First, in the loss of mitochondrial membrane potential using the fluorescent probe JC-1, the percentage of the R2 population in the presence of 10^{-6} M dexamethasone for 48 hours was increased by U0126 from 9.4% to 26.9%. In the loss of mitochondrial membrane potential using the other fluorescent probes $DiOC_6$, the low-intensity population of $DiOC_6$ in the presence of dexamethasone was increased by U0126 from 6.5% to 47.8% (Fig. 3A). The population of cells containing activated caspase-3 in the presence of 10^{-7} M dexamethasone for 48 hours was increased from 10.2% to 25.2% by the concomitant use of 10 μM U0126 (Fig. 3B). As for DNA fragmentation, apoptotic cells in the sub-G_0/G_1 population in the cell-cycle analysis using PI in the presence of 10^{-6} M dexamethasone for 48 hours ware increased by the treatment of 10 μM U0126 from 6.15% to 18.3% (Fig. 3C). Chromatin condensation was induced by the concomitant use of dexamethasone and U0126 (Fig. 3D). These results suggest that the treatment of U0126 relieves glucocorticoid resistance in CC3 cells at the premitochondrial or mitochondrial stage.

To directly demonstrate the hypothesis that ERK inhibits and p38 enhances glucocorticoid-induced T-cell apoptosis, the effects of transient expression of constitutive-active MEK1 or MKK6 were investigated. The low transfection efficiency in T cells made it difficult to analyze the effects of gene expression product by transient transfection. Nucleofector (Amaxa Biosystems, Cologne, Germany) dramatically improved the transfection efficiency. The proportion of cells expressing more than 10-fold the GFP fluorescence after transfection of pEGFP-C1, an EGFP expression vector, was 7.8% ± 2.8% (mean ± SD) (Fig. 4A). Transfection of the expression vectors for constitutive-active MEK1 (pFC-MEK1) or MKK6 (pcDNA3.1-MKK6 SETE) with pEGFP-C1 at a concentration ratio of 4 to 1 ensured the expression of MEK1 or MKK6 in the GFP-expressing cells. Cells transfected with the control vector pcDNA3.1 and pEGFP-C1 at a concentration ratio of 4 to 1 were incubated in the absence or presence of dexamethasone, and the cells expressing more than 10-fold the GFP fluorescence were sorted and analyzed for annexin V binding. Treatment with dexamethasone increased the population of annexin V–binding cells from 3.5% to 30.6% (Fig. 4B). In a similar manner, the expression vectors for constitutive-active MEK1 or MKK6 were transfected with pEGFP-C1. In cells expressing constitutive-active MEK1 or MKK6, 20.9% and 54.3% of cells were positive for annexin V binding, respectively, in the presence of dexamethasone (Fig. 4B). Since U0126 also inhibits ERK5 at higher concentrations, the effect of constitutive-active form of MEK5, a direct activator of ERK5, was also investigated. In cells expressing constitutive-active MEK5 or a control expression vector, 35.8% and 38.9% of cells were positive for annexin V binding, respectively (Fig. 4B). These data from the overexpression study suggest that ERK (ERK1/2) inhibits and p38 enhances glucocorticoid-induced T-cell apoptosis, and that ERK5 shows little effect if any.

In order to investigate the effect of knocking down ERK or p38, CC7 cells were transfected with siRNA for ERK1/2 or p38α/β or FAM-labeled control siRNA by Nucleofector. Since SB203580 inhibits both p38α and p38β among P38 isoforms, the inhibition of p38 by SB203580 is considered to be through p38α/β. Almost all cells transfected with FAM-labeled control siRNA showed FAM fluorescence; the transfection efficiency of siRNAs into CC7 cells was considered to be near 100%.

The mRNA levels of ERK1/ERK2 or p38α/p38β in CC7 cells transfected with siRNA for ERK1/2 or p38α/β, respectively, was analyzed by real-time PCR. Given the mRNA level for the control siRNA 1.0, the relative mRNA levels of ERK1, ERK2, p38α, and p38β were 0.13, 0.12, 0.54, and 0.17, respectively (Fig. 4C).

Treatment with dexamethasone increased the population of annexin V binding–positive cells from 5.5% to 58.8% in cells transfected with control siRNA. In cells transfected with ERK1/2 or p38α/β, 67.5% and 49.2% of cells were positive for Annexin V binding, respectively, in the presence of dexamethasone. In the absence of dexamethasone, siRNA for ERK1/2 or p38α/β showed no effect on annexin V binding, respectively (Fig. 4D).

These data show that knocking down ERK1/2 or p38α/β enhances or inhibits glucocorticoid-induced T-cell apoptosis, respectively.

Analysis of the effect of ERK or p38 on the transactivation or transrepression function of GR

Next, the effects of the activation of ERK or p38 on the transactivation or transrepression function of GR were investigated. First, the transactivation function of GR was analyzed using both $(GRE)_2$-tk-luc and MMTV-luc as reporter plasmids. In cells transfected with the control vector and $(GRE)_2$-tk-luc, dexamethasone treatment increased the relative luciferase activity from 0.01 ± 0.002 to 0.05 ± 0.003. Transfection with the expression vectors for constitutive-active MEK1, MKK3, or MKK6 increased the luciferase activity to 0.10 ± 0.015, 0.08 ± 0.023, and 0.08 ± 0.011, respectively, in the presence of dexamethasone (Fig. 5A). Then, MMTV-luc was used as a reporter plasmid, and similar experiments were performed. In the cells transfected with the control vector or the expression vectors for constitutive-active MEK1, MKK3, or MKK6 in the presence of dexamethasone, the relative luciferase activity was 0.17 ± 0.005, 0.15 ± 0.005, 0.17 ± 0.010, and 0.17 ± 0.008, respectively. In the untreated cells, the luciferase activity was nearly at the background level (Fig. 5B). These data suggest that both ERK and p38 either activate or do not change the GR transactivation function, depending on the target genes.

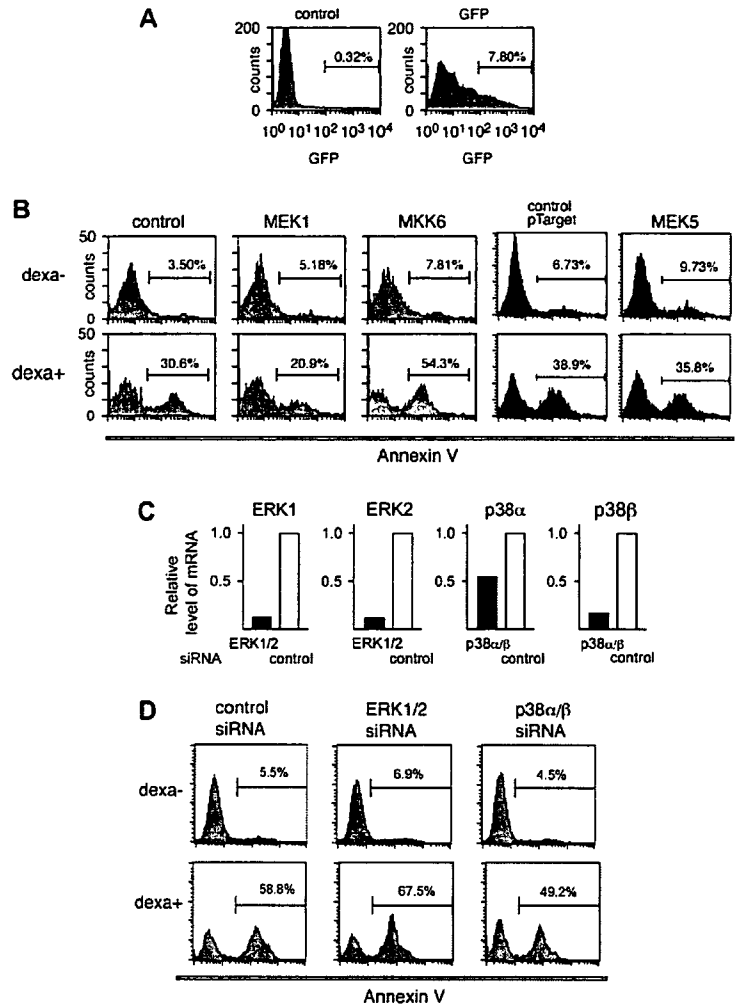

Figure 4. The effects of activation or inhibition of MAP kinase pathways on the externalization of phosphatidylserine. 5×10^6 CC7 cells were transfected with 1 μg of pEGFP-C1 and 4 μg of either a vector plasmid encoding constitutive-active MEK1, MKK6, or MEK5 and a control expression vector, and treated with or without 10^{-6} M dexamethasone for 40 hours. The cells exhibiting more than 10-fold of GFP fluorescence were sorted (**A**) and analyzed for annexin V binding (**B**). The number in each histogram in (**B**) indicates the percentage of annexin V–binding cells. (**C**,**D**) CC7 cells were transfected with siRNA for ERK1/2, p38α/β, or control siRNA and cultured for 48 hours. The comparative mRNA level for ERK1, ERK2, p38α, and p38β was calculated with quantitative real-time PCR method, respectively (**C**). The ratio of annexin V–positive CC7 cells, in which mRNA level of ERK1/2 or p38α/β was downregulated by siRNA, was analyzed (**D**). The dot plots and histograms represent one of three independent experiments. The histograms represent one of three independent experiments.

Then, the transrepression function of GR was investigated by analyzing the degree of repression of TNF-α-activated nuclear factor-κB (NF-κB) activity by dexamethasone. The luciferase activity in the absence of TNF-α in cells transfected with the control vector was set as 1. In the control cells, dexamethasone suppressed the TNF-α-activated NF-κB activity from 9.8 ± 1.5 to 4.4 ± 1.6. In cells transfected with the expression vectors for the constitutive-active MEK1, MKK3, or MKK6, dexamethasone treatment suppressed the TNF-α-activated NF-κB activity from 8.5 ± 1.5, 10.6 ± 2.3, and 10.3 ± 1.7 to 4.0 ± 1.3, 4.3 ± 1.6, and 4.5 ± 0.4, respectively (Fig. 5C). These data suggested that neither ERK nor p38 has any effect on transrepression function of GR. The results of the effects of ERK and p38 on the transactivation or transrepression function of GR could not explain the opposite effects of ERK and p38 on glucocorticoid-induced T-cell apoptosis, suggesting that the main target of ERK and p38 in glucocorticoid-induced T-cell apoptosis is not the level of transcription regulated by GR, although this possibility cannot be excluded.

