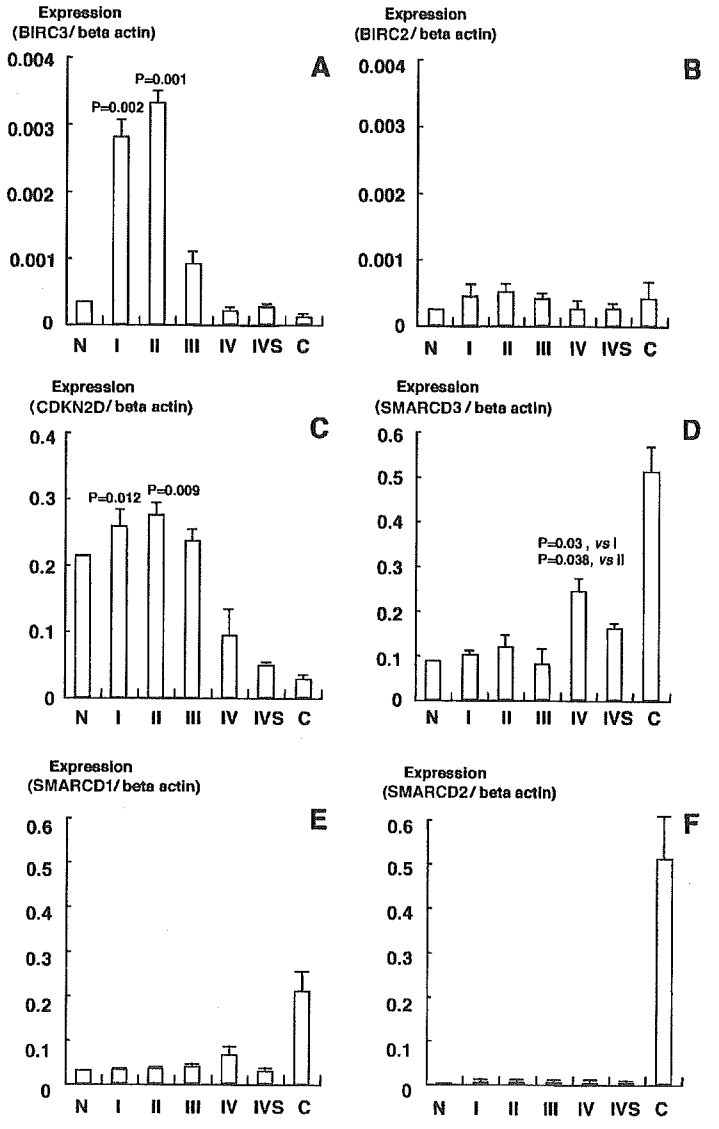


Figure 5. Real-time PCR analysis in neuroblastoma. The mean expression levels of each stage of patients and cell lines were standardized by the β-actin level. The expression of *BIRC3* and *CDKN2D* was significantly higher in stage-1 and -2 tumors than in those of stage 4. *SMARCD3* expression was also significantly higher in stage-4 tumors than in those of stages 1 and 2 according to Welch's test (N, normal adrenal; C, cell lines).

the tumors examined. Most early- and advanced-stage tumors were classified into similar expression profiling subgroups, group 1 and group 2, respectively. However, the remaining tumors in each group revealed obviously different expression patterns. Therefore, these variations in gene expression patterns suggest heterogeneity in the tumors in both the early and the advanced stages of NB. *MYCN* amplification is known to be the best-characterized genetic alteration associated with the prognosis for NB outcome. In this study, one tumor with *MYCN* amplification showed an expression pattern distinct from that of 3 other tumors with *MYCN* amplification, suggesting that *MYCN*-independent pathways exist in the progression of NB.

To gain a better insight into the structure of the microarray data, we used PCA and plotted the data in three-dimensional scaling. In this analysis, histologic subgroups and stages of the disease were found to be clearer classification methods than two-way clustering analysis. Thus, two-way clustering analysis only separated the data by histologic and clinical variation in expression patterns. Because we used only a limited number of tumors, addi-

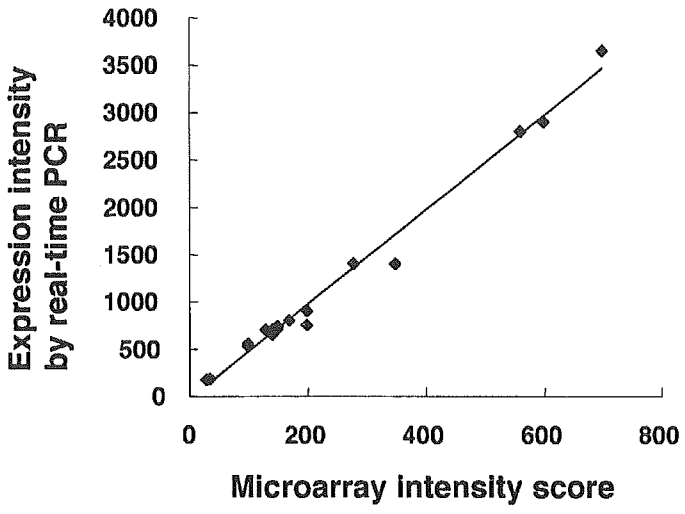


Figure 6. Scatter graph of intensity scores of *BIRC3* by microarray and real-time PCR analyses in 20 samples. Microarray intensity score and expression intensity score by real-time PCR are plotted on a logarithmic scale on the abscissa and ordinate, respectively. The coefficient was as high as 0.968 ($n = 20$, $P < 0.0001$).

tional studies that use a larger number of NB samples are required to provide conclusive data for the microarray analysis and PCA.

As the *BIRC3*, *CDKN2D*, and *SMARCD3* genes have been reported to be associated with apoptosis, the cell cycle, and transcriptional activation, respectively, it is possible that these genes are involved in the progression of NB. Thus, among the genes differentially expressed, we further investigated the *BIRC3*, *CDKN2D*, and *SMARCD3* genes by real-time PCR analysis. The *BIRC3* gene belongs to the inhibitors of apoptosis (IAP) family and has been reported to inhibit the apoptosis pathway by blocking caspase activity (LaCasse et al., 1998; Young et al., 1999). Recently, Survivin, a member of the IAP family, has been demonstrated to inhibit the apoptosis of NB cells (Islam et al., 2000). Moreover, *NAIP*, another member of the IAP family, has been reported to suppress neuronal differentiation and apoptosis in PC12 cells (Gotz et al., 2000). In the present study, *BIRC3* expression was up-regulated in the early-stage tumors, whereas Survivin has been reported to be highly expressed in the advanced stage of NB. These facts suggest that *BIRC3* could play an important role in suppressing neuronal apoptosis in the favorable group of NB, just as Survivin does in unfavorable groups. Moreover, the *BIRC3* gene is on chromosome band 11q21 and has been reported to be fused to the *MALT1* gene in mucosa-associated lymphoid tissue (MALT) lymphoma with t(11;18)(q21;q21) (Dierlamm et al., 1999; Motegi et al., 2000). Generally, MALT lymphoma is characterized by an indolent clinical behavior and a good prognosis (Dierlamm et al., 1999). The *BIRC3–MALT1* fusion products have been thought to lead to inhibition of germinal-center B-cell apoptosis and the subsequent development of MALT lymphomas (Motegi et al., 2000). Thus, this outcome and our results both suggest that *BIRC3* is involved in the genesis and/or progression of several human cancers, especially tumors with a good prognosis. The *BIRC3* gene has high homology with the *BIRC2* gene, and these two genes are located in tandem on 11q21 (Young et al., 1999). The functions and tissue distributions of these two genes appeared similar (Young et al., 1999). However, we found obviously different expression patterns between *BIRC2* and *BIRC3* in NB, indicating that the *BIRC3*, but not the *BIRC2*, gene would be functional in NB.

The *CDKN2D* gene, a cyclin-dependent kinase (CDK) inhibitor, was more highly expressed in the early-stage tumors. This gene is a member of the inhibitors of CDK4 (INK4) family and directly blocks not only CDK4, but also CDK6 (Guan et al., 1999). There is accumulating evidence that genetic alterations of the *CDKN2A* and *CDKN2B* genes, both members of the INK4 family, are involved in the biologic behavior of many different types of human cancers, such as melanoma, lung carcinoma, and acute lymphoblastic leukemia (Ranade et al., 1995; Xiao et al., 1995; Maloney et al., 1999). Furthermore, we previously reported that the *CDKN2A* gene might be a candidate tumor-suppressor gene involved in the progression of NB (Takita et al., 1997, 1998). However, unlike the alterations in the *CDKN2A* and *CDKN2B* genes, alterations of the *CDKN2D* gene are rare in human cancers (Zariwala

et al., 1996), and *CDKN2D*-deficient mice showed no development of tumors (Zindy et al., 2000). Therefore, it is possible that *CDKN2D* is not a potent tumor-suppressor gene in human cancers. However, recently, it has been demonstrated that mice lacking both *CDKN2D* and *CDKN1B* showed ectopic neuronal cell divisions and apoptosis in many parts of the brain that were normally quiescent (Zindy et al., 1999). The *CDKN2D* and *CDKN1B* proteins, therefore, cooperate to prevent cell division within the brain and maintain differentiated neurons in a quiescent state (Zindy et al., 1999). Thus, a high expression of *CDKN2D* in early-stage NB may suggest that this protein plays a role in preventing the cell proliferation of a favorable type of NB cells.

We found that *SMARCD3*, a member of the SWI/SNF complex family, was expressed significantly more in the advanced stage of NB. The SWI/SNF complex is known to be a chromatin-remodeling enzyme, and it has been implicated in the transcriptional activation of a number of genes through chromatin remodeling (Wang et al., 1996). Recently, the *SNF5/INI1* gene, on chromosome band 22q21, a member of the SWI/SNF complex family, was identified as a tumor-suppressor gene for malignant rhabdoid tumor in children (Versteege et al., 1998; Uno et al., 2002). Furthermore, mice lacking the *Snf5/Ini1* gene have been reported to stop developing at the peri-implantation stage, and heterozygous mice develop vertebral tumors showing features of neural-crest-derived cells (Guidi et al., 2001). These findings and the present results suggest that the *SMARCD3* gene might be involved in the genesis and/or progression of NB. Although the *SMARCD3* gene has two homologs, *SMARCD1* and *SMARCD2*, the expression patterns of these two genes were different from *SMARCD3*, indicating the presence of a specific role of *SMARCD3* in NB.

In conclusion, we showed that there are genetic subsets in NB and that some of the genes of interest are differentially expressed, including *BIRC3*, *CDKN2D*, and *SMARCD3*, which have never been reported to be associated with NB. Thus, microarray technology is a good system for identifying such genes. From the microarray technology results together with the results of real-time PCR analysis in additional NB samples, it has been shown that it is likely that, in addition to the *MYCN*, *TRKA*, and *RASH* genes, the *BIRC3*, *CDKN2D*, and *SMARCD3* genes also have an important prognostic value in NB. Only a limited number of cases were analyzed in our study, and a large-scale study would allow a detailed classification of NB.

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# Nuclear Factor of Activated T-cells (NFAT) Rescues Osteoclastogenesis in Precursors Lacking c-Fos*

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**Osteoclasts are specialized macrophages that resorb bone. Mice lacking the AP-1 component c-Fos are osteopetrotic because of a lack of osteoclast differentiation and show an increased number of macrophages. The nature of the critical function of c-Fos in osteoclast differentiation is not known. Microarray analysis revealed that *Nfatc1*, another key regulator of osteoclastogenesis, was down-regulated in $Fos^{-/-}$ osteoclast precursors. Chromatin immunoprecipitation assay showed that c-Fos bound to the *Nfatc1* and *Acp5* promoters in osteoclasts. *In vitro* promoter analyses identified nuclear factor of activated T-cells (NFAT)/AP-1 sites in the osteoclast-specific *Acp5* and *Calcr* promoters. Moreover, in $Fos^{-/-}$ precursors gene transfer of an active form of NFAT restored transcription of osteoclast-specific genes in the presence of receptor activator of the NF-κB ligand (RANKL), rescuing bone resorption. In the absence of RANKL, however, $Fos^{-/-}$ precursors were insensitive to NFAT-induced osteoclastogenesis unlike wild-type precursors. These data indicate that lack of *Nfatc1* expression is the cause of the differentiation block in $Fos^{-/-}$ osteoclast precursors and that transcriptional induction of *Nfatc1* is a major function of c-Fos in osteoclast differentiation.**

AP-1 refers to a family of dimeric transcription factors composed of Fos (c-Fos, Fra1, Fra2, and FosB) and Jun proteins (1, 2). Transcription factors such as nuclear factor-κB (NF-κB)[1] and AP-1, both critically involved in osteoclast differentiation, are activated in the presence of macrophage/colony-stimulating factor (M-CSF, also known as CSF-1) and receptor activator of NF-κB ligand (RANKL) (3, 4). These cytokines induce signals via multiple pathways including mitogen-activated protein kinases, phosphatidylinositol 3-kinase and calcium (3, 5, 6). The essential role for c-Fos during osteoclast differentiation (7–9) is partially explained by the observations that the expression of all Fos family proteins is down-regulated in $Fos^{-/-}$ precursors and that other Fos proteins such as Fra1 can rescue the differentiation of these precursors (10, 11). Therefore, a role of c-Fos appears to enhance production of Fos proteins during osteoclastogenesis. c-Fos also transcriptionally induces β-interferon, which then negatively regulates osteoclastogenesis by down-regulating c-Fos at the protein level (12). Beyond the Fos family, however, c-Fos target genes that rescue osteoclastogenesis in $Fos^{-/-}$ precursors are not known.

