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Identification of estrogen-responsive genes in the GH3 cell line by cDNA microarray analysis

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Abstract

To identify estrogen-responsive genes in somatolactotrophic cells of the pituitary gland, a rat pituitary cell line GH3 was subjected to cDNA microarray analysis. GH3 cells respond to estrogen by growth as well as prolactin synthesis. RNAs extracted from GH3 cells treated with 17 -estradiol (E2) at 10 ^{9} M for 24 h were compared with the control samples. The effect of an antiestrogen ICI182780 was also examined. The array analysis indicated 26 genes to be up-regulated and only seven genes down-regulated by E2. Fourteen genes were further examined by real-time RT-PCR quantification and 10 were confirmed to be regulated by the hormone in a dose-dependent manner. Expression and regulation of these genes were then examined in the anterior pituitary glands of female F344 rats ovariectomized and/or treated with E2 and 8 out of 10 were again found to be up-regulated. Interestingly, two of the most estrogen-responsive genes in GH3 cells were strongly dependent on E2 in vivo. #1 was identified as calbindin-D9k mRNA, with 80- and 118-fold induction over the ovariectomized controls at 3 and 24 h, respectively, after E2 administration. #2 was found to be parvalbumin mRNA, with 30-fold increase at 24 h. Third was c-*myc* mRNA, with 4.5 times induction at 24 h. The levels were maintained after one month of chronic E2 treatment. Identification of these estrogen-responsive genes should contribute to understating of estrogen actions in the pituitary gland.
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Keywords: Estrogen-responsive genes; cDNA microarray; Pituitary; GH3; Rats

1. Introduction

Estrogen regulates multiple functions in different cell types in the anterior pituitary gland [1–3]. In the somatolactotrophs (GH/prolactin cells), it is well documented that estrogen activates prolactin mRNA transcription through the estrogen-responsive element (ERE) located in the 5 -upstream regulatory region [4,5]. The storage and release of prolactin are also regulated by estrogen [6]. In addition to hormone production, estrogen promotes cell proliferation in somatolactotrophs, which is prominent in the rat case [7–9]. Although estrogen-responsive expression of a series of genes must be involved in these biological functions of the pituitary cells, only a few have so far been reported to be regulated by estrogen [2].

GH3 is a widely used rat pituitary somatolactotrophic cell line, originally isolated from the MtT/W5 pituitary tumor, whose growth and prolactin synthesis are stimulated by estrogen [10,11]. There is a variation in the estrogen-responsiveness of this cell line reported in the literature [5,12–15], but the cells obtained from the Health Science Research Resources Bank in Osaka, Japan, display high sensitivity with regard to induction of cell proliferation. In the present study, we performed a gene expression analysis of estrogen action in GH3 cells using the cDNA microarray technique and found many of the identified estrogen-responsive genes to also be similarly regulated in vivo in the anterior pituitary in F344 rats.

2. Materials and methods

2.1. Chemicals

17 -estradiol (E2) was purchased from Sigma Chemicals, St. Louis, MO, USA and ICI182780 was obtained from Tocris Cookson Ltd., Bristol, UK. Each was dissolved in

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ethanol to give stock solutions. Actinomycin D and cycloheximide were purchased from Wakojunyaku KK, Osaka, Japan.

2.2. Cell culture

The pituitary cell line GH3 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in DME/F12 mixed medium (Sigma Chemical Co.) containing penicillin and streptomycin with 10% horse serum (HS, Gibco/Invitrogen Corp., Carlsbad, CA, USA) and 2.5% fetal bovine serum (FBS, Gibco/Invitrogen). Before estrogen treatment, cells were maintained for a week in phenol red-free medium (Sigma Chemicals) containing the same antibiotics along with dextran-charcoal-treated serum. For cell growth assays, GH3 cells were seeded in 24-well plates at 1 10^4 cells/well, and hormones were added the next day. Growth was measured after five days by means of a modified MTT assay with WST-1 (Dojindo Chemicals, Kumamoto, Japan). For microarray analysis, 3 10^6 GH3 cells were seeded in 90 mm dishes and treated with E2 at 10 ^{9}M and/or ICI at 10 ^{7}M and harvested after 24 h treatment. Cells were harvested after addition of Isogen (Wakojunyaku). For mRNA quantification, cells were treated with E2 at 10 ^{12} to 10 ^{9}M and/or ICI182780 at 10 ^{7}M. After the indicated period of time, cells were harvested with cell lysis buffer supplied with an SV-total RNA isolation kit (Promega Co., Madison, WI, USA).

2.3. Animals

Animal experiments were conducted under the guidelines of the 'A Guide for the Care and Use of Laboratory Animals of Hiroshima University'. Female F344 rats were purchased at four weeks of age from Charles River Japan Co. (Kanagawa, Japan). They were maintained with free access to basal diet and tap water. All animals except the intact control underwent surgical ovariectomy upon receipt and implanted with pellets containing 10 mg of E2 subcutaneously as described previously [16]. Animals were sacrificed under ether anesthesia after 3, 8, 24 and 48 h in the short-term experiment. Treatment was extended between 7 and 30 days for the long-term experiment. The pituitary gland and the uterus of each rat were weighed and immediately frozen in liquid nitrogen and stored at 80 C.

2.4. The GeneChip analysis

Total RNAs were extracted with Isogen, a premixed RNA isolation reagent, based on the acid guanidium thiocyanate–phenol–chloroform extraction method. The supplied protocol was followed.

First-strand cDNA was synthesized by incubating 5 g of total RNAs with 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 100 pmol T7-(dT)24 primer [5 -GGCCAGTGAATTGTAATACGAC-TCACTATAGGGAGGCGG-(dT)24-3], 1 first-strand buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT) and 0.5 mM dNTPs at 42 C for 1 h. Second-strand synthesis was performed by incubating the first-strand cDNAs with 10 U *E. coli* ligase (Invitrogen), 40 U DNA polymerase I (Invitrogen), 2 U RNase H (Invitrogen), 1 reaction buffer (18.8 mM Tris–HCl pH 8.3, 90.6 mM KCl, 4.6 mM $MgCl_2$, 3.8 mM DTT, 0.15 mM NAD, 10 mM $(NH_4)_2SO_4$) and 0.2 mM dNTPs at 16 C for 2 h. Ten units of T4 DNA polymerase (Invitrogen) were then added, and the reaction was allowed to continue for another 5 min at 16 C. After phenol–chloroform extraction and ethanol precipitation, the double-stranded cDNA was resuspended in 12 l DEPC-treated dH_2O. Labeling of the dsDNA was achieved by in vitro transcription using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Briefly, the dsDNA was mixed with 1 HY reaction buffer, 1 biotin labeled ribonucleotides (NTPs with Bio-UTP and Bio-CTP), 1 DTT, 1 RNase inhibitor mix and 1 T7 RNA polymerase. The mixture was incubated at 37 C for 4 h. The labeled cRNA was then purified using a RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified cRNA was fragmented in 1 fragmentation buffer (40 mM acetate, 100 mM KOAc, 30 mM MgOAc) at 94 C for 35 min. For hybridization with the GeneChip Rat Genome U34A (Affymetrix), 15 g fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1 eukaryotic hybridization control (1.5 pM BioB, 5 pM BioC, 25 pM BioD and 100 pM Cre), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA and 1 manufacturer-recommended hybridization buffer in a 45 C rotisserie oven for 16 h. Washing and staining were performed with a GeneChip Fluidic Station (Affymetrix) using the appropriate antibody amplification washing and staining protocol. The phycoerythrin-stained arrays were scanned as digital image files and scanned data were analyzed with GeneChip software (Affymetrix) [17].