Analysis of the steady-state protein expression levels of bcl-2 and IAP family members

As described before, apoptosis through mitochondria is regulated by *bcl*-2 family members, which consist of both anti-apoptotic members, such as *bcl*-2 and *bcl*-xL, and

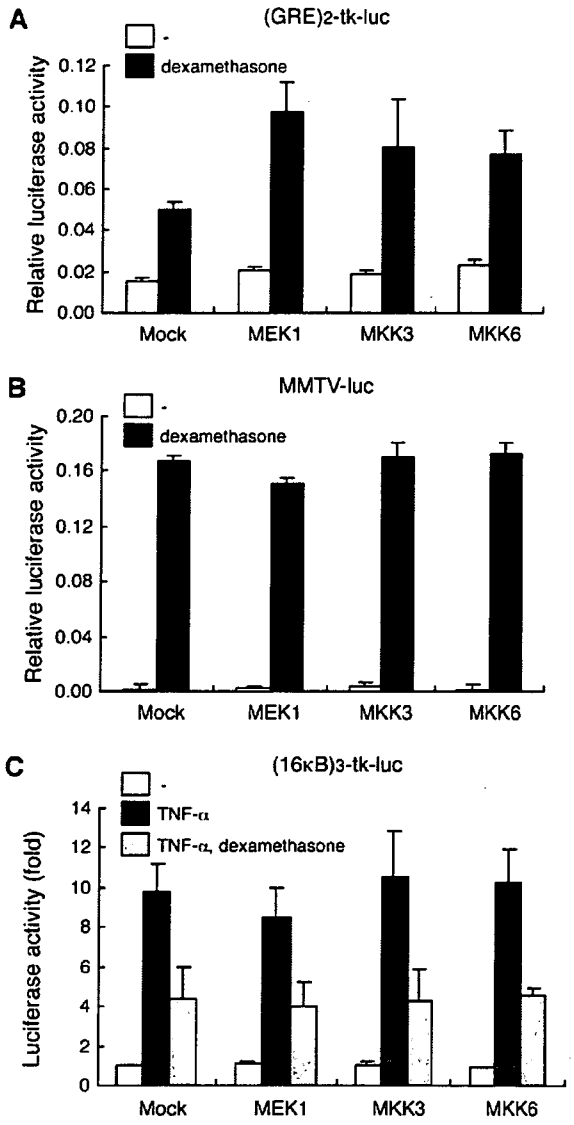

Figure 5. The effect of the activation of ERK or p38 on the transactivation or transrepression function of GR. CC7 cells were transfected with 800 ng GR-dependent luciferase reporter ($(GRE)_2$-tk-luc (A) or MMTV-luc (B)), 200 ng pRL-CMV, 400 ng pCMX-GR, and 600 ng pcDNA3.1 or an expression vector encoding constitutive-active MEK1, MKK3, or MKK6, and then treated with or without 10^{-6} M dexamethasone for 18 hours. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized by the *Renilla* luciferase activity. (C) CC7 cells were transfected with 800 ng NF-κB-dependent luciferase reporter $(16\kappa B)_3$-tk-luc, 200 ng pRL-CMV, 400 ng pCMX-GR, and 600 ng pcDNA3.1 or an expression vector encoding constitutive-active MEK1, MKK3, or MKK6, and then treated with TNF-α in the presence or absence of 10^{-6} M dexamethasone for 24 hours. The luciferase activity in mock-transfected cells with no treatment was set as 1.0.

pro-apoptotic members, such as Bak, Bax, and Bad. Therefore, the steady-state protein expression levels of *bcl*-2, *bcl*-xL, Bak, Bax, and Bad was examined by Western blotting analysis in untreated CC7 cells or CC7 cells treated with U0126 and/or dexamethasone for 24 hours. To lessen the effect of the decrease in the protein level accompanying apoptosis itself, the incubation was performed for 24 hours. The glucocorticoid-induced apoptosis in CCRF-CEM cells is known to become evident after 24 hours [18]. As a result, no remarkable changes in the protein levels were detected. In addition, as other regulators of apoptosis, two IAP family members, c-IAP1 and XIAP, were also analyzed for their protein expression levels; no remarkable changes in the levels of these proteins were detected (Fig. 6).

Effect of U0126 on several dexamethasone-resistant cell lines

As described before, apoptosis was induced in dexamethasone-resistant CC3 cells in the presence of U0126 (Fig. 2B). To examine whether apoptosis is induced in other dexamethasone-resistant cell lines, six human T cell lines, namely Jurkat, JM, RPMI-8402, PEER, CCRF-HSB-2, and HPB-ALL, were tested for annexin V binding in a similar manner. No differences in annexin V binding were found between the presence and absence of 10^{-6} M dexamethasone for each cell line. In the presence of U0126, however, 18.9% ± 4.0% and 28.4% ± 1.3% of PEER and CCRF-HSB-2 cells respectively were positive for annexin V binding in cells, although slight increases in the annexin V binding were detected by treatment with U0126

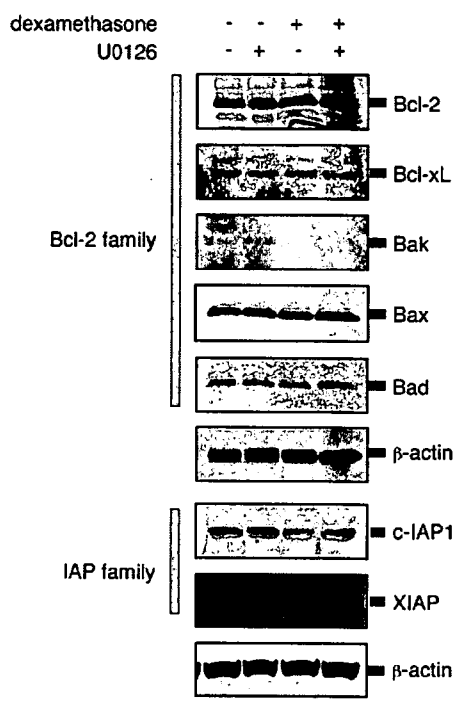

Figure 6. Analysis of the steady-state protein expression levels of *bcl*-2 and IAP family members. CC7 cells were treated with or without 10 μM U0126 in the presence or absence of 10^{-6} M dexamethasone for 24 hours and then lysed with RIPA buffer. A total of 20 μg protein was loaded in each lane. The protein expression levels of *bcl*-2, *bcl*-xL, Bak, Bax, Bad, c-IAP1, XIAP, and β-actin were analyzed with immunoblotting.

alone. No increases in annexin V binding were found in the other four T cell lines by the concomitant use of dexamethasone and U0126 (Fig. 7A). It was investigated whether the treatment of U0126 actually inhibited the activation of ERK in PEER and CCRF-HSB-2 cells, respectively. Phosphorylated ERK1 (p44) and ERK2 (p42) decreased after the treatment of 10 μM U0126 in the absence or the presence of dexamethasone in both cells (Fig. 7B), showing that U0126 inhibits ERK activation in both PEER and CCRF-HSB-2 cells. These results suggest that some of the glucocorticoid-resistant cell lines were converted into glucocorticoid-sensitive cell lines by the inhibition of ERK activation.

Discussion

In this report, the results suggest that ERK inhibits and p38 enhances glucocorticoid-induced T-cell apoptosis at several steps of apoptosis, namely the loss of mitochondrial membrane potential, caspase activation, externalization of phosphatidylserine, DNA fragmentation, and chromatin condensation. This notion was further supported by experiments on the activation of ERK or p38 by transient transfection of constitutive-active MAPKKs or on the inhibition of ERK or p38 by siRNA. Since ERK and p38 are thought to play pivotal roles in differentiation, proliferation, cell survival, and apoptosis in T cell [11,19,20], clarification of the cross-talk with glucocorticoid is extremely intriguing. It may also provide some clues for clarifying the mechanism of glucocorticoid-induced T-cell apoptosis. However, since the roles of both glucocorticoid and MAPKs in apoptosis depend on the cell type, these findings cannot be generalized for all cell types.

Our findings also suggest that ERK and p38 possibly affect the apoptosis process at the premitochondrial or mitochondrial stage (Fig. 8). Among the premitochondrial stages, transcriptional regulation by GR is a possible target of ERK and p38. It was reported that GR was not a direct target of ERK and p38 [21], and that coactivators of GR could be possible targets [22–25]. In our study, both ERK and p38 activated GR-mediated transactivation when $(GRE)_2$-tk-luc was used as the reporter gene, but not when MMTV-luc was used as reporter gene. The effects of ERK and p38 on GR-mediated transactivation might depend on the target genes [26]. On the other hand, neither ERK nor p38 had much effect on the transrepression by GR. In any case, the opposite effects of ERK and p38 on glucocorticoid-induced T-cell apoptosis could not be explained at the level of transcription, although the possibility was not ruled out.

Pro-apoptotic and anti-apoptotic *bcl*-2 family members that regulate apoptosis through mitochondria are other possible targets of ERK and p38. Since the protein levels of several *bcl*-2 members were not changed after treatment with U0126, we could not identify the targets of ERK. One possibility is that the phosphorylation status or subcellular localization of *bcl*-2 family members might be changed by ERK [27,28].

It is known that glucocorticoid induces G1 arrest in the cell cycle in glucocorticoid-induced T-cell apoptosis [29]. It was reported that p38 was necessary for G1 arrest in cycling cells [12]. It was hypothesized that p38 was necessary

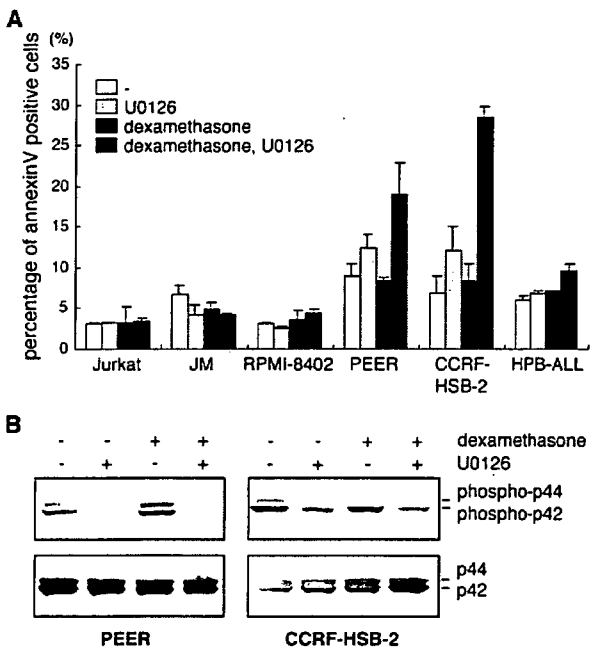

Figure 7. Effects of U0126 on glucocorticoid-induced apoptosis in several dexamethasone-resistant cell lines. **(A)** Six human T cell lines, namely Jurkat, JM, RPMI-8402, PEER, CCRF-HSB-2, and HPB-ALL, were treated with or without 10 μM U0126 in the presence or absence of 10^{-7} M dexamethasone for 48 hours, and then analyzed for annexin V binding. **(B)** PEER and CCRF-HSB-2 cells were treated with or without 10^{-6} M dexamethasone in the presence or absence of 10 μM U0126 for 24 hours. Cell lysate was subjected to immunoblotting with anti-phospho ERK1/2 antibody or anti-ERK1/2 antibody.

