

予測の正確性

	True Value Yes	True Value No
Prediction Yes	29	6
Prediction No	7	34

Sensitivity = 80.6%
 Specificity = 85.0%
 Accuracy = 82.9%
 Likelihood Ratio
 of a positive test = 5.37

definition of sensitivity, specificity and accuracy

	True value Yes	True value No
Prediction Yes	a	b
Prediction No	c	d

$\text{sensitivity} = a/(a+c) = 1 - (\text{pseudo.negativity})$
 $\text{specificity} = d/(b+d) = 1 - (\text{pseudo.ppositivity})$
 $\text{accuracy} = (a+d)/(a+b+c+d)$
 $\text{likelihood ratio} + = \{a/(a+c)\}/\{b/(b+d)\}$

図6 後期 76 サンプルを予測した結果

正確性、感受性、特異性の算出法は表下に示した。

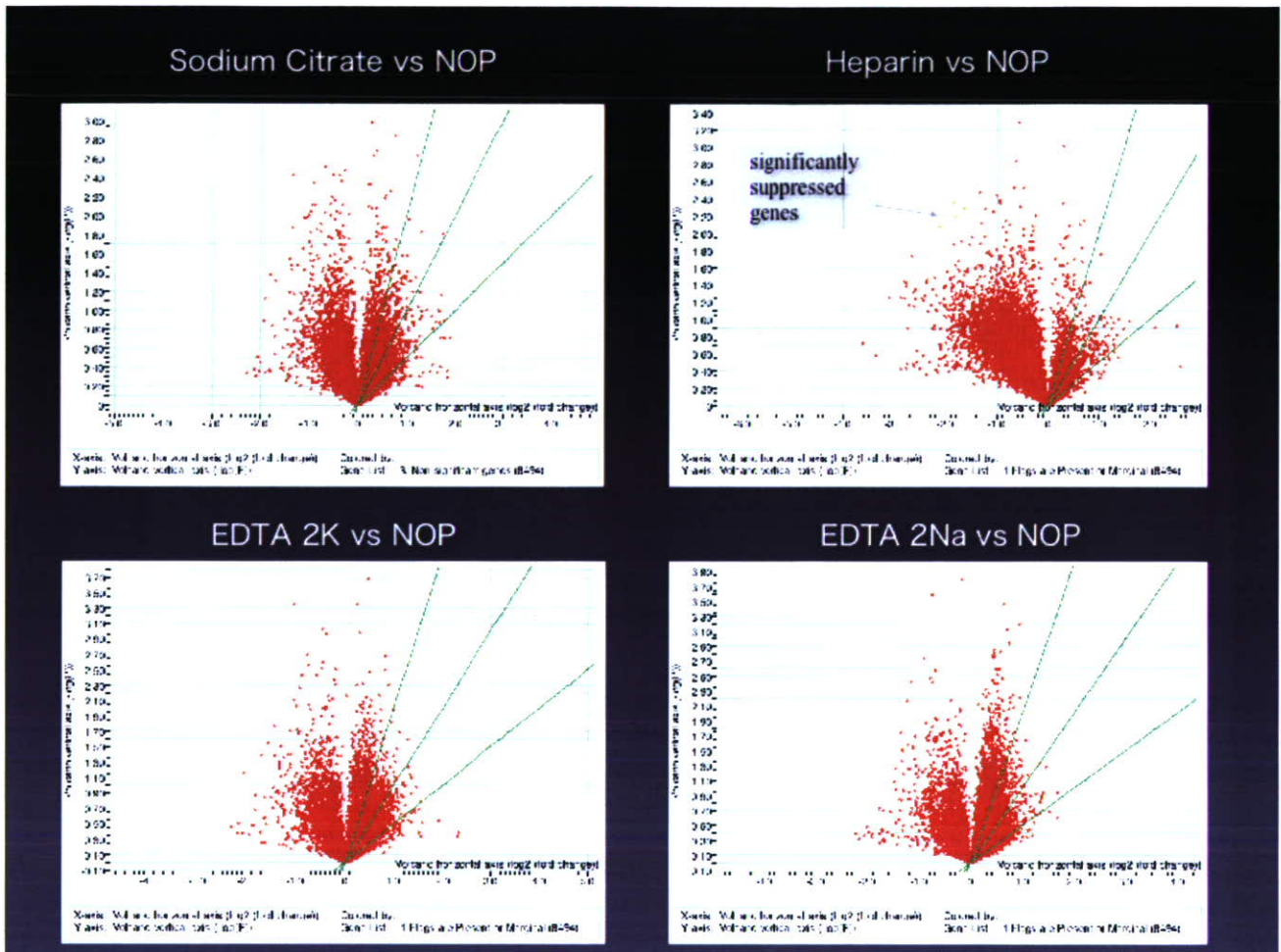


図7 抗凝固剤の遺伝子発現に与える影響

抗凝固剤を使用した時と、無処理(NOP)の細胞の遺伝子発現を、各抗凝固剤を用いて検討した。ヘパリンを用いた時に、無処理と比較して、発現が抑制された遺伝子が4個見られた(図、右上)。