2.5. Quantification of mRNAs by real-time RT-PCR

RNA preparation was carried out with an SV-total RNA isolation kit. One microgram of total RNA was reverse-transcribed with 200 U of MMLV-RT (Invitrogen) and 2.5 pmol of oligo-dT primer (Invitrogen) in 25 l buffer containing 1 mM dNTP, 100 mM Tris–HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 60 mM dithiothreitol and 5 U/ l RNasin with incubation at 37 C for 60 min.

The real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen) and an ABI Prism 7700 (The Perkin–Elmer Co) was employed for quantitative measurement for following the supplied protocol [18]. Specific primer sets with a T_m of about 59 C were designed for each mRNA selected from the microarray analysis (Table 1). The PCR conditions were a 15 min of initial activation step followed by 45 cycles of 15 s at 94 C, 30 s at 50 C and 60 s at

Table 1
Nucleotide sequences of primers for quantitative real-time PCR

Gene	GenBank accession#	Forward	Reverse
#1	K00994	AACCAGCTGTCCAAGGAGGA	CTTCTCCATCATCGTTCTTATCCA
#2	AI175539	TTTCTTCAGGCCCACCATCT	TTGCAGGATGTCGATGACAGA
#3	AI014135	GAACCAATTCTCCTAGCACAAGTG	CACGCCTGTGTTGGGCTAA
#4	AI178971	GGTGTGAAATCCCCAGGGT	CCCTGTCCACTCTGAGCGAC
#5	S81478	GATCAACGTCTCGGCCAATT	GCACAAACACCCTTCCTCCA
#6	D26393	GATTTCTAGGCGGTTCCGGA	ACTCGGAGCACACGGAAGTT
#7	AI230712	TGGCAGAAAAATCAATCCAGC	AAAGCCAGCCCCAAATCAC
#8	AF081366	CATCTGGACAACTGTGCTGGA	GGCACCACACATGAAGGAATT
#9	Y00396	CCGAGCTACTTGGAGGAGACA	AGGCCAGCTTCTCGGAGAC
#10	U02553	GATCAACGTCTCGGCCAATT	GCACAAACACCCTTCCTCCA
#14	U24175	CAGTGGATCGAGAGCCAGC	TGCCCCAGCTTGATCTTCAG
#15	D13623	ACCAAGACCGGTAGCAAGGG	GAAATCCGACGGAAGAGTGC
#21	AA892522	CCTTCGACTCAGCCACAAAAA	ACAGGGTCTTACCCTGCCTTC
#22	L16922	AGCCAGAGCCCACAATATGG	GCAATCATTTCTTCCGGCAC
G3PDH	AB017801	TGAAGGTCGGTGTGAACGGATTTG	TGATGGCATGGACTGTGGTCATGA

72 C. Prior to the quantitative analysis, PCR products were prepared separately and purified by gel electrophoresis. The fragments extracted from the gel were used as standards for quantification. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (The Perkin–Elmer Co.). All mRNA contents were normalized with reference to G3PDH mRNA.

2.6. Statistical analysis

Multiple comparison was made by ANOVA followed by Scheffe's test. Otherwise, Student's *t*-test was applied.

3. Results

3.1. Estrogen-dependent cell proliferation of GH3

The relative cell numbers were measured at day 5 of treatment with E2 at concentrations from 10 ^{13} to 10 ^{9} M (Fig. 1). Significant stimulation of cell proliferation was observed at 10 ^{12} M and the response appeared to reach a maximum at 10 ^{11} M. The sizes of individual cells treated with E2 appeared to be larger than without hormone.

3.2. Estrogen-responsive genes identified by cDNA microarray

Differentially expressed genes based on the ratio of the measured hybridization intensities on GeneChip Rat Genome U34A between control and E2-treated cells are listed in Table 1. A minimal change of two-fold was applied to select up- and down-regulated genes. Two independent experiments were carried out and the genes showing reliable hybridization for both experiments were counted. The genes are listed according to average values of E2 induction. The results of ICI182780 treatment alone or with E2 are also given in Table 2. The genes regulated by E2 but not showing inhibition by ICI182780, which only accounted for four in total, are not included in the table. Interestingly, only 26 genes were categorized as up-regulated and seven as the down-regulated, out of approximately 8000 genes on the chip.

3.3. Confirmation of mRNA changes

From Table 2, the top ten genes and four others (#14, #15, #21 and #22) were selected and subjected to quantification of mRNA levels to confirm the results of cDNA microarray analysis. cDNAs from GH3 cells treated with E2 at 10 ^{12} to 10 ^{9} M and/or ICI at 10 ^{7} M are examined and

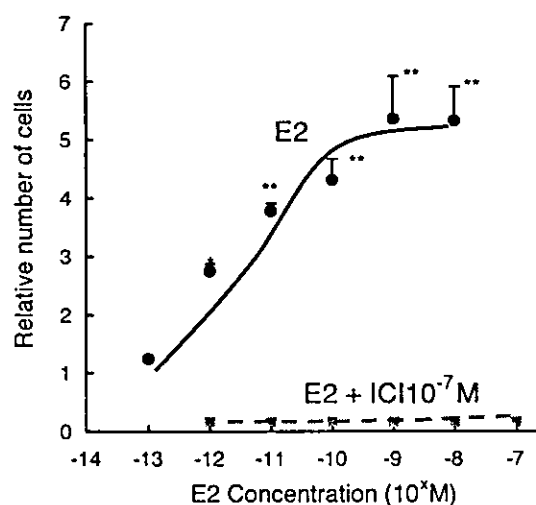

Fig. 1. Effects of 17 -estradiol (E2) and ICI182780 (ICI) on GH3 cell proliferation. Cells were seeded in 24-well plates at 1 10^4 cells per well. After five days treatment with E2 at 10 ^{13} to 10 ^{9} M alone or with ICI at 10 ^{7} M, cell proliferation was measured by a modified MTT assay. Each point represents a mean ± S.E.M. ($n = 4$). Indicates significant differences from the control value at 0.05 and 0.01, respectively.

Table 2
Genes up- and down-regulated by estrogen two or more fold in the microarray study

Genbank accession#		Gene name/blast match	Fold change in expression			
			E2(Exp1)	E2(Exp2)	E2+ICI	ICI
Genes up-regulated						
#1	K00994	Calbindin-D9k	8.12	6.20	0.70	0.33
#2	AI175539	Parvalbumin	7.58	4.54	0.81	0.28
#3	AI014135	Ribosomal RNA	6.23	4.93	1.17	0.94
#4	M17083	Alpha globin	5.23	4.99	0.39	0.59
#5	S81478	3CH134/CL1 ATPase	4.77	4.12	0.97	1.11
#6	D26393	Type II hexokinase	2.75	3.15	0.14	0.49
#7	AI230712	PACE4	2.98	2.73	0.44	0.15
#8	AF081366	K + channel ROMK2.1 isoform	3.21	2.44	0.88	0.20
#9	Y00396	c-*myc* protein	2.99	2.59	0.76	0.35
#10	U02553	Protein tyrosine phosphatase	3.32	2.23	0.67	0.44
#11	AF036548	RGC-32	3.47	2.05	1.12	0.37
#12	U53505	Type II iodothyronine deiodinase	2.26	2.87	0.77	0.34
#13	Y09507	Hypoxia-inducible factor 1	2.60	2.38	1.13	0.69
#14	U24175	Regulator of transcription 5a1	2.77	2.01	0.61	0.46
#15	D13623	p34 protein	2.43	2.32	1.05	1.02
#16	M58040	Transferrin receptor	2.37	2.38	0.73	0.30
#17	AA819776	EST (similar to HSP86)	1.93	2.76	1.82	1.97
#18	AA875126	EST (unknown)	2.33	2.27	0.58	0.70
#19	M14656	Osteopontin	1.89	2.69	1.37	1.22
#20	X67788	Ezrin, p81	2.28	2.23	0.47	0.50
#21	AA892522	EST (unknown)	2.19	2.23	0.60	0.82
#22	L16922	Progesterone receptor	2.30	2.04	0.89	0.67
#23	U57097	APEG-1 protein	2.36	1.97	1.43	1.51
#24	M24852	Neuron-specific protein	1.87	2.45	1.73	1.57
#25	AA817846	EST (similar to D- -hydroxy butyrate dehydrogenase)	1.86	2.37	0.97	0.96
#26	AI169417	Phosphoglycerate mutase type B subunit mRNA	1.98	2.23	0.97	0.92
Genes down-regulated						
	U67080	Zinc finger protein r-MyT3	0.49	0.47	1.38	1.06
	AA799964	EST (unknown)	0.49	0.41	0.51	0.71
	AI639263	EST (unknown)	0.46	0.41	0.68	0.32
	M27925	Synapsin 2a	0.47	0.35	1.31	1.31
	E03229	JP 1991272688-A/2	0.47	0.30	1.31	0.95
	AI237654	Vdup1	0.40	0.35	0.81	0.84
	AA893280	EST (similar to adipose differenti-ation-related protein)	0.47	0.21	0.91	0.98