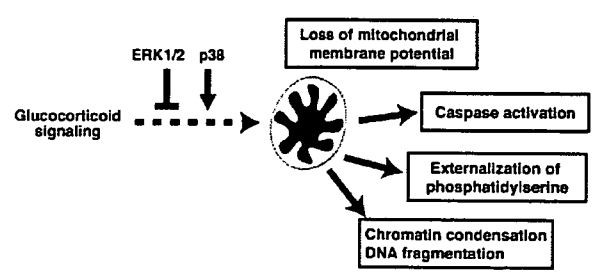

Figure 8. ERK1/2 and p38 possibly affect the apoptotic process at the premitochondrial or mitochondrial stage. After the apoptotic signal reaches the mitochondria, several factors are released from the intermembrane space, leading to activation of the apoptosis cascade. Apoptosis through mitochondria is regulated by *bcl*-2 family members. See the text for details. ERK and p38 are supposed to affect the apoptosis process at the premitochondrial or mitochondrial stage.

for G1 arrest in glucocorticoid-induced T-cell apoptosis, but SB203580 alone had little effect on the cell cycle itself in CC7 cells (Fig. 3C and data not shown). Thus, this hypothesis seems to be unlikely.

ERK is mainly activated by the Ras-Raf pathway [9]. The Ras-Raf-ERK pathway is activated through the stimulation of T-cell receptor or by several growth factors. The finding that ERK inhibits glucocorticoid-induced T-cell apoptosis, although reported by others [26,30,31], explains the fact that apoptosis through T-cell receptor antagonizes glucocorticoid-induced apoptosis . Moreover, it was shown in this report that inhibition of the ERK pathway restored dexamethasone sensitivity in some glucocorticoid-resistant T cells. This is especially significant when considering clinical measures for glucocorticoid resistance. The combination of both inhibitors of the ERK activation pathway and glucocorticoid may help us to cope with glucocorticoid resistance. Regarding the reason why inhibition of ERK did not restore dexamethasone sensitivity in all the glucocorticoid-resistant T cell lines, there are several possibilities. One is that some of the cells may be devoid of functional GR. Another is that the survival of some cells may depend principally on other signals including that of PI3-kinase.

A variety of signals including lipopolysaccharide (LPS), inflammatory cytokines such as TNF-α and IL-1, hormones, G protein–coupled receptors, osmotic and heat shock, and other stresses activate p38 [12]. Some of these stimuli may also be used with glucocorticoid to overcome glucocorticoid resistance.

Experimental procedures

Reagents

Dexamethasone, antibody for β-actin and HRP-conjugated anti-goat IgG were purchased from Sigma (St. Louis, MO, USA), U0126 and SB203580 were from Calbiochem (San Diego, CA, USA), TNF-α was from R&D Systems, Inc. (Minneapolis, MN, USA), and JC-1, $DiOC_6(3)$ and Hoechst 33342 were from Molecular Probes, Inc. (Eugene, OR, USA). The antibodies for ERK1/2, p38 MAPK, JNK/SAPK, phospho-ERK1/2, phospho-p38 MAPK, phospho-JNK/SAPK, Bax, HRP-conjugated anti-mouse IgG, and HRP-conjugated anti-rabbit IgG were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies for *bcl*-2, *bcl*-xL, Bak, Bad, c-IAP1, and XIAP were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). pRL-CMV was from Promega Co. (Madison, WI, USA). pEGFP-C1 was from Clontech (Palo Alto, CA, USA). pcDNA3.1 was from Invitrogen Co. (Carlsbad, CA, USA). pFR-luc, pFC-MEK1, pFC-MKK3, pFC-MEKK, pFA2-Elk1, pFA2-CHOP, and pFA2-cJun were from Stratagene (La Jolla, CA, USA). siRNAs for ERK1, ERK2, p38α, p38β, and FAM-labeled control siRNA were obtained from Ambion (Austin, TX, USA).

Plasmid construction

pcDNA3.1-MKK6-SETE was a kind gift from Dr. Yukiko Gotoh (Tokyo, Japan). Human MEK5 cDNA was amplified by RT-PCR method with ExTaq (Takara, Japan) and cloned into pTarget mammalian expression vector (Promega, Madison, WI, USA). The primers used for the amplification were 5′-ATGCTGTGGCTAGCCCTTGGC-3′ and 5′-TCACGGGGGCCCCTGCTGG-3′. Constitutive active form of MEK5, MEK5-SDTD [32], was constructed by replacing both Ser313 and Thr317 with Asp with Site-directed II mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutagenesis primers were 5′-GCACTCAGCTGGTGAATGATATAGCCAAGGATTATGTTGGAACAAATGC-3′ and 5′-GCATTTGTTCCAACATAATCCTTGGCTATATCATTCACCAGCTGAGTGC-3′. cDNA sequence of MEK5-SDTD was confirmed with sequence analysis. The firefly-luciferase reporter vector and MMTV-luc have been described previously [33]. tk-Luc was constructed by cloning the −109 to +37 region of the herpesvirus thymidine kinase promoter into the *Bgl* II and *Hind* III sites of the pGL3-basic vector (Promega, Madison, WI, USA). A pair of oligonucleotides, 5′-TAGCCCCTTGGAAATTCCGGAGCTTGGAAATTCCGGAGCTTGGA AAT TCCGGAG-3′ and 5′-TCGACTCCGGAATTTCCAAGCTCCGGAATTTCCAAG CTCCGGAATTTCCAAGGGGCTA-3′, were annealed, resulting in a double-stranded oligonucleotide with both a blunt end and a *Xho* I–compatible overhang that was then inserted into the *Sma* I and *Xho* I sites of tk-Luc, giving rise to $(16\kappa B)_3$-tk-Luc containing three copies of the κB sites found in the human ICAM-1 gene promoter [34]. A pair of oligonucleotides, 5′-GTTACAAACTGTTCTGTTACAAACTGTTCT-3′ and 5′-TCGAAGA ACAGTTTGTAACAGAACAGTTTGTAAC-3′, were annealed, resulting in a double-stranded oligonucleotide with both a blunt end and a *Xho* I–compatible overhang that was then inserted into the *Sma* I and *Xho* I sites of tk-Luc, giving rise to $(GRE)_2$-tk-Luc containing two copies of the glucocorticoid response element (GRE) [35].

Cell culture and limiting dilution

All the human T cell lines, except for CCRF-CEM, were provided by the Fujisaki Cell Center (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan). CCRF-CEM was obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Fetal bovine serum was treated with dextran-coated charcoal as described previously [36]. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 292 μg/mL L-glutamine, and 5 μM 2-mercaptoethanol. Regarding the limiting dilution, CCRF-CEM cells were diluted in a volume of 300 μL at a density of 1 cell/mL in 96-well plates. The wells containing a single cell, as observed under a microscope, were selected for isolating subclones.

Detection of apoptosis

Externalization of phosphatidylserine was detected with an annexin V–FITC kit (Immunotech Co., Marseilles, France) or annexin V–PE (Pharmingen, San Diego, CA, USA). Briefly, cells were washed with phosphate-buffered saline (PBS) and resuspended with annexin V binding buffer, followed by the addition of annexin V and PI. The cells were analyzed using an EPICS flow cytometer (Beckman Coulter, Inc., Miami, FL, USA) or a FACScan (BD Biosciences, San Diego, CA, USA), and the percentage of annexin V–binding cells was calculated. Regarding the loss of mitochondrial membrane potential, 2×10^5 CC7 cells were incubated in culture medium containing 5 μM JC-1 or 40 μM $DiOC_6(3)$ at 37°C for 30 minutes, and then analyzed using a FACScan. Activated caspase-3 was detected with PE-conjugated anti-active caspase-3 antibody (Pharmingen). Anti-active caspase-3 antibody recognizes the heterodimer of 17- and 12-kDa subunits that were derived from the 32-kDa proenzyme. Approximately 1×10^6 cells were fixed in PBS containing 1% paraformaldehyde, permeabilized, and stained intracellularly in PBS containing 0.1% saponin and 4% FBS. Cells were then washed twice in PBS containing 4% FBS and analyzed on a FACScan flow cytometer. Regarding the degradation of DNA, 1×10^6 CC7 cells were washed with ice-cold PBS, resuspended with 2 mL of ice-cold 70% ethanol, and then incubated for 4 hours at 4°C. The cells were washed with PBS twice, resuspended with 1 mL of PBS containing 10 μg/mL RNase A, incubated for 30 minutes at 37°C, and propidium iodide was added to a final concentration of 10 μg/mL. The PI intensity was measured for 1×10^5 events using a FACScan, and the percentage of the sub-G_0/G_1 fraction was analyzed using CellQuest software. To assess the apoptotic nuclear morphology, CC7 cells were stained with 2 ng/mL Hoechst 33342, and observed under a confocal microscope (Leica Microsystems, Wetzlar, Germany).

Transfection

CC7 cells were transfected using Nucleofector (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's instruction. Briefly, 5×10^6 CC7 cells were washed with PBS, resuspended with the cell line nucleofector kit V solution, and mixed with plasmid DNA in a total volume of 100 μL. The cell mixture was transferred to a cuvette and transfected with program C-16 using Nucleofector. Transfected cells were cultured in 2 mL RPMI 1640 medium at 37°C for 3 hours, and treated with various stimuli.