NFATc1 is a member of the NFAT (nuclear factor of activated T-cells) family of transcription factors (NFATc1, NFATc2, NFATc3, and NFATc4, as accepted by HUGO and the Genome Data Base, corresponding to NFAT2, NFAT1, NFAT4, and NFAT3, respectively) (13–15). It has been shown to be up-regulated following RANKL treatment and is important for osteoclast differentiation (5, 16, 17). In this study, we explore the cause of the differentiation block in $Fos^{-/-}$ precursors by analyzing transcriptional target genes of c-Fos, especially *Nfatc1*, during osteoclast differentiation.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—ST2-T cells were established by infecting the mouse stromal line ST2 with the retroviral vector expressing RANKL (18). For co-culture, bone marrow cells or splenocytes were seeded at $6 \times 10^5$ cells/cm$^2$ with $6 \times 10^4$/cm$^2$ ST2-T cells and cultured in the presence of $10^{-8}$ M 1,25-dihydroxyvitamin $D_3$ and $10^{-7}$ M dexamethasone. For osteoblast-free culture, non-adherent hematopoietic precursor cells were cultured in the presence of 10 ng/ml recombinant human M-CSF (Genzyme) and 10–30 ng/ml recombinant mouse RANKL (R&D Systems). RAW264.7 cells were obtained from ATCC (TIB-71). Transient transfection was performed using LipofectAMINE (Invitrogen).

*Microarray*—Oligonucleotide microarrays (GeneChip Murine Genome U74Av2, Affymetrix) were used to monitor the relative abundance of transcripts. Gene Expression Omnibus accession numbers: $Fos^{+/+}$ splenocytes (GSM10341), $Fos^{-/-}$ splenocytes (GSM10342), $Fos^{+/+}$ bone marrow (GSM10343), $Fos^{-/-}$ splenocytes expressing ΔNFAT (GSM10344), $Fos^{-/-}$ splenocytes expressing green fluorescent protein (GFP) (GSM10345).

*Western Blotting*—Total cell extracts were prepared in a standard SDS lysis buffer. Nuclear extracts were prepared as described (19).

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[1] The abbreviations used are: NF-κB, nuclear factor-κB; M-CSF, macrophage/colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; NFAT, nuclear factor of activated T-cells; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; IL, interleukin; TRAP, tartrate-resistant acid phosphatase.

*Immunofluorescence*—Mature osteoclasts were prepared from femurs of 3-day-old wild-type mice by curetting with a scalpel into medium. After 1 h incubation, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 and 1% bovine serum albumin in phosphate-buffered saline. Primary antibodies used were anti-NFATc1 (7A6) or anti-NFATc2 (G1-D10).

*Plasmids*—Mouse *Acp5*-luciferase reporter plasmid was constructed in pGL3 vector (Promega) by transferring the promoter regions (−1453 down to the end of intron 1) from pKB5 (a gift from D. Roodman), and the NFAT site mutation was introduced by using the QuikChange kit (Stratagene). ΔNFAT was constructed by assembling PCR-amplified fragments encoding amino acids 1–239 of enhanced GFP (Clontech) followed by Ser-Arg (an XbaI site) and amino acids 317–902 of human NFATc4 (20). The 2.5-kb ΔNFAT fragment was cloned into both the cytomegalovirus-driven expression vector pRK5 and the retroviral vector pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was a gift from G. R. Crabtree. The −797 and −94 *Calcr*-P3-pGL3basic constructs have been described (22). Additional *Calcr*-P3 5′-deletion constructs were generated by PCR using different forward *Calcr* primers with a BglII site and a common reverse *Calcr* primer with a HindIII site. The NFAT site mutations were incorporated into each deletion by sequential PCR reactions using mutant *Calcr* primers and vector primers. The c-Fos expression vector pMX-c-Fos-IRES-GFP was constructed by inserting the BamHI-SalI fragment of mouse c-*Fos* cDNA in pBabe-c-Fos (10). The sequence of each construct was confirmed. The small interfering RNA vectors were based on RVH1 and LTRH1 (23) (a gift from R. Medzhitov). The oligonucleotides encoding the mouse c-Fos small interfering RNA were: RNAi1, 5′-gatcccctgatgttctcgggtttcaattcaagagattgaaacccgagaacatcatttttggaac-3′ and 5′-tcgagttccaaaaatgatgttctcgggtttcaatctcttgaattgaaacccgagaacatcagg-3′; RNAi2, 5′-gatcccctccaagcggagacagatcattcaagagatgatctgtctccgcttggatttttggaac-3′ and 5′-tcgagttccaaaaatccaagcggagacagatcatctcttgaatgatctgtctccgcttggaggg-3′.

*Electrophoretic Mobility Shift Assay (EMSA)*—Oligonucleotide sequences for *Acp5* EMSA were: *Acp5*-120 (the NFAT/AP-1 site in the human *Acp5* promoter), 5′-cgagccctcggagaaactgcatcatcctcg-3′, 5′-tcgacgaggatgatgcagtttctccgagggctcgagct-3′; IL-2 (the distal NFAT/AP-1 site in the human IL-2 promoter), 5′-cgagaaggaggaaaaactgtttcatacagg-3′, 5′-tcgacctgtatgaaacagtttttcctccttctcgagct-3′; consensus AP-1 (the AP-1 site in the human collagenase promoter), 5′-cgagataaagcatgagtcagacacctcg-3′, 5′-tcgacgaggtgtctgactcatgctttatctcgagct-3′; mutated AP-1, 5′-cgagataaagcaagagtctgacacctcg-3′, 5′-tcgacgaggtgtcagactcttgctttatctcgagct-3′.

Oligonucleotide sequences for *Calcr* EMSA were: *Calcr*-2, 5′-ggaacatgacagctcatttccatgttccct-3′; *Calcr*-2mA, 5′-ggaacattcacgctcatttccatgttccct-3′; *Calcr*-2mN, 5′-ggaacatgacagctccgggccatgttccct-3′; AP-1, 5′-cgcttgatgactcagccggaa-3′. Recombinant NFATc1 protein was synthesized *in vitro* from wtNFATc1-pCITE4 plasmid (a gift from N. A. Clipstone). Competition reactions included a 200-fold molar excess of unlabeled oligonucleotides.

*Real-time Reverse Transcriptase-PCR*—*Calcr* transcripts were quantitated on ABI PRISM 7000 (Applied Biosystems) using SYBR Green and were normalized to c-*fms* and *Gapdh* transcripts for co-cultures and osteoblast-free cultures, respectively.

*Bone Resorption Assay*—The surface of bone slices was visualized by backscattered electron imaging using a scanning electron microscope (S-2500CX, Hitachi). The extent of bone resorption was quantified with Metamorph (Universal Imaging).

*Chromatin Immunoprecipitation*—The chromatin immunoprecipitation assay was performed as described previously (24). Polyclonal anti-c-Fos antibody (Ab-2, Oncogene) or normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) were used. Immunoprecipitated DNA fragments were quantified by real-time PCR on ABI PRISM 7000. The PCR primers used were: *Nfatc1*-disF046, 5′-cgcccatgcaatctgttagtaa-3′; *Nfatc1*-disR248, 5′-gccctgagaaagctactctccc-3′; *Acp5*-1474F, 5′-tgcccagtacacattaccatcag-3′; *Acp5*-1715R, 5′-ggaccaaaagcggtgatcac-3′.

## RESULTS

*NFATc1 Is Down-regulated in $Fos^{-/-}$ Osteoclast Precursors*—We set out to identify genome-wide novel c-Fos target genes in the osteoclast lineage. Three osteoclastogenic cultures derived from wild-type and $Fos^{-/-}$ splenocytes and wild-type bone marrow were prepared and co-cultured with stromal ST2-T cells. By day 6, the wild-type cultures produced abundant multinucleated osteoclasts, which were tartrate-resistant acid phosphatase (TRAP)-positive, whereas no such cells were generated in the $Fos^{-/-}$ culture (data not shown). The co-cultured cells were harvested *in toto*, and gene expression was analyzed by microarrays. The genes in which expression was detectable in wild-type cultures but absent or very low in the $Fos^{-/-}$ culture are summarized in Table I. The numbers for wild-type splenocytes (Sp+/+), $Fos^{-/-}$ splenocytes (Sp−/−), and wild-type bone marrow cells (Bm+/+) are GeneChip scores indicating RNA levels. In the $Fos^{-/-}$ culture, the expression of *Nfatc1* was undetectable, and the expression of known osteoclast marker genes (25) was reduced. These include *Acp5* (encoding TRAP), *Ctsk* (cathepsin K), *Car2* (carbonic anhydrase 2), *Mmp9* (matrix metalloproteinase 9), and *Calcr* (calcitonin receptor). However, *Marco*, a macrophage receptor, was not reduced in the $Fos^{-/-}$ culture (Table I).

TABLE I
*Identification of c-Fos target genes by transcriptional profiling*
ΔNFAT/GFP, -fold increase between ΔNFAT- and GFP-expressing $Fos^{-/-}$ splenocytes in osteoclastogenic cultures. A, absent; NC, no change.

| Accession No. | Gene | Sp+/+ | Sp−/− | Bm+/+ | ΔNFAT/GFP |
|---|---|---|---|---|---|
| M99054 | *Acp5* | 3836 | 462 | 2580 | 4.0 |
| AJ006033 | *Ctsk* | 2862 | 61 | 2495 | 8.4 |
| M25944 | *Car2* | 971 | 14 | 719 | 3.2 |
| AF087434 | *Nfatc1* | 752 | A | 325 | 3.5 |
| U69535 | *Sema4d* | 707 | A | 309 | 2.0 |
| AV239570 | *Mmp9* | 620 | 80 | 451 | NC |
| AA656014 | *Tm7sf* | 593 | 30 | 295 | NC |
| AW125713 | Unknown | 544 | 60 | 331 | NC |
| U87814 | *Pstpip1* | 528 | A | 207 | NC |
| AV251613 | Unknown | 357 | A | 120 | 6.5 |
| U18542 | *Calcr* | 337 | A | 395 | 12.2 |
| X53929 | *Dcn* | 320 | A | 321 | NC |
| L22545 | *Col18a1* | 254 | A | 188 | 7.4 |
| AF029215 | Antigen for MRC OX-2 | 214 | A | 111 | 2.7 |
| AF042487 | *Kcnn4* | 208 | A | 117 | NC |
| U18424 | *Marco* | 108 | 761 | 223 | NC |

To confirm the differential expression at the protein level, Western blot analysis was performed using total protein extracts prepared on day 6 from the osteoclastogenic co-cultures. Consistent with the RNA data, NFATc1 was not detectable in the $Fos^{-/-}$ culture (Fig. 1*A*). The size variation of NFATc1 in the wild-type bone marrow culture may be caused by either degradation products or by different isoforms of NFATc1 (26, 27). Next we examined the subcellular localization of NFATc1 in mature osteoclasts freshly isolated from the femurs of wild-type mice. Immunofluorescence microscopy showed nuclear staining of NFATc1 but not NFATc2 in mature multinucleated osteoclasts generated *in vivo* (Fig. 1*B*). Western blot analysis of nuclear extracts from the macrophage-osteoclast precursor RAW264.7 cells demonstrated that nuclear NFATc1 was detectable only after RANKL stimulation (Fig. 1*C*). These data suggest that RANKL stimulates NFATc1 synthesis via c-Fos.

*c-Fos Binds to the Nfatc1 Promoter*—Putative c-Fos binding sites have been mapped in the promoter region of *Nfatc1* (Fig. 2*A*) (27, 28). To examine whether the *Nfatc1* promoter could be directly regulated by c-Fos in osteoclast precursors, we performed a chromatin immunoprecipitation assay using primary wild-type bone marrow cells and $Fos^{-/-}$ splenocytes treated with RANKL. The *Nfatc1*-P1 promoter fragment containing the distal block of homology between human and mouse sequences (27) was specifically precipitated with an anti-c-Fos antibody in samples prepared from wild-type cells treated with RANKL (Fig. 2*B*). The proximal block of homology could not be analyzed because of difficulty in PCR amplification. We also tested whether the *Acp5* promoter (Fig. 3*A*) was precipitated by anti-c-Fos antibody (Fig. 2*B*). In wild-type cells, c-Fos is present on the *Acp5* promoter in the absence of RANKL, and c-Fos occupancy of the *Acp5* promoter increases after RANKL treatment.