Gene are listed in order of average E2 fold change in Experiments 1 and 2. **Four E2 up-regulated genes were not inhibited by ICI, which are not included in this table (The GenBank accession numbers of these are AI138070, AA866485, D84480 and X74293).
Indicates genes originally listed as ESTs but found to have perfect match by BLAST.

the results were summarized in Fig. 2. Although the fold increases of E2 induced gene expression were slightly lower than in the microarray analysis, up-regulation and inhibition by ICI182780 were confirmed except with three genes, #3, #6 and #15, which showed no responses. Time dependence of gene expression induced by E2 was also examined and the results are summarized in Fig. 3. As expected, some of the genes were expressed early after E2 administration and others increased gradually. Since the microarray analysis was carried out at only one time point, 24 h after E2 treatment, early responding and quickly muting genes would not be expected to be identified.

To determine E2 in inducing the transcription of genes #1 and #2, GH3 cells were treated with E2 in the presence of 0.5 g/ml of actinomycin D (a transcription inhibitor) and 10 g/ml cycloheximide (a translation inhibitor) for 3 and 24 h (Table 3). Increase in mRNA levels by E2 was blocked

Table 3
Effects of cycloheximide and actinomycin D on E2-induced mRNA change of calbindin D9k and parvalbumin in GH3 cells

	3 h	24 h
Gene#1: calbindin D9k		
Control	5.45 ± 0.70	4.02 ± 0.33
CHX	4.03 ± 0.11	3.74 ± 0.27
ActD	1.01 ± 0.21	1.13 ± 0.23
Gene#2: parvalbumin		
Control	1.81 ± 0.41	4.52 ± 0.94
CHX	2.51 ± 0.19	8.34 ± 0.37
ActD	0.93 ± 0.09	1.58 ± 0.31

Cell were treated with E2 at 10 ^{9}M for 3 and 24 h with or without cycloheximide (CHX) at 10 g/ml or actinomycin D (ActD) at 0.5 g/ml. The inductions by E2 were calculated for each treatment (mean ± S.E.M., $n = 4$).
Indicates significant induction at 0.05 and 0.01, respectively.
Indicates significant induction at 0.05 and 0.01, respectively.

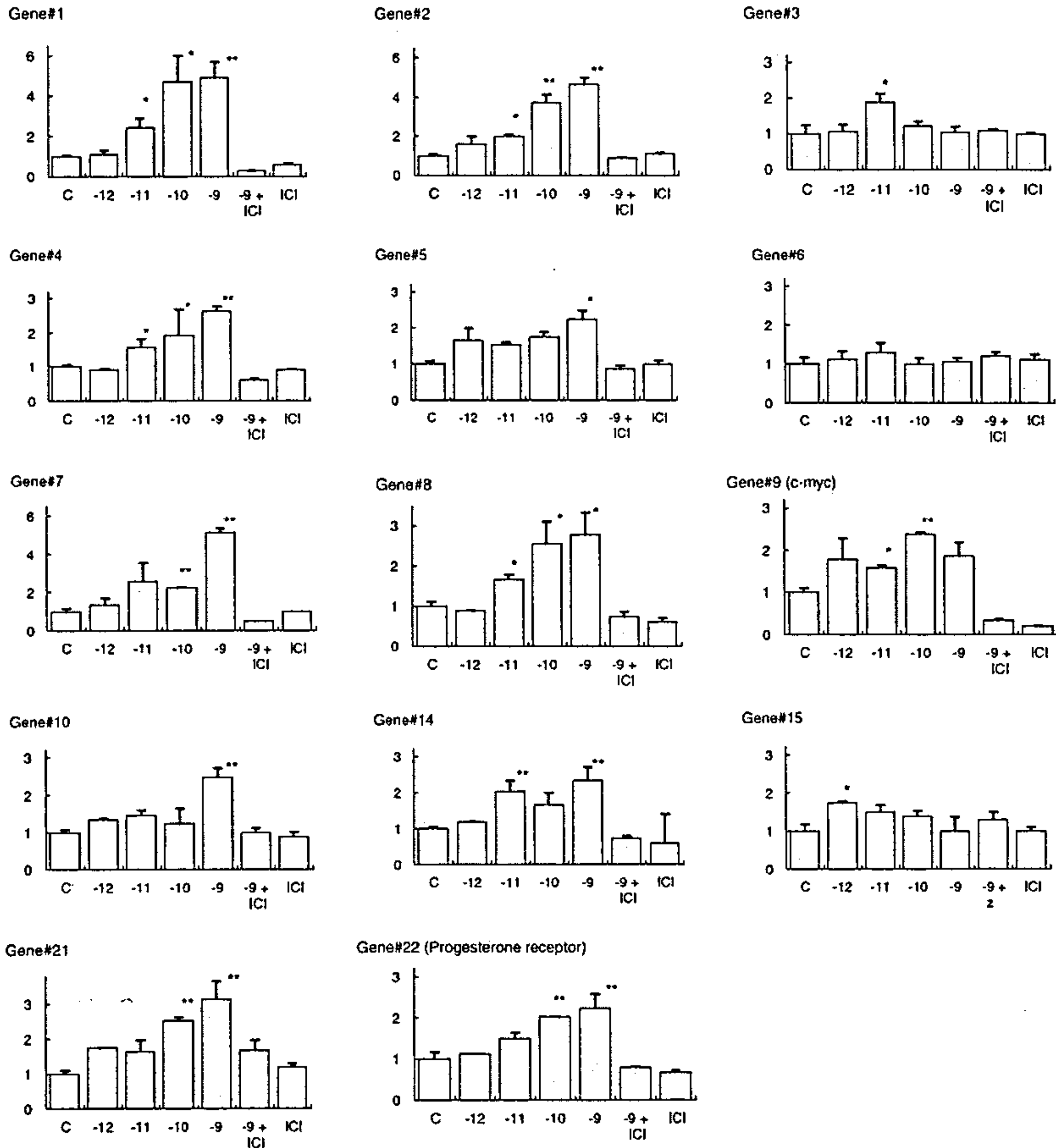

Fig. 2. Dose-dependent changes in gene expression levels measured by quantitative real-time RT-PCR. Cells were treated with different concentrations of E2 at 10^{-12} to 10^{-9} M and/or a single dose of ICI 182780 (ICI) at 10^{-7} M for 24 h. All mRNA contents were normalized with reference to G3PDH mRNA. The fold changes were calculated based on the gene expression in the cells treated with vehicle. Each point is an average of two independent experiments.

by actinomycin D but not by cycloheximide, which indicates that E2 regulates these genes at the transcriptional level.