Real-time PCR

After total RNA was purified with RNeasy kit (Qiagen, Tokyo, Japan), first-strand cDNA synthesis was performed with QuantiTect reverse transcription kit (Qiagen). Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The primers used were follows: 5′-CCTTCCAACCTGCTGCTCAAC-3′ and 5′-TTCTGTCAGGAACCCTGTGTGATC-3′ for ERK1, 5′-ACCTGCTGGA CCGGATGTTAA-3′ and 5′-GGTGAGC-CAGCGCTTCCT-3′ for ERK2, 5′-TGTGAATGAA-GACTGTGAGCTGAAGATT-3′ and 5′-GCCACGTAGC CTGTCATTTCATC-3′ for p38α, and 5′-CTTGGAAG-GATGCTGGTGCT-3′ and 5′-CCTCAACGCTCTCATCA-TATGG-3′ for p38β. Samples were subjected to real-time PCR with SYBR Premix Ex Taq (TAKARA) using LightCycler (Roche, Mannheim, Germany). Using comparative delta Ct method, the mRNA level of target gene was calculated.

Luciferase assay

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Two $\times 10^5$ CC7 cells were washed with PBS and lysed with 100 μL of passive lysis buffer. Fifty μL of the lysate was subjected to the luciferase assay. The luciferase activity was detected with a Lumat LB 9507 luminometer (Berthold Technology Co., Bad Wildbad, Germany).

Immunoblotting

Cells were washed with PBS and lysed with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], Complete Mini Protease Inhibitor Cocktail [Roche Diagnostics, Mannheim, Germany] in PBS). The lysates were collected and centrifuged at 15,000 rpm for 15 minutes at 4°C. The protein concentrations were measured with BCA protein assay (Pierce, Rockford, IL, USA). A total of 20 μg protein was subjected to SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated in blocking buffer (5% nonfat skim milk in PBS or Tris-buffered saline (TBS) containing 0.05% Tween-20) for 1 hour. The membrane was incubated for 2 hours in the primary antibody, washed with PBS or TBS containing 0.05% Tween-20, and then incubated for 1 hour in the secondary antibody. The protein expression levels were visualized with an ECL Plus kit (Amersham Biosciences, Buckinghamshire, England) and a STORM8600 image analyzer (Amersham Biosciences).

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REVIEW

Differentiation and Regeneration of Adrenal Tissues: An Initial Step toward Regeneration Therapy for Steroid Insufficiency

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Abstract. In animal experiments, adrenal cortical tissue has been successfully regenerated through xenotransplantation of cloned adrenocortical cells, suggesting that the intraadrenal stem cells required for such tissue formation may be present in the adrenal cortex. Stable expression of Ad4BP/SF-1, a key factor for adrenal and gonadal development and steroidogenesis, has been shown to direct embryonic stem cells toward the steroidogenic lineage. However, this steroidogenic capacity was very limited since progesterone was only produced in the presence of an exogenous substrate. Bone marrow mesenchymal cells are thought to contain pluripotent progenitor cells, which differentiate into multiple lineages. We have demonstrated that adenovirus-mediated forced expression of SF-1 in long-term cultured bone marrow cells can produce steroidogenic cells with the capacity for *de novo* synthesis of various steroid hormones in response to ACTH. This discovery may represent the first step in autologous cell transplantation therapy for patients with steroid hormone deficiency.

Key words: Bone marrow cell, Ad4BP/SF-1, Steroidogenic cell, Transplantation, Regeneration, Gene therapy
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ALTHOUGH hormone replacement therapy for patients with adrenal or gonadal failure has been well established, most of these patients require the therapy for their entire lifetime. In addition, these patients are always at risk of developing side effects. The situation is more serious in patients with congenital steroid deficiency, since the replacement therapy has to be started soon after birth and must be continued throughout the patient's lifetime. Furthermore, some patients with endocrine tumors that overproduce biological hormones are not completely cured, even after surgery, or their symptoms are not well controlled by various drugs. Therefore, an innovative therapy that will solve these problems has been anticipated.

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Currently, no clinical applications of regeneration medicine or gene therapy have been reported in the whole field of endocrinology, including adrenal or gonadal diseases. However, studies on the ontogenesis and differentiation mechanisms of adrenal and gonadal tissues have been rapidly increasing [1, 2]. Most of the genes responsible for the synthesis and metabolism of steroid hormones have already been revealed [1–5] (Fig. 1). Ad4BP/SF-1, whose name is derived from "Ad4-binding protein" (Ad4BP) or "steroidogenic factor-1" (SF-1), is known to be a master regulator in the transcriptional regulation of these steroidogenic enzymes as well as an essential factor for adrenal and gonadal development [1–5]. Based on these data, several experimental trials of gene therapy or regeneration medicine for adrenal or gonadal tissues have gradually accumulated using *in vitro* culture systems or animal models. In this review, we provide an overview of recent progress in elucidating the differentiation and

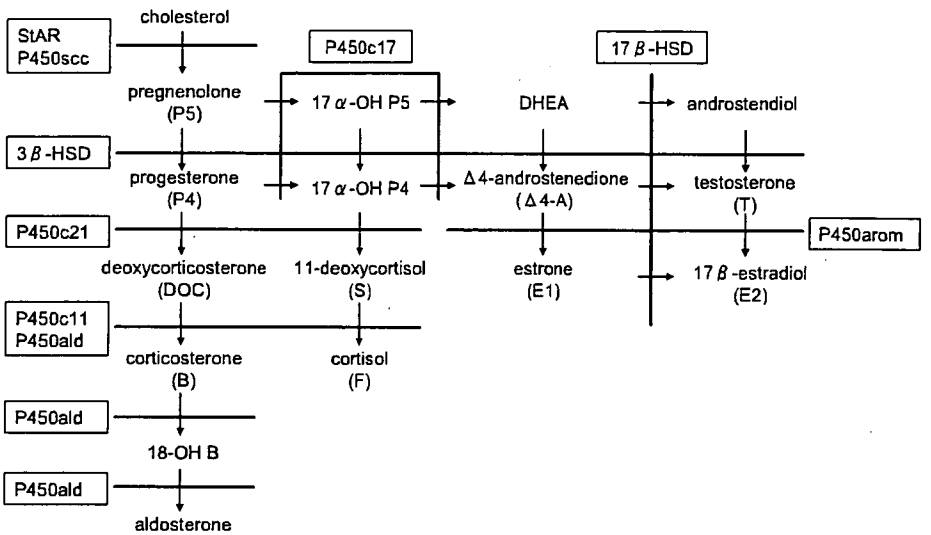

Fig. 1. Steroidogenic Pathways.
StAR, P450scc and 3β-HSD, are expressed in both the adrenals and gonads of humans and mice. P450c21, P450c11 and P450ald are exclusively expressed in the adrenals, but not the gonads of both humans and mice. P450c17 is expressed in both the adrenals and gonads of humans, but in only the gonads of mice. 17β-HSD type 3 in mice and in humans are mostly expressed in the testis.

regeneration of adrenal and gonadal tissues, including our trial of steroidogenic cell regeneration from mesenchymal bone marrow cells (BMCs).

Outline of the differentiation mechanism of the adrenal cortex and gonads

The adrenal cortex, gonads, reproductive tracts and kidneys are derived from the intermediate mesoderm. The mesoderm forms the mesonephros as a basement structure in the early developmental stages of the adrenal cortex and gonads. The early development of the adrenal cortex and gonads is derived from mesenchymal cells attached to the coelomic cavity lining adjacent to the urogenital ridge. These cells originate from the identical origin, namely adrenogenital primordium cells [6]. Thereafter, the common precursor cells divide into two distinct characteristic types of cells, designated adrenal precursor cells and bipotential gonadal precursor cells, and finally develop into the adrenal cortex or testes and ovaries, respectively, under the regulation of many transcriptional factors, including Ad4BP/SF-1 [1, 2]. Signaling pathways via not only transcription factors but also growth factors, such as FGF, have been suggested to be involved in gonadal development [7]. The fetal adrenal cortex is evident from 6–8 weeks of gestation. In the fetal cortex, there are two distinct layers, namely an inner prominent fetal zone and an outer definitive zone, that differentiate into the adult adrenal gland [8]. The fetal cortex occupies 80% of the adrenal cortex area and secretes large amounts of DHEA and DHEA-sulfate, which become substrates for placental estrogen synthesis. The fetal zone shrinks rapidly after birth. In the definitive zone at birth, the zona glomerulosa and zona fasciculata can already be distinguished, and both layers subsequently develop and proliferate. The zona reticularis becomes clear from about 3 years after birth and is almost completed by puberty [8]. On the other hand, sexual dimorphism of the gonads begins between 6 and 7 weeks of gestation with the development of seminiferous cords in the fetal testes. In contrast, histological differentiation of the fetal ovaries is not apparent until 6 months of gestation. As described below, the molecular mechanism of sexual differentiation has been extensively, if not completely, revealed for these two decades of life and clearly indicates that the nuclear receptor Ad4BP/SF-1 is a type of master regulator that controls the overall stream of the developmental mechanism (Fig. 2).

In humans, it is generally accepted that sex determination is controlled by the presence or absence of the Y chromosome, which carries a gene encoding the