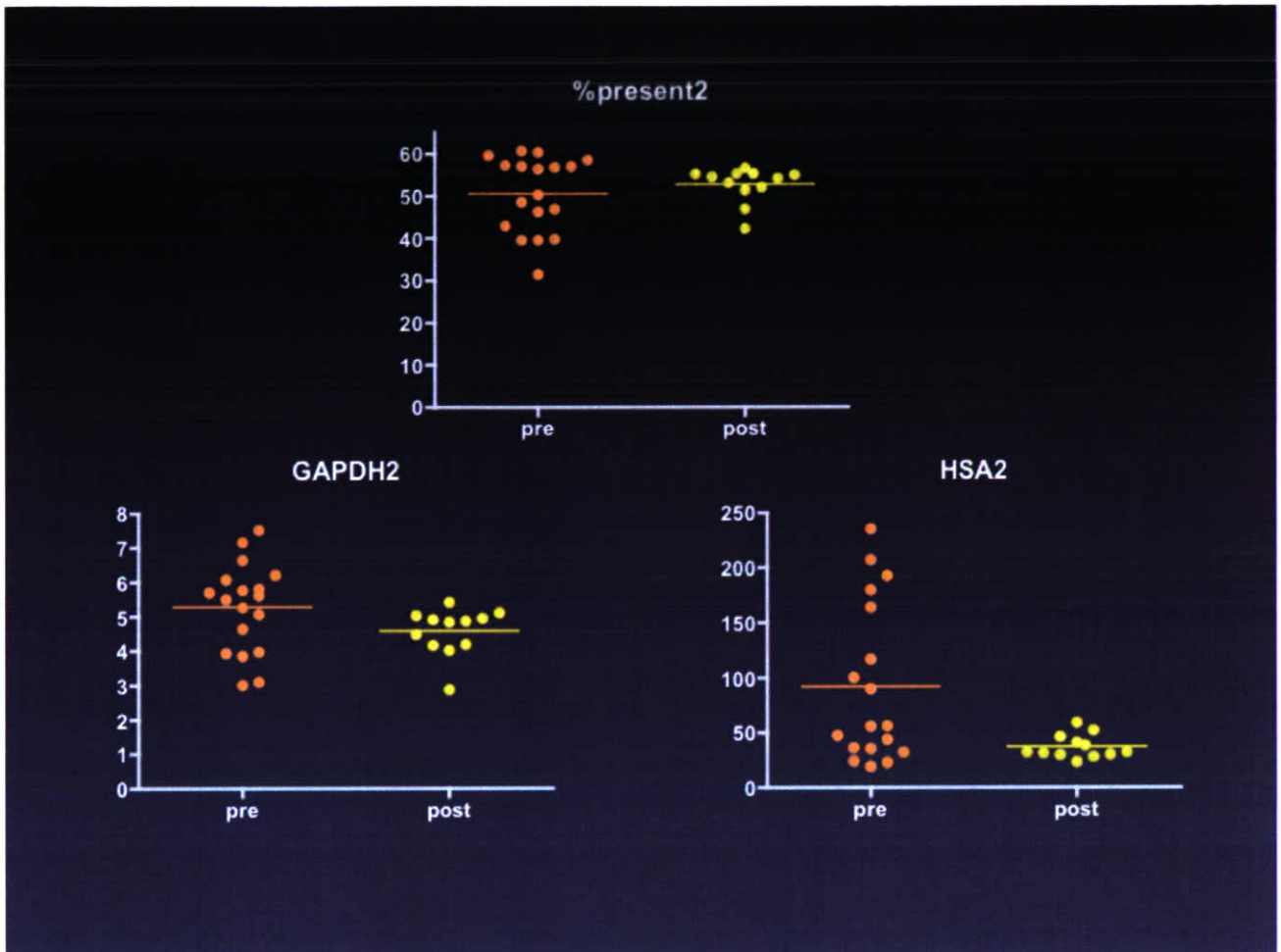


図8 薬物投与前採血(pre)と薬物投与後採血(post)で得られたデータの比較

Chip にハイブリ後、得られた品質管理項目について比較した。

臨床検体Chip解析結果 in vitroとin vivo(血液検体)との比較

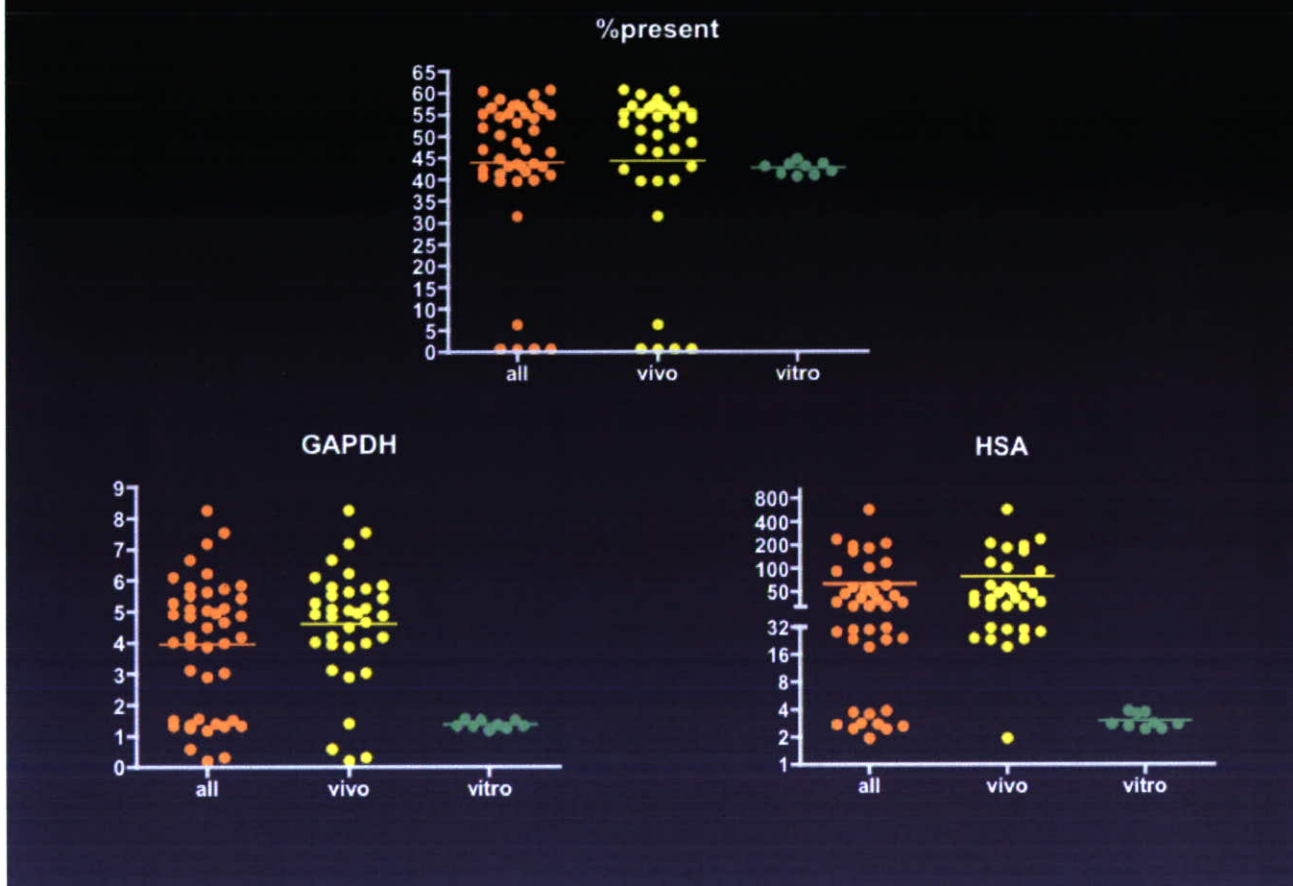


図9 臨床検体で得られた Chip データと培養細胞より得られた Chip データの品質比較

三酸化ヒ素（亜ヒ酸）による遺伝子発現変化の解析

K-meansクラスタ法による分類

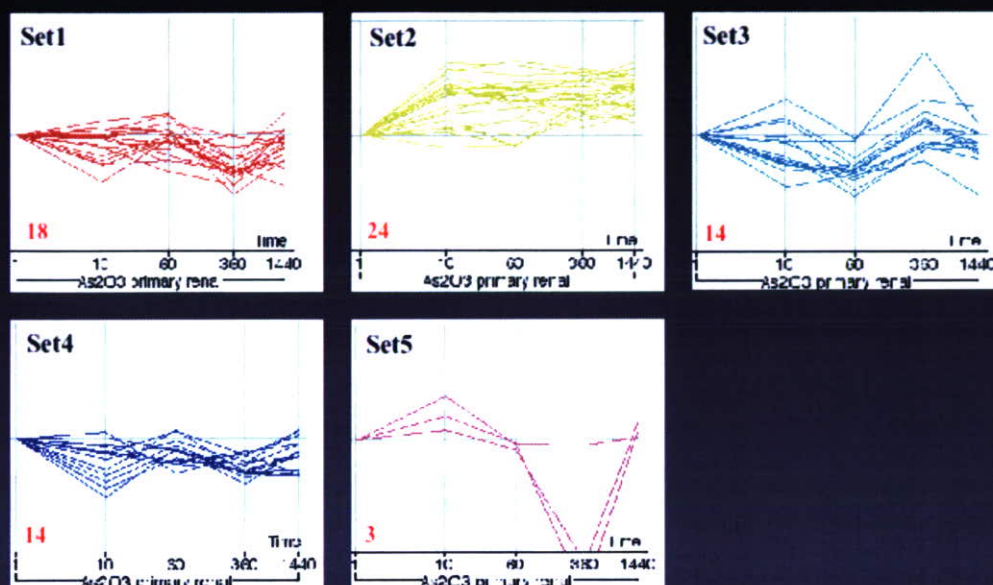


図10 三酸化ヒ素により発現が変化した遺伝子の K-means クラスタ法による分類
クラスターSet2 の中に今回着目した HMOX-1 遺伝子が含まれていた。