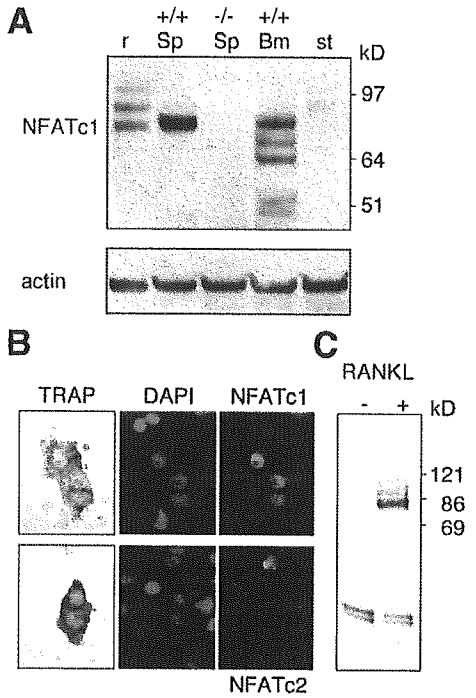


FIG. 1. **Expression of NFATc1.** *A*, NFATc1 is undetectable by Western blotting in $Fos^{-/-}$ osteoclastogenic culture. Splenocytes (*Sp*) and bone marrow cells (*Bm*) were co-cultured with ST2-T cells (*st*) for 6 days under osteoclastogenic conditions. Protein extracts *in toto* were analyzed using anti-NFATc1 monoclonal antibody (7A6). +/+ and −/− indicate wild-type and $Fos^{-/-}$ cultures, respectively. *r*, positive control Ramos cell extract. *B*, nuclear localization of NFATc1 in freshly isolated mature multinucleated osteoclasts. Immunofluorescence microscopy was performed with the anti-NFATc1 and anti-NFATc2 (G1-D10) antibodies. *TRAP*, TRAP activity stain; *DAPI*, 4′,6-diamidino-2-phenylindole (a nuclear stain). Note that the NFATc2-positive cell is TRAP-negative. *C*, Western blot analysis of nuclear extracts prepared from RAW264.7 cells cultured in the absence (−) or presence (+) of RANKL for 4 days using the anti-NFATc1 antibody.

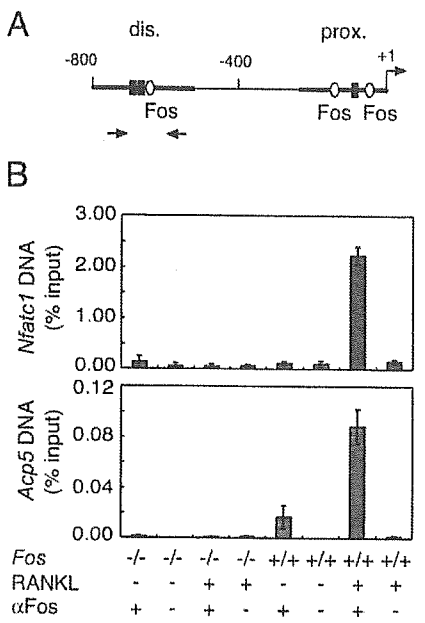


FIG. 2. **c-Fos binds to the *Nfatc1* promoter.** *A*, schematic presentation of putative Fos (*open ovals*) and NFAT (*closed rectangles*) binding sites in the mouse *Nfatc1* promoter (27, 28). The numbering is relative to the proximal transcriptional start site directed by promoter P1. Distal (*dis.*) and proximal (*prox.*) blocks of homology are shown in *thick lines*. *Arrows*, PCR primers. *B*, chromatin immunoprecipitation assay. Non-adherent $Fos^{-/-}$ splenocytes and wild-type bone marrow cells were treated with or without RANKL for 20 h before cross-linking. Precipitation was performed with IgG or anti-c-Fos antibody (*αFos*), and the amounts of precipitated DNA relative to total input DNA were quantified by a real-time PCR for *Nfatc1* (*top*) and *Acp5* (*bottom*; see Fig. 3*A* for PCR primers).

These results suggest that c-Fos binds to the *NFATc1* and *Acp5* promoters during osteoclastogenesis.

*Acp5 and Calcr Promoters Contain Functional NFAT/AP-1 Sites*—It is known that AP-1 composed of Fos/Jun dimers and NFAT transcription factors can cooperatively bind to promoter regions of various genes including the IL-2 gene (29, 30). To study the molecular mechanisms by which NFAT is involved in the regulation of osteoclast-specific gene expression we examined promoter sequences of *Acp5* and *Calcr*, two potential c-Fos target genes (Table I). First we searched for such composite binding sites for NFAT and AP-1 (NFAT/AP-1 sites) in mouse, human, and pig *Acp5* promoter sequences. Two short stretches were highly conserved around the multiple transcription start sites, which we termed *Acp5*-160 and *Acp5*-120, respectively (*Acp5* elements located at −160 and −120 relative to the 3′ end of exon 1). Sequences of the conserved *Acp5*-120 were found to be similar to the prototypical 15-bp NFAT/AP-1 site in the IL-2 promoter (15) (Fig. 3*A*). *Acp5*-160 contains the binding site for the microphthalmia transcription factor (31). From EMSA results, the binding activity at *Acp5*-120 was indistinguishable from that observed with the IL-2 site (Fig. 3*B*). Binding of c-Fos to the *Acp5* promoter in osteoclasts was demonstrated by chromatin immunoprecipitation assay (Fig. 2*B*). Then we mutated the putative NFAT site in *Acp5*-120 from GGAGAA to GGCCCG in *Acp5*-luciferase reporter plasmids. Both human and mouse wild-type *Acp5*-luciferase constructs were most efficiently activated with NFATc4 compared with NFATc1, NFATc2, and NFATc3 in transient transfection assays (data not shown). Thus we constructed a constitutively active nuclear form of NFATc4, ΔNFAT (20), fused to GFP. When the wild-type and mutant reporter plasmids were co-transfected with a ΔNFAT expression plasmid into RAW264.7 cells, ΔNFAT activated the wild-type *Acp5* promoter more efficiently than the mutant promoter (Fig. 3*C*). Furthermore, the *Acp5* promoter activity was enhanced by a co-transfected c-Fos expression vector and suppressed by small interfering RNA vectors for c-Fos in transient transfection assays (Fig. 3*D*). These data suggest that *Acp5*-120 is a functional NFAT/AP-1 binding site in the *Acp5* promoter.

Next we searched for NFAT/AP-1 sites in the mouse osteoclast-specific *Calcr*-P3 promoter (22) and found eight putative NFAT/AP-1 sites in the −797 *Calcr*-P3 promoter (Fig. 4*A*). Co-transfection of both the *Calcr*-P3-luciferase reporter plasmids and the ΔNFAT-expression vector into RAW264.7 cells resulted in a 30-fold increase in promoter activity above constitutive levels. Sequential 5′ deletion of the *Calcr*-P3 promoter demonstrated that the −178 construct containing the putative NFAT/AP-1 sites 1–4 was sufficient for full activity. Site-specific mutagenesis of each NFAT site from GGAAAN to GGCCCG revealed that site 2 at −93 was critical and that sites 1, 3, and 4 appear to cooperate with site 2 (Fig. 4*A*). In EMSA, site 2 was bound by NFATc1 using either RANKL-stimulated RAW264.7 nuclear extracts or *in vitro* translation products (Fig. 4*B*). Next we transfected RAW264.7 cells with *Calcr*-P3-luciferase reporters and treated them with the calcium ionophore A23187. We observed that the *Calcr* promoter activities were enhanced only when site 2 was present presumably

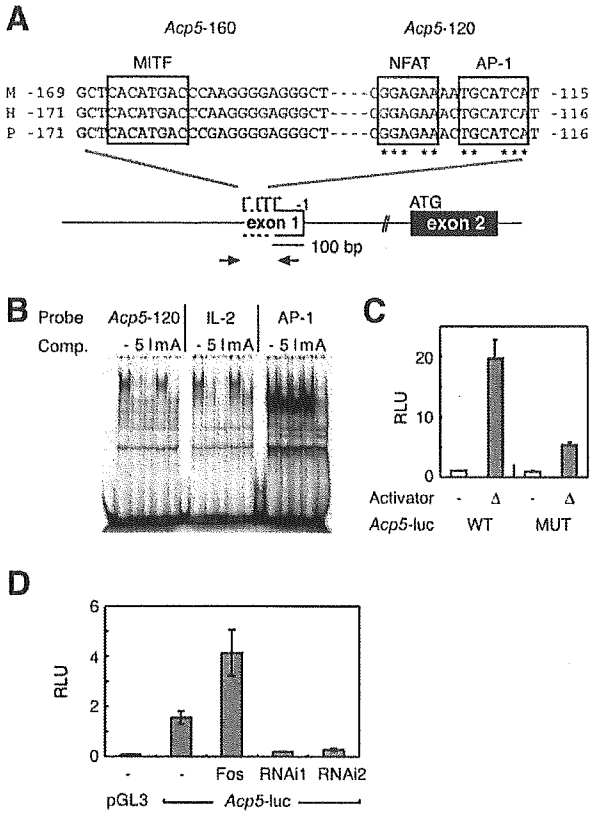


FIG. 3. **Identification of NFAT/AP-1 site in the *Acp5* promoter.** *A*, conserved *Acp5* promoter sequences in mouse (*M*), human (*H*), and pig (*P*). *Asterisks* indicate nucleotides identical to the 15-bp mouse IL-2 distal NFAT/AP-1 site. *Arrows* indicate PCR primers for the chromatin immunoprecipitation assay in Fig. 2*B*. *B*, EMSA using *Acp5*-120 (*5*), IL-2 distal NFAT/AP-1 site (*I*) and AP-1 consensus (*A*) or mutant (*m*) sites for probes and competitors (*Comp.*). Nuclear extracts containing NFAT binding activity were prepared from osteoclastogenic co-culture. *C*, transient transfection assay in RAW264.7 cells using wild-type (*WT*) and mutant (*MUT*) mouse *Acp5* promoter-luciferase constructs. The activator plasmid expressing ΔNFAT (Δ) was co-transfected. *RLU*, relative light units (normalized to co-transfected *Renilla* luciferase activity and relative to the unstimulated wild-type promoter). *D*, the *Acp5* promoter-luciferase construct was activated by c-Fos expression vector (*Fos*) and suppressed by Fos small interfering RNA vectors (*RNAi1*, *RNAi2*) in transient co-transfection in RAW264.7 cells.

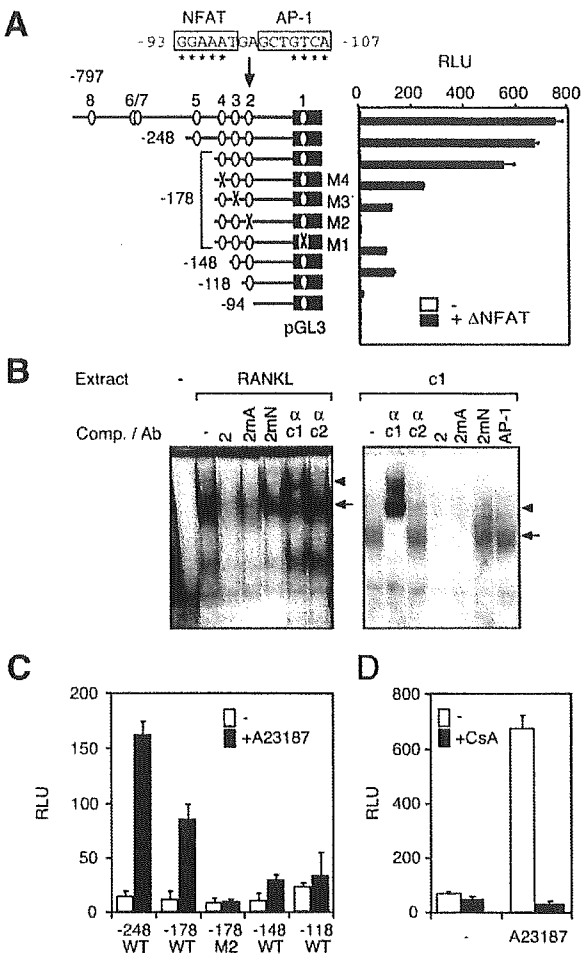


FIG. 4. **Identification of NFAT/AP-1 sites in the *Calcr*-P3 promoter.** *A*, a series of *Calcr*-P3 constructs was tested in RAW264.7 cells. The numbering is relative to the transcriptional start. *Open ovals* are sites 1–8 (*2*2), and *X* is a site-specific mutant. *Asterisks* indicate nucleotides that are identical between *Calcr*-P3 site 2 and the IL-2 site. Relative light units (*RLU*) are normalized to micrograms of protein and are relative to unstimulated pGL3, a promoterless luciferase vector. *B*, EMSA using *Calcr*-P3 site 2. The DNA-binding protein source was either nuclear extracts prepared from RAW264.7 cells cultured in the absence (−) or presence of RANKL or *in vitro* translated NFATc1 (*c1*). *Arrows* and *arrowheads* indicate specific NFAT binding activity and supershifts, respectively. The oligonucleotide competitors were wild-type site 2 (*2*), site 2 with the putative AP-1 site mutated (*2mA*), site 2 with the NFAT site mutated (*2mN*), and wild-type AP-1 (*AP-1*). Supershifts were done with antibodies to NFATc1 (*αc1*) and NFATc2 (*αc2*). *C*, induction of *Calcr*-P3 by A23187 (1 μM), which was added to transfected RAW264.7 cells 2 h before harvest. *WT*, wild type. *D*, transient transfection assay in RAW264.7 cells using the −319 *Calcr*-P3-luciferase construct. Cyclosporin A (1 μg/ml) was added 1 h before transfection, and A23187 (1 μM) was added for 4 h before harvest.

through activation of endogenous NFAT (Fig. 4*C*). Furthermore, the stimulatory effect of A23187 was blunted by pretreatment of the cells with cyclosporin A (Fig. 4*D*). These data suggest that site 2 is the critical NFAT site in the *Calcr* promoter.