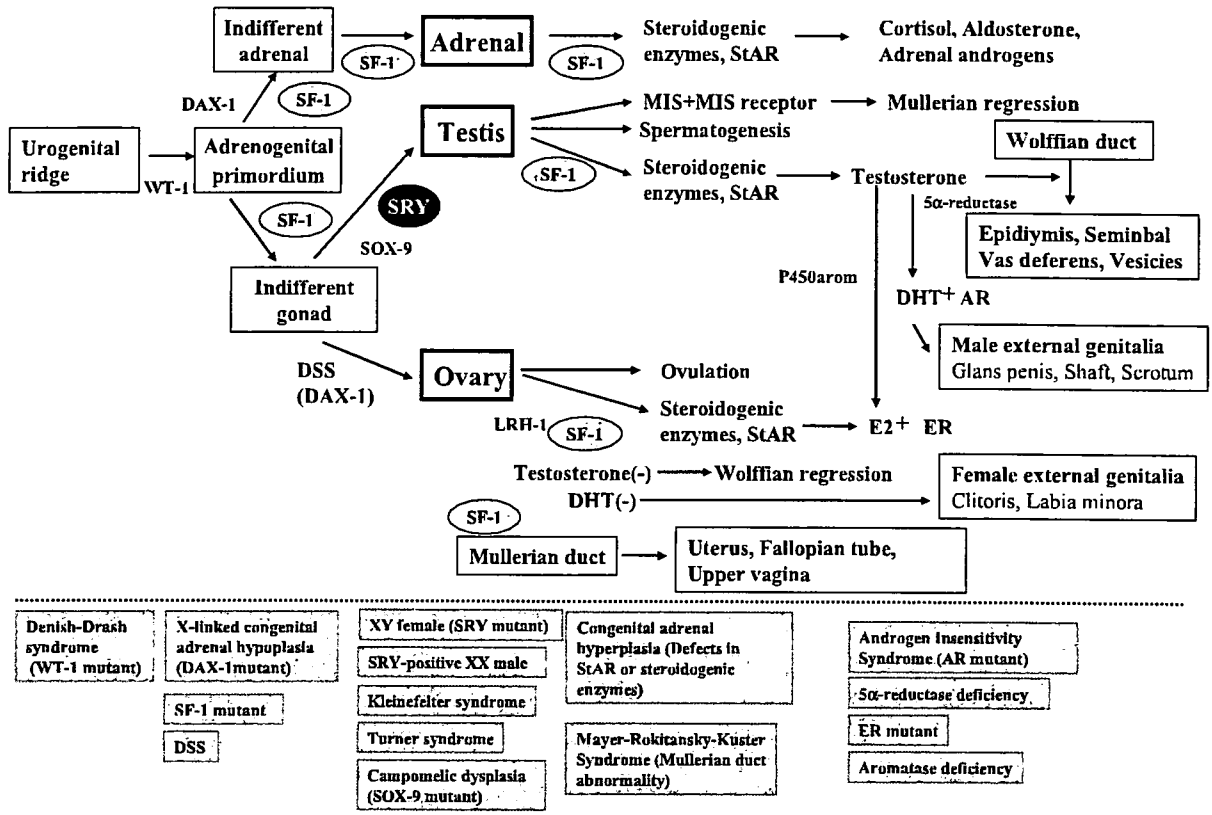

Fig. 2. Outline of the sexual differentiation cascade and related diseases.
SF-1, steroidogenic factor 1; StAR, steroidogenic acute regulartory protein; WT-1, Wilmus' tumor-related gene; DSS, dosage-sensitive sex reversal; DAX-1, DSS-adrenal hypoplasia congenital gene; SOX9, SRY-related genes HMG-box 9; AR, androgen receptor; ER, estrogen receptor; E2, estradiol; DHT, dihydrotestosterone; LRH-1, liver receptor homologue 1; MIS, Mullerian inhibiting substance

testis-determining factor (TDF) on its short arm. The TDF has been isolated and designated SRY [9, 10]. SRY encodes a protein of the high-mobility group [HMG] box type, that probably acts to influence chromatin structure and other genes in the sex-determination cascade. Specifically, SRY initiates the testis-determining pathway by inducing the expression of a series of downstream factors, including SF-1, Wilms' tumor-related gene (WT-1), dosage-sensitive sex reversal-adrenal hypoplasia congenital gene (DAX-1) and SRY-related genes HMG-box 3 (SOX3) and HMG-box 9 (SOX9), thus resulting in a male phenotype. On the other hand, individuals without TDF develop into a female phenotype, since the bipotential gonad cannot differentiate into the testes, and instead differentiates into the ovaries. Indeed, mutations in WT1, SF-1, SRY or any of the downstream genes can impair testicular development in humans [1] (Fig.2).

On the other hand, little is known about the so-called ovary-determination factor. DAX-1 is considered to be one of the candidates for this factor since it antagonizes SRY function. The expression level of DAX-1 affects sex determination and leads to the dosage-sensitive sex reversal (DSS) phenotype in humans. This assumption is supported by a transgenic study in which male gonads expressing larger amounts of DAX-1 than wild-type gonads showed delayed and incomplete testicular differentiation in mice carrying a weak SRY allele [11].

Ad4BP/SF-1 was initially identified independently by Morohashi and Parker as a ubiquitous transcription factor for steroidogenic hormones [1–5]. Ad4BP/SF-1 binds as a monomer to its responsive element located in the promoter of steroidogenic genes and enhances their transcriptional levels [1–5]. In addition, the cAMP-protein kinase A (PKA) signal pathway can

strongly potentiate Ad4BP/SF-1 transactivation activity [12]. Since knockout mice for Ad4BP/SF-1 show agenesis of both the adrenal glands and the gonads [13, 14] as well as decreased expression levels of luteinizing hormone (LH and follicle stimulating hormoneFSH in the pituitary gonadotroph [14, 15], Ad4BP/SF-1 has been considered to be an essential factor for differentiation of the pituitary-adrenal or -gonadal axis. DAX-1 is also an important factor for the development of this axis since patients with DAX-1 mutations show X-linked adrenal hypoplasia congenital (AHC) and hypogonadotropic hypogonadism [16, 17]. DAX-1 is considered to be involved in the differentiation process of the fetal adrenal gland, since the adrenal glands obtained at autopsy from patients with AHC are very similar to those in the fetal adrenal cortex and do not include the permanent (adult) cortex structure [18].

Although their exact roles in humans are unknown, many other factors, such as, Wnt4, Cited 2, PBX-1 [1], M33 [19], Fgf 9 [20], Dhh [21] and Gata4 [22], are also known to be involved in adrenal or gonadal development since disruption or overexpression of these genes in mice causes sex reversal or delayed sexual differentiation [2].

Transplantation, regeneration and gene therapy for the adrenal or gonadal glands

Candidate diseases for such cutting-edge medicine will be various diseases involving adrenal enzyme deficiencies (single gene diseases), chronic adrenal failure, adrenal cancers, intractable Cushing syndrome, etc.

(1) Trial of transplantation, regeneration and gene therapy for the adrenal glands

Hornsby *et al.* reported that transplantation of cloned cells from bovine adrenocortical cells into severe combined immunodeficiency (SCID) mice resulted in the formation of adrenal gland-like tissue, which successfully compensated for adrenal failure after bilateral adrenalectomy [23]. Furthermore, these authors succeeded in immortalizing the cloned cells by expressing telomerase reverse transcriptase (TERT) and noted similar successful compensation of adrenal failure in adrenalectomized SCID following transplantation of the immortalized cells [24]. These reports indicate the existence of intraadrenal stem cells. Although the above mechanism is currently unclear, it is speculated that xenotransplanted adrenal cell grafts may promote angiogenesis at the transplantation site, leading to accelerated cell proliferation to form the adrenal gland [25]. In addition, mice survived for more than 16 days and the normal adrenocortical structure was observed when human adrenal cells and 3T3-L1 cells, which produce FGF, were co-transplanted into the subcapsular region of the kidneys of adrenalectomized mice [26]. Interestingly, in a trial of autologous transplantation of single adrenocortical layer cells into the subcapsular region of the kidneys of adrenalectomized mice, transplantation of zona glomerulosa cells resulted in the formation of new adrenocortical tissue, which was composed of cells that were histologically similar to those of the zona glomerulosa and zona fasciculata. However, when zona fasciculata cells were transplanted, no such structure was formed [27]. These findings are very interesting with respect to the stem cell origin of the adrenal cortex and the zonation. An undifferentiated zone between the zona glomerulosa and zona fasciculata has been suggested to be an adrenocortical stem cell zone, which expresses SF-1 [28]. Cell migration and differentiation are thought to occur from within this stem cell zone, although factors regulating this regeneration process remain unknown. While adrenal cell transplantation into the subcapsular region of the kidneys leads to good regeneration of adrenal cortical tissue, cell transplantation into the skin has also been reported to be possible when a collagen gel is used [29]. Although previously denied, autologous transplantation of adrenocortical cells or tissues does actually cause innervation, which is correlated with the degree of recovery of the steroid production [30].

(2) Trial of steroid-producing cell regeneration from embryonic stem cells (ESCs)

Crawford *et al.* demonstrated that stable SF-1 expression in ESCs resulted in a steroidogenic capacity and adenosine 3,5-cyclic monophosphate (cAMP) or retinoic acid-dependent inducibility of cytochrome P450scc, leading to progesterone production [31]. In this experiment, SF-1 initiated the program toward steroidogenic cells through a process that clearly did not require embryoid body formation. However, this steroidogenic capacity was restricted at the stage of progesterone synthesis, and did not occur as *de novo*

synthesis, since the addition of an exogenous substrate, 20α-hydroxycholesterol, which bypasses the mitochondrial outer membranes, was required for progesterone production. In addtion, the converted cells were not responsive to adrenocorticotropic hormone (ACTH) or human chorionic gonadotropin (hCG). Nevertheless, these results clearly indicate that SF-1 is a key factor in steroidogenic cell differentiation. The diffierence between steroidogenic ES cells and steroidogenic tissue may partly arise because SF-1 is not expressed until around E9 [32], after the urogenital mesoderm has been separated from pluripotent cellular lineage. Early differentiation step before SF-1 expression may be necessary to drive to form normal steroidogenic cells. Apart from the steroidogenic capacity, ES cells has been shown to differentiate into functional germ cells that can participate in spermatogenesis when transplanted ito reconstituted testicular tubules [33].

(3) Trial of steroid-producing cell regeneration from BMCs

A number of studies have suggested that, following transplantation into different tissues, BMCs may contribute to the regeneration of hematopoietic or mesenchymal lineages in multiple organs [34–37]. Although these potential effects may partly occur through spontaneous cell fusion with recipient cells [38, 39], BMCs certainly contain pluripotent progenitor cells, which can differentiate into multiple lineages. We therefore tested whether the introduction of SF-1 into mouse BMCs could produce steroidogenic cells [40].

We expanded a relatively pure BMC population by culturing the cells for 120–180 days, and then investigated the steroidogenic properties of the cells after infection with Adx-bSF-1. Flowcytometric analyses revealed that the steroidogenic BMCs were negative for the surface markers CD45 (hematopoietic cells marker), CD11b (monocyte/macrophage marker) and CD44 (a potential marker for mouse mesenchymal cells) but positive for c-kit and Sca-1, which are hematopoietic and mesenchymal stem cell/progenitor markers. Surprisingly, the BMCs infected with Adx-bSF-1 produced significant amounts of progesterone (P4), deoxycorticosterone (DOC), corticosterone (B), 17α-hydroxyprogesterone (17α-OH P4), 11-deoxycortisol (S), dehydroepiandrosterone (DHEA), Δ4-androstenedione (Δ4-A) and testosterone (T), while those infected with Adx-LacZ did not (Fig. 3A). Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of the cells revealed the presence of steroidogenic acute regulatory protein (StAR), P450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD), P450c11, P450c17 and 17β-HSD type 3 mRNAs in BMCs infected with Adx-bSF-1, but in those infected with Adx-LacZ (Fig. 3B). Interestingly, the BMCs originally expressed adrenocorticotropic hormone receptor (ACTH-R), a receptor type transcribed in adipose tissues [41] but not in adrenal glands [42]. In fact, ACTH dose-dependently stimulated the production and secretion of progesterone by BMCs infected with Adx-bSF-1 (Fig. 4A), but not by BMCs infected with Adx-LacZ. The induction of the mRNAs for the respective steroidogenic enzymes following treatment with 2.4 μM ACTH was also confirmed by real-time PCR [40]. Surprisingly, the significant production of DOC was observed until at least 112 days (Fig. 4B), despite the fact that the half-life of the adenovirus was 2–3 weeks and there was no induction of endogenous mouse SF-1. This unexpected result may be explained by the possibility that continuous bSF-1 expression after adenovirus infection, even at low levels, is sufficient to maintain long-term steroid production by BMCs. Another possibility is that SF-1 expression may be indispensable for the initiation of steroidogenesis via the induction of steroidogenic enzymes, but not critical for its maintenance.