三酸化ヒ素曝露によるHMOX1 mRNA発現量の変化

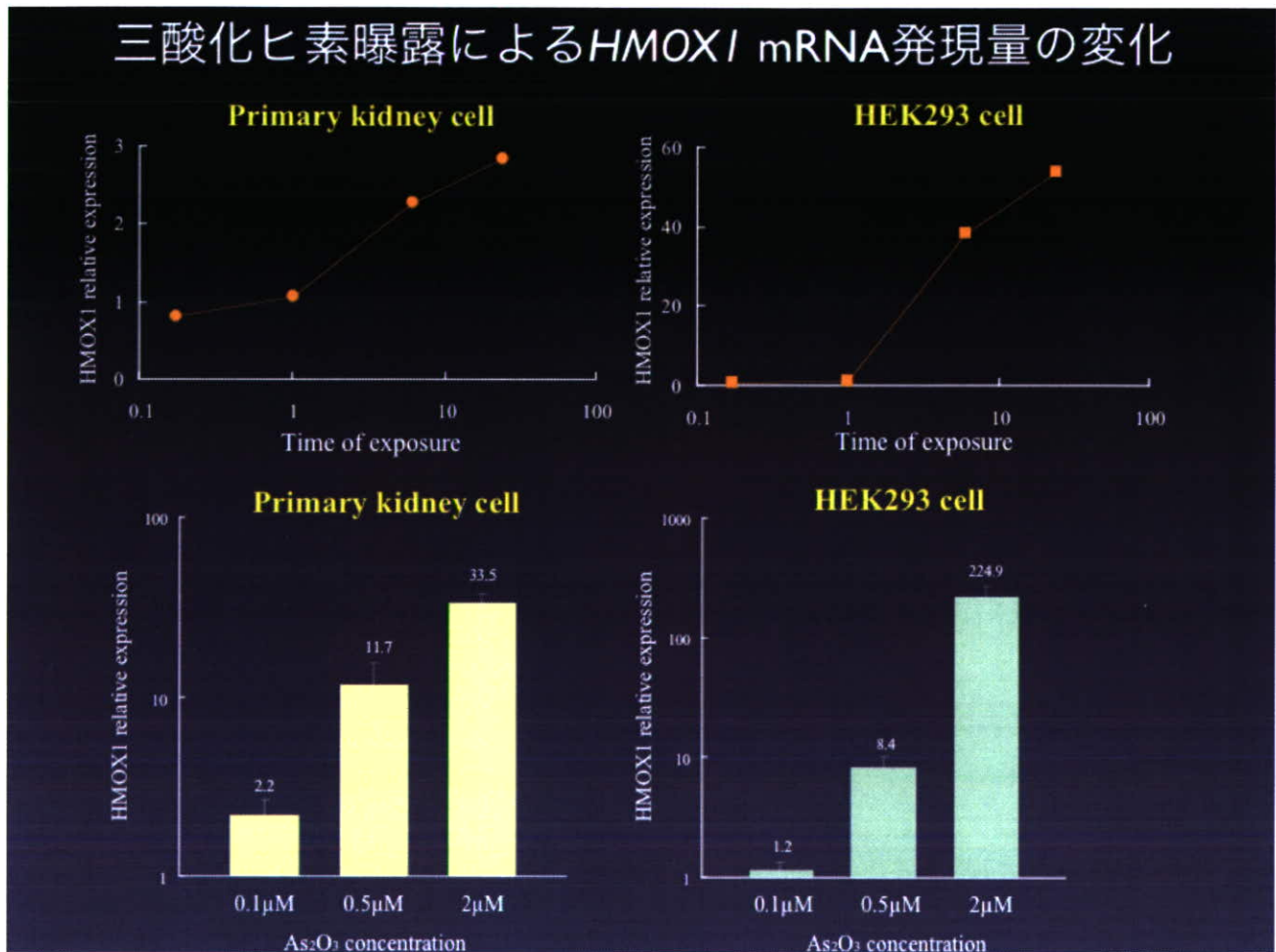


図11 三酸化ヒ素曝露によるHMOX-1遺伝子 mRNAの発現量変化

上段は時間依存性、下段は濃度依存性を調べた。いずれも三酸化ヒ素曝露により有意に発現が亢進している。

三酸化ヒ素によるSuperoxide anion生成

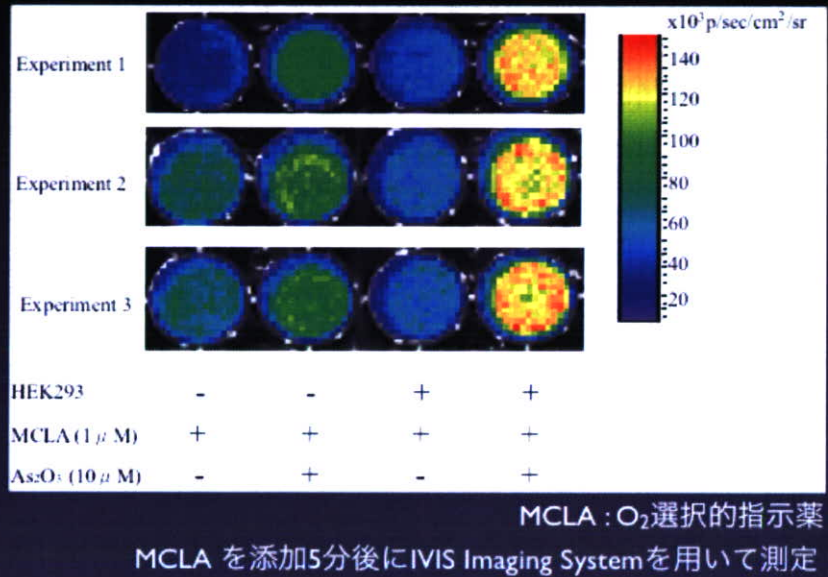


図12 三酸化ヒ素による活性酸素生成

MCLA は活性酸素を検出する試薬であり、細胞、三酸化ヒ素、MCLA の全てが存在する時に活性酸素が発生していた。

三酸化ヒ素による腎毒性に対する 抗酸化剤である α -リポ酸の効果

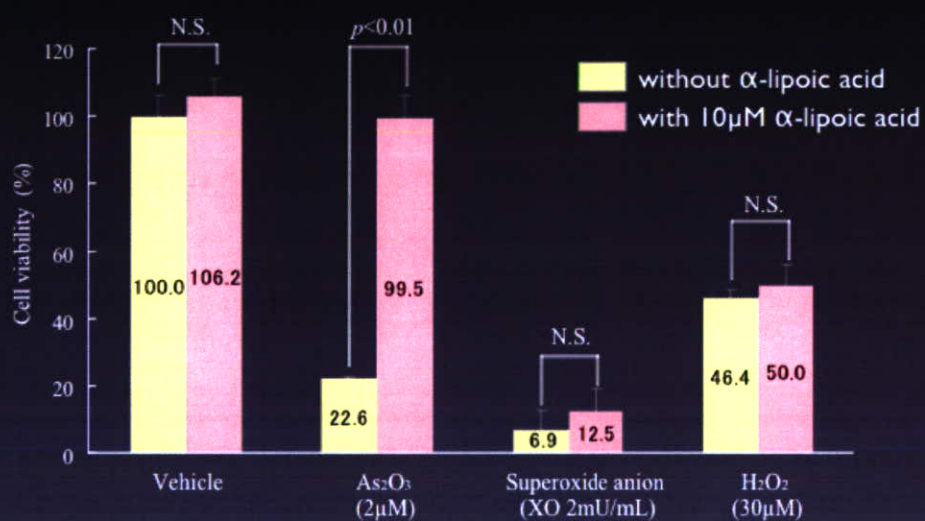


図13 抗酸化剤である α -リポ酸の三酸化ヒ素による細胞障害に対する効果

三酸化ヒ素併用時にのみ α -リポ酸の細胞保護効果がある。

三酸化ヒ素と α -リポ酸の腎機能に与える影響

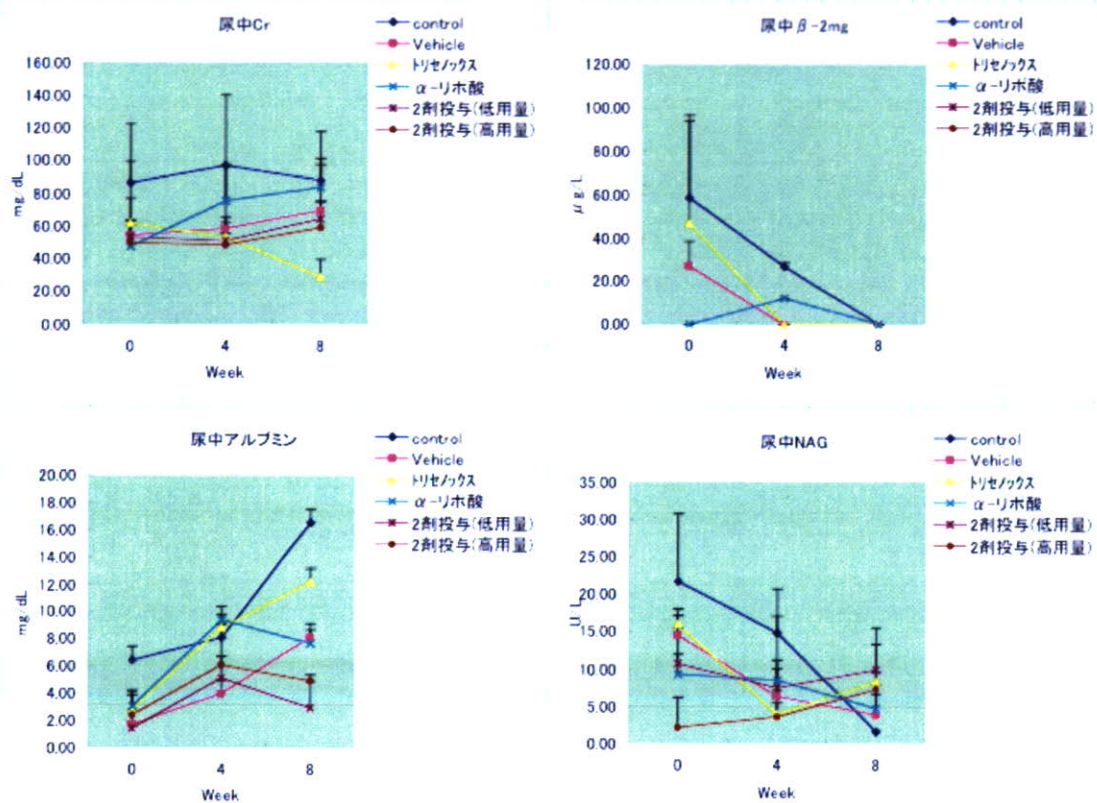


図14 三酸化ヒ素と α -リポ酸投与の腎機能に与える影響

ラットに三酸化ヒ素(トリセノックス)と α -リポ酸を反復投与し、投与前、4週後、8週後に各項目を測定した。

三酸化ヒ素処理時の α -リポ酸投与の生存曲線に与える影響

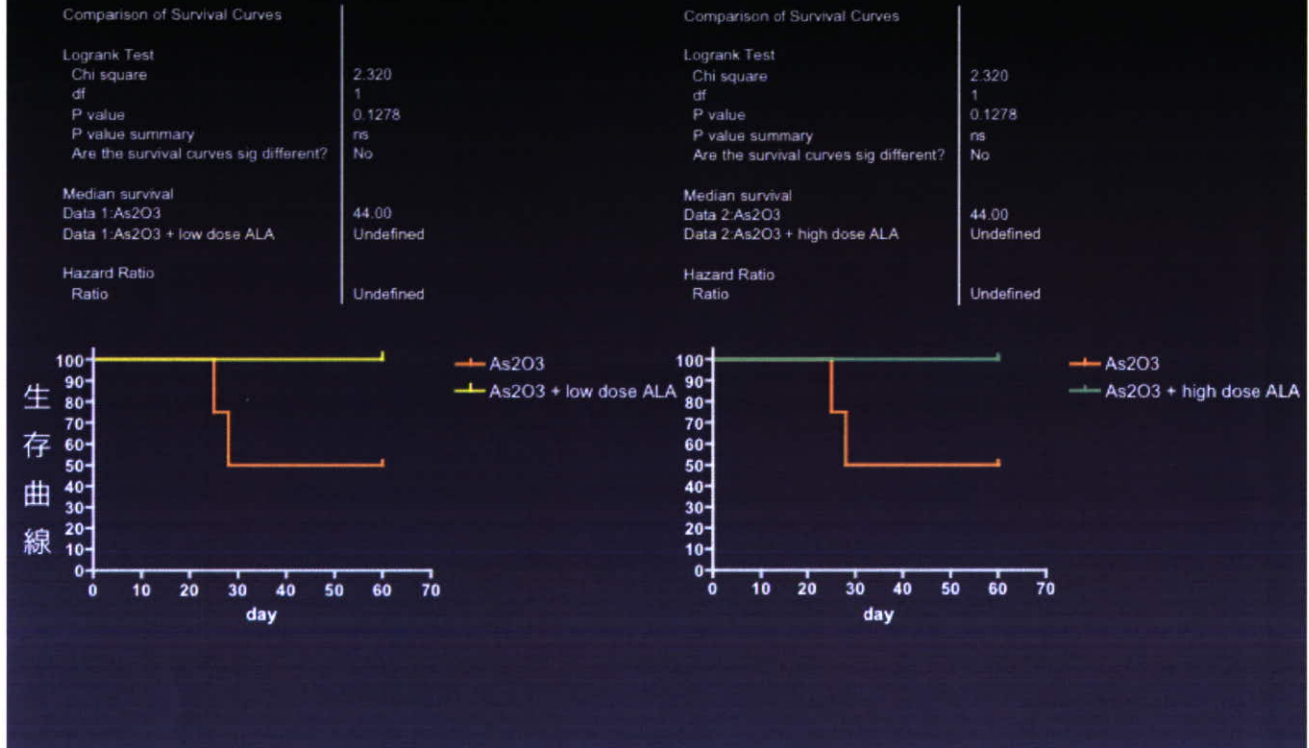
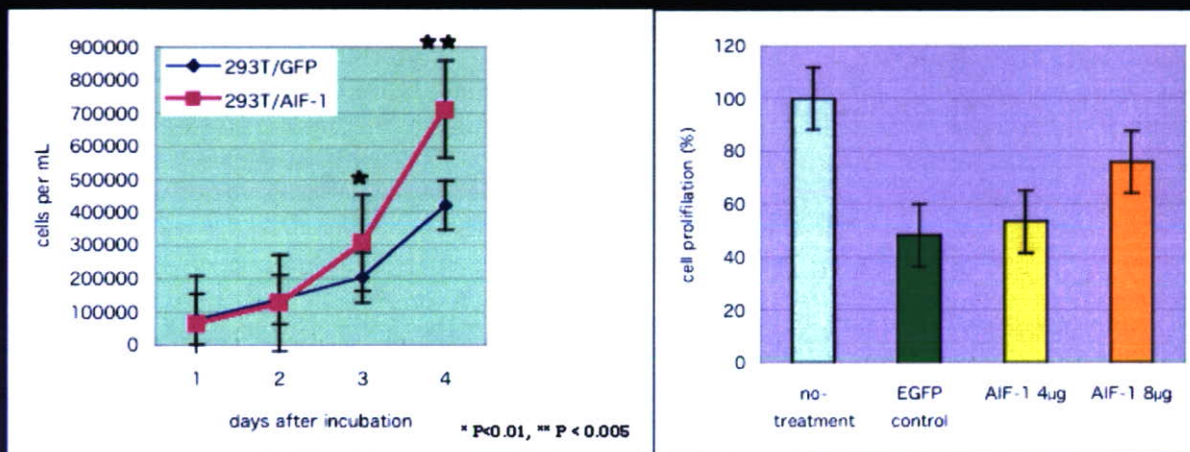


図15 三酸化ヒ素単独投与群と α -リポ酸併用群の生存曲線の比較

低用量(左)と高用量(右)の α -リポ酸はいずれも死亡を抑制していた。

AIF-1を導入した293T細胞の細胞増殖性と活性に対する影響



AIF-1導入細胞の細胞増殖性

AIF-1細胞の細胞活性

図16 AIF-1 導入細胞の細胞増殖と細胞活性へ与える影響

左のグラフは細胞数をカウントし、右のグラフは細胞活性を WST-1 により測定した。いずれの場合も AIF-1 が強発現している事により、コントロールに比べて、細胞増殖・活性ともに増加傾向を示した。

