to those in I-BMSCs and A-BMSCs, proving that group A genes are common markers for BMSCs from various bones.

The mRNA levels of melanoma cell adhesion molecule (MCAM) (group B) in F-BMSCs and T-BMSCs were lower than those in I-BMSCs, but higher than those in A-BMSCs or fibroblasts (Fig. 2). The mRNA levels of thrombin receptor (F2R) and leptin receptor (LEPR) (group C) in F-BMSCs and T-BMSCs were similar to those in I-BMSCs, and lower than those in A-BMSCs (Fig. 2), suggesting that the gene expression profile in long-bone BMSCs is similar to that in I-BMSCs, but differs from that in A-BMSCs.

In previous studies, we showed that mRNA levels of adrenomedullin (ADM), apolipoprotein D (APOD), C-type lectin superfamily member 2 (CLEC2B), collagen type XV α1 (COL15A1), CUG triplet repeat RNA-binding protein 2 (CUGBP2), and MMP1 were lower in A-BMSCs or I-BMSCs than in fibroblasts.[27] These mRNA levels also proved to be lower in F-BMSCs and T-BMSCs than in fibroblasts (Fig. 2).

In Figure 2, sample numbers were different among A-BMSCs ($n=6$), I-BMSCs ($n=14$), T-BMSCs ($n=3$), F-BMSCs ($n=3$), skin fibroblasts ($n=5$), and gum fibroblasts ($n=1$). To reduce this variability, we examined the expression levels of marker genes in an additional six A-BMSC lines and an additional three gum fibroblast lines. The sample numbers had little effect on the expression levels of these genes (data not shown).

Effects of passage and age on marker gene expression levels

If the passage of BMSC cultures markedly affects gene expression levels of marker genes, such markers may be less useful in clinical studies, so we examined the marker gene expression levels as a function of the passage number (Fig. 3). In this study, the passage number markedly altered the expression of several marker genes, including serine (or cysteine) proteinase inhibitor, clade I, member 1 (SERPINI1); CD74 antigen (CD74); and transglutaminase 2 (TGM2). The marker genes in Figure 3B did not always show significantly higher expression levels in I-BMSCs than in fibroblasts at some passage numbers. However, LIF, insulin-like growth factor 1 (IGF1), proteoglycan 1 (PRG1), matrix Gla protein (MGP), bone morphogenetic protein 4 (BMP4), connective tissue growth factor (CTGF), potassium channel tetramerization domain containing 12 (KCTD12), insulin-like growth factor binding protein 7 (IGFBP7), tribbles homolog 2 (TRIB2), VEGF, and dynein (DYNC1I1) showed higher expression levels in I-BMSCs than in fibroblasts, irrespective of the passage number (Fig. 3A). In contrast, the expression levels of fibroblast markers were consistently lower in BMSCs than in fibroblasts, irrespective of the passage number (Fig. 3C).

To confirm the usefulness of the markers, we examined the effect of age on the marker gene expression levels in I-BMSCs from 14 individual lines. No significant differences in the expression levels of marker genes were observed between young I-BMSC lines (I-BMSC-y, $n=7$, age 18–39 years) and old I-BMSC lines (I-BMSC-o, $n=7$, age 53–81 years) (upper panel, Fig. 4), and between young A-BMSC lines (A-BMSC-y, $n=6$, age 19–25 years) and old A-BMSC lines (A-BMSC-o, $n=6$, age 43–63 years) (lower panel).

Use of the marker genes in clinical studies

We are now doing clinical studies on regenerative medicine for periodontal diseases using autologous BMSCs.[19,32] In a previous report, we showed that SERPINI1, tissue factor pathway inhibitor 2 (TFPI2), major histocompatibility complex class II DR alpha (HLADRA), major histocompatibility complex class II DR beta (HLADRB), and CD74 expression levels were higher in BMSC lines from a few patients than in fibroblasts,[27] but it was unclear whether these and the other common markers would be useful in clinical studies for a large number of patients. In the present study, we compared mRNA levels of 13 genes of group A-1, 7 genes of group A-2, and 1 gene of group B in I-BMSC lines from patient 1 (age 63 years, male), patient 2 (39 years, male), patient 3 (64 years, female), patient 4 (46 years, female), patient 5 (25 years, male), patient 6 (56 years, female), patient 7 (52 years, female), patient 8 (22 years, male), and patient 9 (40 years, female) with those in fibroblast lines (Table 2). We found that LIF, IGF1, PRG1, MGP, BMP4, CTGF, KCTD12, IGFBP7, TRIB2, HLADRB, HLADRA, CD74, TGM2, DYNC1I1, and MCAM expression levels were consistently much higher (greater than fourfold) in the BMSC lines than in fibroblasts, although HLADRB and HLADRA levels showed very large interindividual differences (>100-fold). Adducin 3 (ADD3), SERPINI1, Rho GDP dissociation inhibitor beta (ARHGDIB), and TFPI2 expression levels were not always higher in patients' BMSCs than in fibroblasts, and laminin alpha 3 (LAMA3) and VEGF expression levels in some BMSC lines were only two- to fourfold higher than in fibroblasts.

In addition, six fibroblast markers also proved useful for quality examination of patients' BMSCs (Table 2). APOD, COL15A1, CUGBP2, and MMP1 levels, in particular, were much lower (<5%) in all BMSC lines than in fibroblasts, so these may be reliable negative markers for BMSCs.

Secreted levels of several markers

Since enzyme-linked immunosorbent assay (ELISA) provides verification that differential gene expression translates to changes in protein levels, we examined the secreted levels of several markers in I-BMSC and fibroblast cultures by ELISA (Fig. 5). We found that the LIF and VEGF levels in I-BMSC cultures were consistently higher than those in fibroblast cultures, whereas the pro-MMP1 levels in fibroblast cultures were consistently higher than those in I-BMSC cultures.