3.4. Expression of genes in the pituitary gland

Expression of estrogen regulated genes in GH3 cells was further investigated in the anterior pituitary gland. First, mRNA expression of eleven-responsive genes was examined in short-term (24 h) and long-term (30 days) E2-treated ovariectomized F344 rats. Findings for estrogen-dependent increase for each gene are summarized in Table 4 as fold change of mRNA in E2-treated animals over that in the ovariectomized controls. All the genes except #4 were up-regulated in pituitary tissue by the short-term and long-term treatment of E2. Estrogen dependence of expression of gene #1 (calbindin-D9k) and gene #2 (parvalbumin) was extremely strong, over 100-fold induction being noted. For these and gene #9 (c-*myc*), more detailed time-dependent analysis was carried out. In Fig. 4, each mRNA level was calculated based on the level in ovariectomized rats at day

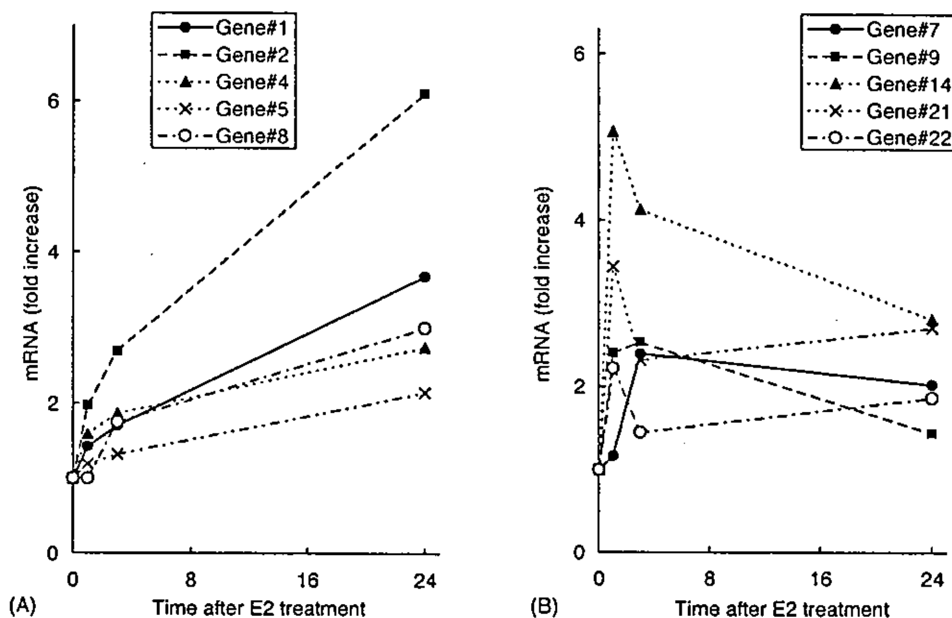

Fig. 3. Time-dependent change in gene expression levels measured by quantitative real-time RT-PCR. All mRNA contents were normalized with reference to G3PDH mRNA. Cells were treated with E2 at 10 ^{9} M for 0, 1, 3 and 24 h. Each point represents a mean ± S.E.M. ($n = 4$). · Indicates significant differences from the control values at 0.05 and 0.01, respectively.

Table 4
Estrogen-responsive genes identified by the microarray study in the pituitary tissues in ovariectomized F344 rats

Gene	GenBank accession#	Fold change in expression	
		24 h	1 month
#1	K00994	118	95.0
#2	AI175539	28.9	70.0
#4	M17083	1.1	0.6
#5	S81478	2.3	2.0
#7	AI230712	2.9	4.7
#8	AF081366	9.9	2.0
#9	Y00396	4.5	17.7
#10	U02553	3.1	1.6
#14	U24175	2.4	4.0
#21	AA892522	2.0	5.1
#22	L16922	4.2	9.4

Ovariectomized F344 rats were treated subcutaneously with pellets containing E2 for 1 and 30 days. The gene expression was measured by quantitative real-time RT-PCR in pituitary tissue and the fold changes were calculated based on the mRNA level in ovariectomized controls at time 0 ($n = 5$).

0. All the three mRNAs, for calbindin-D9k, parvalbumin and c-*myc*, were induced significantly within 3 h of subcutaneous E2 administration, although the increase was most prominent for calbindin-D9k, with a 72-fold elevation. Higher levels were still maintained after a month of chronic E2 treatment.

4. Discussion

The GH3 cell line has been widely used to investigate the functions of somatolactotrophic cells, since regulation of its GH and prolactin production appears to be physiologically relevant with dependence on thyroid hormones, estrogen and glucocorticoid [11,12,19]. In the present study, we applied microarray analysis and identified a number of estrogen-responsive genes.

In terms of GH3 estrogen-responsiveness, there are two distinct parameters, prolactin synthesis and cell proliferation. However, reported sensitivity to estrogen has varied in the literature [4,13–15,20]. The inter-laboratory variation may be due partly to differences in strain, since GH3 has a rather old origin and has been widely used. Technical problems with charcoal treatment of serum for removing estrogenic substances may have had an impact in some cases [21]. The estrogenic activity of phenol red or related contaminants in common culture media was not recognized until Katzenellenbogen's group provided a convincing evidence [22]. Prior to the present microarray analysis, GH3 cells were examined in our culture conditions and found to be very sensitive to estrogen, exhibiting induction of cell proliferation in response to E2 at a concentration as low as 10 ^{12} M. The high sensitivity on cell proliferation appears typical for pituitary cell lines, like the MtT/E-2 cell line we have established and another lactotrophic cell line, PR1 [4,23]. ER is the major type of ER expressed in GH3 cells with a ratio to ER of 380:1 according to quantitative PCR (data not shown).

Recently, estrogen-responsive genes have been investigated by cDNA microarray in human breast cancers and the normal uterus [24,25]. However, the pituitary gland has not been explored for estrogen-responsive genes by this approach, to our knowledge. In the present microarray analysis, a relatively small number of genes were found to be

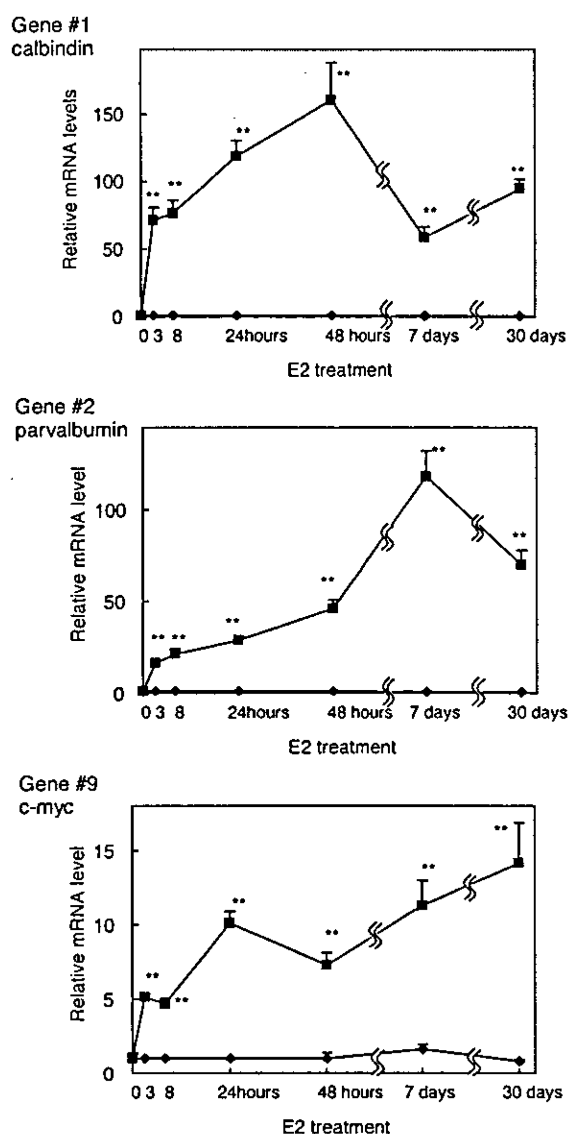

Fig. 4. Time-dependent analysis of three estrogen-responsive genes in the pituitary in vivo. Ovariectomized F344 rats were treated subcutaneously with pellets containing E2 for 3, 8, 24, and 48 h and 7 and 30 days. Gene expression was measured by quantitative real-time RT-PCR in pituitary tissue and fold changes were calculated based on the mRNA level in the ovariectomized controls at time zero. All mRNA contents were normalized with reference to G3PDH mRNA. Each point and bar represent mean ± S.E.M. ($n = 5$), · Indicates significant differences from the control values at 0.05 and 0.01, respectively.