*NFAT Rescues Osteoclast Differentiation in $Fos^{-/-}$ Precursors*—To test whether NFAT activity could rescue osteoclastogenesis in the absence of c-Fos, we introduced GFP or the GFP fusion ΔNFAT into $Fos^{-/-}$ splenocytes by retroviral gene transfer. Infected cells were co-cultured with the ST2-T cells under osteoclastogenic conditions. At day 6, mRNA was harvested and microarray analysis was performed to compare gene expression between GFP and ΔNFAT virus-infected $Fos^{-/-}$ cells. Strikingly, expression of ΔNFAT activated about two-thirds of the genes that failed to be induced in $Fos^{-/-}$ cells including *Acp5*, *Calcr*, *Ctsk*, and endogenous *Nfatc1* (Table I, ΔNFAT/GFP). This indicated that the differentiation block was to a large extent overcome by ΔNFAT in the absence of c-Fos when RANKL from ST2-T cells was present. Indeed, whereas abundant GFP-positive cells were observed by day 6 with both viruses, TRAP-positive cells were generated only with ΔNFAT virus (Fig. 5*A*). Next we tested whether the ΔNFAT-expressing $Fos^{-/-}$ osteoclasts could resorb bone. Although resorption pits were not visible on bone slices in cultures of GFP virus-infected $Fos^{-/-}$ splenocytes, co-cultures containing ΔNFAT virus-infected $Fos^{-/-}$ cells generated multiple resorption pits (bone surface resorbed, 2.3 ± 0.5%) (Fig. 5*B*). Next we compared the rescue efficiency in two types of osteoclastogenic cultures, co-culture using ST2-T cells and osteoblast-free cultures using only soluble M-CSF and RANKL. In co-cultures, the rescue with ΔNFAT was comparable with that with c-Fos as judged by

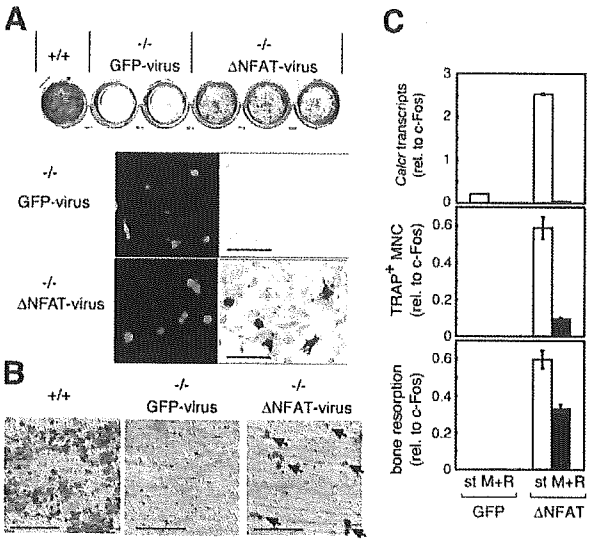


FIG. 5. **Rescue of osteoclast formation with ΔNFAT.** *A*, TRAP staining of osteoclastogenic co-cultures derived from wild-type (+/+) splenocytes and GFP and ΔNFAT virus-infected $Fos^{-/-}$ splenocytes (−/−). *Below*, GFP fluorescence and TRAP stain are shown in higher magnification. The *bars* represent 100 μm. *B*, bone resorption assay. Infected cells were co-cultured with ST2-T cells on bovine bone slices for 10 days, and pit formation was examined in a double-blind study with backscattered electron imaging. The *bars* represent 0.75 mm. *Arrows* indicate bone resorption pits. *C*, rescue activity of ΔNFAT virus relative to that of c-Fos virus. *st*, osteoclastogenic co-cultures using ST2-T cells. *M+R*, osteoblast-free cultures using soluble M-CSF and RANKL. $Fos^{-/-}$ splenocytes were infected with GFP, c-Fos, or ΔNFAT viruses. *Calcr* transcripts were quantitated by real-time PCR; TRAP-positive multinucleated cells (*MNC*) were counted, and the resorbed area (%) was measured on bone surfaces.

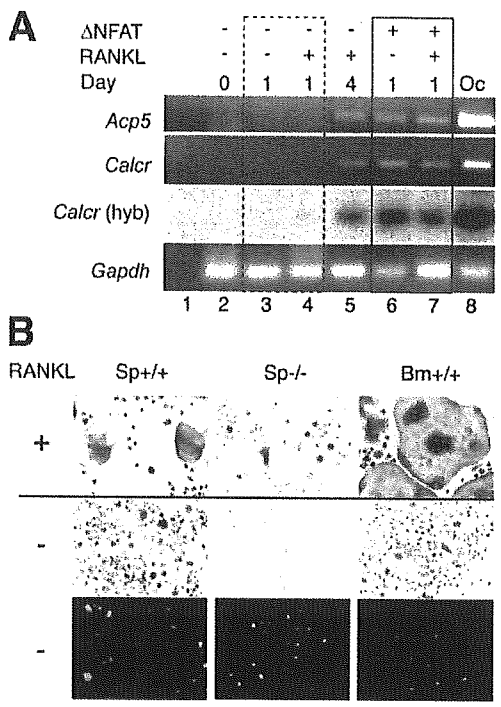


FIG. 6. **Rescue of osteoclast formation with ΔNFAT in the absence of RANKL.** *A*, transient transfection of ΔNFAT-expression vector induces endogenous *Acp5* and *Calcr* expression in RAW264.7 cells untreated or treated with 30 ng/ml RANKL as indicated and harvested at the days depicted. Osteoclasts generated in co-culture served as positive control (*lane 8*) and no RNA as negative control (*lane 1*). Transcripts were analyzed by reverse transcriptase-PCR with primers for *Acp5*, *Calcr* (all isoforms), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The reverse transcripts-PCR products for *Calcr* were also detected by hybridization with an internal probe (*hyb*). *B*, rescue of TRAP-positive $Fos^{-/-}$ cells by ΔNFAT requires RANKL. M-CSF-dependent macrophages derived from wild-type and $Fos^{-/-}$ splenocytes (*Sp+/+* and *Sp−/−*) and wild-type bone marrow cells (*Bm+/+*) were infected with the GFP ΔNFAT virus and then were cultured for 3 more days in the presence or absence of RANKL. The *bottom row* shows expression of GFP ΔNFAT fusion protein.

*Calcr* expression, TRAP-positive cell numbers, and resorption (Fig. 5*C*, *st*). In contrast, in the absence of stromal cells the rescue with ΔNFAT was lower than that of c-Fos based on all three parameters (Fig. 5*C*, *M+R*). Apart from the differences in efficiency in both cultures, ΔNFAT substituted at least in part for the osteoclastogenic function of c-Fos to the extent that $Fos^{-/-}$ splenocytes formed bone resorption pits in the presence of RANKL. In addition, gene transfer of the human full-length *NFATc1* also rescued $Fos^{-/-}$ osteoclastogenesis *in vitro* (data not shown). These data collectively indicate that the lack of NFATc1 is a major reason for the differentiation block in $Fos^{-/-}$ osteoclast precursors.

*NFAT Rescue of $Fos^{-/-}$ Precursors Is RANKL-dependent*—To examine the role of RANKL in NFAT-induced osteoclast formation, we introduced the GFP fusion ΔNFAT into RAW264.7 cells by transient transfection. This resulted in the induction of the endogenous *Acp5* and *Calcr* genes as early as 1 day after transfection even in the absence of RANKL (Fig. 6*A*). This is consistent with the reported osteoclastogenic activity of NFATc1 in the absence of RANKL (5). Next, we tested whether ΔNFAT could rescue $Fos^{-/-}$ precursors in the absence of RANKL. ΔNFAT produced bone-resorbing TRAP-positive cells from wild-type precursors (*Bm+/+*, *Sp+/+*) in the absence or presence of RANKL (Fig. 6*B*). However, $Fos^{-/-}$ precursors (*Sp−/−*) hardly produced any TRAP-positive cells upon introduction of ΔNFAT in the absence of RANKL (Fig. 6*B*), and no bone resorption pits were observed (data not shown). These data suggest that the rescue of $Fos^{-/-}$ cells with NFAT activity requires receptor activator of NF-κB (RANK) signaling.

## DISCUSSION

It has been established that the lack of c-Fos expression results in a differentiation block in the osteoclast lineage (7–9). However, whether this is a cumulative effect of numerous deregulated c-Fos target genes or an effect of one critical c-Fos target gene is unclear. Our results show that the absence of *Nfatc1* expression in $Fos^{-/-}$ precursors is the major cause of the differentiation block because an active form of NFAT alone rescued osteoclast-specific gene expression and bone resorptive function.

We have identified NFAT/AP-1 sites in the *Acp5* and *Calcr* promoters. EMSA showed that NFAT and AP-1 cooperatively bind to the *Acp5* NFAT/AP-1 site, and the chromatin immunoprecipitation assay indicated that c-Fos binds to the *Acp5* promoter in osteoclasts. These observations are consistent with the idea that NFATc1 and c-Fos synergize to activate the *Acp5* promoter (5). On the other hand, the rescue of osteoclastogenesis by ΔNFAT alone in the absence of c-Fos clearly demonstrates that c-Fos is not essential for activation of the *Acp5* promoter. To activate these promoters in $Fos^{-/-}$ precursors, ΔNFAT may interact with Jun-Jun homodimers (32) or may act alone in the absence of cooperative partners (33). EMSA using $Fos^{-/-}$ cell extract in combination with *in vitro* translated NFATc1 will help to address this issue. Although binding of

NFAT to the *Calcr* promoter was unambiguously demonstrated by EMSA, binding of AP-1 to the *Calcr* promoter needs to be rigorously tested in the future.

Importantly, osteoclast-specific gene expression is not entirely rescued with ΔNFAT. Those genes for which expression is not rescued, for example *Mmp9*, may be more strictly dependent on c-Fos or additional c-Fos-dependent transcription factors. Curiously, the rescue activity of ΔNFAT was similar to that of c-Fos when *Fos*$^{-/-}$ precursors were co-cultured with ST-2 but was lower than that of c-Fos when soluble M-CSF and RANKL were used. Therefore, c-Fos dependence appears to increase as stromal factors decrease. One of the stromal factors involved might be the ligand of osteoclast-associated receptor (OSCAR) (34). Whereas in wild-type precursors ΔNFAT or NFATc1 induce osteoclast differentiation even in the absence of RANKL (5), ΔNFAT expression in *Fos*$^{-/-}$ precursors failed to rescue osteoclast differentiation in the absence of RANKL. This suggests that in the absence of RANKL, NFAT requires c-Fos, presumably as a binding partner or possibly indirectly to exert its osteoclastogenic function. It also suggests that RANKL may induce an alternative partner for NFAT that can substitute for c-Fos function in *Fos*$^{-/-}$ cells.

Taken together, these results demonstrate that a major function of c-Fos during osteoclast formation is to trigger a transcriptional regulatory cascade by producing and cooperating with NFATc1, thereby activating a number of target genes involved in osteoclast differentiation and function. These yet to be identified novel target genes together with *Nfatc1* may provide additional drug targets for bone diseases including osteoporosis and rheumatoid arthritis.

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# Identification of Soluble $NH_2$-Terminal Fragment of Glypican-3 as a Serological Marker for Early-Stage Hepatocellular Carcinoma

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## ABSTRACT

**For detection of hepatocellular carcinoma (HCC) in patients with liver cirrhosis, serum α-fetoprotein has been widely used, but its sensitivity has not been satisfactory, especially in small, well-differentiated HCC, and complementary serum marker has been clinically required. Glypican-3 (GPC3), a heparan sulfate proteoglycan anchored to the plasma membrane, is a good candidate marker of HCC because it is an oncofetal protein overexpressed in HCC at both the mRNA and protein levels. In this study, we demonstrated that its $NH_2$-terminal portion [soluble GPC3 (sGPC3)] is cleaved between $Arg^{358}$ and $Ser^{359}$ of GPC3 and that sGPC3 can be specifically detected in the sera of patients with HCC. Serum levels of sGPC3 were 4.84 ± 8.91 ng/ml in HCC, significantly higher than the levels seen in liver cirrhosis (1.09 ± 0.74 ng/ml; $P < 0.01$) and healthy controls (0.65 ± 0.32 ng/ml; $P < 0.001$). In well- or moderately-differentiated HCC, sGPC3 was superior to α-fetoprotein in sensitivity, and a combination measurement of both markers improved overall sensitivity from 50% to 72%. These results indicate that sGPC3 is a novel serological marker essential for the early detection of HCC.**

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide, and its incidence is still increasing (1). Because HCC develops from cirrhotic liver after chronic infection with hepatitis virus B or C, patients with liver cirrhosis (LC) are advised to undergo periodical screening of serum α-fetoprotein (AFP) levels and liver ultrasound for the purpose of early detection of cancer (2). AFP is a glycoprotein expressed abundantly in fetal liver but not in normal adult liver and is re-expressed by HCC as it dedifferentiates from a premalignant lesion in the cirrhotic liver through well-differentiated (WD) and moderately differentiated (MD) HCC to poorly differentiated HCC (3). AFP has been used as a serum marker of HCC for more than 40 years. However, ultrasound imaging has been more effective lately in early detection of small WD HCC, in which AFP has yet to be elevated (4), highlighting the clinical need for novel sensitive serum markers for WD HCC.