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Numata A, Shimada K, Kamezaki K, Haro T, Kakumitsu H, Shide K, Kato K, Miyamoto T, Yamashita Y, <u>Oshima Y</u> , Nakajima H, Iwama A, Aoki K, Takase K, Gondo H, Mano H, Harada M.	Signal transducers and activators of transcription 3 augments the transcriptional activity of CCAAT/enhancer-binding protein a in granulocyte colony-stimulation factor signaling pathway	J Biolo Chem	280	12621-12629	2005
<u>大島康雄</u> 、 <u>藤村昭夫</u>	日本人組織を用いたトキシコゲノミクス研究	臨床薬理	36	11-12	2005
H. Ando, <u>Y. Oshima</u> , H. Yanagihara, Y. Hayashi, T. Takamura, S. Kaneko, and <u>A. Fujimura</u> .	Profile of rhythmic gene expression in the livers of obese diabetic KK-A(y) mice.	Biochem Biophys Res Commun	346	1297-1302	2006
A. Sasaki, <u>Y. Oshima</u> , S. Kishimoto, and <u>A. Fujimura</u> .	Individual differences in gene expression in primary cultured renal cortex cells derived from Japanese subjects.	IPSJ Transactions on Bioinformatics	47	67-72	2006
A. Sasaki, <u>Y. Oshima</u> , and <u>A. Fujimura</u> .	An approach to elucidate potential mechanism of renal toxicity of arsenic trioxide	Exp Hematol	35	252-262	2007

研究成果による特許権等の知的財産権の出願・登録状況

出願番号	発明の名称
特願 2007-47053	腫瘍治療における三酸化二ヒ素の副作用低減剤及び解毒剤
特願 2007-72357	遺伝子発現情報に基づく薬物有害反応の予測方法

健康危険情報

なし

Signal Transducers and Activators of Transcription 3 Augments the Transcriptional Activity of CCAAT/Enhancer-binding Protein α in Granulocyte Colony-stimulating Factor Signaling Pathway*

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The Janus kinase (Jak)-Stat pathway plays an essential role in cytokine signaling. Granulocyte colony-stimulating factor (G-CSF) promotes granulopoiesis and granulocytic differentiation, and Stat3 is the principle Stat protein activated by G-CSF. Upon treatment with G-CSF, the interleukin-3-dependent cell line 32D clone 3(32Dcl3) differentiates into neutrophils, and 32Dcl3 cells expressing dominant-negative Stat3 (32Dcl3/DNStat3) proliferate in G-CSF without differentiation. Gene expression profile and quantitative PCR analysis of G-CSF-stimulated cell lines revealed that the expression of C/EBP α was up-regulated by the activation of Stat3. In addition, activated Stat3 bound to CCAAT/enhancer-binding protein (C/EBP) α , leading to the enhancement of the transcriptional activity of C/EBP α . Conditional expression of C/EBP α in 32Dcl3/DNStat3 cells after G-CSF stimulation abolishes the G-CSF-dependent cell proliferation and induces granulocytic differentiation. Although granulocyte-specific genes, such as the G-CSF receptor, lysozyme M, and neutrophil gelatinase-associated lipocalin precursor (NGAL) are regulated by Stat3, only NGAL was induced by the restoration of C/EBP α after stimulation with G-CSF in 32Dcl3/DNStat3 cells. These results show that one of the major roles of Stat3 in the G-CSF signaling pathway is to augment the function of C/EBP α , which is essential for myeloid differentiation. Additionally, cooperation of C/EBP α with other Stat3-activated proteins are required for the induction of some G-CSF responsive genes including lysozyme M and the G-CSF receptor.

The proliferation and differentiation of hematopoietic progenitor cells are regulated by cytokines (1). Among these, gran-

ulocyte colony-stimulating factor (G-CSF)¹ specifically stimulates cells that are committed to the myeloid lineage (2). The importance of G-CSF to the regulation of granulopoiesis has been confirmed by the observation of severe neutropenia in mice carrying homozygous deletions of their G-CSF or G-CSF receptor genes (3, 4). Cytokines activate several intracellular signaling pathways, and the Janus kinase (Jak) signal transducers and activators of transcription (Stat) pathway is essential for cytokine function (5, 6). The binding of G-CSF to cell surface G-CSF receptors activates Jak1, Jak2, and Tyk2 followed by the activation of Stat1, Stat3, and Stat5 (7–9). Stat3 is the principle protein activated by G-CSF (8, 10). Phosphorylated Stats translocate from the cytoplasm into the nucleus and induce transcription of their target genes within a short period of time. 32Dcl3 cells differentiate to neutrophils following treatment with G-CSF. In contrast to their parental cells, 32Dcl3 cells expressing dominant-negative Stat3 (32Dcl3/DNStat3) proliferate in the presence of G-CSF, but they maintain immature morphologic characteristics without evidence of differentiation (11). Additionally, transgenic mice with a targeted mutation of their G-CSF receptor that abolishes G-CSF-dependent Stat3 activation show severe neutropenia with an accumulation of immature myeloid precursors in their bone marrows (12). To clarify the role of Stat3 in the G-CSF signaling pathway, we wished to identify target genes of Stat3.

We found that the levels of CCAAT/enhancer-binding protein (C/EBP) α mRNA were up-regulated following G-CSF stimulation in 32Dcl3 but were unchanged in 32Dcl3/DNStat3. In addition, the activation of Stat3 augmented the function of C/EBP α , which is the essential transcriptional factor for myeloid differentiation. G-CSF-induced granulocytic differentiation was restored in 32Dcl3/DNStat3 cells by the conditional expression of C/EBP α . These results show that one of the major

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¹ The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; IL, interleukin; C/EBP α , CCAAT/enhancer-binding protein; NGAL, neutrophil gelatinase-associated lipocalin precursor; Jak, Janus kinase; Stat, signal transducers and activators of transcription; DNStat3, dominant-negative Stat3; IRES, internal ribosome entry site; GFP, green fluorescent protein; ER, endoplasmic reticulum; IFN, interferon; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter; LUC, luciferase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; TK, thymidine kinase; 4-HT, 4-hydroxytamoxifen.

roles of Stat3 in the G-CSF signaling pathway is to enhance the function of C/EBP α .

MATERIALS AND METHODS

Cell Culture, Expression Plasmid, and Cytokines—32D clone 3 (32Dcl3) and 32Dcl3/DNStat3 cells (DNStat3 deletes the transactivation domain of Stat3) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (ICN, Osaka, Japan), penicillin/streptomycin (Invitrogen), recombinant murine interleukin-3 (IL-3) (Kirin Brewery, Takasaki, Japan), and recombinant human G-CSF (Chugai Pharmaceutical, Tokyo, Japan). 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine.

For the construction of pTag2A-G-CSF receptor, the human G-CSF receptor cDNA (13) (pHQ3, kindly provided by S. Nagata and R. Fukunaga) was excised from the pBluescript vector and inserted into the FLAG-tagged mammalian expression plasmid pCMV-Tag2A (Clontech). pcDNA3-rat C/EBP α was described before (14). Stat3 cDNA was amplified by PCR and inserted into pCMV-HA vector (Clontech). Stat3c cDNA was elicited from RCMV-Stat3c (15), kindly given from Dr. Darnell, and inserted into pcDNA3.1 (Clontech). For the construction of pMY-IRES-GFP/C/EBP α -ER, full-length human C/EBP α cDNA was fused in-frame with ligand-binding domain (amino acids 281–599) of mouse estrogen receptor harboring a mutation (G525R) that confers selective responsiveness to 4-hydroxytamoxifen (4-HT). A reporter construct of a minimal TK promoter with CEBP-binding sites (pC/EBP)2TK was described previously (14).

Murine recombinant leukemia inhibitory factor, natural IFN- α , and recombinant IFN- γ were purchased from Sigma, HyCult Biotechnology (Uden, The Netherlands), and Peprotech (Rocky Hill, NJ), respectively. For Western blotting, 32Dcl3 cells or 32Dcl3/DNStat3 cells were deprived of IL-3 for 12 h. Then cells were stimulated with G-CSF (10 ng/ml), IL-3 (10 ng/ml), leukemia inhibitory factor (10 ng/ml), IFN- α (1,000 units/ml), or IFN- γ (1,000 units/ml) for 30 min.

Microarray Analysis—32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokine in RPMI 1640 containing 10% fetal bovine serum for 8 h and then stimulated with 10 ng/ml G-CSF. Total RNA was extracted, by the acid guanidinium method, from 32Dcl3 and 32Dcl3/DNStat3 cells before or after the stimulation for 2 h with G-CSF. Double-stranded cDNA synthesized from the total RNA (20 μ g/sample) was then used to prepare biotin-labeled cRNA for the hybridization with GeneChip MGU74Avs2 microarrays (Affymetrix, Santa Clara, CA) harboring oligonucleotides corresponding to ~6000 known genes as well as ~6000 expressed sequence tag sequences. Hybridization, washing, and detection of signals on the arrays were performed with the GeneChip system (Affymetrix).