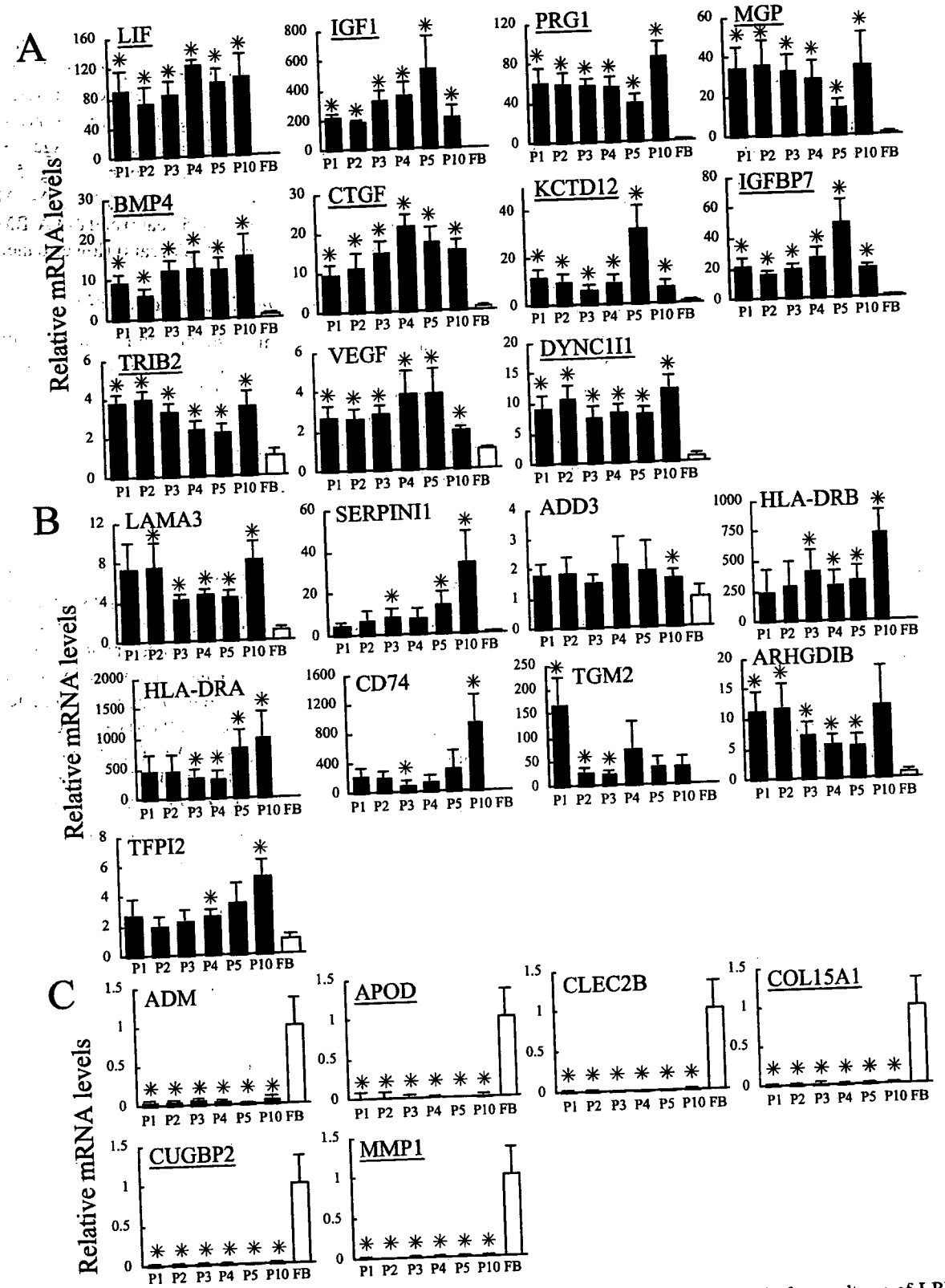

FIG. 3. Effect of passage number on marker gene expression levels. RNA was harvested separately from cultures of I-BMSC lines from four donors and fibroblast lines from four donors. (**A**) Most group A genes showed consistently higher expression levels in four I-BMSC lines than in four standard fibroblast lines (FB, open columns) at passages 1, 2, 3, 4, 5, and 10 ($*p < 0.05$). (**B**) Some group A genes showed significantly higher expression levels in four I-BMSC lines than in four fibroblast lines only at some passage numbers ($*p < 0.05$). (**C**) The expression levels of fibroblast marker genes were consistently lower in four I-BMSC lines than in four fibroblast lines at passages 1, 2, 3, 4, 5, and 10 ($*p < 0.05$). The values are mean ± SEM for four cultures. Underlined are the markers we had selected for optimal assays, considering the effects of *in vivo* location of BMSCs and passage number, magnitude of increase in expression levels, and interindividual differences (see "Discussion" section).