The steroid profile of the cultured BMCs showed a mixed pattern of mouse adrenal and gonadal steroidogenesis, namely simultaneous production of DOC, B, DHEA, Δ4-A and T. Interestingly, P450c17 is expressed in the human adrenal gland, but not in the mouse adrenal gland. Therefore, the significant expression of P450c17 in BMCs indicates a mixed steroid profile beyond the species level, suggesting the possibility of a common origin for steroidogenic tissues, namely stem cells. Although little is known about the origin of the stem cells, a previous study on the expression profile of SF-1 indicated that the undifferentiated adrenal cortex and gonads of early stage fetuses originate from a common adrenogenital primordium [6]. The multipotency of BMCs to differentiate into either adrenal or gonadal steroidogenic cells provides an important model for future clarification of the mechanisms of tissue-, zone- or cell-specific adrenal and gonadal steroidogenic cell differentiation. One plausible speculation may be that bone marrow-

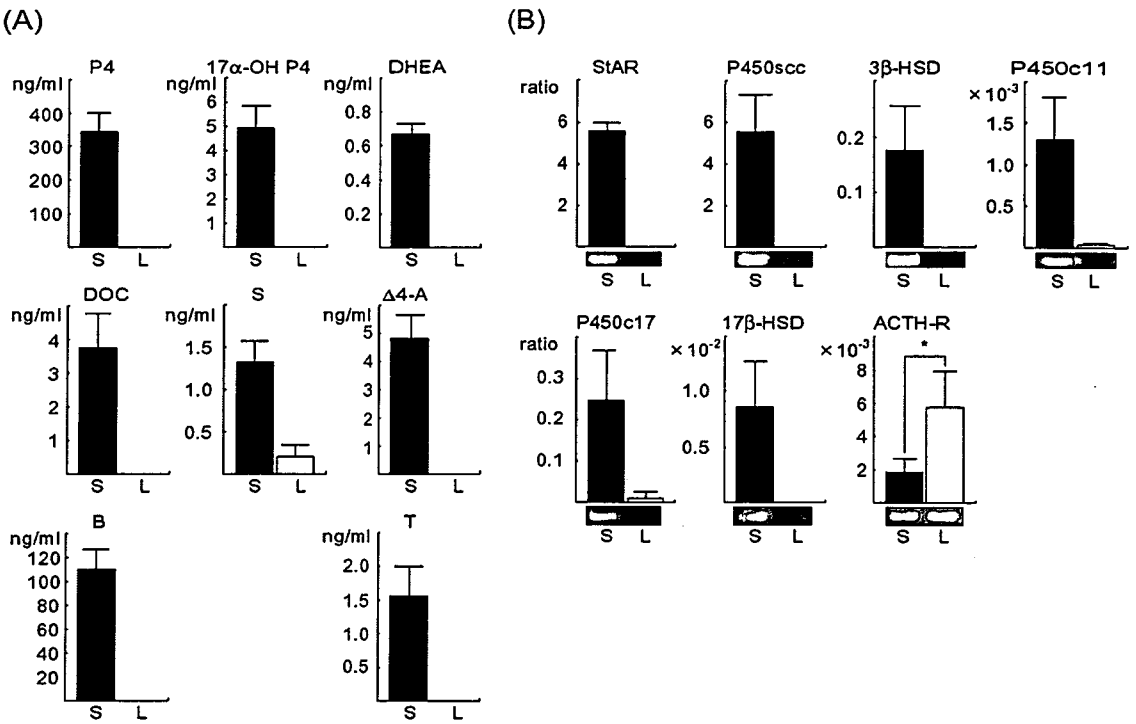

Fig. 3. Characteristics of the BMCs infected with Adx-bSF-1 (from ref. 39).
(A) Basal secretion of progesterone (P4), deoxycorticosterone (DOC), corticosterone (B), 17α-hydroxyprogesterone (17α-OHP4), 11-deoxycortisol (S), dehydroepiandrosterone (DHEA), Δ4-androstenedione (Δ4-A) and testosterone (T) in medium obtained from long-term cultured BMCs prepared from GFP mice. The cells were infected with Adx-bSF-1, or Adx-LacZ as a control, and cultured for 7 days. The steroid contents accumulated in the medium over the next 4 days were then measured. Values represent the mean ± SD (n = 3). The steroid contents in the medium from the control cells are undetectable, except for S. S (black column) and L (white column) indicate BMCs transfected with Adx-bSF-1 and Adx-LacZ, respectively.
(B) Real-time PCR analysis of StAR, P450scc, 3β-HSD, P450c11, P450c17, 17β-HSD type 3 and ACTH-R. The relative mRNA expression levels were calibrated by the corresponding β-actin level. The relative ratios to the expression levels in the control Y-1 cells are expressed for StAR, P450scc and 3β-HSD, while the relative ratios to the expression levels in the mouse adrenal glands are expressed for P450c11 and ACTH-R, and the relative ratios to the expression levels in the mouse testes are expressed for P450c17 and 17β-HSD type3. S (black column) and L (white column) indicate BMCs transfected with Adx-bSF-1 and Adx-LacZ, respectively. No significant PCR products for StAR, P450scc, 3β-HSD, P450c11, P450c17 and 17β-HSD type 3 are obtained from control cells infected with Adx-LacZ. Values represent the mean ± SD (n = 3). * $P<0.05$. The specific PCR bands obtained after more than 40 cycles of amplification are shown in ethidium bromide stained-agarose gels in the lower figures.

derived stem cells settle in the adrenocortical stem cell zone, where SF-1 expression may become possible.

Although the character of mouse mesenchymal stem cells has not been fully clarified and remains controversial [43, 44], steroid-producing cells may originate from multipotent and immature stem cells. Importantly, long-term cultured BMCs were able to differentiate into an osteoblastic phenotype following appropriate treatment, suggesting that the character of the steroidogenic cells may be much closer to mesenchymal BMC lineages [45]. However, the exact origin of these steroidogenic cells remains unclear and warrants further investigation.

Similar to our data, a recent report demonstrated that cAMP stimulation of a cloned mouse BMC line, KUM9, stably expressing SF-1 induced cell differentiation into cells with a testis-type steroidogenic capacity [46]. In addition, mouse BMCs were reported to be able to differentiate to Leydig-type cells in an *in vivo* environment, although a possibility of cell fusion cannot be completely excluded [46].

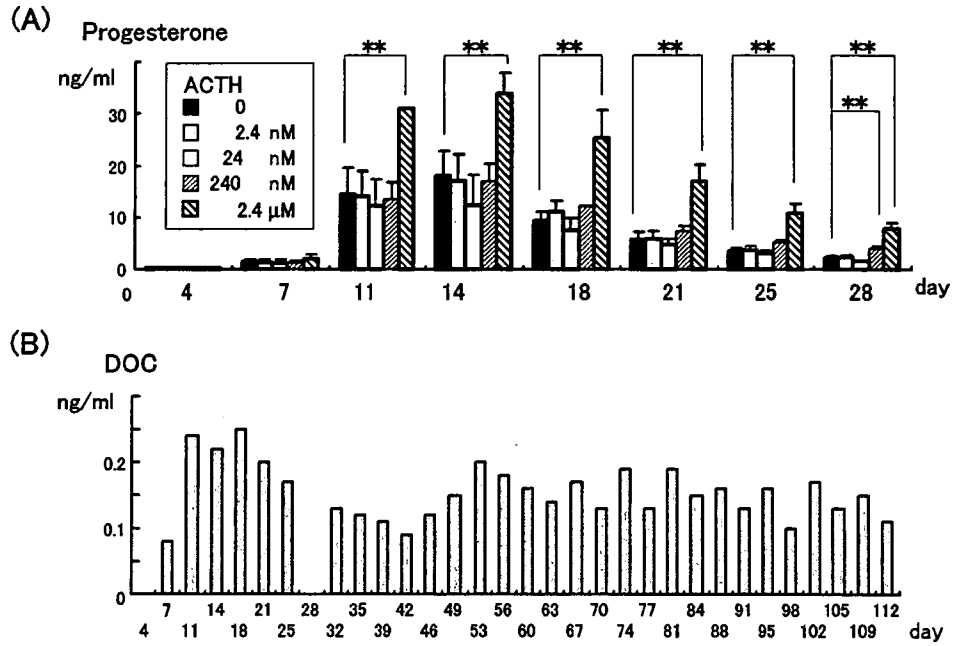

Fig. 4. Responsiveness to ATCH (A) and long-term steroidogenesis (B) (partly extracted from ref. 39)
(A) Effect of ACTH on the secretion of progesterone by cultured BMCs prepared from GFP mice. After infection with Adx-bSF-1 or Adx-LacZ (day 0), cells were treated with 2.4 nM to 2.4 μM ACTH on days 0, 4, 7, 11, 14, 18, 21, 25 and 28. Before the addition of ACTH, the medium was collected and the steroid concentration was measured. Values represent the mean ± SD (n = 3). ** $P<0.01$ vs. the control (absence of ACTH).
(B) Long term steroidogenesis. Time course of basal deoxycorticosterone (DOC) secretion into the medium by long-term cultured BMCs from GFP mice. Cells were transfected with Adx-bSF-1 or Adx-LacZ and cultured. Values represent the means of duplicate dishes. The black columns indicate the steroids secreted by the cells infected with Adx-bSF-1. The secretion of DOC was undetectable in the medium obtained from BMCs infected with Adx-LacZ (data not shown).