Quantitative Real-time Reverse Transcription-PCR Assay—32Dcl3 and 32Dcl3/DNStat3 cells maintained in IL-3, were washed twice with PBS and starved of cytokines for 8 h and then stimulated with 10 ng/ml G-CSF. Cells were harvested at the indicated times, and total RNA was isolated using Isogen (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. One microgram of extracted RNA was transcribed in a 20- μ l cDNA synthesis reaction using an RNA PCR kit (AMV) (Takara, Tokyo, Japan). Real-time PCR for C/EBP α , G-CSF receptor, lysozyme M, neutrophil gelatinase-associated lipocalin precursor (NGAL), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed by a TaqMan assay on an ABI 7000 system. PCR primers and probes were designed as follows: murine C/EBP α , sense, 5'-CCA TGT GGT AGG AGA CAG AGA CCT A-3', and antisense, 5'-CTC TGG GAT GGA TCG ATT GTG-3'; probe FAM-5'-CGG CTG GCG ACA TAC AGT ACA CAC AAG-3'-TAMRA, and sense, 5'-CCA AGA AGT CGG TGG ACA AGA-3', and antisense, 5'-CGG TCA TTG TCA CTG GTC AAC T-3'; probe FAM-5'-AGC ACC TTC TGT TGC GTC TCC ACG TT-3'-TAMRA; murine G-CSF receptor, sense, 5'-CTA AAC ATC TCC CTC CAT GAC TT-3', and antisense, 5'-GGC CAT GAG GTA GAC ATG ATA CAA-3'; probe FAM-5'-CAT CTT CTC TGT CCC CAC CGA CCA A-3'-TAMRA; murine lysozyme M, sense, 5'-TGC CTG TGG GAT CAA TTG C-3', and antisense, 5'-ATG CCA CCC ATG CTC GAA T-3'; probe 5'-FAM-CAG TGA TGT CAT CCT GCA GAC CA-TAMRA-3'; murine NGAL, sense, 5'-GGC CTC AAG GAC GAC AAC A-3', and antisense, 5'-CAC CAC CCA TTC AGT TGT CAA T-3'; probe 5'-FAM-CAT CTT CTC TGT CCC CAC CGA CCA A-TAMRA-3', and murine GAPDH sense, 5'-ACG GCA AAT TCA ACG GCA-3', and antisense, 5'-AGA TGG TGA TGG GCT TCC-3'; probe 5'-FAM-AGG CCG AGA ATG GGA AGC TTG TCA TC-TAMRA-3'. PCR amplifications were performed in a 50- μ l volume, containing 4 μ l of cDNA template, 50 mM KCl, 10 mM Tris-HCl(pH 8.3), 10 mM EDTA, 60 mM, 200 μ M dNTPs, 3

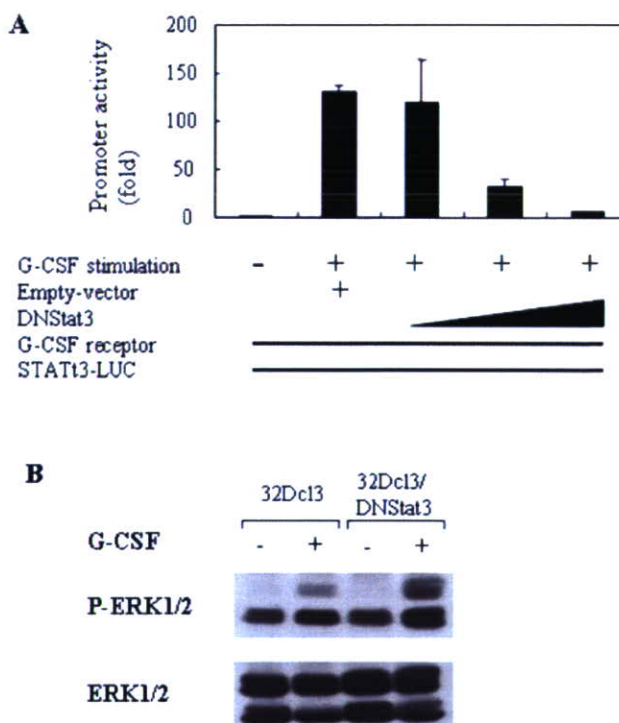


FIG. 1. The effect of dominant-negative Stat3 on G-CSF signaling pathway. A, transient transfection in 293T cells with a reporter construct with α 2-macroglobulin promoter (*STAT13-LUC*), dominant-negative Stat3, and G-CSF receptor. Twelve hours after transfection, cells were stimulated with 10 ng/ml G-CSF. Promoter activity was measured as luciferase activity 36 h after transfection. The vertical axis number is the fold induction when compared with control. B, 32Dcl3 cells or 32Dcl3/DNStat3 cells were cultured with IL-3 and then deprived of IL-3 for 12 h. Cells were treated with the G-CSF for 30 min and lysed. Post-nuclear supernatants were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. p-ERK1/2, phosphorylated ERK1/2.

mm MgCl₂, 200 nM each primer, 0.625 units of AmpliTaqGold, and 0.25 units of AmpErase uracil N-glycosylase. Each amplification reaction also contained 100 nM appropriate detection probe. Each PCR amplification was performed in duplicate, using conditions of 50 °C for 2 min preceding 95 °C for 10 min followed by 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). In each reaction, GAPDH was amplified as a housekeeping gene to calculate a standard curve and allow for the correction for variations in target sample quantities. Relative copy numbers were calculated for each sample from the standard curve after normalization to GAPDH by the instrument software.

Conditional C/EBP α Expression—pMY-IRES-GFP/C/EBP α -ER was transfected into 32Dcl3 and 32Dcl3/DNStat3 cells by electroporation. 5×10^6 cells were transfected with 20 μ g of expression vector, and GFP-positive cells were sorted by FACS Vantage (BD Biosciences). Expression of C/EBP α was determined by Western blotting analysis (see below).

Luciferase Assay—293T cells were transfected by the calcium phosphate precipitation method in 6-well plates, and luciferase activity was assayed using a luminometer Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's protocol. Each expression plasmid amount was 50–100 ng/well, and the same amount of empty expression vector was used as control, respectively. Results of reporter assays represent the average values for relative luciferase activity generated from five independent experiments.

Flow Cytometry— 1×10^7 cells were incubated with 5 μ l of recombinant phosphatidylethanolamine-conjugated rabbit anti-murine Gr1 monoclonal antibody (BD Biosciences) for 30 min at 4 °C, washed twice in PBS, and analyzed on a FACS Calibur (BD Biosciences).

Immunoprecipitation and Immunoblotting—Cells were lysed with lysis buffer, and lysates were immunoprecipitated with anti C/EBP β (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (8). Total cell lysates or the immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes

TABLE I
Microarray analysis

32Dcl3 and 32Dcl3/DNStat3 cells were starved of cytokines for 8 h and then stimulated or left unstimulated with 10 ng/ml G-CSF. Total RNA was extracted from each fraction and was subjected to the hybridization with high-density oligonucleotide microarrays (MGU74Av2). Fold induction means a rate of increase in gene expression level by G-CSF stimulation. Candidate genes were identified as transcripts that were up-regulated in 32Dcl3 cells and down-regulated or unchanged in 32Dcl3/DNStat3 cells after G-CSF stimulation.

Gene product name	Abbreviation	Accession number	Fold induction	
			32Dcl3	32Dcl3/DNStat3
B-cell leukemia/lymphoma α	Bcl2	L31532	35.6	0.0629
CyclinE1	Ccne1	NM007633	29.7	0.690
Serotonin-gated ion channel	5HT3	M74425	27.2	0.592
KIF3B protein	kif3b	D26077	21.5	0.921
Protein kinase, serine/arginine-specific 1	Srpkl	AB012290	18.7	0.321
MAP kinase-interacting serine/threonine kinase 1	Mknk1	Y11091	15.7	0.845
Protein tyrosine phosphatase	Ptpn13	D83966	12.4	0.964
Transferrin receptor	Trfr	X57349	10.6	0.964
Lymphocyte antigen 57	Ly57	AF068182	9.62	0.968
Macrophage stimulating 1 receptor	Mst1r	X74736	8.83	0.762
Mitogen-activated protein kinase kinase 7	MKK7	AB005654	8.14	0.980
RAR-related orphan receptor alpha	Rora	U53228	7.94	0.861
Hemoglobin Y, β -like embryonic chain	Hbb-y	V00726	7.38	0.375
Runt related transcription factor 1	Runx1	NM009821	7.01	0.226
Microtubule-associated protein 6	Mtap6	Y14754	5.06	0.885
CCAAT/enhancer binding protein α	C/EBP α	M62362	2.05	0.840
Ecotoropic viral integration site 1	Evi1	M21829	1.55	0.239
Integrin alpha L	Itgal	M60778	1.35	0.567
Ninjurin 1	Ninj1	U91513	1.34	0.783
Interleukin 17 receptor	IL17R	U31993	1.24	0.449
Mucosal addressin	MAdCAM	D50434	1.14	0.527
Carbon catabolite repression 4 homolog	Ccr4	X16670	1.06	0.0768
Friend leukemia integration 1	Fli1	X59421	1.01	0.305

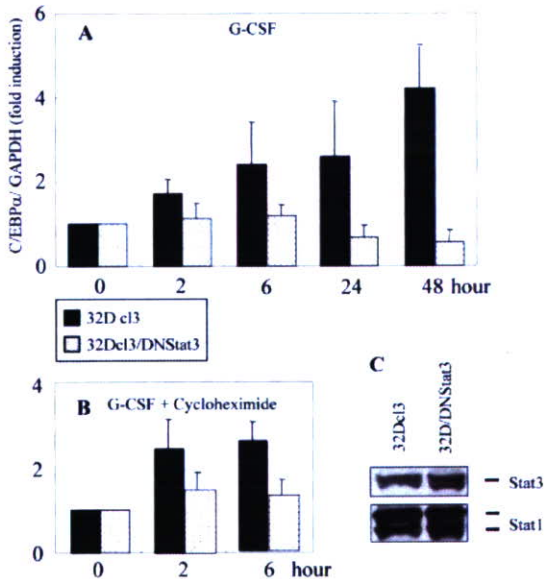


FIG. 2. Expression of C/EBP α mRNA in G-CSF-stimulated 32Dcl3 and 32Dcl3/DNStat3 cells. A and B, 32Dcl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF (A) or 10 ng/ml G-CSF and 10 μ g/ml cycloheximide (B). Total RNA was isolated from both cell lines at the indicated times and transcribed to cDNA, which was subjected to real-time PCR for murine C/EBP α . The numbers given on the vertical axis represent the fold induction of the ratios of GAPDH-normalized expression values when compared with those before G-CSF stimulation. Results are expressed as mean fold of two independent experiments.

were probed using the indicated antibodies followed by an IgG-horse-radish peroxidase-conjugated secondary antibody (Amersham Biosciences) and visualized with the ECL detection system (Amersham Biosciences). Anti-phospho-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA). Anti-phospho-Stat1 and -Stat5 antibodies were obtained from New England Biolabs (Beverly, MA), and anti-Stat1, -Stat3, and -C/EBP α antibodies were purchased from Santa Cruz Biotechnology. Membranes were probed using and visualizes with the

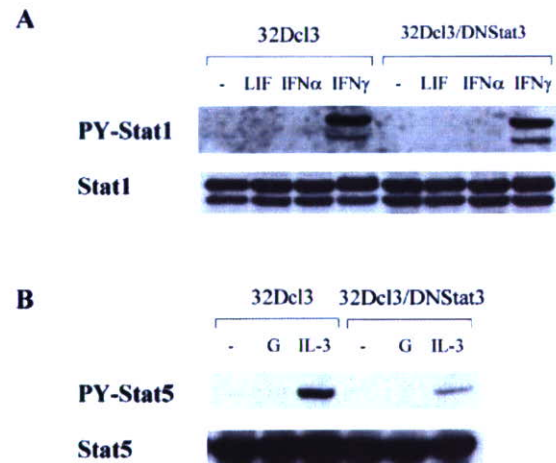


FIG. 3. The effect of the abrogation of Stat3 on other cytokine signaling pathway. 32Dcl3 cells or 32Dcl3/DNStat3 cells were cultured with IL-3 and then deprived of IL-3 for 12 h. Cells were treated with the indicated cytokines for 30 min and lysed. Post-nuclear supernatants were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. LIF, leukemia inhibitory factor.