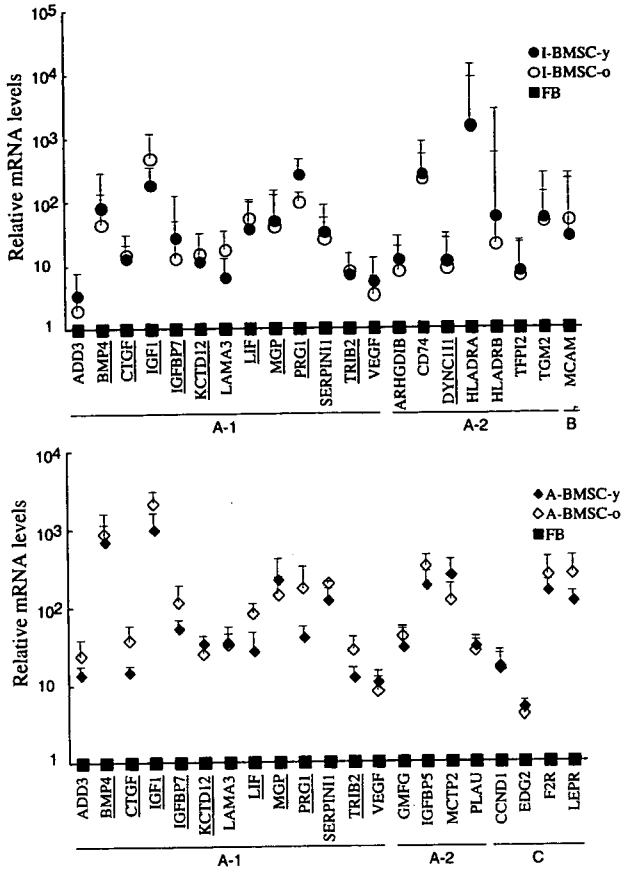

FIG. 4. Effect of age on marker gene expression levels. The expression levels (mean ± SEM) of marker genes in young I-BMSCs (closed circles, $n = 7$, age 18–39 years) and old I-BMSCs (open circles, $n = 7$, age 53–81 years) relative to standard fibroblasts (closed squares, $n = 6$, age 17–47 years), along with young A-BMSCs (closed diamonds, $n = 6$, age 19–25 years) and old A-BMSCs (open diamonds, $n = 6$, age 43–63 years), were determined. RNA was isolated from BMSC cultures at passages 5–8. No marker genes showed any significant difference between young I-BMSCs and old I-BMSCs or between young A-BMSCs and old A-BMSCs. Underlined are the markers we had selected for optimal assays, considering the effects of *in vivo* location of BMSCs and passage number, magnitude of increase in expression levels, and interindividual differences (see "Discussion" section).

DISCUSSION

Identity and purity of transplantable cells are essential factors for the efficacy and evaluation of tissue engineering and cell therapy, but optimal assays for *ex vivo*–expanded BMSCs have not been established. While several DNA microarray studies have revealed the gene expression profile of BMSCs,[33–40] and some studies have identified a number of BMSC markers that may distinguish BMSCs from fibroblasts,[27,33,34] it remained unclear whether these would be reliable markers in clinical studies, since previous studies had quantified expression of a small number of candidate marker genes with a small number of examined BMSC lines. It also remained unknown whether these markers were common for BMSCs from various bones. Different microenvironments, shapes, and sizes of various bones may affect the expression of BMSC markers, and "some" BMSC markers may depend upon the *in vivo* location of bone.

In this study, we demonstrated that the differentiation potential of F-BMSCs and T-BMSCs (long-bone BMSCs) is similar to that of I-BMSCs, but different from that of A-BMSCs (Fig. 1). Nonetheless, all BMSC lines expressed numerous common markers at similar mRNA levels, whereas several markers showed discrete expression levels between A-BMSCs and other BMSCs (Fig. 2). The distinct gene expression profile of A-BMSCs could be accounted for by origin: alveolar/jaw bone originates in the ectoderm (neural crest), whereas the ilium, tibia, and femur originate in the mesoderm.

We referred to stromal cells, which were isolated from bone marrow aspirates, adhered on the substrate, and proliferated in monolayer cultures, as BMSCs, although these cells are often called mesenchymal stem cells. The definition of mesenchymal stem cells is controversial, but we define mesenchymal stem cells as stromal cells that have multilineage differentiation potential, including osteogenic, chondrogenic, and adipogenic potentials. I-BMSCs, T-BMSCs, and F-BMSCs showed this multipotentiality, indicating that these cells were indeed mesenchymal stem cells, whereas A-BMSCs had poor chondrogenic and adipogenic potentials. The reason for this discrepancy is unknown, but in the presence of common markers for BMSCs, the chondrogenic and adipogenic potentials of A-BMSCs may be suppressed by some genes *in vivo* location dependently.

In this study, we found that many markers were common for all BMSCs, while only one marker (MCAM) was positive for I-BMSCs, T-BMSCs, and F-BMSCs, and negative for A-BMSCs. The expression of MCAM may be linked with multipotentiality or could be suppressed in some bones—irrespective of multipotentiality. This point needs further investigation. However, the common markers will be useful for identification of BMSCs with osteogenic potential, and the expression of these markers did not suppress the multilineage differentiation potential of I-BMSCs, T-BMSCs, or F-BMSCs. Since BMSCs from various bones will be used for treatment of various diseases, common markers may be useful in routine tests. In addition, some markers that are directly linked with multipotentiality may be useful in the future. The identification of markers functionally linked with multipotentiality will be an important issue in future studies.

Most of the common and *in vivo* location–dependent markers were involved in signal transduction by growth factors/cytokines (IGF1, LIF, BMP4, VEGF, CTGF, IGFBP5, IGFBP7, F2R, LEPR, etc.) or in remodeling of extracellular matrix (LAMA3, MGP, PRG1, SERPINI1, TGM2, TFPI2, etc.). Whether the expression levels of these markers are related to the differentiation potential of BMSCs will also be investigated in the future.

TABLE 2. MESSENGER RNA LEVELS OF MARKER GENES IN *EX VIVO* EXPANDED BMSC POPULATIONS BEFORE TRANSPLANTATION

		Pt. 1 *P1*	*Pt. 2* *P2*	*Pt. 3* *P1*	*Pt. 4* *P2*	*Pt. 5* *P1*	*Pt. 6* *P2*	*Pt. 7* *P2*	*Pt. 8* *P2*	*Pt. 9* *P2*	*FB (n=6)*
Group	*Gene symbol*	*Relative mRNA levels*									
A-1	LIF	70	22	239	68	221	1330	265	47	195	1±1
	IGF1	111	146	131	174	81	260	453	548	71	1±1
	PRG1	63	147	151	156	62	142	134	110	92	1±0
	MGP	51	41	52	54	17	69	221	12	327	1±1
	BMP4	158	8	18	11	41	34	60	6	53	1±1
	CTGF	10	29	29	9	15	14	16	18	16	1±0
	KCTD12	26	13	9	15	7	19	12	14	20	1±0
	LAMA3	33	4	12	5	8	16	29	13	16	1±0
	IGFBP7	9	8	15	15	19	5	11	10	19	1±1
	SERPINI1	41	4	6	4	2	4	4	11	4	1±1
	TRIB2	10	7	8	10	8	7	7	9	11	1±0
	VEGF	3	4	8	5	3	9	5	13	2	1±0
	ADD3	2	2	2	2	1	2	2	2	2	1±1
A-2	HLADRB	15	502	3394	28	5124	1002	996	83	749	1±0
	HLADRA	264	512	3279	4429	1064	160	146	37	79	1±0
	CD74	85	187	473	823	170	462	360	16	104	1±1
	TGM2	33	133	29	185	55	34	49	38	105	1±1
	DYNC1I1	7	6	7	10	4	12	9	14	9	1±1
	ARHGDIB	3	3	29	3	10	19	3	1	5	1±1
	TFPI2	4	2	8	13	11	13	8	3	6	1±0
B	MCAM	12	16	40	74	21	84	57	69	24	1±0
FB marker genes	ADM	1×10^{-3}	3×10^{-3}	2×10^{-3}	1×10^{-3}	3×10^{-3}	1×10^{-3}	5×10^{-3}	3×10^{-3}	3×10^{-3}	1±0
	APOD	9×10^{-3}	6×10^{-3}	1×10^{-3}	3×10^{-3}	6×10^{-4}	7×10^{-4}	1×10^{-3}	5×10^{-3}	7×10^{-3}	1±0
	CLEC2B	2×10^{-3}	8×10^{-3}	4×10^{-3}	2×10^{-3}	5×10^{-3}	4×10^{-3}	1×10^{-3}	1×10^{-2}	4×10^{-3}	1±0
	COL15A1	9×10^{-3}	3×10^{-2}	3×10^{-3}	7×10^{-4}	2×10^{-4}	7×10^{-3}	4×10^{-4}	1×10^{-2}	4×10^{-4}	1±0
	CUGBP2	3×10^{-2}	2×10^{-2}	3×10^{-2}	3×10^{-2}	2×10^{-2}	3×10^{-2}	3×10^{-2}	5×10^{-2}	6×10^{-2}	1±0
	MMP1	2×10^{-2}	8×10^{-3}	1×10^{-2}	1×10^{-2}	2×10^{-1}	6×10^{-2}	8×10^{-3}	8×10^{-3}	8×10^{-3}	1±0