regulated by estrogen with confirmation in most cases by quantitative real-time PCR. Suppression by ICI of E2-induced gene expression was also confirmed. The degrees of change were similar with real-time PCR analysis and GeneChip data and although we selected up-regulated genes after 24 h of estrogen exposure, some genes proved to be rapidly regulated (Fig. 3(B)) including these for the progesterone receptor and c-*myc*. Estrogen-responsive induction of progesterone receptor is well documented for the primary target, the uterus, as well as in the anterior pituitary gland [26,27]. Estrogen activation of c-*myc* also has been reported in the anterior pituitary gland and breast cancer cells [28,29]. A total of seven genes could be listed as down-regulated but they were not analyzed further, since all of them displayed relatively small degrees of change to 0.34–0.48 of the control values. Other known estrogen-responsive genes in the pituitary gland, such as prolactin and TGF were not on the array used in the present study.

Interestingly, the in vivo expression of two genes, calbindin-D9k and parvalbumin, was found to be highly induced by E2 both in the short and longer term, which may suggest that hypothalamus or other indirect endocrine pathways would be involved in regulating genes in addition to the direct transcriptional activation. Calbindin-D9k is a vitamin D-dependent intestinal calcium-binding protein that is detectable in the duodenum, uterus and placenta [30–32]. Another vitamin D-dependent calcium-binding protein, calbindin-D28k, expressed in kidney and brain has no homology with calbindin-D9k either at the nucleotide or at the transcript levels [33]. The calbindin-D9k gene has been reported to contain a 15-base-pair imperfect palindrome with high homology to the estrogen- and glucocorticoid-responsive elements (ERE and GRE) [34]. Although there is no evidence that this protein is regulated by estrogen in the intestine through this motif, it is possible that the imperfect ERE is functional for the hormone-dependent transcription in the pituitary gland. Parvalbumin is another calcium-binding protein that belongs to the EF-hand calcium-binding protein like calbindin-D9k [35]. It is abundant in fast contracting/relaxing muscle fibers, where it plays a role as a calcium buffer and is also found in neurons as well as in endocrine glands including pituitary, thyroid, adrenals, testes and ovaries [36]. It has been postulated that parvalbumin can prevent cell death due to calcium overload in neurons. Although its expression is developmentally regulated in muscle, brain and other tissues, no evidence indicating hormonal regulation has been reported [37,38]. The 5 flanking region of the gene seems to function as the promoter but it does not contain any motifs for estrogen-dependent transcription [39,40].

Since RNA was extracted from whole anterior pituitary tissue in the present study, it is not clear which types of cell actually contributed to the increase in mRNA levels. Chronic treatment of rats with E2 is known to result in the development of lactotrophic tumors [5]. The F344 strain is the most sensitive to E2 and somatolactotrophs of the pituitary become hyperplastic after exposure for a week and steadily proliferate thereafter. In the present study, major response of GH3 cells was cell proliferation so that some of the identified genes might be expected to be mitosis-related and involved in estrogen-induced pituitary hyperplasia/tumorigenesis. Although up-regulation of the calbindin-D9k and parvalbumin gene are evident on long-term treatment of E2, there was no obvious correlation with the time period for pituitary hyperplasia in contrast to the c-*myc* expression which steadily increase.

In conclusion, the present microarray analysis allowed identification of a number of estrogen-responsive genes in GH3 cells whose regulation appears biologically relevant in the pituitary gland in vivo. The actual significance of two calcium-binding proteins discovered to be prominently induced by E2 remains to be explored in the future.

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SENESCENT B LYMPHOPOIESIS IS BALANCED IN SUPPRESSIVE HOMEOSTASIS: DECREASE IN INTERLEUKIN-7 AND TRANSFORMING GROWTH FACTOR-β LEVELS IN STROMAL CELLS OF SENESCENCE-ACCELERATED MICE

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Senescent B Lymphopoiesis Is Balanced in Suppressive Homeostasis: Decrease in Interleukin-7 and Transforming Growth Factor-β Levels in Stromal Cells of Senescence-Accelerated Mice

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The suppression of the B cell population during senescence has been considered to be due to the suppression of interleukin-7 (IL-7) production and responsiveness to IL-7; however, the upregulation of transforming growth factor-β (TGF-β) was found to contribute to B cell suppression.To investigate the mechanism of this suppression based on the interrelationship between IL-7 and TGF-β during senescence, senescence-accelerated mice (SAMs), the mouse model of aging, were used in this study to elucidate the mechanisms of B lymphopoietic suppression during aging. Similar to regular senescent mice, SAMs showed a decrease in the number of IL-7–responding B cell progenitors (i.e., colony-forming unit pre-B [CFU-pre-B] cells in the femoral bone marrow [BM]). A co-culture system of B lymphocytes and stromal cells that the authors established showed a significantly lower number of CFU-pre-B cells harvested when BM cells were co-cultured with senescent stromal cells than when they were co-cultured with young stromal cells. Interestingly, cells harvested from a senescent stroma and those from the control culture without stromal cells were higher in number than those harvested from a young stroma, thereby implying that an altered senescent stromal cell is unable to maintain self-renewal of the stem cell compartment. Because TGF-β is supposed to suppress the proliferative capacity of pro-B/pre-B cells, we added a neutralizing anti-TGF-β antibody to the co-culture system with a pro-B/pre-B cell-rich population to determine whether such suppression may be rescued. However, unexpectedly, any rescue was not observed and the number of CFU-pre-B cells remained unchanged when BM cells were co-cultured with senescent stromal cells compared with the co-culture with young stromal cells, which essentially showed an increase in the number of CFU-pre-B cells ($P < 0.001$ in 5 μg/ml). Furthermore, TGF-β protein level in the supernatant of cultured senescent stroma cells was evaluated by enzyme-linked immunoabsorbent assay, but surprisingly, it was found that TGF-β concentration was significantly lower than that of cultured young stromal cells. Thus, TGF-β activity was assumed to decline particularly in a senescent stroma, which means a distinct difference between the senescent suppression of B lymphopoiesis and secondary B lymphocytopenia. Concerning proliferative signaling, on the other hand, the level of *IL-7* gene expression in cells from freshly isolated BM decreased significantly with age. Therefore, the acceleration of proliferative signaling and the deceleration of suppressive signaling may both be altered and weakened in a senescent stroma (i.e., homeosupression). Exp Biol Med 229:494–502, 2004

Key words: aging; B-lymphopoiesis; interleukin-7; transforming growth factor-β; senescence-accelerated mice; homeosuppression

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Introduction

Aging is accompanied by changes in the immune system, leading to a decrease in the overall cellular and humoral responsiveness (1). Because the most marked age-associated change in the immune system is the rapid involution of the thymus after puberty, most of the decline in humoral immunity has been attributed to changes in the T-cell compartment rather than to an intrinsic primary B cell

deficit. Consequently, attention has been focused on age-associated changes in T lymphocytes and their functions (2). However, it has recently been clarified that there are also deficiencies in B cell development in the bone marrow (BM) of aged animals (3–6). Alterations in B cell development may include both the skewing of *V*-gene utilization, particularly in cells responsive to phosphoryl choline, and the decrease in the generation of various developmental B cell subsets. The altered representation of these subsets appears to be a consequence of a developmental arrest of the maturation of pro-B cells and the earliest stage of surface Ig-positive cells (7). Age-related changes in the B cell development may account for the deterioration of the immune system in senescent mice.