ECL detection system (Amersham Biosciences).

Proliferation Assay—32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokine for 8 h and then stimulated with 10 ng/ml G-CSF. The number of viable cells was determined by trypan blue dye exclusion using a hemocytometer. [3 H]Thymidine incorporation assays were also performed. Briefly, cells (1×10^5) in 100 μ l of medium stimulated with murine IL-3 (1.0 ng/ml) or recombinant human G-CSF (10 ng/ml) were cultured for 48 h. During the final 4 h, [3 H]thymidine (1 μ Ci/well) was added. Cells were then harvested by filtration, and radioactivity was counted by scintillation spectrophotometer.

RESULTS

G-CSF-induced Intracellular Signal Response in 32Dcl3/DNStat3 Cells—32Dcl3 cells differentiate into neutrophils following treatment with G-CSF, but 32Dcl3 cells expressing a

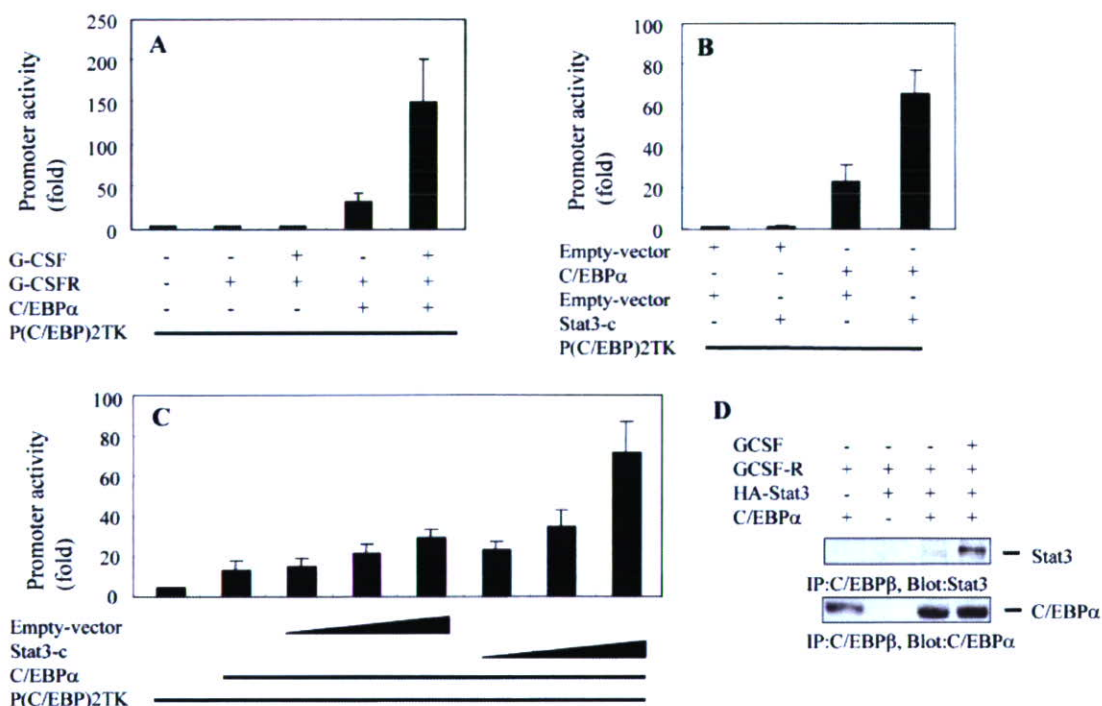


FIG. 4. Activated Stat3 makes complex with C/EBP α , leading to the enhancement of C/EBP α -induced transcription. *A*, transient transfection in 293T cells with a reporter construct of a minimal TK promoter with CEBP-binding sites (*p(C/EBP)2TK*), C/EBP α , and G-CSF receptor (*G-CSFR*). Twelve hours after transfection, cells were stimulated with 10 ng/ml G-CSF. Promoter activity was measured as luciferase activity 36 h after transfection. The vertical axis number is the fold induction when compared with control. *B* and *C*, transient transfection in 293T cells with a reporter construct of a minimal TK promoter with CEBP-binding sites (*p(C/EBP)2TK*), C/EBP α , Stat3c, and control vectors. Promoter activity was measured as luciferase activity 24 h after transfection. The vertical axis number is the fold induction when compared with control. *D*, transient transfection in 293T cells with a construct of G-CSF receptor, HA-Stat3, and C/EBP α and control vectors. After 24 h, cells were lysed and immunoprecipitated (IP) with anti C/EBP β . Cells were stimulated with G-CSF during the final 9 h in the culture. The immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Stat3 was detected by immunoblotting.

dominant-negative Stat3 (32Dcl3/DNStat3) proliferate following G-CSF treatment. These cells maintain immature morphologic characteristics without evidence of differentiation (11). First, we examined the effect of dominant-negative Stat3, carboxyl-truncated Stat3 that lacked 55 amino acids including the transactivation domain. We transfected reporter construct of STAT3-LUC, in which the α 2-macroglobulin promoter (16) drives expression of the luciferase (LUC) reporter gene and G-CSF receptor, together with empty vector (pcDNA3) or DNStat3 to 293T cells. After 12 h of transfection, cells were stimulated with 10 ng/ml G-CSF. Cells were cultured for more 24 h, and luciferase assay was performed. As shown in Fig. 1A, G-CSF induced the transcriptional activity of Stat3 by 150-fold, and DNStat3 inhibited this G-CSF-induced Stat3 activation in a dose-dependent manner.

G-CSF mainly induces the phosphorylation of Stat3, but it also phosphorylates Stat1 and Stat5 in some cells among the Stats family (8) and induces the activation of MAP kinases. In both 32Dcl3 cells and 32Dcl3/DNStat3 cells, neither Stat1 nor Stat5 was phosphorylated in response to G-CSF (data not shown). As for the MAP kinase activation, the degree of the phosphorylation of ERK1/2 by G-CSF stimulation in 32Dcl3/DNStat3 cells was stronger than that in 32Dcl3 cells (Fig. 1B).

Identification of Genes Regulated by Stat3 in the G-CSF Signaling Pathway by Oligonucleotide Array Analysis—To identify Stat3-regulated genes involved in granulocytic differentiation, we compared gene expression change in both cell lines using microarray analysis. 32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved in RPMI 1640 containing 10% fetal bovine serum lacking cytokine for 8 h and then stimulated with 10 ng/ml G-CSF.

Total RNA was isolated from 32Dcl3 cells and 32Dcl3/DNStat3 cells treated with G-CSF after 0 and 2 h, transcribed to biotin-labeled cRNA, and hybridized to GeneChip MGU74Av2 arrays to compare the expression profile of ~12,000 murine genes. The fold induction in the expression level of each gene was calculated as the ratio of GAPDH-normalized fluorescence intensity value of G-CSF-stimulated cells when compared with those before G-CSF stimulation. As shown in Table I, we could identify a set of candidate genes for Stat3 targets, expression of which was up-regulated in 32D cl3 cells but down-regulated or unchanged in 32Dcl3/DNStat3 cells. Such Stat3-dependent expression profiles were confirmed in triplicate experiments.

C/EBP α Is a Target Gene for Stat3 in G-CSF Signaling Pathway—Among the identified genes, it was decided to focus further efforts on C/EBP α . C/EBP α has been shown to be critical for early granulocytic differentiation (17–19), and the factors regulating its activity are unclear. The expression of C/EBP α was examined by real-time quantitative reverse transcription-PCR. C/EBP α mRNA levels are rapidly up-regulated in 32Dcl3 cells, being elevated 2.39-fold after 6 h and 4.20-fold after 48 h (Fig. 2A). In contrast to 32Dcl3 cells, the C/EBP α mRNA levels were not changed in 32Dcl3/DNStat3 cells after G-CSF stimulation (Fig. 2A). A similar expression pattern was seen in separate experiments with independently designed primers and probes (data not shown). Levels of C/EBP α mRNA were unaffected by cycloheximide treatment (Fig. 2B). The expression level of the sum of Stat3 plus dominant-negative Stat3 in 32Dcl3/DNStat3 cells is a little larger than that of Stat3 in 32Dcl3 cells (Fig. 2C).

Activated Stat3 Binds to C/EBP α and Enhances the Transcription Activity of C/EBP α —We next examined the effect of