Relative mRNA levels in BMSC cultures at passage 1 or 2 (P1 or P2) were determined by real time quantitative RT-PCR, and were normalized in BMSC lines from 9 patients (Pt.1–Pt.9) against the values obtained with 6 fibroblast lines.

Abbreviations: FB, Fibroblast; P, Passage numbers.

Underlines: We selected these markers for optimal assays, considering the effects of *in vivo* location of BMSC and passage, magnitude of increase in expression levels and inter-individual differences (see Discussion).

We first identified the above markers from BMSC cultures at the fifth to eighth passage, but we confirmed, using nine patients' BMSC lines, that the mRNA levels of the marker genes were also much higher (greater than fourfold) in BMSCs at the first or second passage than in fibroblasts, except for ADD3, SERPINI1, ARHGDIB, and TFPI2; LAMA3 and VEGF expression levels in some BMSC lines were only two- to fourfold higher than in fibroblasts (Table 2). In subsequent studies, a passage-dependent difference was observed for several markers (Fig. 3), but LIF, IGF1, PRG1, MGP, BMP4, CTGF, KCTD12, IGFBP7, TRIB2, and DYNC1I1 showed higher expression levels in BMSCs, irrespective of the passage number, than in fibroblasts (Fig. 3A); these may be reliable markers for *ex vivo*–expanded BMSCs. On the other hand, LAMA3, SERPINI1, ADD3, HLADRB, HLADRA, CD74, TGM2, ARHGDIB, and TFPI2 did not show consistently higher expression levels in BMSCs than in fibroblasts at some passage numbers, and HLADRA and HLADRB showed very large interindividual differences (>100-fold), so these are probably not reliable markers (Fig. 3B and Table 2). Proliferation, senescence, or adaptation to culture conditions may have affected the expression of some markers, passage number dependently. We also showed that donor age had little effect on the expression levels of the marker genes in BMSCs (Fig. 4), which will be of great importance in clinical studies for patients of various ages.

We selected BMSC markers from many candidates for optimal assays, considering interindividual differences (Table 2), magnitude of increase in expression levels relative to fibroblasts (Table 2), and the effects of passage (Fig. 3) and *in vivo* location of BMSCs (Table 1 and Fig. 2). LIF, IGF1, PRG1, MGP, BMP4, CTGF, KCTD12, IGFBP7, TRIB2, and DYNC1I1 proved to be reliable positive mark-

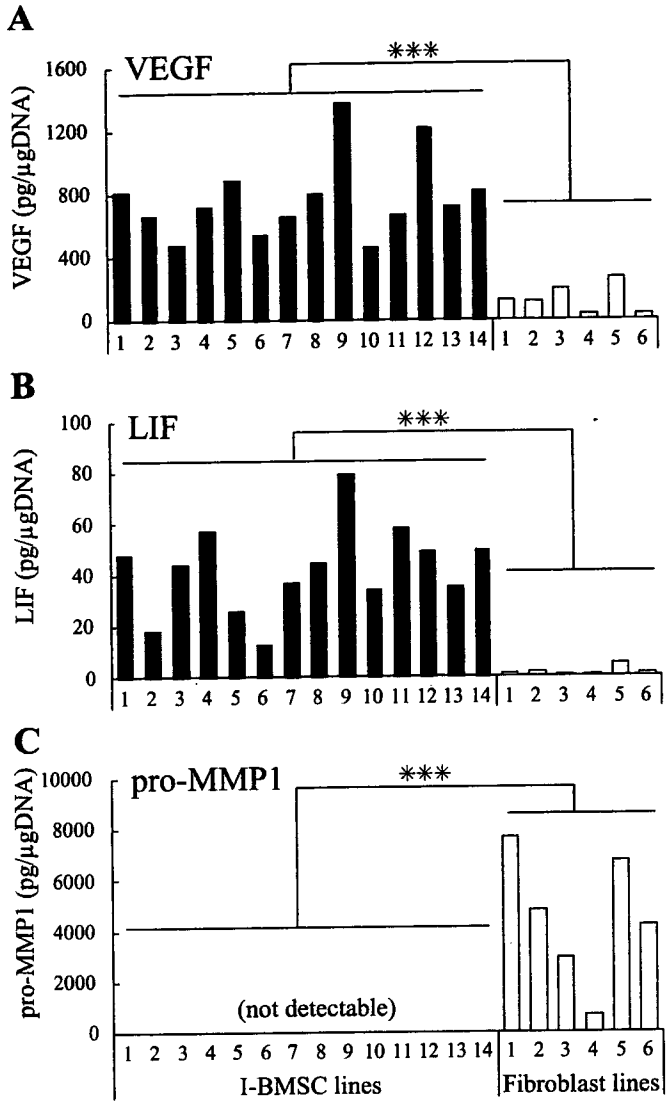

FIG. 5. Quantification of marker protein levels by ELISA. (**A**) VEGF, (**B**) LIF, and (**C**) pro-MMP1 levels in the culture medium of I-BMSC lines (closed columns) or fibroblast lines (open columns) were determined by ELISA. The media were isolated from BMSC cultures at passages 5–8. The secreted protein levels were significantly different (***$p < 0.001$) between BMSCs and fibroblast lines.