B lymphopoiesis is suppressed during senescence not only in mice but also in humans. A decrease in interleukin-7 (IL-7) production by stromal cells and a simultaneous reduction in B lymphocyte reactivity to IL-7 are considered as a possible background for this negative senescent regulation. Furthermore, in addition to senescence, B cells were noted to be regulated by two pathways not only for IL-7 but also for transforming growth factor-β (TGF-β) in regular mice; that is, not only the downregulation of the former but also the upregulation of the latter simultaneously play a role in suppression regulation (8). This is in good agreement with the observation that the supplementation of IL-7 could not compensate for the B cell suppression. In this study, possible senescence-associated alterations in the productions of IL-7 and TGF-β are examined in senescence-accelerated mice (SAMs).

Senescence-accelerated mice provide a unique model system for studying senescence or aging in higher organisms, because they exhibit a marked acceleration of aging, which has been confirmed to be the same manner as that observed in the regular mice. Senescence-accelerated mice are characterized by the early onset of aging (mean life span of 40 weeks under conventional conditions), loss of general behavioral activity, increased skin coarseness, and spinal lordokyphosis (9). Because the number of splenic cells starts to decrease at approximately 30 weeks old, the SAMs used were, in general, 30 weeks old or slightly older. Although one must carefully interpret the results of studies using SAMs because the mechanism of "accelerated aging" may not be associated with that of "normal aging," results of previous studies conducted by other researchers and ourselves indicate that SAMs are a suitable model for predicting the possible mechanism of aging in hematopoietic systems (10–14).

The purposes of this study are to confirm the status of B lymphopoiesis in SAMs compared with that in other regular strains and to elucidate the mechanism of age-related changes in B lymphopoiesis in SAMs. Here, we examined age-related changes in the number and function of B cell progenitors in the BM and their supportive microenvironment.

Materials and Methods

Mice. A senescence-prone substrain of the AKR/J mouse, SAMs/-P-1 (9), from The Jackson Laboratory in Bar Harbor, ME, was kindly provided by Dr. Toshio Takeda, Emeritus, the Chest Disease Research Institute, Kyoto University. The mice were bred and maintained at the experimental animal facility of the National Institute of Health Sciences under pathogen-free conditions. Male SAMs designated as "young (8–12 weeks old)" or "senescent (30–36 weeks old)" were used in the present study; these ages were selected because the number of splenic cells and/or hemopoietic progenitor cells start to decrease at approximately 30 weeks of age (11).

Preparation of BM Cells. The BM cell suspensions were prepared by repeatedly flushing the cells from femurs and dispersing them by trituration through a 23-gauge hypodermic needle with the Iscove-modified Dulbecco medium (IMDM; Invitrogen Corp., Carlsbad, CA) or RPMI 1640 medium (Invitrogen).

***In Vitro* Colony Assays.** Colony-forming unit pre-B (CFU-pre-B) cells were assayed by suspending mononuclear cells in 1-ml aliquots of the recombinant IL-7 (rIL-7)–supplemented MethoCult M 3630 medium (Stem Cell Technologies Inc., Vancouver, Canada) in 35-mm, plastic petri dishes. Femoral BM cells from three mice per group were pooled and assayed. A MethoCult M 3630 medium consisting of 1 ml of the semisolid IMDM medium containing 1% methylcellulose, 30% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), 0.1 m*M* 2-mercaptoethanol (2-ME), 2 m*M* L-glutamine, and 10 ng/ml of rIL-7 (R&D Systems, Inc., Minneapolis, MN) was used. Granulocyte-macrophage colony-forming units (GM-CFUs) were assayed by suspending mononuclear cells in the alpha medium containing 1% methyl cellulose, 30% FBS, 1% bovine serum albumin, 1 m*M* 2-ME, and 10 ng/ml of granulocyte-macrophage colony-stimulating factor (Genzyme, Cambridge, MA) and plating 1-ml aliquots in 35-mm, plastic dishes. Both CFU-pre-B cells and GM-CFUs in culture plates in triplicate were incubated at 37°C in a fully humidified atmosphere of 5% carbon dioxide in air. Aggregates of 50 or more cells in 7-day cultures were counted as colonies. Aggregates ranging from 10 to 49 cells were counted as clusters.

Co-culture of Stromal Monolayers and Pro-B/Pre-B Cell-Rich Populations. Stromal monolayers were prepared by culturing BM cells derived from young or senescent SAMs at 1×10^6/ml in 96-well Coster 3596 or 24-well Falcon 3047 flat-bottomed plates in 0.2 or 1 ml of the RPMI 1640 medium supplemented with 20% FBS. Confluent adherent layers were formed after 7 days. To obtain pro-B/pre-B cell-rich populations, the bulk culture of pooled BM cells from young SAMs stimulated with rIL-7 was performed as described previously (4). Briefly, BM cells from young SAMs were cultured at 1×10^6 cells/ml in RPMI 1640 supplemented with 20% FBS, 2×10^{-5} *M*

2-ME, 1% L-glutamine, and 2 ng/ml of murine rIL-7 (Genzyme) and plated in six-well Coster 3516 culture trays. Nonadherent cells were harvested after 4 days of culture. This bulk culture provided a highly rich (>10-fold) source of IL-7–responsive B220$^+$, CD43$^+$, IgM$^-$, pro-B/pre-B cells (data not shown). Pro-B/pre-B cell-rich populations were suspended at 5×10^4/ml in RPMI 1640 supplemented with 20% FBS, 2×10^{-5} *M* 2-ME, 1% L-glutamine, and 1 ng/ml of murine rIL-7. Aliquots (0.1 or 1.0 ml) of this cell suspension were added to established stromal cell monolayers in 96- and 24-well flat-bottomed trays, respectively, and co-cultured at 37°C in a fully humidified atmosphere of 5% carbon dioxide in air. Nonadherent cells were harvested after 3 days, counted, and cloned using the CFU-pre-B colony assay system.

Extraction of Total RNA and Polymerase Chain Reaction (PCR). Total RNA was extracted from BM cells using the TRIsol reagent (Invitrogen) according to the manufacturer's instructions. First, messenger RNA (mRNA) was reverse transcribed using superscript (Life Technologies, Grand Island, NY) and random hexamers. Next, PCR amplification of complementary DNA (cDNA) was performed with the graded dilution of cDNA for semi-quantitative evaluation of IL-7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression under the following conditions: IL-7 cDNA, 95 °C for 1 min, 55°C for 2 mins, and 72°C for 3 mins for 35 cycles; and GAPDH cDNA, 94°C for 30 secs, 60°C for 30 secs, and 72°C for 1 min for 20 cycles. Murine IL-7 and GAPDH primers were synthesized based on a published cDNA sequence (15):

IL-7 (sense) 5'-GCCTGTCACATCTGAGTGGC-3'
IL-7 (antisense) 5'-CAGGAGGCATCCAG-GAACTTCTG-3'
GAPDH (sense) 5'-TGAAGGTCGGTGTGAACG-GATTTGGC-3'
GAPDH (antisense) 5'-CATGTAGGCCATGAGGTC-CACCAC-3'

The expected amplified PCR products were 496 and 982 base pairs long for IL-7 and GAPDH, respectively. The PCR products were photographed using the Bio-Rad 2000 gel documentation system (Bio-Rad Laboratories, Hercules, CA), and intensities of expressions were evaluated by ImageGauge version 3.11 (Science Lab 98 for Windows; Fuji Film, Tokyo, Japan). In this experiment, GAPDH expressions were not altered among the experimental groups.