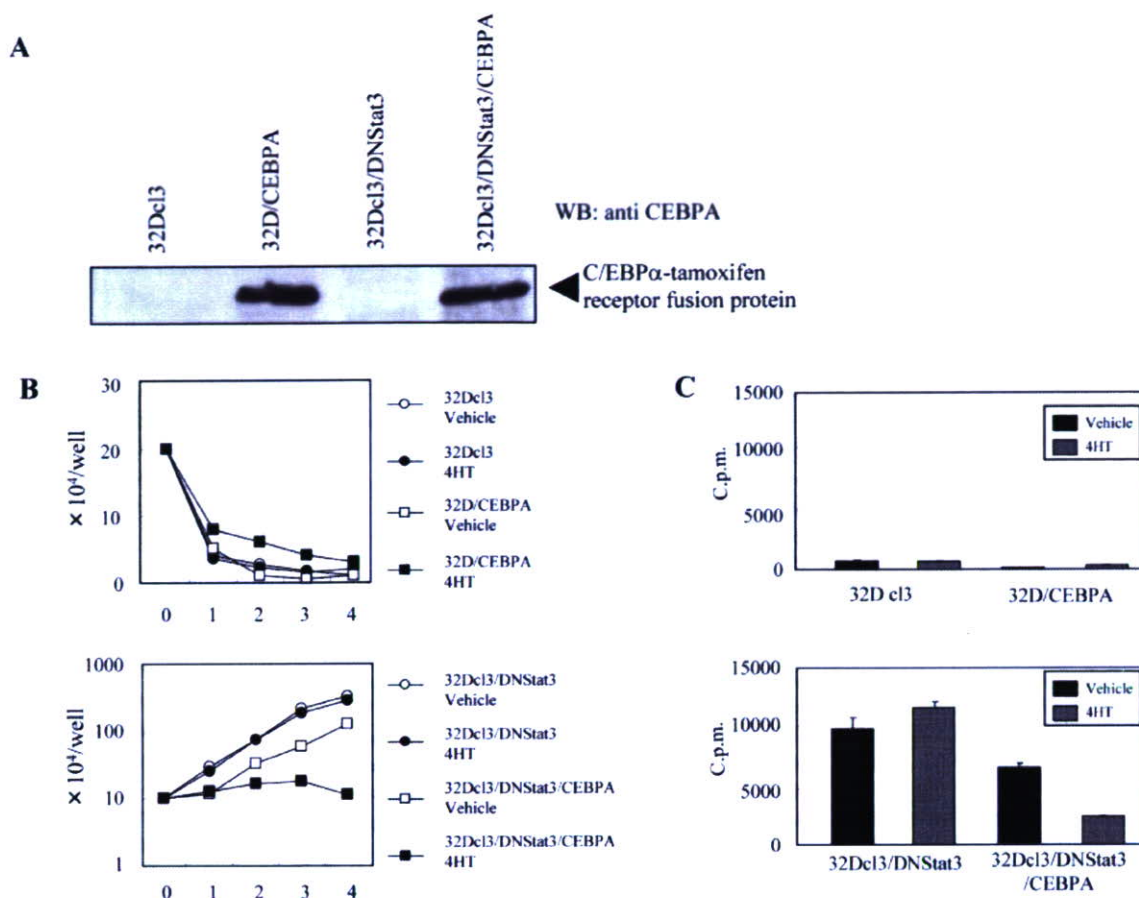


FIG. 5. Proliferation of 32Dcl3 and 32Dcl3/DNStat3 by restoration of C/EBP α . *A*, the expression vector pMY-IRES-GFP/C/EBP α -ER was transfected into 32Dcl3 and 32Dcl3/DNStat3 cells. The expression of C/EBP α -ER was examined by Western blotting (WB) using anti-C/EBP α polyclonal antiserum. Lane 1, 32Dcl3; lane 2, 32D/CEBPA; lane 3, 32Dcl3/DNStat3; lane 4, 32Dcl3/DNStat3/CEBPA. *B*, growth curve of 32Dcl3, 32Dcl3/CEBPA cells (upper panel), and 32Dcl3/DNStat3, 32Dcl3/DNStat3/CEBPA cells (lower panel). Cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μ M 4-HT or vehicle. Viable cells were counted daily by trypan blue dye exclusion method at the indicated times. The numbers given on the vertical axis represent the mean cell counts ($\times 10^4$ /well) of triplicate wells. Standard deviations (S.D.) were less than 15% of each mean. Three independent experiments were performed, and similar results were obtained. Data shown are representative of these results. *C*, 3 H incorporation assays in 32Dcl3, 32Dcl3/CEBPA (upper panel) and 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells (lower panel). Cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μ M 4-HT or vehicle for 48 h. During the final 4 h, 1 μ Ci of [3 H]thymidine was added, cells were harvested by filtration, and radioactivity was counted by scintillation spectrophotometer. Results are expressed as mean cpm of triplicate wells \pm S.D. Three independent experiments were performed, and similar results were obtained. Data shown are representative of these results.

Stat3 abrogation on the balance of intracellular signals in other cytokine pathways. Although Stat1 was not phosphorylated by leukemia inhibitory factor stimulation in neither 32Dcl3 cells nor 32Dcl3/DNStat3 cells, its activation in response to IFN- γ occurred at the same degree in both 32cl3 cells and 32Dcl3/DNStat3 cells (Fig. 3A). As for the Stat5 activation, the phosphorylation of Stat5 by IL-3 stimulation in 32Dcl3 cells was stronger than that in 32Dcl3/DNStat3 cells (Fig. 3B). These data indicated that there was the possibility that abrogation of Stat3 signaling can alter the balance of intracellular signals in other cytokine signaling pathways. The transcription of C/EBP α is regulated by C/EBP α itself (20, 21). Then we examined whether activated Stat3 in G-CSF signaling enhance C/EBP α activity or not.

We transfected a reporter construct of a minimal TK promoter with CEBP-binding sites (p(C/EBP)2TK), C/EBP α , and G-CSF receptor to 293T cells. After 12 h of transfection, cells were stimulated with 10 ng/ml G-CSF. Cells were cultured for more 24 h, and a luciferase assay was performed. C/EBP α up-regulated the C/EBP α -dependent gene expression, and the G-CSF stimulation enhanced this C/EBP α -dependent gene expression (Fig. 4A). Next we examined the effect of constitutive

active Stat3 (Stat3C) on the augmentation of C/EBP α transcriptional activity instead of the G-CSF stimulation. We transfected reporter construct p(C/EBP)2TK, C/EBP α , and Stat3C to 293T cells. After 24 h of transfection, luciferase assay was performed. Stat3C augmented the C/EBP α -dependent gene expression, although Stat3C alone had no influence on the luciferase activity (Fig. 4, B and C).

As p(C/EBP)2TK contains only a C/EBP α -binding site and does not contain a Stat3-binding sequence, the possibility that Stat3C makes a complex with C/EBP α and augments the function of C/EBP α is raised. Then we transfected C/EBP α , Stat3, and G-CSF receptor to 293T cells and stimulated cells with G-CSF for 6 h. There is no detectable level of endogenous C/EBP α or C/EBP β protein in 293T cells. Cells were lysed and immunoprecipitated with C/EBP β antibody (this antibody cross-reacts with C/EBP α). As shown in Fig. 4D, immunoprecipitants with anti-C/EBP β contain Stat3. In addition, the complex formation between C/EBP α and Stat3 is augmented by G-CSF stimulation, indicating that activated Stat3 makes the complex with C/EBP α .

C/EBP α Restores G-CSF-induced Granulocytic Differentiation in 32Dcl3/DNStat3 Cells—To analyze the role of Stat3-

FIG. 6. Morphologic features of 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells. Granulocytic differentiation of 32Dcl3/DNStat3 cells after induction of C/EBP α is shown. Cells were maintained in IL-3 and washed twice with PBS and then starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μ M 4-HT or vehicle for 5 or 8 days. The cells were cytospun and stained with May-Grunwald and Giemsa stain (original magnification, \times 400).

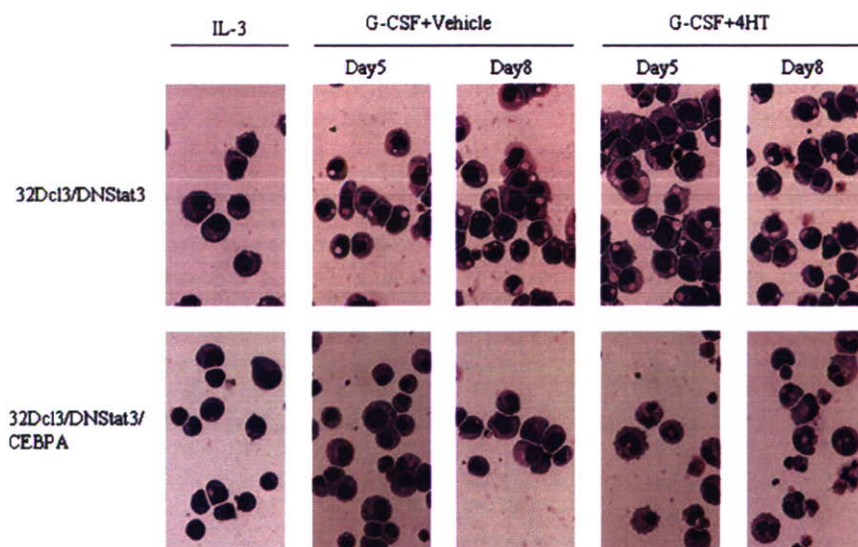


TABLE II
Differential count of 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells

32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells were maintained in IL-3 and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μ M 4-HT or vehicle for 5 days. Differential count was performed by May-Grunwald and Giemsa stain. Values are the mean \pm S.D. percent of cells from three independent experiments. Myelocyte includes promyelocytes, myelocytes, and metamyelocytes. Band(seg) includes band and segmented neutrophils.

Cells	G-CSF+Vehicle	G-CSF+4HT
32Dcl3/DNStat3		
Myeloblasts	98.0 \pm 0	99.3 \pm 0.47
Myelocytes	1.3 \pm 0.94	0.67 \pm 0.47
Band(seg)	0.67 \pm 0.94	0 \pm 0
32Dcl3/DNStat3/CEBPA		
Myeloblasts	90.7 \pm 3.3	3.0 \pm 2.8
Myelocytes	5.0 \pm 0.82	54.3 \pm 2.4
Band(seg)	4.3 \pm 2.6	42.7 \pm 0.47

regulated C/EBP α function in the G-CSF signaling pathway, we transfected a C/EBP α -tamoxifen receptor fusion protein (C/EBP α -ER) into 32Dcl3 and 32Dcl3/DNStat3 cells (32Dcl3/CEBPA cells, 32Dcl3/DNStat3/CEBPA cells, respectively). The expression of C/EBP α -ER in these cells was verified by Western blotting (Fig. 5A). C/EBP α -ER localizes to the cytoplasm and is in an inactive form in the absence of tamoxifen. Upon treatment with tamoxifen, it translocates from cytoplasm to nucleus and becomes active. 32Dcl3, 32Dcl3/CEBPA, 32Dcl3/DNStat3, and 32Dcl3/DNStat3/CEBPA cells were cultured with G-CSF in the presence or absence of tamoxifen, and cell proliferation was examined by both counting viable cells and [3 H]thymidine incorporation. 32Dcl3/DNStat3 proliferated in response to G-CSF, and proliferation was not affected by the presence of tamoxifen. Conversely, G-CSF-induced proliferation of 32Dcl3/DNStat3/CEBPA cells in the presence of tamoxifen was dramatically reduced (Fig. 5, B and C).

32Dcl3/DNStat3 cells maintain morphologically immature characteristics and proliferate without granulocytic differentiation after G-CSF stimulation. We examined the morphological changes in 32Dcl3 and 32Dcl3/DNStat3 cells induced by G-CSF after translocation of C/EBP α from the cytoplasm to the nucleus. When tamoxifen was added to medium containing G-CSF, 32Dcl3/DNStat3/CEBPA cells rapidly began to differentiate into granulocytes, and 5 days later, about 40% of the cells were morphologically similar to mature neutrophils. In contrast, 32Dcl3/DNStat3/CEBPA cells cultured in G-CSF-con-

taining medium without tamoxifen appeared immature with blast-like morphologic features (Fig. 6, Table II). To quantitatively analyze the difference in granulocyte maturation in 32Dcl3/DNStat3/CEBPA cells stimulated by G-CSF in the presence of tamoxifen, the mature granulocyte marker Gr-1 was monitored by FACS analysis. 32Dcl3 cells differentiate into Gr-1-positive neutrophils in response to G-CSF (Fig. 7A). As shown in Fig. 7D, Gr-1-positive cells were increased by the addition of tamoxifen in 32Dcl3/DNStat3/CEBPA cells treated with G-CSF, although low levels were detected in the absence of tamoxifen.