ers for BMSCs, whereas APOD, COL15A1, CUGBP2, and MMP1 were reliable negative markers (underlined genes in Tables 1 and 2, and Figs. 2–4). The positive and negative marker sets should thus be combined in a routine test. We also found that LIF, VEGF, and pro-MMP1, as determined by ELISA, were useful in distinguishing BMSCs from fibroblasts (Fig. 5). A combination of ELISA and RT-PCR may provide more accurate results.

It is interesting to compare BMSC markers identified in this study with those in other studies.[33,34,40] Wagner *et al.*[33] selected 25 marker genes that were expressed at higher levels in bone marrow mesenchymal stem cells, adipose tissue–derived mesenchymal stem cells, and cord blood–derived mesenchymal stem cells than in fibroblasts. Of the 25 genes, 17 were examined in our DNA microarray analyses, but Wagner's positive mesenchymal stem cell markers did not show any significant difference in the present study, although MMP1 was a negative marker in both Wagner's and our studies. Brendel *et al.*[34] found that 21 genes were up-regulated in human BMSCs compared with fibroblasts, using DNA microarray containing 9,600 probes. Of the 21 genes, 12 genes were included in our DNA microarray probes, but Brendel's BMSC markers did not show any significant difference in the present study, except for IGFBP5: IGFBP5 and IGFBP7 were included in our BMSC-marker list (Table 1 and Fig. 2). In addition, Wagner and Brendel identified negative markers, which were expressed at higher levels in fibroblasts than in BMSCs or mesenchymal stem cells, but these negative markers were not included in our list of fibroblast markers except for MMP1 (Table 2 and Fig. 2).

These varying results may be due to different chips, culture conditions, and cells used: Wagner used HS68 fibroblasts, whereas we used gum-derived and skin-derived fibroblasts. In addition, Wagner searched for common makers in various mesenchymal stem cell lines from bone marrow, adipose tissue, and cord blood, whereas we searched for common markers in bone marrow BMSC lines from various bones. Neither Wagner nor Brendel confirmed the differential expression of all candidate genes by quantitative RT-PCR, but in our experience, DNA microarray analysis often produces false positives and false negatives.

In preliminary studies with GeneChip (U133 plus 2.0 Array, Affimetrix) containing ~54,000 probes, we extended our studies of BMSC markers and found that some of Wagner's markers, including PLOD2, CSPG2, HOXA5, and TM4SF1, were higher in I-BMSC lines than in fibroblast lines, and some of Brendel's markers, including PLOD2, IGFBP5, ABLIM1, COL4A2, DLX5, EYA2, ID3, and IRX3, were also higher in I-BMSC lines than in fibroblast lines (data not shown). In addition to the markers listed in Tables 1 and 2, these may also be useful for identification of BMSCs. Reproducibility in different laboratories and further characterization of the additional markers may prove useful in the future.

Recently, Song *et al.*[40] identified a number of "stemness" genes that might be essential to maintain adult stem cell multipotency, and "differentiation" genes that might be essential to maintain the differentiation state. The "stemness" genes were expressed in undifferentiated BMSCs and dedifferentiated mesenchymal stem-like cells that were obtained from cultures of BMSC-derived osteoblasts, BMSC-derived chondrocytes, or BMSC-derived adipocytes. These genes were down-regulated in BMSC-derived osteoblasts, BMSC-derived chondrocytes, and/or BMSC-derived adipocytes. Song did not determine the expression levels of these genes in fibroblasts, but LIF, PRG1, TFPI2, MCTP2, and PLAU were commonly included in both their and our BMSC

marker lists, and MMP1 and COL15A1 were included in both their "differentiation" gene list and our negative BMSC marker list (Table 2). Song also examined the effects of siRNA against several marker genes on proliferation and differentiation of BMSCs, an approach that may promote studies on the functional aspects of BMSC markers.

In conclusion, we identified reliable markers for bone marrow BMSCs by real-time RT-PCR with low-density array cards, considering the effects of *in vivo* location of BMSCs and passage numbers, magnitude of increase in expression levels, and interindividual differences. We believe that these markers will be useful in a routine test for BMSCs in tissue engineering and cell therapy.

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Research Article

Transforming Growth Factor-β Promotes Survival of Mammary Carcinoma Cells through Induction of Antiapoptotic Transcription Factor DEC1

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Abstract

Transforming growth factor-β (TGF-β) signaling facilitates tumor growth and metastasis in advanced cancer. In the present study, we identified differentially expressed in chondrocytes 1 (DEC1, also known as SHARP2 and Stra13) as a downstream target of TGF-β signaling, which promotes the survival of breast cancer cells. In the mouse mammary carcinoma cell lines JygMC(A) and 4T1, the TGF-β type I receptor kinase inhibitors A-44-03 and SB431542 induced apoptosis of cells under serum-free conditions. Oligonucleotide microarray and real-time reverse transcription-PCR analyses revealed that TGF-β induced DEC1 in these cells, and the increase of DEC1 was suppressed by the TGF-β type I receptor kinase inhibitors as well as by expression of dominant-negative TGF-β type II receptor. Overexpression of DEC1 prevented the apoptosis of JygMC(A) cells induced by A-44-03, and knockdown of endogenous DEC1 abrogated TGF-β–promoted cell survival. Moreover, a dominant-negative mutant of DEC1 prevented lung and liver metastasis of JygMC(A) cells *in vivo*. Our observations thus provide new insights into the molecular mechanisms governing TGF-β–mediated cell survival and metastasis of cancer. [Cancer Res 2007;67(20):9694–703]