Many previous studies have identified genes up-regulated in HCC compared with surrounding noncancerous lesions using differential display or cDNA subtraction (5–8). Recently, microarray studies on HCC presented gene lists containing a number of overexpressed genes (9–14). However, to determine whether a gene is a good candidate as a serological marker of WD HCC, it is crucial to determine the following: (*a*) whether it is overexpressed in WD HCC; (*b*) whether it is not expressed abundantly in other normal organs; and (*c*) whether it is detectable in the serum.

Overexpression of *GPC3* mRNA in HCC has been reported by ourselves and several other groups (15–18). Moreover, frequency of *GPC3* mRNA overexpression was significantly higher than that of elevated serum level and mRNA level of AFP in small HCC (16). We also observed frequent overexpression of *GPC3* in WD HCC compared with AFP with microarray analysis.[8] Together with minimal expression in normal organs (16, 19), GPC3 has, undoubtedly, previously existed as an attractive candidate marker of HCC. We showed previously using a monoclonal antibody (mAb) that GPC3 protein is also highly expressed in HCC (15). In this study, we further characterized GPC3 protein using a panel of newly generated mAbs and investigated whether it could be detected specifically in the sera of the patients with HCC. Finally, we successfully established a detection system for the soluble fragment of GPC3 (sGPC3) and confirmed its usefulness as a novel biomarker for HCC.

## MATERIALS AND METHODS

**Serum Samples.** Serum samples were collected at Tokyo University Hospital with informed consent from 69 patients with HCC and 38 patients with LC, defined according to the following criteria: patients with a pathological diagnosis of HCC after surgery or with evidence of tumor stain on computed tomography or angiography were diagnosed with HCC; and patients diagnosed with LC were limited to those who had no history of HCC and no ultrasound evidence of tumor for more than 6 months from the day of serum collection.

**Purification of Recombinant GPC3 Proteins.** For protein expression, we used modified pCXN vector that contained dihydrofolate reductase expression unit as a selection marker. Original pCXN vector (20) was generously provided by J. Miyazaki (Osaka University Medical School, Osaka, Japan). An expression vector for GPC3 that lacks the COOH-terminal hydrophobic glycosylated phosphatidylinositol (GPI)-anchoring domain, GPC3ΔGPI, was constructed by introducing cDNA corresponding to amino acid residues 1–563 of GPC3 into modified pCXN with a FLAG tag added at the COOH terminus. An expression vector for GPC3ΔGPI without heparan sulfate, GPC3ΔGPIΔHS, was constructed by changing $Ser^{495}$ and $Ser^{509}$ to Ala to abolish the heparan sulfate attachment site. These constructs were stably transfected into Chinese hamster ovary cells deficient in the dihydrofolate reductase gene. Culture media containing GPC3ΔGPI-FLAG or GPC3ΔGPIΔHS-FLAG recombinant proteins were collected and loaded to DEAE ion-exchange chromatography DEAE Sepharose FF (Amersham Bioscience, Tokyo, Japan). After washing, eluted protein solutions were applied to anti-FLAG M2 antibody beads (Sigma, St.

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Louis, MO). Proteins eluted with solution containing 200 μg/ml FLAG peptide (Sigma) were subjected to gel filtration chromatography with HiLoad 26/60 Superdex200pg (Amersham Bioscience). Finally, recombinant protein was concentrated using DEAE Sepharose FF.

**Generation of Anti-GPC3 mAbs.** We used recombinant GPC3ΔGPI as an immunogen. Spleen cells were isolated and fused with mouse myeloma P3-X63Ag8U1 cells (American Type Culture Collection, Manassas, VA). Hybridomas were selected by ELISA against the purified recombinant GPC3ΔGPIΔHS-FLAG, followed by cloning with limited dilution. Three mouse mAbs (A1836A, M18D04, and M19B11) were used in this study. For epitope mapping of these mAbs, a pGEX-5X (Amersham Biosciences) construct for the $NH_2$-terminal portion of GPC3 (amino acids 25–358) was expressed in *Escherichia coli* BL21 Codon Plus (DE3) pLys (Stratagene, La Jolla, CA) as a glutathione *S*-transferase-fusion protein and subject to immunoblotting analysis.

**Immunoblotting.** Total cell lysates were obtained after lysis in 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.1% SDS with protease inhibitor mixture (Sigma). Culture supernatant was obtained from serum-free medium used for culture of hepatoma cells. Proteins were separated with 12% SDS-PAGE and transferred to polyvinylidene difluoride Hybond P membrane (Amersham Biosciences). The membrane was treated with 2% nonfat milk in TBS containing 0.05% Tween 20 (TBST) followed by incubation with anti-GPC3 mAb in TBST and subsequent incubation with horseradish peroxidase-conjugated secondary antibody (dilution, 1:5000; Amersham Biosciences) in TBST. The protein was visualized using the enhanced chemiluminescence plus detection system (Amersham Biosciences).

**Immunoprecipitation.** We first prepared antibody beads by covalently linking 25 μl of protein G-Sepharose (Amersham Biosciences) and 50 μg of anti-GPC3 mAb M18D04 or M19B11 with 20 mM dimethyl pimelimidate (ICN Aurora, Aurora, OH). We then added 50 μl of sera from the patients or culture media of HuH7 cells diluted in 250 μl with PBS to 25 μl of antibody beads and incubated them for 2 h at 4°C. After extensive washing with PBS, antibody beads were boiled for 5 min in 50 μl of SDS-PAGE loading buffer containing 10% 2-mercaptoethanol, and subsequently, immunoblotting was performed.

**Sandwich ELISA.** One μg of anti-GPC3 mAb A1836A per well was immobilized to 96-well plate Maxisorp (Nalge Nunc International, Roskilde, Denmark) and stabilized with Immunoassay Stabilizer (Advanced Biotechnologies Inc., Columbia, MD). Twenty-five μl of sera or standard were diluted with 100 μl of buffer containing 20% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR), 1% BSA (Oriental Yeast Co., Ltd., Osaka, Japan), and 2% mouse ascites Hyb-3423 (Institute of Immunology, Tokyo, Japan) in 50 mM Tris-Cl (pH 8.0), 0.15 M NaCl, and 1 mM EDTA and incubated at room temperature for 2 h. After washing, 25 μl of biotinylated antibody solution containing anti-GPC3 mAbs M18D04 (1.88 μg/ml) and M19B11 (3.75 μg/ml) and 100 μl of horseradish peroxidase-labeled streptavidin (Vector Laboratories Inc., Burlingame, CA) were added to the plate and incubated twice at room temperature for 30 min. TMB Soluble Reagent and Stop Buffer (Scy Tek Laboratories, Inc., Logan, UT) were added as substrate, and absorbance at 450 nm was read with EIA Reader (Corona Electric Co., Ltd., Ibaraki, Japan). Recombinant GPC3ΔGPI was used as a standard sample in each assay.

**Amino Acid Sequence Analysis.** Recombinant GPC3ΔGPI and GPC3ΔGPIΔHS were purified and separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane ProBlott (Applied Biosystems, Foster City, CA). The membrane was stained with CBB R-250, and sections containing bands of $M_r$ 40,000 and $M_r$ 30,000 were cut out separately. These polyvinylidene difluoride membrane sections were washed with a solution including 50% acetonitrile and 0.1% trifluoroacetic acid and applied to an ABI 492 Protein Sequencer (Applied Biosystems) to sequence the $NH_2$ terminus of the protein. Because the $NH_2$ terminus of the $M_r$ 40,000 protein was blocked, the membrane was further incubated in acetate with 0.6 mg/ml 3-bromo-3-methyl-2-nitrophenyl-mecapto-3*H*-indole (ICN Biomedicals Inc., Irvine, CA) at 80°C for 1 h in the dark to chemically cleave the protein at the COOH terminus of tryptophan residues. After washing twice with 80% acetate and once with 10% methanol, the peptide was analyzed using an ABI 492 Protein Sequencer. The detected sequence was aligned using FASTS software available online,[9] and the protein was identified.

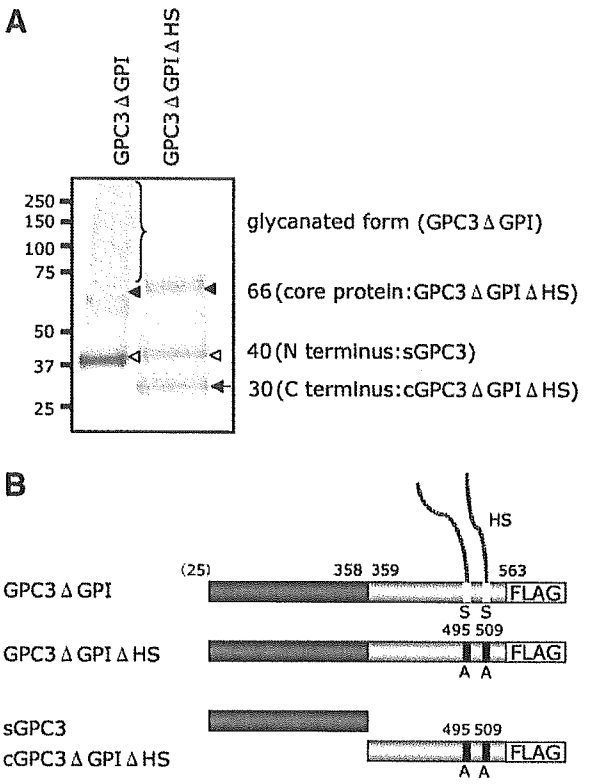


Fig. 1. Characterization of recombinant glypican-3 (GPC3) proteins. *A*, CBB R-250-stained SDS-PAGE of purified recombinant GPC3. *Brace*, glycanated GPC3 (smearing), GPC3ΔGPI; *closed arrowhead*, core protein of GPC3 ($M_r$ 66,000) that lacks heparan sulfate glycosaminoglycan, GPC3ΔGPIΔHS; *open arrowhead*, sGPC3 ($M_r$ 40,000); *arrow*, cGPC3ΔGPIΔHS ($M_r$ 30,000). *B*, schematic diagram of recombinant proteins. *Numbers above the boxes* indicate amino acid residue number. Note that $NH_2$-terminal residue 25 is putative and indicated in *parentheses*. *HS*, heparan sulfate glycosaminoglican.

## RESULTS

**The $NH_2$-Terminal Portion of GPC3 Is Cleaved between $Arg^{358}$ and $Ser^{359}$ *in Vitro*.** We have previously generated mAb K6534 raised against a peptide corresponding to amino acids 355–371 of GPC3 protein, and we demonstrated, for the first time, overexpression of its core protein in HCC with immunoblotting using this antibody (15). Another antibody is required to construct a sandwich ELISA system for serum examination of GPC3, so we started generating high-affinity mAbs using recombinant GPC3ΔGPI as an immunogen. While purifying the immunogen from the culture supernatant of Chinese hamster ovary cells, we observed a $M_r$ 40,000 band (Fig. 1*A*) in addition to the $M_r$ 66,000 band that corresponds to core protein of GPC3 as observed with K6534 (15).

Because the $NH_2$ terminus of this $M_r$ 40,000 band was modified, as revealed by initial amino acid sequencing, we performed sequencing of internal amino acids of the band after cleavage at the COOH terminus of tryptophan residues to verify its origin. We detected six cycles of three amino acid residues VRY, EPX, YES, ITY, LPX, and QSV, each cycle corresponding to the first to sixth residue following tryptophan (W), respectively. After alignment with FASTF algorithm, these sequences matched with the (W)VPETPV (amino acid 51–57), (W)YCSYCQ (amino acid 261–267), and (W)REYILS (amino acid

[9] http://fasta.bioch.virginia.edu/.

296–302) partial sequences of GPC3, respectively, indicating that this band is derived from an $NH_2$-terminal portion of GPC3. We designated this soluble cleaved fragment of GPC3 as sGPC3.

To further characterize sGPC3, we next tried to precisely identify the undetermined cleavage site by sequencing the residual COOH-terminal portion of GPC3 (designated cGPC3). However, the corresponding band was not visible by SDS-PAGE, presumably due to attachment of heparan sulfate glycosaminoglycan, leading to smearing (Fig. 1*A*). After substituting the two heparan sulfate attachment sites of the expression construct and purifying the resultant GPC3ΔGPIΔHS, we could observe a band of $M_r$ 30,000, as expected (Fig. 1*A*). The $NH_2$-terminal sequence of this band was identified as SAYYPEDLF, identical to amino acids 359–367 of GPC3. Thus, the cleavage site was identified as being between $Arg^{358}$ and $Ser^{359}$ (Fig. 1*B*). We do not have precise information on the $NH_2$-terminal sequence of sGPC3 due to modification, but considering that amino acid 1–24 is a putative signal sequence, sGPC3 is likely to consist of amino acids 25–358 with an estimated molecular weight of 38,100, consistent with the $M_r$ 40,000 band observed in SDS-PAGE (Fig. 1*A*).