Effect of Anti–TGF-β Antibody on Growth of Pro-B/Pre-B Cell-Rich Population Co-Cultured with Stromal Cells. To examine the effect of TGF-β produced by stromal cells on the growth of pro-B/pre-B cells, a neutralizing monoclonal antibody (mAb) to TGF-β (mouse IgG_1 isotype, R&D Systems) at dilutions ranging from 1–10 μg/ml was added to the co-culture system. The mouse IgG_1 isotype (R&D Systems) was used as mock control. The number of nonadherent cells in the co-cultures was determined 3 days later.

Determination of Level of TGF-β Protein Produced by Cultured Stromal Cells. Stromal monolayers were prepared by culturing BM cells from young and senescent SAMs at 1×10^6/ml in 24-well Falcon 3047 flat-bottomed plates in 1 ml of the RPMI 1640 medium supplemented with 20% FBS. Confluent adherent layers were obtained after 7 days. The supernatant in the culture plates was removed; and then 1 ml of RPMI 1640, supplemented with 20% FBS, 2×10^{-5} *M* 2-ME, and 1% L-glutamine were added to the culture plates. The culture medium was collected after 7 days of culture and was used for the determination of the level of the TGF-β protein produced by stromal cells. The TGF-β concentration in the culture medium was determined using a TGF-β–specific enzyme-linked immunoabsorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions. All the samples were assayed in triplicate. The samples were acid activated (16) by adding 1/5 vol of 1 *N* hydrochloride at room temperature and neutralized after 10 mins by adding of 1/5 vol of 1.2 *N* NaOH in 0.5 *M* HEPES, and the mixture was diluted with the same volume of calibrator diluents in the ELISA kit.

Statistical Analysis. Data were analyzed using the analysis of variance (ANOVA). Values were considered significantly different at $P < 0.05$.

Results

Decrease in Number of B Cell Progenitors (Pre-B Cells). Age-related changes in the numbers of B lymphocytes and hematopoietic progenitors differ from each other. Table 1 summarizes the results of the triplicate experiments. The number of femoral GM-CFU cells from 30-week-old and 36-week-old senescent mice assayed on the basis of their colony-forming ability increased to 112% and 109%, respectively, that of GM-CFU from 12-week-old mice. In contrast, the CFU-pre-B colony assay, using Day 7 B cell colonies as the end point, was used to determine the number of IL-7–responsive B cell progenitors in young and senescent BM cells. The numbers of femoral CFU-pre-B cells from 30-week-old and 36-week-old mice decreased to 75.7% and 65.0%, respectively, that from 12-week-old mice. Furthermore, the decrease in the number of CFU-pre-B cells from femoral BM in senescent mice could not be counteracted by increasing IL-7 concentration in the culture medium 4-fold or by extending the culture period (data not shown).

Significant Decrease in Number of Large Pre-B Colonies. Among B cell colonies of various sizes, we noted that the number of relatively larger B cell colonies decreased significantly with aging (Fig. 1). The number of cells per colony ranged from 50–5000. Therefore, CFU-pre-B cell colonies in Table 1 were categorized according to their size, namely, small (50–200 cells), intermediate (201–

Table 1. Age-Related Changes in Number of Hematopoietic Progenitor Cells in Senescence-Accelerated Mice[a]

	Mean ± SEM of triplicate experiments (%)		
	12-week-old mice	30-week-old mice	36-week-old mice
Femoral GM-CFU cells	72,762 ± 672	81,250 ± 2811 (112%)	79,058 ± 4763 (109%)
Femoral CFU-pre-B cells	13,868 ± 516	10,505 ± 1083* (75.7%)	9017 ± 220** (65.0%)

[a] GM-CFU, granulocyte-macrophage colony-forming unit; CFU-pre-B, colony-forming unit pre-B.
* $P < 0.05$; ** $P < 0.001$.

3000), and large (>3000 cells). As shown in Figure 1, the numbers of large, intermediate, and small B cell colonies for all groups decreased with age (58.3% for large colonies, 75.8% for intermediate colonies, 78.4% for small colonies in 30-week-old mice relative to those in 12-week-old mice; 22.1% for large colonies, 52.0% for intermediate colonies, and 76.5% for small colonies in 36-week-old mice relative to those in 12-week-old mice). The decrease in the numbers was statistically most significant for large colonies in 30- and 36-week-old mice relative to those to 12-week-old control ($P < 0.001$ and $P < 0.005$, respectively) and also for intermediate colonies in 36-week-old mice relative to those in 12-week-old control ($P < 0.05$).

Decrease in IL-7 Expression Level in BM. As observed in the regular senescent mice, the number of pre-B cell progenitors also decreased in SAMs. Therefore, the expression level of IL-7, which is known to be a pre-B cell stimulator, was evaluated in BM cells. IL-7 expression level decrease during senescence (17–19). In this study, IL-7 mRNA expression level was evaluated by reverse transcriptase (RT)–PCR. The BM stromal cell–derived IL-7 is a positive regulator of *in vivo* B lymphopoiesis. As shown in Figure 2, the IL-7 mRNA expression level in BM cells from senescent mice was 6.2% that from young mice. These findings are comparable to those in the literature (17–19). Further experiments using SAMs were designed.

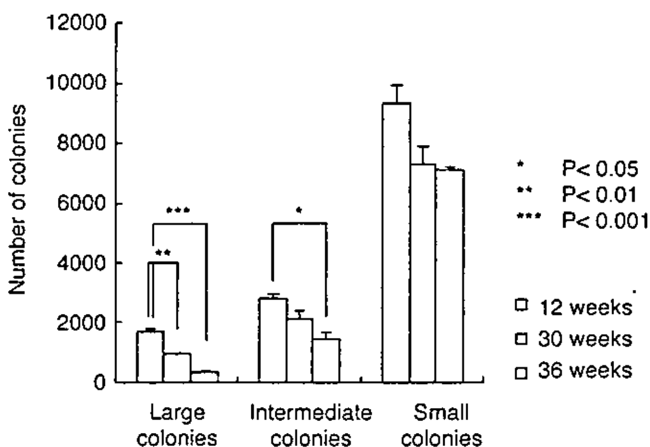

Figure 1. Age-related changes in number of B cell colonies of large, intermediate, and small sizes (mean ± SEM of three replicate experiments). Femoral bone marrow (BM) cells from three mice per group were harvested and pooled. The preparation of BM cell suspensions is described in the "Materials and Methods" section. Colony-forming unit pre-B (CFU-pre-B) cells were assayed by suspending mononuclear cells in 1-ml aliquots of the recombinant interleukin-7 (rIL-7)–supplemented MethoCult M 3630 medium (Stem Cell Technologies Inc., Vancouver, Canada) in 35-mm, plastic petri dishes. Culture plates in triplicate for CFU-pre-B cells were incubated at 37 °C in a fully humidified atmosphere of 5% carbon dioxide in air. According to the size of CFU-pre-B cell colonies shown in Table 1, colonies were categorized as follows: small (50–200 cells), intermediate (201–3000), and large (>3000 cells).

Decrease in Proliferative Capacity of B Cell Progenitors, Pre-B Cell Response to IL-7. The decrease in IL-7 expression level is also associated with the decrease in the responsiveness of pre-B cells to IL-7. To evaluate such responsiveness, we used a recloning assay to determine the proliferative capacity of the progeny of CFU-pre-B cells. Cells derived from 36 large colonies were pooled and recloned for 7 days in a semisolid medium supplemented with rIL-7. Table 2 shows the results of the recloning study. The numbers of secondary colonies, which included small (50–200 cells) and intermediate (201–3000) colonies and clusters (10–49 cells) generated from individual large primary colonies and derived from 30-week-old and 36-week-old femoral BMs, decreased significantly to 69.0% and 2.7%, respectively, that of secondary colonies grown from large primary colonies derived from 12-week-old femoral BM. Furthermore, cells from small primary colonies derived from either young or senescent mouse BM formed no secondary colonies. In B lymphopoiesis, unlike in the case of myelogenous progenitors, the results indicate that the responsiveness of CFU-pre-B cells to IL-7 decreases with age.