Introduction

Apoptosis is an important mechanism of negative regulation of cancer development and metastasis (1). During the process of metastasis, apoptosis occurs through various mechanisms; after the detachment of tumor cells from the extracellular matrix and the neighboring cells at primary tumor sites, forms of cell death known as anoikis and amorphosis occur. In the bloodstream, cell death occurs through immune surveillance and/or destruction by mechanical stress. At sites of secondary metastasis, cell death occurs after extravasation during the phase of formation of micrometastasis. These findings suggest that the metastatic potential of cancer cells is closely associated to their resistance to apoptosis.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates the growth, differentiation, and apoptosis of various types of cells. TGF-β transduces signals through two distinct serine-threonine kinase receptors, termed type I (TβR-I) and type II (TβR-II). TβR-I is activated by TβR-II on ligand binding and transduces signals through various proteins, of which Smad proteins are the major signal transducers for TGF-β (2, 3). Activated TβR-I phosphorylates receptor-regulated Smads (R-Smads; i.e., Smad2 and Smad3), which interact with common mediator Smad (Smad4) and translocate to the nucleus. Nuclear Smad complexes bind to various transcription factors and transcriptional coactivators and regulate transcription of target genes. Transcriptional corepressors, including c-Ski and SnoN, inhibit TGF-β signaling through interaction with Smad complexes. Inhibitory Smads, including Smad6 and Smad7, are induced by TGF-β and bone morphogenetic proteins (BMP), bind to type I receptors, and prevent phosphorylation of R-Smads, resulting in inhibition of TGF-β family signaling.

TGF-β exerts both inhibitory and stimulatory effects on the progression of tumors (4, 5). In early stages of carcinogenesis, TGF-β serves as a tumor suppressor through inhibition of cell growth. Thus, mutations of signal components of TGF-β, including TβR-II and Smad4, have been reported to be responsible for progression of certain gastrointestinal tumors (6, 7). However, some tumor cells escape the growth inhibition induced by TGF-β, and TGF-β facilitates the progression and metastasis of tumors in advanced stages of cancer. TGF-β induces epithelial-to-mesenchymal transdifferentiation (EMT) in mammary epithelial cells. TGF-β also acts on the tumor microenvironment, where it stimulates angiogenesis and tissue fibrosis and causes local and systemic immunosuppression, leading to progression and metastasis of tumors.

TGF-β induces apoptosis of various types of cells, and the mechanisms of TGF-β–induced apoptosis have been extensively studied. Smad proteins play critical roles in execution of TGF-β–induced death in certain types of cells (8). Several genes regulated by Smad transcriptional complexes have been reported to be involved in TGF-β–mediated apoptosis, including *DAP-kinase*, *SHIP*, and *GADD45β* (8). In addition, certain molecules including the adaptor protein Daxx have been reported to be directly activated by TβR-II and to induce cell death through activation of c-Jun NH_2-terminal kinase (9). Most of these molecules regulate the expression of members of the Bcl-2 family and activate various caspases. On the other hand, it is becoming evident that TGF-β

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also promotes cell survival under certain conditions (8). TGF-β has been shown to promote cell survival through activation of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway, which plays a key role in mediating cell survival downstream of tyrosine kinase signaling, including that mediated by insulin-like growth factor or platelet-derived growth factor (PDGF).

Several studies have revealed recently that targeting of TGF-β signaling in breast cancer cells by various strategies, such as use of soluble TβR-II antagonist, neutralizing TGF-β antibody, or small-molecule type I receptor kinase inhibitors, can prevent cancer metastasis (10–13). Consistent with this, we showed previously that systemic gene transfer of Smad7 inhibits the metastasis of mouse mammary carcinoma JygMC(A) cells in an *in vivo* experimental model (14). Thus, TGF-β is considered one of the molecular targets in the treatment of breast cancer metastasis. However, little is known about the downstream signaling pathways of TGF-β involved in the process of metastasis of breast cancer.

Here, we present the first evidence that the roles played by TGF-β signaling in survival of breast cancer cells are mediated by induction of differentially expressed in chondrocytes 1 (DEC1, also known as SHARP2 and Stra13). DEC1 is a basic helix-loop-helix transcription factor, which is frequently overexpressed in certain cancers, including breast carcinomas (15). Correlation between the expression of DEC1 and tumor grade in breast cancer has been reported (16). We found that TGF-β induces DEC1 and prevents apoptosis of mouse mammary carcinoma cells. In addition, we show that a dominant-negative mutant of DEC1 (dnDEC1) prevents lung and liver metastasis of breast cancer cells *in vivo*. Our observations thus provide new insight into the molecular mechanisms governing TGF-β–mediated survival of tumors and may aid the development of new strategies for cancer therapy.

Materials and Methods

Cell culture and reagents. Mouse mammary carcinoma 4T1 cells and mouse mammary epithelial NMuMG cells were obtained from American Type Culture Collection. 4T1 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. NMuMG cells were maintained in DMEM containing 10% FBS, 10 μg/mL insulin, and antibiotics. Mouse mammary carcinoma JygMC(A) cells were cultured as described previously (14). Cells were grown in a 5% CO_2 atmosphere at 37°C. The TβR-I kinase inhibitor used in the present study, A-44-03, is a dihydrochloride salt form of A-77-01 (17). SB431542 was obtained from Sigma. LY294002 and STI571 (imatinib mesylate/Gleevec) were purchased from Calbiochem and Novartis Pharma, respectively. The contents of one capsule of STI571 were dissolved in 17 mL of distilled water, centrifuged, filtered, and used as 10 mmol/L stock solution (18).

Apoptosis assays. For detection of cytosolic DNA ladder formation, both floating cells and adherent cells were collected and lysed with a lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L EDTA, 0.5% Triton X-100]. Cell extracts were incubated with 0.1 mg/mL RNase A and 0.2 mg/mL proteinase K at 42°C for 1 h. DNA was purified by standard phenol-chloroform extraction and ethanol precipitation. Dry DNA pellets were then resuspended in TE containing 0.2 mg/mL RNase A, and samples were electrophoretically separated on 2% agarose gel containing 0.01% ethidium bromide. For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays, cells were fixed in 4% paraformaldehyde. After permeabilization in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate, reagents of the *In situ* Cell Death Detection kit (TMR red, Roche Diagnostics) were added. The nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence was examined using an IX71 microscope (Olympus) and measured by the Integrated Intensity Program of MetaVue (Molecular Devices Corp.).