**Soluble GPC3 Is a Major Form of GPC3 Specifically Detected in the Sera of Patients with HCC.** We succeeded in generating a number of high-affinity mAbs specific for GPC3 and classified these antibodies into two groups, N-mAbs and C-mAbs, according to their epitopes within amino acids 25–358 or 359–563, respectively (data not shown). These antibodies could also recognize endogenous GPC3 protein in immunoblotting: core protein ($M_r$ 66,000) and glycanated form (smearing) of GPC3 were detected by both N-mAbs and C-mAbs; whereas sGPC3 ($M_r$ 40,000) was detected only by N-mAbs (Fig. 2*A*). An additional $M_r$ 50,000 band was detected strongly in the cell lysate of HepG2 with both N-mAbs and C-mAbs (Fig. 2*A*). This band was only weakly detectable in HuH6 cells and was undetectable in five other hepatoma cell lines (Fig. 2, *A* and *C*; data not shown), suggesting cell-specific variations in the processing of the protein. In the culture supernatant, sGPC3, rather than a core protein or a glycanated form of GPC3, was the major form of GPC3 detected (Fig. 2*A*).

Based on the above *in vitro* finding, we speculated that sGPC3, instead of core protein of GPC3, might be the major form of GPC3 in the sera of HCC patients. To avoid possible interference on immunoblotting by significant migration of albumin or immunoglobulin in the serum, we performed immunoprecipitation before immunoblottng using three N-mAbs (Fig. 2*B*). sGPC3 alone was successfully detected by immunoprecipitation with M18D04 (Fig. 2*C*) or M19B11 (data not shown) followed by immunoblotting with A1836A in the sera of patients with HCC, but not in sera from normal liver (NL). These results clearly demonstrate that sGPC3 is the major diagnostic target specifically detectable in the sera of HCC patients.

**Soluble GPC3 Is Useful as a Serological Marker of WD HCC and MD HCC.** We next constructed a sandwich ELISA system with these three antibodies to measure the serum level of sGPC3 (Fig. 3*A*). To verify the specificity of the assay, we performed immunoblotting of 10 sera samples from HCC with sGPC3 levels ranging from 4.0 to 55.0 ng/ml and 3 samples from NL with sGPC3 levels of <0.1 mg/ml. We detected only sGPC3 in all 10 HCC samples, whereas no band was detected in 3 samples from NL, indicating high sensitivity and specificity of the assay (Fig. 3*B*). When we examined sera from 69 cases with HCC, 38 cases with LC, and 96 cases with NL, the level of sGPC3 (mean ± SD) was 4.84 ± 8.91 ng/ml for HCC, 1.09 ± 0.74 ng/ml for LC, and 0.65 ± 0.32 ng/ml for NL and was significantly higher in HCC than in NL ($P < 0.001$, Student's $t$ test) or in LC ($P < 0.01$; Fig. 3*C*).

We then evaluated sGPC3 as a general marker for HCC in comparison with AFP. Initial analysis of the receiver-operating characteristic curve using the data from 69 cases with HCC and 38 cases with LC suggested that, used in isolation, sGPC3 is not as good as AFP: the calculated area under the receiver-operating characteristic curve was 0.729 for sGPC3 and 0.799 for AFP (Fig. 3*D*). The sensitivity and specificity of sGPC3 for the diagnosis of HCC (cutoff value, 2.0 ng/ml) were 51% and 90%, respectively, whereas those of AFP measured in parallel (cutoff value, 20 ng/ml) were 55% and 90%, respectively. AFP and sGPC3 were not correlated ($r = 0.13$), and combination measurement of both markers markedly improved sensitivity to 72%.

HCC may be divided into two subgroups correlating to the extent of disease: (*a*) one first treated by surgery, mainly with a solitary tumor or few tumors; and (*b*) the second treated with transcatheter arterial chemoembolization, mostly with multiple and advanced tumors. The serum level of sGPC3 was 2.61 ± 2.69 ng/ml for the former group,

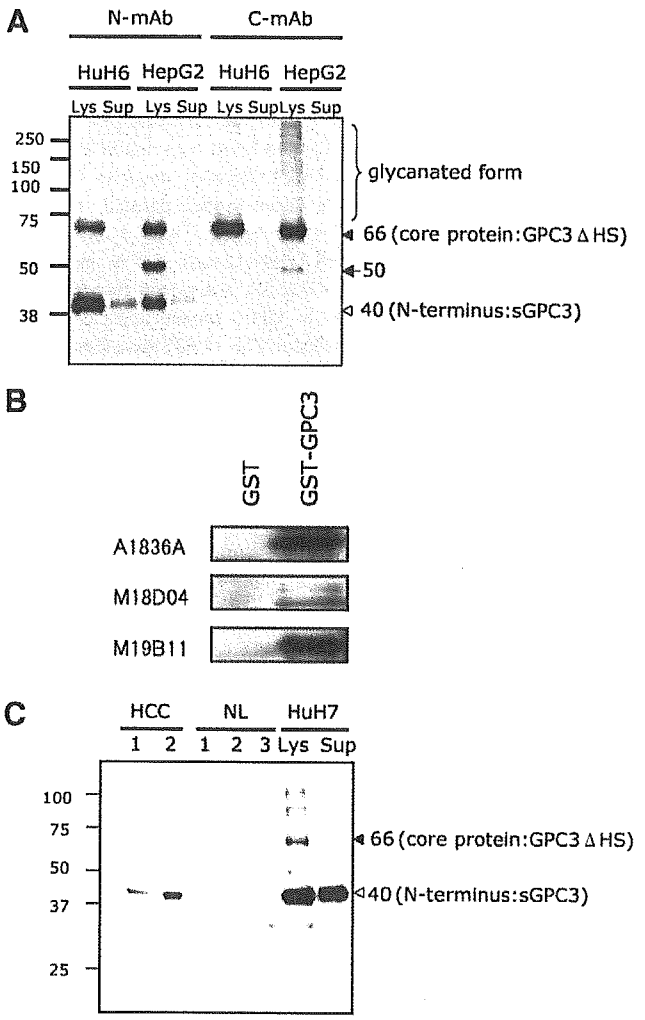


Fig. 2. Characterization of endogenous glypican-3 (GPC3) proteins with monoclonal antibodies (mAbs). *A*, representative immunoblotting of endogenous GPC3 in the cell lysate and culture supernatant with N-mAb and C-mAb. HepG2 and HuH6 were analyzed. Note that soluble GPC3 (sGPC3) alone is detected in the culture supernatant. *Brace*, glycanated GPC3 (smearing); *closed arrowhead*, core protein of GPC3 ($M_r$ 66,000); *open arrowhead*, sGPC3 ($M_r$ 40,000); *arrow*, uncharacterized processed fragment of GPC3 ($M_r$ 50,000). *Lys*, lysate; *Sup*, supernatant of culture media. *B*, immunoblotting analysis with anti-GPC3 antibodies A1836A, M18D04, and M19B11 recognized glutathione *S*-transferase-sGPC3 but did not recognize glutathione *S*-transferase. *C*, detection of sGPC3 alone in the sera of the patients with hepatocellular carcinoma. Sera from two patients with hepatocellular carcinoma and three healthy adults (NL) were analyzed by immunoprecipitation with M18D04 followed by immunoblotting with A1836A. HuH7 cells were analyzed as a reference. *Closed arrowhead*, core protein of GPC3 ($M_r$ 66,000); *open arrowhead*, sGPC3 ($M_r$ 40,000).

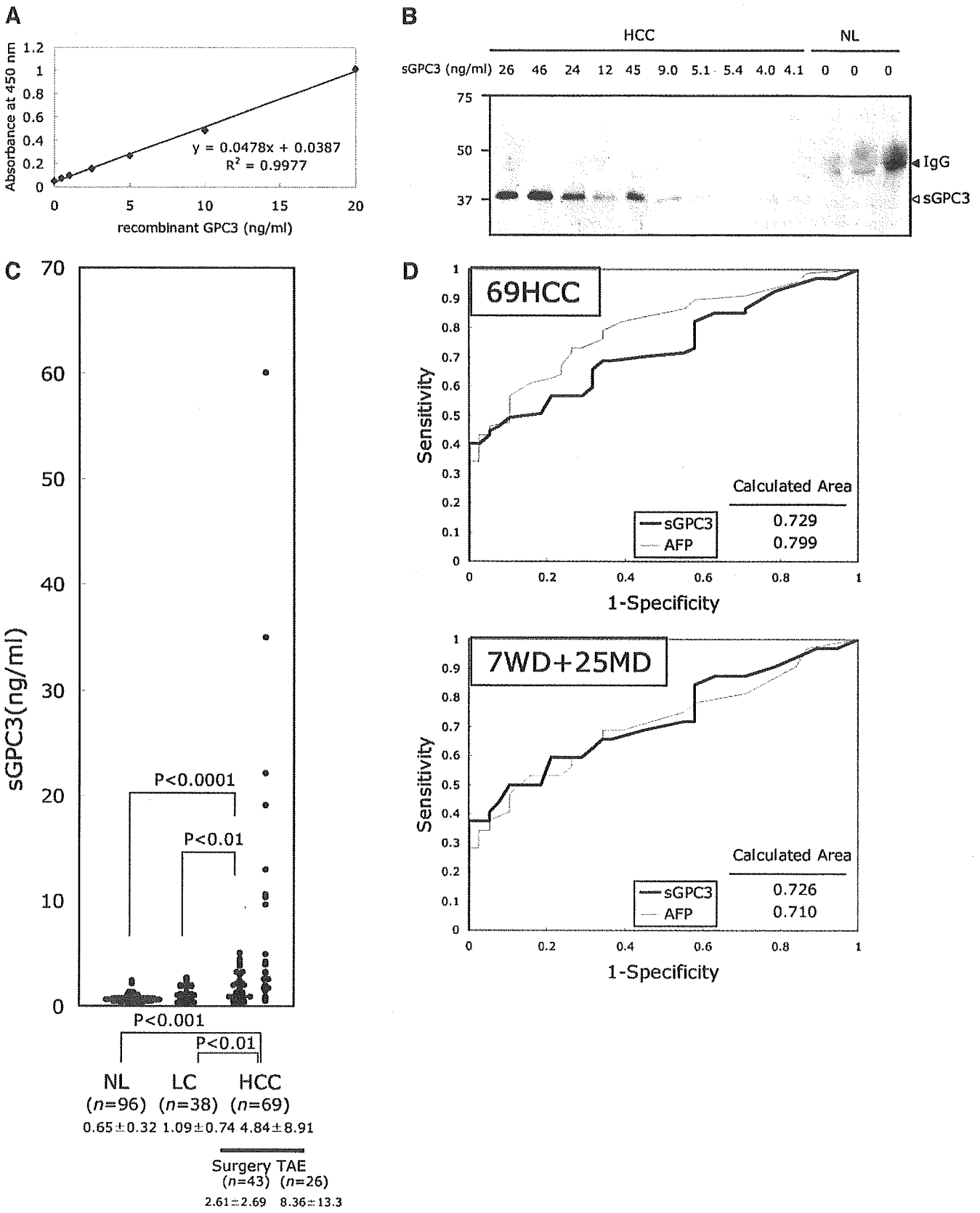


Fig. 3. Evaluation of soluble glypican-3 (sGPC3) as a serological marker of hepatocellular carcinoma (HCC). *A*, standard curve of sandwich ELISA. *B*, high specificity of sandwich ELISA. Specific detection of sGPC3 alone solely in the sera with elevated sGPC3 level measured with sandwich ELISA. Sera from 10 patients with HCC and 3 healthy adults (*NL*) were analyzed by immunoprecipitation with M18D04 followed by immunoblotting with A1836A. Serum sGPC3 level is indicated for each sample. *Open arrowhead*, sGPC3 ($M_r$ 40,000); *closed arrowhead*, IgG. *C*, distribution of sGPC3 in the sera of patients with normal liver, liver cirrhosis (*LC*), and HCC (surgery and transcatheter arterial chemoembolization subgroup). Mean ± SD (ng/ml) of serum sGPC3 is indicated. Number of samples is indicated as *n*. *D*, receiver-operating characteristic curve analysis of sGPC3 (*thick line*) and α-fetoprotein (*thin line*). *Top panel*, all of the 69 HCCs and 38 cases of LC were included in the analysis. *Bottom panel*, 32 HCCs (including 7 well-differentiated and 25 moderately differentiated HCCs) and 38 cases of LC were analyzed. Area under the receiver-operating characteristic curve is indicated.

significantly higher than that for NL ($P < 0.0001$, Student's $t$ test) or LC ($P < 0.01$), and 8.36 ± 13.3 ng/ml for the latter group (Fig. 3*C*), suggesting that the serum level of sGPC3 is elevated in an earlier stage and rises as HCC progresses. We then evaluated sGPC3 as a marker for HCC in relatively early-stage disease. When 43 cases treated by surgery were confined to 32 cases with relatively early-stage HCC (7 cases with WD HCC and 25 cases with MD HCC), calculated areas under the receiver-operating characteristic curve for sGPC3 and AFP were 0.726 and 0.710, respectively, indicating that sGPC3 is superior to AFP (Fig. 3*D*). The sensitivity of sGPC3 and AFP for the diagnosis of WD HCC and MD HCC was 50% and 47%, respectively. Moreover, combination measurement of both markers in WD HCC and MD HCC also markedly improved sensitivity to 72%. These results clearly demonstrate the utility of sGPC3 as a serological marker for HCC, especially for relatively early-stage HCC, and its complementarity to AFP.