Decrease in Maintenance Capacity of Stromal Cells for B Lymphopoiesis. Although the pre-B progenitor cells were altered during senescence, a decrease in the maintenance capacity of stromal cells for B cell lineages may also be of importance in association with hematopoietic senescence (17, 18, 20). Using a co-culture system in 24-well flat-bottomed trays, we determined whether the capacity of stromal cells to support B lymphopoiesis is altered with age. Interestingly and unexpectedly, the number of lymphocytes recovered from the coculture of pro-B/pre-B cells with young stromal cells was significantly lower than that recovered from senescent stromal cells (Fig. 3A). In contrast, the total number of CFU-pre-B cells recovered from the co-culture with young stromal cells was significantly higher than that recovered from the co-culture with senescent stromal cells (Fig. 3B).

Decrease in TGF-β Production by Senescent Stromal Cells. On the basis of the above-mentioned co-

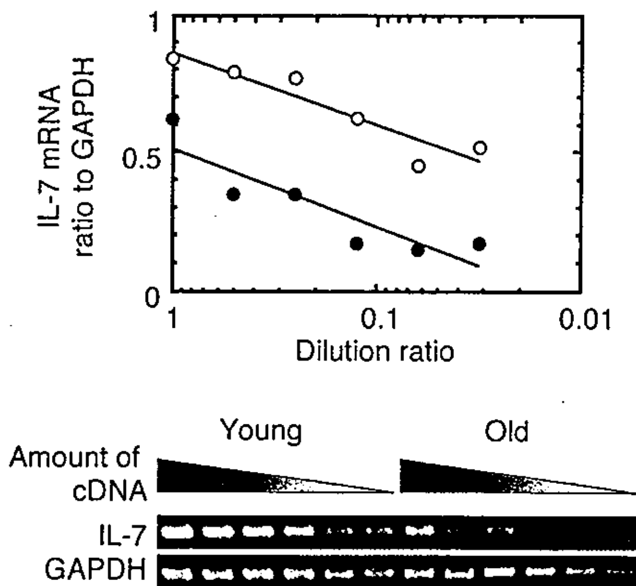

Figure 2. Expression level of interleukin-7 (IL-7) messenger RNA (mRNA) in bone marrow (BM) cells freshly isolated from young and old senescence-accelerated mice (mean ± SEM of three replicate experiments). Vertical bars for SEM are within the symbols. Total RNA was extracted from BM cells using the TRIsol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The extracted mRNA was reverse transcribed using Superscript (Life Technologies, Grand Island, NY) and random hexamers. The reverse-transcribed complementary DNAs (cDNAs) are then amplified by polymerase chain reaction (PCR) using specific primers for murine IL-7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The conditions and primer sequences used for the PCR amplification of cDNAs are shown in the "Materials and Methods" section of reference 15. The expected amplified PCR products were 496 and 982 base pairs long for IL-7 and GAPDH, respectively. In this experiment, GAPDH expressions were not altered among the experimental groups.

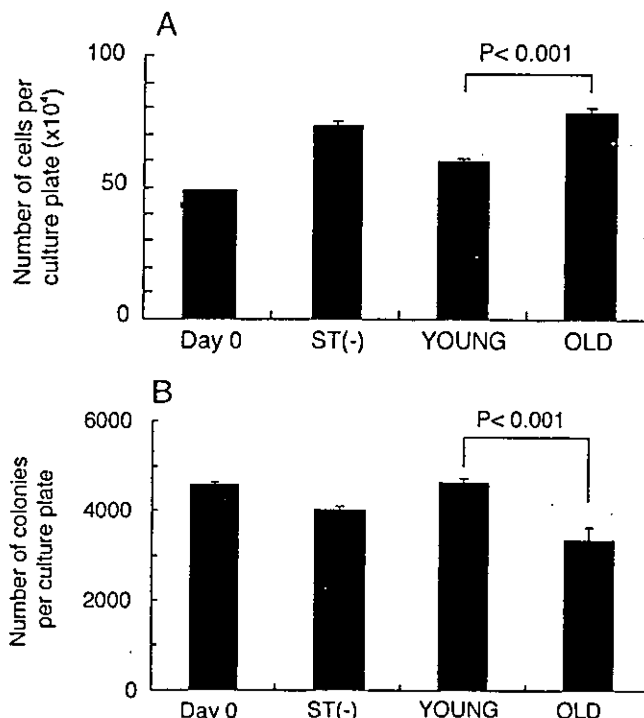

Figure 3. (A) Co-culture of pro-B/pre-B cell-enriched populations with stromal cells: change in the number of nonadherent cells (mean ± SEM of three replicate experiments). (B) Co-culture of pro-B/pre-B cell-rich populations with stromal cells: change in total number of CFU-pre-B cells (mean ± SEM of three replicate experiments). Stromal monolayers were prepared by culturing bone marrow (BM) cells from young or senescent senescence-accelerated mice (SAMs) at 1×10^6/ml in 96-well Coster 3596 or 24-well Falcon 3047 flat-bottom plates in 0.2 or 1 ml of the RPMI 1640 medium supplemented with 20% fetal bovine serum. Confluent adherent layers were formed after 7 days. To obtain Pro-B/pre-B cell-rich populations (>10-fold) (i.e., interleukin-7 [IL-7]-responsive B220$^+$, CD43$^+$, IgM$^-$, pro-B/pre-B cells), pooled BM cells from young SAMs stimulated with recombinant IL-7 were cultured, as described in the "Materials and Methods" section (4). Nonadherent cells were harvested and counted. Day 0 indicates nonadherent cell number at the beginning of co-culture; ST (-), nonadherent cell number after culture with IL-7 alone; YOUNG, nonadherent cell number after co-culture with young stromal cells in the presence of IL-7; OLD, nonadherent cell number after co-culture with senescent stromal cells in the presence of IL-7.

culture data, we propose a hypothesis that the CFU-pre-B inhibitory activity of BM cells may reside predominantly in young stromal cells rather than in senescent stromal cells. Because stromal cells produce BM-derived TGF-β (21) and are also a negative regulator of B lymphopoiesis (22–24), we investigated whether TGF-β production by stromal cells is reduced with age. Figure 4 shows percent changes in the number of the same seeded BM cells cocultured with young stromal cells (open circles) or senescent stromal cells (closed circles) in 96-well flat-bottomed trays after adding a graded dose of a neutralizing mAb to TGF-β. The high-dose group (10 μg/ml and more; data not shown) exhibited a toxic effect, but the lower-dose group showed a significant difference of responses between the young and senescent groups, implying that the proliferation of the senescent

Table 2. Secondary B Cell Colonies Derived From One Large Colony-Forming Unit B Cell Colony[a]

Donor mouse age (week)	Mean ± SEM of triplicate experiments					
	Large	Intermediate	Small	Cluster	No. of total colonies[b] (with cluster)[c]	% to a/% to b
12	ND	1.4 ± 0.6	5.8 ± 0.7	7.2 ± 0.6	7.2 ± 0.6 (14.4 ± 1.0)	100%/100%
30	ND	ND	2.3 ± 0.5	7.4 ± 1.7	2.3 ± 0.5* (9.7 ± 1.4)**	24%/69%
36	ND	ND	ND	0.4 ± 0.2	ND (0.4 ± 0.2)**	—/2.7%

[a] Percentages for 30-week-old or 36-week-old mice compared with 12-week-old mice are shown in parentheses. ND, not detected.
[b] Number of total colonies by large, intermediate, and small B cell colonies without clusters.
[c] Number of total colonies with clusters.
* $P < 0.005$; ** $P < 0.01$.