Quantitative real-time reverse transcription-PCR. Total RNAs were extracted using Trizol reagent (Invitrogen). First-strand cDNAs were synthesized using the SuperScript First-Strand Synthesis System (Invitrogen) with oligo(dT) primers. Quantitative real-time reverse transcription-PCR (RT-PCR) was done as described previously (13). The primer sequences were as follows: mouse DEC1: 5′-GAAGCACGTGAAAGCATTGACA (forward) and 5′-CCCGACAAATCACCAGCTTG (reverse); mouse plasminogen activator inhibitor-1 (PAI-1): 5′-CCACAAAGGTCTCATGGACCAT (forward) and 5′-TGAAAGTGTTGTGCCCTCCAC (reverse); and mouse hypoxanthine phosphoribosyltransferase 1 (HPRT1): 5′-CTGGTTAAGCAGTACAGCCCCA (forward) and 5′-GGTCCTTTTCACCAGCAAGCT (reverse). All samples were run in duplicate in each experiment. Values were normalized to mouse HPRT1.

Immunoblotting. Cells were lysed after various treatments with NP40 lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 1% aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride]. The supernatants were measured for protein concentrations, and those with equal amounts of total proteins were applied to 8.5% SDS-gel electrophoresis, followed by semidry transfer of the proteins to Pall FluoroTrans W membrane (Pall Life Sciences). Nonspecific binding of proteins to the membrane was blocked by incubation in TBS-T buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20] containing 5% skim milk. Separated proteins were immunoblotted with anti-Flag antibody (M2; Sigma), anti–phospho-Smad2 ($Ser^{465/467}$) antibody (Cell Signaling Technology), or anti-Smad2/3 antibody (BD Transduction Laboratories). Detection of immunoblotted proteins was done by enhanced chemiluminescence.

Construction of recombinant adenoviruses and adenoviral infection. Recombinant E1-deleted adenoviral vectors carrying cDNAs encoding β-galactosidase (*LacZ*) reporter gene, Smad6, Smad7, and c-Ski were described previously (19). cDNAs encoding Flag-tagged dominant-negative TβR-II (dnTβR-II), which lacks the intracellular domain of TβR-II, and Flag-tagged human DEC1 were subcloned into the pAxCAwtit cassette cosmid (Takara). Each cosmid carrying the expression unit and adenovirus DNA terminal protein complex was cotransfected into E1 transcomplemental cell line 293 cells. The recombinant adenoviruses generated by homologous recombination were isolated. For adenoviral infection, 5×10^5 of JygMC(A) cells per well in six-well plates were infected with adenovirus vectors at 30 to 300 plaque-forming units per cell.

Microarray analysis. mRNAs were extracted from JygMC(A) cells treated without or with TGF-β3 and those infected with Ad-*LacZ* or Ad-Smad7. Total RNAs were used to prepare cDNA and conduct oligonucleotide microarray analysis using GeneChip Mouse Genome 430 2.0 Array (Affymetrix) according to the manufacturer's instructions. FileMaker Pro software (Filemaker, Inc.) was used for statistical analysis.

RNA interference and oligonucleotides. Stealth small interfering RNA (siRNA) duplex oligoribonucleotides against mouse DEC1 were synthesized by Invitrogen. JygMC(A) cells were transfected in the presence of 150 pmol of either siRNA or control siRNA in a 500 μL volume with 7.5 μL LipofectAMINE RNAiMAX reagent (Invitrogen) per well of a six-well plate according to the manufacturer's protocols. To confirm knockdown of DEC1, cells were harvested 24 h after siRNA transfection and subjected to quantitative real-time RT-PCR.

Generation of JygMC(A) cells stably expressing dnDEC1. To establish JygMC(A) cells that stably express the dnDEC1 (Jyg-dnDEC1), a human DEC1 mutant lacking the basic region was cloned into pCAG-IRES-Puro expression vector (14). The pCAG-Flag-dnDEC1-IRES-Puro plasmid was introduced into JygMC(A) cells using LipofectAMINE 2000 reagent (Invitrogen). Stable clones were obtained by puromycin (Sigma) selection (8 μg/mL) in the culture medium, and several clones were then isolated by limiting dilution. JygMC(A) cells stably expressing the empty vector pCAG-empty-IRES-Puro (Jyg-empty) were used as a control.

***In vivo* experiment using JygMC(A) cells stably expressing dnDEC1.** To investigate *in vivo* tumor growth and metastasis of parental JygMC(A), Jyg-empty, or Jyg-dnDEC1, a mouse experimental model of metastasis was used. All animal procedures were done in the animal experiment laboratory of the Japanese Foundation for Cancer Research (JFCR) according to the guidelines proposed by the Science Council of Japan. Female BALB/c *nu/nu*

mice (4 weeks of age) were purchased from Charles River Japan. Mice were maintained under specific pathogen-free conditions. Parental JygMC(A), Jyg-empty, or Jyg-dnDEC1 cells (10^7 cells) were xenografted into the mammary fat pad of each mouse ($n > 6$ mice per group). Primary tumor growth and metastases were examined as described previously (14). Statistical differences to controls were validated by the two-sided Student's t test. $P < 0.05$ was considered significant.

Results

Antiapoptotic effects of exogenous and endogenous TGF-β on murine normal epithelial and breast cancer cells. Murine breast carcinoma JygMC(A) cells undergo cell death when cultured in low concentrations of serum. Interestingly, TGF-β promoted the survival of the JygMC(A) cells under these conditions, and the low-molecular-weight TβR-I kinase inhibitor A-44-03 further enhanced the death of JygMC(A) cells (Fig. 1*A*). Regulation of cell survival by TGF-β signaling was further investigated by DNA ladder formation and TUNEL assay. DNA ladder formation was observed under serum-free conditions, but not in the presence of 10% serum (Fig. 1*B*). TGF-β suppressed DNA ladder formation, whereas A-44-03 enhanced it under these conditions, indicating that inhibition of TGF-β signaling leads to cell death by apoptosis in JygMC(A) cells. Apoptosis of JygMC(A) cells was also observed in a TUNEL assay under serum-free conditions, and the cell survival–promoting effect of TGF-β was confirmed (Fig. 1*C* and *D*). Another low-molecular-weight TβR-I kinase inhibitor SB431542, which is less potent than A-44-03 (17), also induced apoptosis of JygMC(A) cells, although 10 μmol/L SB431542 was required to induce their apoptosis in the presence of 1 ng/mL TGF-β3 (Supplementary Fig. S1*A* and *B*). These findings suggest that exogenous TGF-β promotes the survival of JygMC(A) cells and that endogenous TGF-β produced by JygMC(A) cells functions as a prosurvival factor for these cells.