## DISCUSSION

GPC3 (alternatively called OCI-5 or MXR-7) is a heparan sulfate proteoglycan. The structural characteristics of the glypican family are (*a*) a core protein of approximately $M_r$ 60,000, (*b*) binding to the membrane through GPI anchor, (*c*) heparan sulfate glycosaminoglycan attachment at Ser-Gly sequence within the COOH-terminal portion, and (*d*) a highly conserved pattern of 14 Cys residues (19). *GPC3* was originally isolated as a gene that is developmentally expressed in fetal rat intestine (21, 22). Mutation of *GPC3* is found in Simpson-Golabi-Behmel syndrome characterized by an overgrowth phenotype, hence its putative function was associated with an apoptotic effect (23). Silencing of *GPC3* in some types of cancer (24–26) is in line with this notion.

Overexpression of *GPC3* mRNA in HCC has been reported by ourselves and several other groups (15–18), although the role of GPC3 in carcinogenesis or progression of HCC has yet to be determined. In general, transcription level and protein level do not necessarily correlate. We have succeeded previously in generating an anti-GPC3 mAb against a peptide within the COOH-terminal portion, and we demonstrated using the antibody that the expression level of GPC3 core protein correlated well with its transcription level and that GPC3 was also overexpressed at protein level for the first time (15). Difficulties in making high-affinity antibodies against GPC3 (27), presumably due to its complex structure derived from disulfide bonds between 14 Cys residues, prohibited further analysis. We tried to generate high-affinity mAbs again by using recombinant GPC3 protein expressed in mammalian cells as an immunogen, and we finally succeeded in generating numerous high-affinity mAbs; to our knowledge, this is the first establishment of mAbs that can react with sGPC3. We did not recognize sGPC3 in a previous study (15) because we used a mAb against a relatively COOH-terminal portion (amino acids 355–371).

In the present work, we have precisely characterized GPC3 and demonstrated that the $M_r$ 40,000 protein, sGPC3, derives from the $NH_2$-terminal portion of GPC3 and is cleaved between $Arg^{358}$ and $Ser^{359}$. The $M_r$ 40,000 protein was previously described by Mast *et al.* (19), who were searching for the binding protein on the plasma membrane of HepG2 cells for tissue factor pathway inhibitor. They purified a $M_r$ 40,000 protein from culture supernatant of HepG2 cells and showed that it was derived from the $NH_2$-terminal portion of GPC3. They did not identify a cleavage site for the protein, unlike our study, but it is highly likely that the soluble protein they observed is sGPC3. They described purification of a $M_r$ 40,000 protein only when protease inhibitors were used throughout the procedure, strongly suggesting that GPC3 cleavage is mediated by a protease (19). In addition, they found that washing the cells with dextran sulfate or heparin released significantly higher amounts of GPC3 than seen before treatment, strongly suggesting that most GPC3 is noncovalently attached to the cell surface after cleavage of the GPI anchor, but not in the culture supernatant (19). Our finding that sGPC3 alone is the major form of GPC3 in the culture supernatant of hepatoma cells and the serum of patients with HCC is consistent with these findings.

Very recently, two other groups reported elevated levels of GPC3 in the serum of HCC patients. The results still seem preliminary, although they are quite similar to ours. Here, we have made significant improvements in the reliability of the assay. Nakatsura *et al.* (28) used a polyclonal antibody raised against 303–464 amino acids of GPC3 in their analysis. The specificity of their ELISA is to be confirmed because it is not sandwich ELISA, despite the many nonspecific bands the antibody detected in their immunoblotting. Moreover, the standard used in the assay was not recombinant GPC3 but a supernatant of HepG2 cells that is a mixture of many heterogeneous proteins. It is possible that they are measuring a mixture of nonspecific but HCC-related proteins. Capurro *et al.* (29) used a polyclonal antibody and a mAb, both raised against the last 70 amino acids of the COOH-terminal portion of GPC3, to detect glycanated GPC3 in serum with their sandwich ELISA. However, the major detectable form of GPC3 in serum is sGPC3, which cannot be detected with these antibodies against the COOH-terminal portion, as shown clearly in the present study. In fact, we examined many combinations of mAbs in our sandwich ELISA, but we could detect signal only when we used a combination of two N-mAbs (data not shown). Furthermore, the only evidence reported previously for the extracellular localization of glycanated GPC3 is immunoblotting of HepG2 cell culture supernatant, rather than serum from HCC patients. Here, we demonstrated that sGPC3 is in the culture supernatant and serum of the HCC patients using both immunoblotting and sandwich ELISA with the same combination of mAbs. One possible interpretation of the result, obtained by Capurro *et al.*, is that they are detecting some short fragments derived from a COOH-terminal portion but not the glycanated form of GPC3, and this issue should be further investigated.

We have delineated the usefulness of sGPC3 as highly sensitive to early-stage HCC. In addition, there were several cases with elevated serum sGPC3 among LC patients, although not included in this study, where HCC developed within 6 months after serum examination or some tumor was already detected by ultrasound without final diagnosis of HCC by computed tomography or angiography. We have also demonstrated the complementarity of sGPC3 to another HCC marker, AFP. These findings promise future bedside use of sGPC3 as a serological marker of HCC. Another attractive aspect of GPC3 is that the membrane-anchored portion is a potential target for antibody therapy. In this context, diagnosis with serum sGPC3 is useful not only in early detection of HCC but also for future identification of patients with high sGPC3 levels for tailor-made HCC therapy. Thus, further investigation into the clinical aspects of GPC3 in HCC is warranted.

## ACKNOWLEDGMENTS

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# STAT3 and MITF cooperatively induce cellular transformation through upregulation of c-*fos* expression

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**The signal transducer and activator of transcription (STAT) family proteins are transcription factors critical in mediating cytokine signaling. Among them, STAT3 is frequently activated in a number of human cancers and transformed cell lines and is implicated in tumorigenesis. However, although constitutively activated STAT3 mutant (STAT3C) leads to cellular transformation, its transformation potential such as colony-forming activity in soft-agar is much weaker than that of v-src. To identify tumorigenic factors that cooperatively induce cellular transformation with STAT3C, we screened the retroviral cDNA library. We found that the microphthalmia-associated transcription factor (MITF), an essential transcription factor for melanocyte development and pigmentation, induces anchorage-independent growth of NIH-3T3 cells in cooperation with STAT3C. Microarray analysis revealed that c-*fos* is highly expressed in transformants expressing STAT3C and MITF. Promoter analysis and chromatin immunoprecipitation assay suggested that both STAT3 and MITF can cooperatively upregulate the c-*fos* gene. In addition, the transformation of NIH-3T3 cells by both MITF and STAT3C was significantly suppressed by a dominant-negative AP-1 retrovirus. These data indicate that MITF and STAT3 cooperatively induce c-*fos*, resulting in cellular transformation.**

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**Keywords:** MITF; STAT3; c-*fos*; cellular transformation

## Introduction

The signal transducer and activator of transcription (STAT) family proteins were identified in the last decade as transcription factors essential for mediating virtually all cytokine signaling (Darnell, 1997; Stark *et al.*, 1998). These proteins become activated through tyrosine phosphorylation. In addition to their central roles in normal cell signaling, recent studies have demonstrated that constitutively activated STAT signaling, especially STAT3, directly contributes to oncogenesis (Bromberg and Darnell, 2000). For example, all *src*-transformed cell lines exhibit constitutively activated STAT3 (Yu *et al.*, 1995), and dominant-negative STAT3 suppresses *src* transformation without having any effect on *ras* transformation (Turkson *et al.*, 1998). More directly, Bromberg *et al.* (1999) demonstrated that a constitutively activated form of STAT3, STAT3C, which has two substituted cysteine residues within the C-terminal loop of the SH2 domain, resulting in a spontaneous transcriptionally active dimer, causes cellular transformation scored by colony formation in soft-agar and tumor formation in nude mice. Thus, the activated STAT3 molecule by itself can mediate cellular transformation. Extensive surveys of primary tumors and cell lines derived from tumors have indicated that an inappropriate activation of STAT3 occurs at a surprisingly high frequency in a wide variety of human cancers (Bowman *et al.*, 2000). However, until now, mutations in the STAT3 gene have not been identified in these cancers, hence it remained to be determined how endogenous STAT3 is constitutively activated and what kinds of genes are involved in tumorigenicity induced by constitutively activated STAT3.

The microphthalmia-associated transcription factor (MITF) is a basic helix–loop–helix leucine zipper (b-HLH-Zip) transcription factor that plays a critical role in the differentiation of various cell types, including neural crest-derived melanocytes, mast cells, osteoclasts, and optic cup-derived retinal pigment epithelium. MITF mutations in humans produce auditory–pigmentary syndromes, such as Waardenburg syndrome type IIa and Tietz syndrome, characterized by mast cell defects, inner ear problems, and abnormal, patchy pigmentation of the hair and skin. In mice, the *mi* allele protein with the deletion of 216R in the basic region is known as a dominant-negative form through the sequestration of wild-type partners in non-DNA-binding dimmers. In addition to the complete absence of melanocytes, MITF dominant-negative mutants exhibit osteopetrosis (Kitamura *et al.*, 2002). MITF consists of at least five

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isoforms, including MITF-A, MITF-B, MITF-C, MITF-H, and MITF-M, and MITF-M is the melanocyte-specific type (Tachibana, 1997; Udono *et al*., 2000; Shibahara *et al*., 2001). MITF regulates the expression of melanocyte differentiation markers, including tyrosinase, tyrosinase-related protein, and dopachrome tautomerase (DCT), all of which are required for pigmentation (Carreira *et al*., 2000). MITF is one of the genes involved in tumor growth and the metastasis of melanoma (Vachtenheim *et al*., 2001, Nyormoi and Bar-Eli, 2003). However, transcriptional target genes of MITF that regulate melanoma tumorigenicity or metastasis have not yet been elucidated. Moreover, since MITF alone has low or no oncogenic activity, a cofactor(s) that cooperatively functions with MITF may be necessary for the transformation of melanocytes.

In this study, we first demonstrated that STAT3 and MITF cooperatively induce cellular transformation *in vitro*. We also identified c-*fos* as a target gene of STAT3 and MITF using microarray analysis. The induction of the c-*fos* gene is necessary for the anchorage-independent growth of NIH-3T3 cells transformed with STAT3C and MITF. Our study provides a novel role of STAT3 in melanocyte proliferation and tumor growth of melanoma.

## Results

### *Screening for STAT3C cofactors for cellular transformation*

We and others have shown that NIH-3T3 cells expressing STAT3C or wild-type STAT3, which is activated by the type C hepatitis virus (HCV) core protein, possess a colony-forming potential in soft-agar and tumorigenicity in nude mice (Yoshida *et al*., 2002). However, the number and size of the colonies and tumor size by the expression of active STAT3 are much smaller than those of NIH-3T3 cells transformed with v-src (Bromberg *et al*., 1999; Yoshida *et al*., 2002). Therefore, the constitutive activation of STAT3 may not be sufficient for full transformation. With this in mind, we screened cofactors that induce full transformation in cooperation with activated STAT3 by using retrovirus cDNA transfer (Kitamura *et al*., 1995). NIH-3T3 cells expressing STAT3C (STAT3C-3T3) were infected with the HeLa cell retroviral cDNA library ($2 \times 10^6$ independent clones) and plated into soft-agar medium. After 3 weeks of incubation, two large colonies were formed and the integrated cDNAs were recovered by PCR and sequenced. One colony contained MITF cDNA with N-terminal 104 amino acids deletion compared with MITF-M (ΔN-MITF), and the other colony included full-length granulin cDNA that has been shown to induce colony formation in soft-agar in NIH-3T3 cells (Zanocco-Marani *et al*., 1999). The ATG of the exon 3 of the MITF gene was utilized as the first AUG codon in ΔN-MITF (Figure 1a). N-terminal truncation resulted in a missing N-terminal glutamine-rich region, but ΔN-MITF retained DNA-binding and transactivation domains.

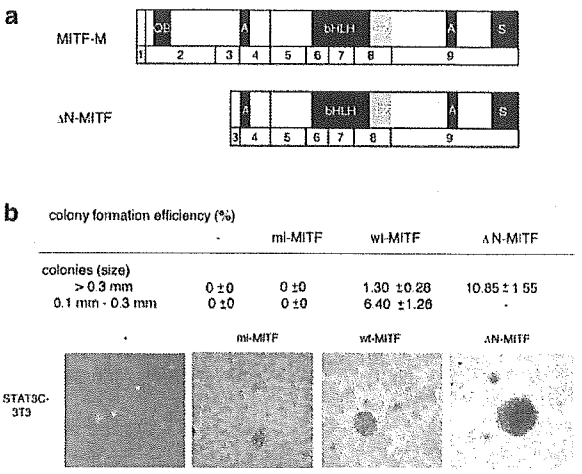



**Figure 1** Transforming potential with a combination of MITF and STAT3C. (a) Structures of MITF-M and our screening clone, ΔN-MITF. The numbers shown under MITF isoforms indicate exons. The glutamine-rich basic region (QB), the transcriptional activation domain (A), the bHLH-LZ structure, and the serine-rich domain (S) are indicated. (b) STAT3C-transformed 3T3 (STAT3C-3T3) cells were infected with the pMX empty vector, pMX-mi-MITF, wt-MITF, or ΔN-MITF and plated into soft-agar medium. On day 21, colonies were counted and photographed

### *MITF-induced anchorage-independent growth in cooperation with STAT3C*

The inserted cDNAs subcloned into the retroviral vector, pMX-IRES-EGFP, were introduced into parental NIH-3T3 cells or STAT3C-3T3 cells and then plated into soft-agar medium. N-terminal-truncated MITF induced the cellular transformation of STAT3C-3T3, but not parental NIH-3T3 cells, whereas granulin cDNA induced anchorage-independent cell growth in both NIH-3T3 cells and STAT3C-3T3 cells (Figure 1b and data not shown). Therefore, ΔN-MITF has the potential to induce the anchorage-independent growth of NIH-3T3 cells in cooperation with STAT3C. We also found that full-length (wt-) MITF could lead to anchorage-independent growth of NIH-3T3 cells in cooperation with STAT3C (Figure 1b). However, ΔN-MITF showed greater colony-forming activity, both in size and number, than wt-MITF.