C/EBP α Up-regulates Genes That Are Related to Granulocytic Differentiation—In a conditional expression system, induction of C/EBP α leads to expression of granulocyte-specific genes, such as neutrophil primary granule genes (lysozyme M, NGAL) and the G-CSF receptor gene (17). In 32Dcl3/DNStat3 cells, the expression of these genes following G-CSF stimulation was inhibited (Fig. 8, A, C, and E). Interestingly, only NGAL was up-regulated by G-CSF in 32Dcl3/DNStat3/CEBPA cells following the restoration of C/EBP α (Fig. 8B). Conversely, the expression of lysozyme M and the G-CSF receptor were not changed by the restoration of C/EBP α (Fig. 8, D and F). These data suggest that regulatory factors in addition to C/EBP α may be involved in the induction of expression of granulocyte-specific genes by G-CSF.

DISCUSSION

G-CSF plays a pivotal role in granulopoiesis and granulocytic differentiation. The binding of G-CSF to its receptor leads to the activation of the Jak-Stat pathway, phosphatidylinositol-3 kinase pathway, and Ras-MAP kinase cascade (22). In the Jak-Stat pathway, G-CSF activates Jak1, Jak2, and Tyk2 followed by the activation of Stat1, Stat3, and Stat5 (7, 8).

Dominant-negative Stat3 inhibits G-CSF-induced transcriptional activity of Stat3 (Fig. 1A), as does G-CSF-induced granulocytic differentiation *in vitro* (11). Also, more transgenic mice with a targeted mutation of their G-CSF receptor that abolishes G-CSF-dependent Stat3 activation show severe neutropenia with an accumulation of immature myeloid precursors in their bone marrows (12). Consequently, Stat3 is thought to play an essential role in G-CSF-induced granulocytic differentiation.

32Dcl3 cells differentiate into neutrophils after treatment with G-CSF, and 32Dcl3/DNStat3 cells (32Dcl3 cells expressing dominant-negative Stat3) proliferate in G-CSF without differentiation. The degree of the phosphorylation of ERK1/2 by

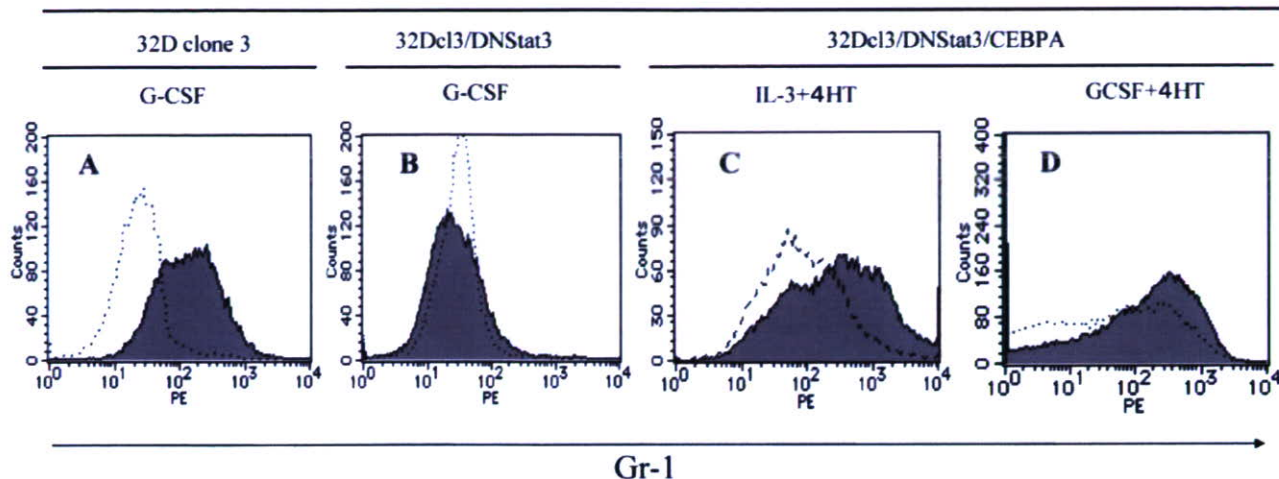


FIG. 7. The expression of Gr-1 on 32Dcl3, 32Dcl3/DNStat3, and 32Dcl3/DNStat3/CEBPA cells. 32Dcl3 (A) and 32Dcl3/DNStat3 cells (B) maintained in IL-3 (broken line) were starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF for 5 days (solid line). 32Dcl3/DNStat3/CEBPA (C) cells maintained in IL-3 were starved of cytokine for 8 h and stimulated with 1.0 ng/ml IL-3 (C) or 10 ng/ml G-CSF (D) plus 0.5 μ M 4-HT (solid line) or vehicle (broken line) for 5 days.

G-CSF stimulation in 32Dcl3/DNStat3 cells was stronger than that in 32Dcl3 cells (Fig. 1B). We reported that Stat3 null bone marrow cells displayed a significant activation of ERK1/2 after G-CSF stimulation than wild-type bone marrow cells did using Stat3 conditional deficient mice (23). Then the augmented phosphorylation of ERK1/2 in response to G-CSF in 32Dcl3/DNStat3 cells might be caused by the functional abrogation of Stat3 in 32Dcl3/DNStat3 cells.

We compared gene profiles between two cell lines, 32Dcl3 and 32Dcl3/DNStat3 cells, to identify target genes of Stat3 in G-CSF signaling. We found that C/EBP α mRNA levels are rapidly up-regulated in 32Dcl3 cells following G-CSF treatment; these levels are increased 2.39-fold after 6 h and 4.20-fold after 48 h of treatment. In contrast to 32Dcl3 cells, C/EBP α mRNA levels are not changed in 32Dcl3/DNStat3 cells after G-CSF stimulation (Fig. 2A). The observation that cycloheximide does not inhibit G-CSF-induced increases in C/EBP α transcript levels (Fig. 2B) suggests that C/EBP α is induced by G-CSF directly downstream of Stat3. Dahl *et al.* (24) also reported that G-CSF induced the expression of C/EBP α in IL-3-dependent progenitors. SOCS3 is one of the major target genes of Stat3. We previously reported that the expression level of SOCS3 protein in Stat3-deficient bone marrow cells is a trace, and it is not augmented by G-CSF stimulation (23). Contrary to this suppression of SOCS3 in Stat3-deficient cells, the induction of SOCS3 by G-CSF is not abolished in 32Dcl3/DNStat3 cells (data not shown).

The phosphorylation of ERK1/2 by G-CSF is stronger and the phosphorylation of Stat5 by IL-3 is weaker in 32Dcl3/DNStat3 cells when compared with those in 32Dcl3 cells, although Stat1 phosphorylation by IFN- γ was not changed between these two cells (Figs. 1B and 3). Then there is the possibility that the transfection of dominant-negative Stat3 affects other signaling pathways in 32Dcl3/DNStat3 cells, resulting in the change of C/EBP α regulation. To clarify whether Stat3 directly up-regulates C/EBP α in the G-CSF signaling pathway in 32Dcl3 cells or not, we examined the effect of Stat3C on the transcription of C/EBP α . C/EBP α up-regulated the C/EBP α -dependent gene expression, and the G-CSF stimulation enhanced this C/EBP α -dependent gene expression (Fig. 4A). Strikingly, Stat3C augmented the C/EBP α -dependent gene expression as G-CSF stimulation did (Fig. 4, B and C). This means that G-CSF-induced up-regulation of C/EBP α -dependent gene expression is, at least partly, due to the activation of Stat3.

Two possibilities arise for the mechanism of the induction of C/EBP α transcription by activated Stat3 in the G-CSF signaling pathway. One is that activated Stat3 binds to the promoter region of C/EBP α and induces the transcription of C/EBP α . Analysis of the reported murine C/EBP α promoter sequence (20) identified no Stat-responsive elements (TTN5AA) (25, 26), but we found six Stat-responsive elements between 6 and 4 kb upstream of the C/EBP α transcription initiation site. Activated Stat3 might bind these Stat-responsive elements between 6 and 4 kb upstream of the C/EBP α transcription initiation site. The other possibility is that activated Stat3 might form the complex with C/EBP α and augment the transcriptional activity of C/EBP α because C/EBP α itself is the only protein reported to activate the murine C/EBP α promoter (20, 21). When a minimal TK promoter with CEBP-binding sites (p(C/EBP)2TK) together with C/EBP α was transfected to 293T cells, C/EBP α up-regulated C/EBP α -dependent gene expression. Activated Stat3 (Stat3C) enhanced this C/EBP α -dependent gene expression in collaboration with C/EBP α , although only Stat3C has no transcriptional activity on p(C/EBP)2TK (Fig. 4, B and C). In addition, the stimulation of G-CSF allows Stat3 to make the complex with C/EBP α (Fig. 4D). Then activated Stat3 by G-CSF makes the complex with C/EBP α and augments the transcriptional activity of C/EBP α . This is one of the reasons why induction of C/EBP α transcript through Stat3 activation by G-CSF occurred in 32Dcl3 cells. Several reports have described factors that repress C/EBP α promoter activity, such as SP1 (27), AP2A (28), or MYC (29). We show here for the first time that Stat3 augments the C/EBP α promoter activity.

Intracellular transcript levels of several genes were changed following G-CSF treatment downstream of Stat3 activation (Table I). To better identify the role of C/EBP α in Stat3-mediated signaling in G-CSF-induced granulocyte differentiation, C/EBP α -ER (C/EBP α -tamoxifen receptor fusion protein) was stably expressed in 32Dcl3 and 32Dcl3/DNStat3 cells. C/EBP α -ER translocates from the cytoplasm to the nucleus and becomes activated upon treatment with tamoxifen. Strikingly, transfection of C/EBP α -ER into 32Dcl3/DNStat3 cells abolished proliferation and induced myeloid differentiation by G-CSF without Stat3 activation (Figs. 5, B and C, and 6). These data indicate that C/EBP α activation induced by G-CSF through Stat3 plays an essential role in stopping the cell proliferation and inducing the differentiation to the myeloid lineage.