A trial of gene therapy for adrenal diseases (*in vitro* and animal models)

Congenital adrenal hyperplasia is caused by genetic defects in adrenal steroidogenic enzymes, and represents a good target for gene therapy. Tajima *et al.* reported that direct injection of an adenovirus containing P450c21 into the adrenal glands of 21-hydroxylase-deficient mice resulted in a high level of intraadrenal expression of P450c21 for 2–7 days after the injection, although the expression level gradually decreased thereafter [47]. It was confirmed that the zonation of the adrenal glands disappeared after the injection. Although long-lasting expression was not obtained, the data suggest a possible application to gene therapy for 21-hydroxylase deficiency. Another previous trial used a sheep model to introduce a desirable gene in the fetal period by direct injection of an adenovirus into the umbilical vein under an ultrasonic wave guide and confirmed a high level expression of the transduced gene in the liver and adrenal glands [48]. These observations suggest a possible application to gene therapy for congenital adrenal hyperplasia (CAH) in the fetal period. Adenoviruses are easily incorporated into the liver, but they also show comparatively good uptake into the adrenal glands [49]. A detailed investigation into the effects of various adenovirus variants regarding the introduction efficiency and responsiveness to ACTH of bovine adrenocortical cells has been reported [50]. This report states that although the E1/E3-deficient type of adenovirus was optimal for the introduction efficiency into bovine adrenocortical cells, it lowered their responsiveness to ACTH. On the other hand, the E3/E4-deficient type showed low introduction ability, but the cells maintained a good responsiveness to ACTH. The authors concluded that since steroids are stress-responsive hormones, the E4-deficient adenovirus should be recommended for adrenal diseases based on the importance of ACTH responsiveness.

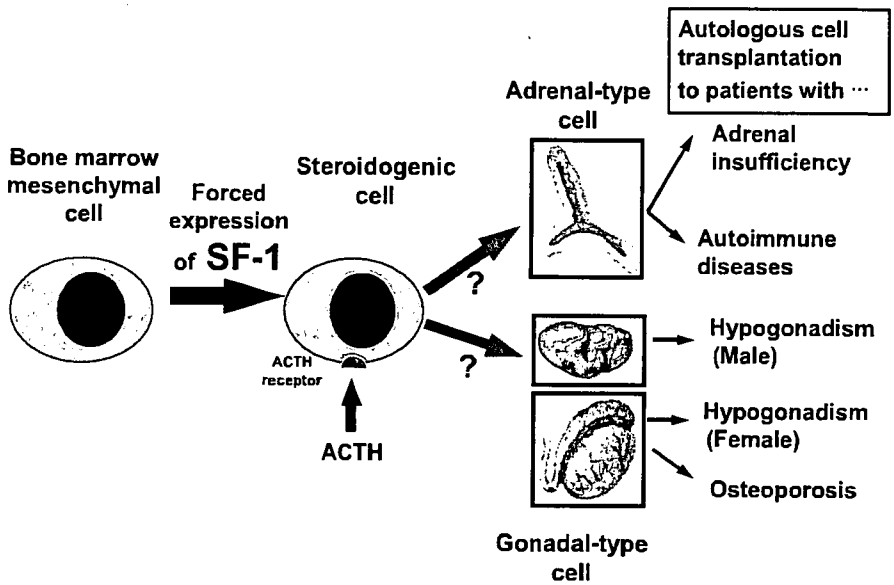

Fig. 5. Future directions for clinical applications involving steroidogenic cells produced from BMCs.

Chuman *et al.* found that the promoter of P450c11 induced a suicide gene in human adrenocortical cancer cells. Furthermore, they constructed a system to augment adrenal-specific expression of this suicide gene by combination with the enhancer element of P450c11. When they introduced a herpes simplex virus thymidine kinase (HSV-TK) into this expression system, HSV-TK was strongly induced by cAMP and ACTH [51]. HSV-TK has the ability to change non-toxic drugs, such as ganciclovir, to toxic phosphorylated chemical compounds. In fact, when HSV-TK is specifically expressed in adrenal cancer cells, the cells become sensitive to ganciclovir. These observations are interesting since they suggest a possible application to gene therapy for adrenal cancer.

Conclusions and future directions

Regeneration medicine for the adrenal cortex by xenograft transplantation of cloned adrenocortical cell clusters may be a promising therapy, but it is still very preliminary and based on animal experiments. Much future effort to obtain vascularized functional tissues and overcome immunological rejection will be needed. Our finding that introduction of SF-1 into long-term cultured BMCs leads to transformation into steroidogenic cells, which are in a basal state as well as responsive to ACTH and capable of producing multiple steroid hormones, will also provide an innovative therapeutic tool for patients suffering from steroid insufficiency like adrenal insufficiency and male or female hypogonadism, since it indicates that autologous BMC transplantation is possible. In addition, this tool may be also applicative to the therapy of autoimmune diseases which sometimes requires glucocorticoids (Fig. 5). However, it remains unclear whether Ad4BP/SF-1 is sufficient to realize ideal steroidogenic cells, since many other factors are involved in cell differentiation and steroidogenesis (Fig. 2). Ad4BP/SF-1 is clearly essential for the production of steroidogenic stem cells, as evidenced by our study and also by the fact that the phenotype of homozygous SF-1 KO mice shows agenesis of both adrenal glands and gonads [13, 14], but this may not be sufficient to produce adrenal- or gonadal-specific cell types. However, a more predominant dosage-sensitive effect of SF-1 on adrenal rather than gonadal formation has been suggested from a finding of the predominant reduction of adrenal size in heterozygous SF-1 KO mice [52]. A similar finding has recently been reported in an experiment that Ad4BP/SF-1 transgenic mice harboring varied expression levels of SF-1 among tissues were applied to rescue homozygous Ad4BP/SF-1 KO mice; the transgenic mice failed to rescue the adrenal gland, but successfully rescued the gonad and spleen [53].

Therefore, one approach to direct the steroidogenic cell lineage might be to control Ad4BP/SF-1 expression levels or Ad4BP/SF-1 transcriptional activity in BMC cells. Relatively less expression of Ad4BP/SF-1 might lead to the production of gonad-type steroidogenic cells but this might not be enough to provoke adrenal-type steroidogenic cells. However, it is also important that efforts be made to identify unknown factors that might determine the tissue-specific steroidogenic cell type; such factors would be useful to modify the cell type of BMC cells, adrenal or gonadal type cells. The multipotency of BMCs to differentiate into adrenal or gonadal steroidogenic cells may provide a key model for solving the mechanism of the determination of the direction of steroidogenic cell type differentiation into the adrenal cortex or gonads in the future (Fig. 5).

It may be possible to source the mesenchymal stem cells from adipose tissue. However, our results are very preliminary and for future clinical applications, we need to reconsider the choice of vector from the viewpoints of safety and efficacy. As far as adenoviruses are concerned, the situation does not appear to be so optimistic with respect to their safety in humans and limited efficacy due to their short half-life. An alternative choice to solve these problems would be to transform mesenchymal stem cells into steroidogenic cells by some humoral factors without exogenous expression of any gene including SF-1. Research in this field has only just begun and is expected to develop extensively in the near future, in order to relieve the many patients who are forced to take hormone replacement therapy.

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Atrazine-Induced Aromatase Expression Is SF-1 Dependent: Implications for Endocrine Disruption in Wildlife and Reproductive Cancers in Humans

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BACKGROUND: Atrazine is a potent endocrine disruptor that increases aromatase expression in some human cancer cell lines. The mechanism involves the inhibition of phosphodiesterase and subsequent elevation of cAMP.

METHODS: We compared steroidogenic factor 1 (SF-1) expression in atrazine responsive and nonresponsive cell lines and transfected SF-1 into nonresponsive cell lines to assess SF-1's role in atrazine-induced aromatase. We used a luciferase reporter driven by the SF-1–dependent aromatase promoter (ArPII) to examine activation of this promoter by atrazine and the related simazine. We mutated the SF-1 binding site to confirm the role of SF-1. We also examined effects of 55 other chemicals. Finally, we examined the ability of atrazine and simazine to bind to SF-1 and enhance SF-1 binding to ArPII.

RESULTS: Atrazine-responsive adrenal carcinoma cells (H295R) expressed 54 times more SF-1 than nonresponsive ovarian granulosa KGN cells. Exogenous SF-1 conveyed atrazine-responsiveness to otherwise nonresponsive KGN and NIH/3T3 cells. Atrazine induced binding of SF-1 to chromatin and mutation of the SF-1 binding site in ArPII eliminated SF-1 binding and atrazine-responsiveness in H295R cells. Out of 55 chemicals examined, only atrazine, simazine, and benzopyrene induced luciferase via ArPII. Atrazine bound directly to SF-1, showing that atrazine is a ligand for this "orphan" receptor.

CONCLUSION: The current findings are consistent with atrazine's endocrine-disrupting effects in fish, amphibians, and reptiles; the induction of mammary and prostate cancer in laboratory rodents; and correlations between atrazine and similar reproductive cancers in humans. This study highlights the importance of atrazine as a risk factor in endocrine disruption in wildlife and reproductive cancers in laboratory rodents and humans.

KEY WORDS: aromatase, atrazine, breast cancer, cAMP, *CYP19*, endocrine disruptor, hermaphroditism, prostate cancer, SF-1. *Environ Health Perspect* 115:720–727 (2007). doi:10.1289/ehp.9758 available via *http://dx.doi.org/* [Online 5 February 2007]

Atrazine, a triazine herbicide used primarily in corn production (Frank and Sirons 1979), is the most common pesticide contaminant of groundwater and surface water (Fenelon and Moore 1998; Hennion M, Pichon V, Legeay P, Cohen M, unpublished data; Kolpin et al. 1998; Lode et al. 1995; Miller et al. 2000; Müller et al. 1997; Solomon et al. 1996; Thurman and Cromwell 2000). In addition to its high use, ubiquitous contamination of aquatic environments, persistence, and mobility, atrazine is a concern because it is a potent endocrine disruptor in wildlife and laboratory rodents.