We then compared the cellular morphology of transfectants. It has been reported that the forced expression of MITF in NIH-3T3 cells results in refractile cell morphology, which resembled dendritic cells and melanocytes (Tachibana, 1997). We also observed that ΔN-MITF-infected NIH-3T3 cells showed dendritic cell-like morphological changes (Figure 2a). However, as shown in Figure 2b, STAT3C-3T3 cells expressing wt-MITF or ΔN-MITF displayed some of the morphological changes associated with fibroblast transformation, that is, elongated shape and rounding.

### *Constitutive activation of STAT3 in melanoma cells*

We then examined STAT3 activation in melanoma cells in which MITF plays an important role in transformed

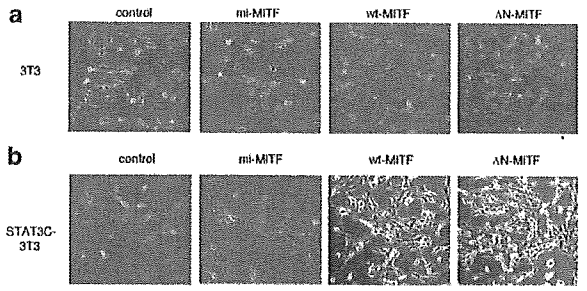



**Figure 2** Cytology of MITF-infected 3T3 and STAT3C-3T3 cells. 3T3 and STAT3C-3T3 were infected with the pMX empty vector, pMX-mi-MITF, wt-MITF, or ΔN-MITF. wt-MITF and ΔN-MITF induced morphological change in both 3T3 (**a**) and STAT3C-3T3 (**b**)

phenotypes. As shown in Figure 3a, some melanoma cell lines, B16F10, G361, MMAc, and HMV-II, showed constitutive phosphorylation of STAT3. We examined whether MITF induced the constitutive activation of STAT3. Immunoblotting with an anti-phosphorylated STAT3-specific antibody revealed that phosphorylation occurred in tyrosine 705 (Y705) of STAT3 in STAT3C-3T3 cells (Figure 3b). wt-MITF and ΔN-MITF did not affect phosphorylation states of STAT3 in NIH-3T3 cells (Figure 3b). As shown in Figure 3c, MITF had little effect on or rather suppressed STAT3-dependent APRE-luciferase activity. These data indicate that STAT3 is often constitutively activated in melanoma cells, but the mechanism is probably independent of MITF expression. Furthermore, MITF-transactivation activity was not affected by STAT3C (data not shown). Therefore, we speculated that an oncogenic target gene(s) could be induced by the cooperative action of STAT3 and MITF.

### *Microarray screening for target genes of STAT3C and MITF*

To identify target genes of MITF and STAT3, a microarray-based screen was undertaken. Total RNA was isolated from ΔN-MITF-infected NIH-3T3 (ΔN-MITF-3T3), STAT3C-3T3, and ΔN-MITF-infected STAT3C-3T3 (ΔN-MITF/STAT3C-3T3) cells and subjected to Affymetrix microarray analysis (about 12000 genes). As summarized in Figure 4a, seven genes in ΔN-MITF/STAT3C-3T3 cells were identified as more than 10-fold upregulated genes compared with ΔN-MITF-3T3 and STAT3C-3T3 cells. Most of the genes were mast cell or melanocyte-specific genes and chemokines, and the upregulation of these genes was confirmed by RT–PCR analysis (Figure 4b). Among these seven genes, the upregulation of c-*fos* is particularly interesting because c-*fos* is a component of the AP-1 transcription factor and known to be an oncogene. The functions of AP-1, composed of Fos family proteins (c-Fos, Fra-1, Fra-2, and FosB) and Jun family proteins (c-Jun, JunB, and JunD), were shown to play important roles not only in normal cell growth but also in several transformed cells induced by oncogenes (Ui *et al*., 2000). Therefore,

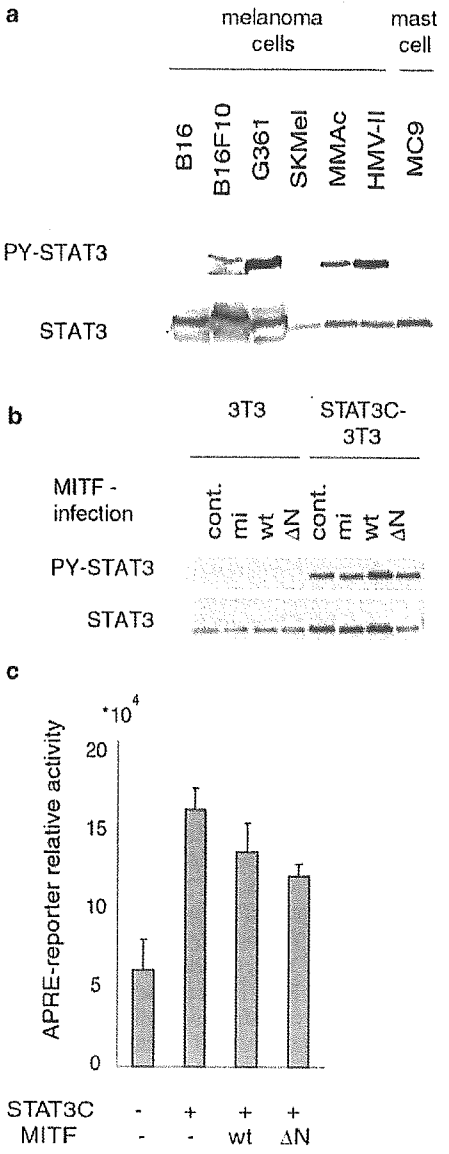
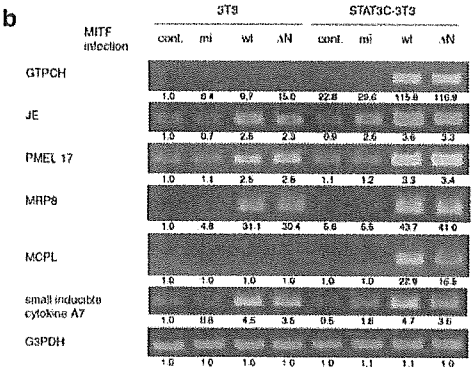



**Figure 3** MITF and STAT3C do not directly activate each other. (**a** and **b**) Phosphorylation of STAT3 was detected by Western blotting with anti-phosphorylated Tyr705 of the STAT3-specific antibody. Lysate from cell lines of melanoma cells and mast cells (**a**) or 3T3 and STAT3C-3T3 cells infected with the pMX empty vector, pMX-mi-MITF, wt-MITF, or ΔN-MITF (**b**) were examined. (**c**) HEK293 cells were transfected with a plasmid mixture containing the APRE-luciferase reporter gene (0.04 $\mu$g) and the $\beta$-galactosidase gene (0.1 $\mu$g). To examine the MITF-dependent APRE-luciferase activity, cDNA of STAT3C (0.2 $\mu$g) and MITF (0.1 $\mu$g) was also introduced. Data normalized with the $\beta$-galactosidase activity from triplicate experiments are shown

we confirmed the upregulation of the c-*fos* gene by Northern blotting. As shown in Figure 4c, c-*fos* was consecutively expressed in ΔN-MITF/STAT3C-3T3 cells, but was not detected in quiescent 3T3 cells. We also detected the endogenously high expression of c-*fos*

**a** Target genes of ΔN MITF and STAT3C

| Acc. Number | gene |
|---|---|
| L09737 | GTP cyclohydrolase 1 |
| M19681 | platelet-derived growth factor-inducible protein (JE) |
| U14133 | pmel 17 |
| M83218 | intracellular calcium binding protein (MRP-8) |
| M57401 | mast cell protease-like protein |
| X70058 | small inducible cytokine A7 |
| V00727 | c-fos oncogene |



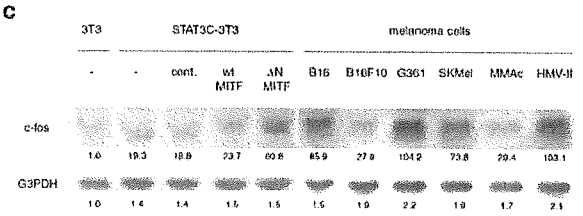




**Figure 4** Expression of genes upregulated by MITF and STAT3C. (**a**) In microarray analysis, seven genes in 3T3 cells expressing both ΔN-MITF and STAT3C were found as >10-fold upregulated genes compared with STAT3C-3T3 or ΔN-MITF single transfectants. (**b**) Upregulation in six of the genes detected by microarray analysis. The mRNA expression level was evaluated by the RT–PCR method in 3T3 or STAT3C-3T3 cells infected with the pMX empty vector, pMX-mi-MITF, wt-MITF, or ΔN-MITF. The intensity of the PCR band was quantified with NIH image software. (**c**) Northern blotting analysis of the c-*fos* oncogene. 3T3 cells, STAT3C-3T3 cells uninfected or infected with empty vector, wt-, or ΔN-MITF viruses, and six kinds of melanoma cell lines were examined with RNA probes of c-*fos* and G3PDH

in several melanoma cell lines (Figure 4c), suggesting that the constitutive expression of c-*fos* contributes to the oncogenesis of melanoma.

### *STAT3 and MITF directly bind to the promoter region of* c-fos

It has been shown that the c-*fos* proximal promoter region contains a single STAT3-binding site in the SIE region (Shibuya *et al*., 1994). We also noticed many MITF-binding motifs (CANNTG; E-box) (Tsujimura *et al*., 1996) in the promoter region (Figure 5a). To confirm that c-*fos* activation was cooperatively induced by MITF and STAT3C, a reporter gene assay using c-*fos* promoter luciferase constructs (Hatakeyama *et al*., 1992) was carried out. The transcriptional activity of c-*fos* was significantly increased by the transient expression of wt- or ΔN-MITF and STAT3C in HEK293 cells. MITF-induced c-*fos* promoter activation was further stimulated by leukemia inhibitory factor (LIF), which activates endogenous STAT3 (Figure 5b and data not shown).

Next, we examined the region of the c-*fos* promoter responsible for the interaction of MITF and STAT3. The c-*fos* promoter construct contains five potential MITF-binding sites. Using mutated or truncated forms of the c-*fos* promoter, we found that the SIE region is important for activation by STAT3 and an MITF-binding motif in the SRE region is essential for promoter activation by MITF (Figure 5c).

To confirm the direct binding of STAT3 and MITF to the c-*fos* promoter region, DNA-binding assay (Figure 5d) as well as chromatin immunoprecipitation (ChIP) assay (Figure 5e) were performed. First, nuclear extracts from 293T cells transfected with Myc-tagged ΔN-MITF and STAT3C or from cells stimulated with or without LIF were incubated with beads conjugated with oligonucleotides of the human c-*fos* promoter sequence, including the SIE and the SRE (55 mer). As shown in Figure 5d, MITF (lanes 2, 4, and 6) as well as both phosphorylated endogenous STAT3 (lanes 3 and 4) and STAT3C (lanes 5 and 6) bound to the oligonucleotides of the c-*fos* promoter region *in vitro*. Non-phosphorylated STAT3 without LIF stimulation (lanes 1 and 2) did not bind to the oligonucleotide beads, suggesting a specific interaction of activated STAT3 and the DNA.

For ChIP assay (Figure 5e), the crosslinked chromatins from wt-MITF/STAT3C-3T3 cells and ΔN-MITF/STAT3C-3T3 cells as well as melanomas (G361 and HMV-II) in which STAT3 was consecutively phosphorylated were immunoprecipitated with STAT3- or MITF-specific antibodies. The crosslinked protein was then removed from DNA by proteolysis. Finally, the immunoprecipitated DNA was analysed by PCR to detect the c-*fos* promoter region. As shown in Figure 5e, the anti-STAT3 antibody and the anti-MITF antibody precipitated the c-*fos* promoter SIE region and the MITF-binding motif in the SRE region, respectively.

### *Dominant-negative mutant of AP-1 inhibited cellular transformation of MITF/STAT3C-3T3*

To investigate the contribution of the c-*fos* gene in the anchorage-independent growth of MITF/STAT3C-3T3 cells, we introduced a dominant-negative mutant of AP-1 into these cells. We used a retrovirus carrying SupJunD-1, which has an N-terminal deletion of the transactivation domain of c-Jun. We have shown that the SupJunD-1 virus suppresses the transactivation activity of AP-1 and inhibits colony formation in soft agar of various types of tumor cells (Ui *et al*., 2000). As shown in Figure 6, the dominant-negative AP-1