A U.S. Environmental Protection Agency (EPA) laboratory first concluded that atrazine was an endocrine disruptor in the year 2000:

> Atrazine tested positive in the pubertal male screen that the EDSTAC [U.S. EPA Endocrine Disruptor Screening and Testing Advisory Committee] is considering as an optional screen for endocrine disruptors. (Stoker et al. 1999)

Among other endocrine-disrupting effects (Babic-Gojmerac et al. 1989; Cooper et al. 1999, 2000; Cummings et al. 2000; Friedmann 2002; Kniewald et al. 1979, 1995; Narotsky et al. 2001; Shafer et al. 1999; Šimic et al. 1991; Stoker et al. 1999, 2000), atrazine disrupts androgen- and estrogen-mediated processes. Atrazine has a low affinity for androgen and estrogen receptors (Roberge et al. 2004; Tennant et al. 1994) and, thus, is not a receptor agonist or antagonist. Atrazine reduces androgen synthesis and action via several mechanisms (Babic-Gojmerac et al. 1989; Kniewald et al. 1979, 1980, 1995; Šimic et al. 1991) and it increases estrogen production (Crain et al. 1997; Heneweer et al. 2004; Keller and McClellan-Green 2004; Sanderson et al. 2000, 2001, 2002; Spano et al. 2004).

Atrazine increases aromatase levels by binding to and inhibiting phosphodiesterase (Roberge et al. 2004; Sanderson et al. 2000, 2001), resulting in elevated cAMP in some human cancer cell lines. Elevated cAMP results in increased transcription of the aromatase gene *CYP19* [cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1); UniGene Hs.654384 (UniGene 2007) or GenBank NM_000103 (GenBank 2007)], increased aromatase activity, and, ultimately, increased estrogen production. The molecular mechanism is not completely understood, however, and effects vary between cell types (Morinaga et al. 2004). In this article we address the role of the important transcription factor, steroidogenic factor 1 (SF-1), in atrazine-induced aromatase expression.

Materials and Methods

Experiments. Experiment 1: SF-1 levels in atrazine-responsive and -nonresponsive cells. To test the hypothesis that SF-1 is required for atrazine-induced aromatase expression, we examined SF-1 levels in atrazine-responsive H295R adrenocortical carcinoma cells and nonresponsive KGN granulosa cells. Endogenous SF-1 mRNA was analyzed by real-time polymerase chain reaction (PCR) with the relative copies of SF-1 to β-actin in KGN cells set to 1. The relative copies of SF-1 to β-actin in H295R cells were calculated accordingly. SF-1 protein levels were confirmed by Western blot analysis.

Experiment 2: Induction of luciferase activity by atrazine and simazine via aromatase promoter II in H295R cells. We transfected H295R and NIH/3T3 cells with a 4.0 kb aromatase promoter II (ArPII) luciferase reporter (pGL3-ArPII4.0) with or without cotransfection with human SF-1 (pcDNA3.1-hSF-1). This experiment was designed to determine whether SF-1 would increase the

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ability of atrazine to induce gene expression via ArPII using a luciferase promoter.

Experiment 3: Other environmental contaminants. We used a luciferase activity assay [NIH/3T3 cells co-transfected with 4.0 kb ArPII reporter (pGL3-ArPII4.0) and human SF-1 (pcDNA3.1-hSF-1)] to screen 55 environmental contaminants. Cells were exposed to the solvent (DMSO) or 10^{-6} mol/L forskolin [protein kinase A (PKA) agonist] with or without each of 55 chemicals at 10^{-5} mol/L (except for 10^{-7} mol/L tributyltin and 10^{-7} mol/L triphenyltin) for 48 hr before the luciferase assay was performed.

Experiment 4: Atrazine induction of aromatase expression and activity in KGN ovarian cells. We used KGN ovarian cells to examine aromatase expression in response to atrazine exposure. SF-1 infection of KGN cells was used to determine whether this transcription factor was sufficient to support atrazine-induced aromatase in this otherwise atrazine nonresponsive cell line.

Experiment 5: Atrazine-enhanced SF-1 binding to ArPII. We conducted a chromatin-immunoprecipitation (ChIP) assay to determine whether atrazine and simazine increased binding of SF-1 to ArPII.

Experiment 6: Binding of atrazine to SF-1. We used surface plasmon resonance (SPR) to examine the ability of atrazine to bind directly to SF-1 as a second mechanism by which atrazine could induce gene expression via ArPII. We used quartz crystal balance techniques to confirm results of the SPR study.

Chemical standards. Chemicals standards for testing in cell lines were obtained from Wako Pure Chemical Co. (Osaka, Japan) and Accu Standard, Inc. (New Haven, CT, USA). We examined the effects of these chemicals, at a concentration of 10^{-5} mol/L (except for 10^{-7} mol/L tributyltin and 10^{-7} mol/L triphenyltin). The chemicals were dissolved in ethanol (except for aldicarb, atrazine, and simazine, which were dissolved in DMSO) at the original concentration of 10^{-2} mol/L. The adenylyl cyclase activator forskolin was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and was dissolved in DMSO.

Cell lines. The human ovarian granulosa-like tumor cell line (KGN) was established and maintained as previously reported (Nishi et al. 2001). H295R adrenal carcinoma cells (ATCC CRL 2128), NIH/3T3 mouse fibroblasts (ATCC CRL1658), and sf21 insect cells (ATCC CRL1711) were all obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown under culture conditions prescribed by the ATCC.

Plasmids and constructs. We constructed a luciferase reporter containing 4.0 kb of the 5′-flanking sequence of *ArPII* as previously described (Fan et al. 2005a). A 4-kb human SF-1 promoter luciferase reporter was established previously (Oba et al. 2000). We purchased the Renilla luciferase reporter plasmid pRL-CMV from Promega (Madison, WI). An adenovirus construct expressing bovine SF-1 (adeno-SF-1) and another expressing β-galactosidase (adeno-LacZ) were prepared as described previously (Gondo et al. 2004).

Quantification of mRNA. Total RNA from cells was isolated and reverse-transcribed to cDNA; relative aromatase mRNA copy numbers to β-actin were then analyzed by real-time PCR following the protocol described previously (Fan et al. 2005b). Primers for human aromatase were 5′-ACG CAG GAT TTC CAC AGA AGA G-3′ (forward) and 5′-CTT CTA AGG CTT TGC GCA TGA C-3′ (reverse). Primers for human β-actin were 5′-AAA CTA CCT TCA ACT CCA TC-3′ (forward) and 5′-ATG ATC TTG ATC TTC ATT GT-3′ (reverse). Endogenous human SF-1 mRNA copies in H295R and KGN cells were also determined by the same method. Primers for SF-1 were 5′-CAG CCT GGA TTT GAA GTT CC-3′(forward) and 5′-TTC GAT GAG CAG GTT GTT GC-3′ (reverse).

Western blots. Western blots were conducted using standard techniques. The anti-SF-1 antibody used was provided by K. Morohashi (National Institution for Basic Biology, Okazaki, Japan).

Relative luciferase reporter assay. For the 55 environmental chemical-screening studies, we co-transfected each of three 10-cm^2 dishes containing 70% confluent NIH-3T3 cells with 1.5 μg ArPII reporter (PGL3-ArPII4.0), 1.5 μg pcDNA3.1-hSF-1, and 10 ng pRL-CMV by Effectence transfection reagent (QIAGEN, Miami, FL, USA) following the manufacturer's protocol. Twenty-four hours after transfection, cells in all three dishes were trypsinized, mixed together, and reseeded to 24-well plates (approximately 1.0×10^5 cells/well). DMSO, 10^{-6} mol/L forskolin, or 10^{-5} mol/L test chemicals plus forskolin was added to the cells, and a luciferase assay was performed 48 hr later. The basic technical details for other relative dual-luciferase assays in this study are as follows. On the first day, 0.75×10^5 cells/well in .0.5 mL growth medium were seeded into 24-well plates. On the second day, 0.4 μg PGL3-ArPII, 1.0 ng pRL-CMV, and 0.1 μg pcDNA3.1-hSF-1, or an equal-molar amount of empty vector pcDNA3.1, were transiently co-transfected to each well using the Effectence transfection reagent. On the third day, the culture medium was replaced with fresh medium in the presence of proper chemicals or its solvent DMSO. On the fifth day (48 hr after chemical treatment), the cells were lysed in 100 μL/well passive lysis buffer, and the luciferase assay was performed in accordance with the protocol of the Dual-Luciferase Reporter Assay System (Promega) using a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The firefly luciferase activity produced by PGL3-ArPII in identically treated triplicate samples was normalized for the renilla luciferase activity produced by pRL-CMV. The data shown represent at least three independent experiments.

Aromatase assay. We measured ^3H_2O released upon conversion of [1β-^{3}H] androstenedione to estrone to measure aromatase activity, as described previously (Mu et al. 2000). Briefly, the cells were cultured in a 24-well dish in Dulbecco's modified Eagle's medium/Ham's F-12 with 10% fetal bovine serum in the presence of atrazine, simazine, or DMSO and incubated for 40 hr. The cells were then incubated with [1β-^{3}H] androstenedione for an additional 6 hr. The medium was extracted with chloroform and centrifuged. The aqueous phase was then mixed with 5% charcoal/0.5% dextran and incubated for 30 min. The mixture was subsequently centrifuged, and the supernatant was added to 5 mL scintillation fluid and assayed for radioactivity. The amount of radioactivity in ^3H_2O was standardized from the protein concentration determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

ChIP assay. ChIP assays were performed using the ChIP assay kit from Upstate Biotechnology (Lake Placid, NY, USA) following the protocol provided by the manufacturer, with some modifications. Briefly, H295R cells were seeded in 10-cm^2 dishes and treated with 10^{-5} mol/L atrazine, simazine, or DMSO for 48 hr. Cells were then cross-linked with 1% formaldehyde for 60 min, washed with chilled phosphate-buffered saline (PBS), resuspended in 200 μL SDS lysis buffer, and sonicated six times for 10 sec each at 60% maximum setting of the sonicator (Handy Sonic-UR-20P; TOMY SEIKO Co., Ltd., Tokyo, Japan). Sonicated cell supernatant was diluted 10-fold, and 1% (20 μL) of the total diluted lysate was used for total genomic DNA as input DNA control. The rest (1,980 μL) was then subjected to immunoclearing by 75 μL salmon sperm DNA/protein A agarose–50% slurry for 30 min at 4°C. Immunoprecipitation was performed overnight at 4°C with 3 μg anti-SF-1 antibody (courtesy of K. Morohashi, National Institution for Basic Biology, Okazaki, Japan). For the negative control, we used normal rabbit IgG (Santa Cruz Biotechnology) instead of the antibody. Precipitates were washed sequentially for 5 min each in low-salt, high-salt, and lithium chloride immune complex wash buffers, and finally washed twice with Tris/EDTA buffer. Histone complexes were then eluted from the antibody by freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$). Histone-DNA cross-links (including the input samples) were reversed by 5 M NaCl at 65°C for 4 hr. DNA fragments were extracted with a