To determine whether the survival-promoting effect of TGF-β is limited to JygMC(A) cells, we examined TGF-β–mediated survival of other mammary epithelial cells (i.e., murine breast cancer 4T1 cells and murine normal epithelial NMuMG cells). Similar to JygMC(A) cells, A-44-03 induced apoptosis of 4T1 cells (Fig. 2*A* and *B*). Although A-44-03 failed to significantly induce apoptosis of NMuMG cells, TGF-β potently enhanced their survival. It is important to note that Smad2 was significantly phosphorylated in the absence of exogenous TGF-β in JygMC(A) cells and 4T1 cells, but only very weakly in NMuMG cells (Fig. 2*C*, compare *lane 2* and *lane 5* with *lane 8*). These findings suggest that cell survival mediated by autocrine TGF-β signaling may be related to tumor growth and metastasis.

Roles of PDGF receptor and Akt signaling in the promotion of cell survival by TGF-β. To elucidate the mechanisms by which TGF-β promotes the survival of JygMC(A) cells, we did cDNA microarray analysis. Smad7 inhibits TGF-β family signaling through direct binding to activated type I receptors as well as by other mechanisms (20). The *Smad7* gene was infected into JygMC(A) cells by an adenoviral vector, and genes regulated by TGF-β signals were determined with the Affymetrix GeneChip Mouse Genome 430 2.0 Array (Supplementary Table S1). The results of the microarray analysis were confirmed by quantitative real-time RT-PCR analysis. Among 59 genes regulated by TGF-β, we were interested in the *Pdgfb* gene (encoding PDGF-B chain) because it has been reported to mediate TGF-β–induced proliferation of glioma cells, and epigenetic regulation of the human *PDGFB* gene is closely related to the prognosis of glioblastoma (21). We confirmed by real-time RT-PCR that expression of PDGF-B mRNA

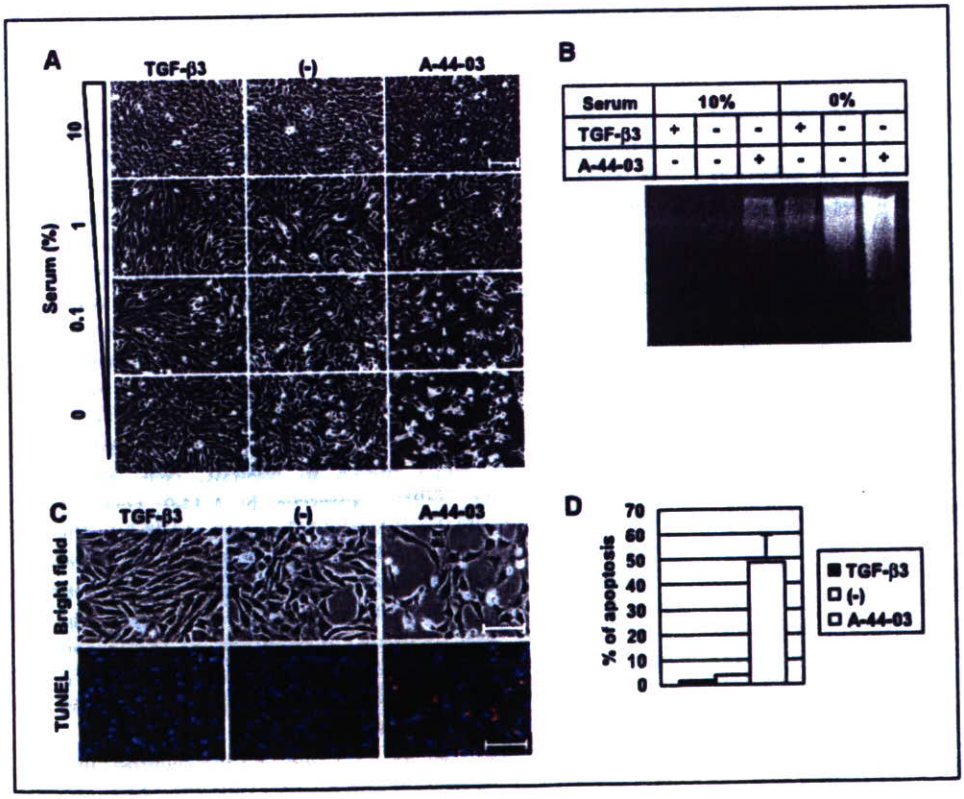


Figure 1. Antiapoptotic effects of TGF-β on JygMC(A) cells. *A*, JygMC(A) cells were treated with TGF-β3 (1 ng/mL) or A-44-03 (1 μmol/L) in the presence of various concentrations of FBS (between 0% and 10%) for 48 h. Cells were observed under phase-contrast microscopy. *Bar*, 100 μm. *B*, JygMC(A) cells were treated with TGF-β3 (1 ng/mL) or A-44-03 (1 μmol/L) in the presence or absence of FBS for 48 h. Apoptotic cell death was assessed by DNA fragmentation assay. Characteristic DNA ladders were observed after ethidium bromide staining. *C*, JygMC(A) cells were treated with TGF-β3 (1 ng/mL) or A-44-03 (1 μmol/L) in the presence of FBS for 48 h. Cells were fixed and observed under phase-contrast microscopy (*top*) or subjected to TUNEL staining (*bottom*). Cell nuclei were counterstained with DAPI. *Red*, TUNEL; *blue*, DAPI. *Bar*, 100 μm. *D*, the percentage of TUNEL-positive cells among DAPI-positive cells in *C* was determined. *Columns*, mean of triplicate determinations; *bars*, SD.

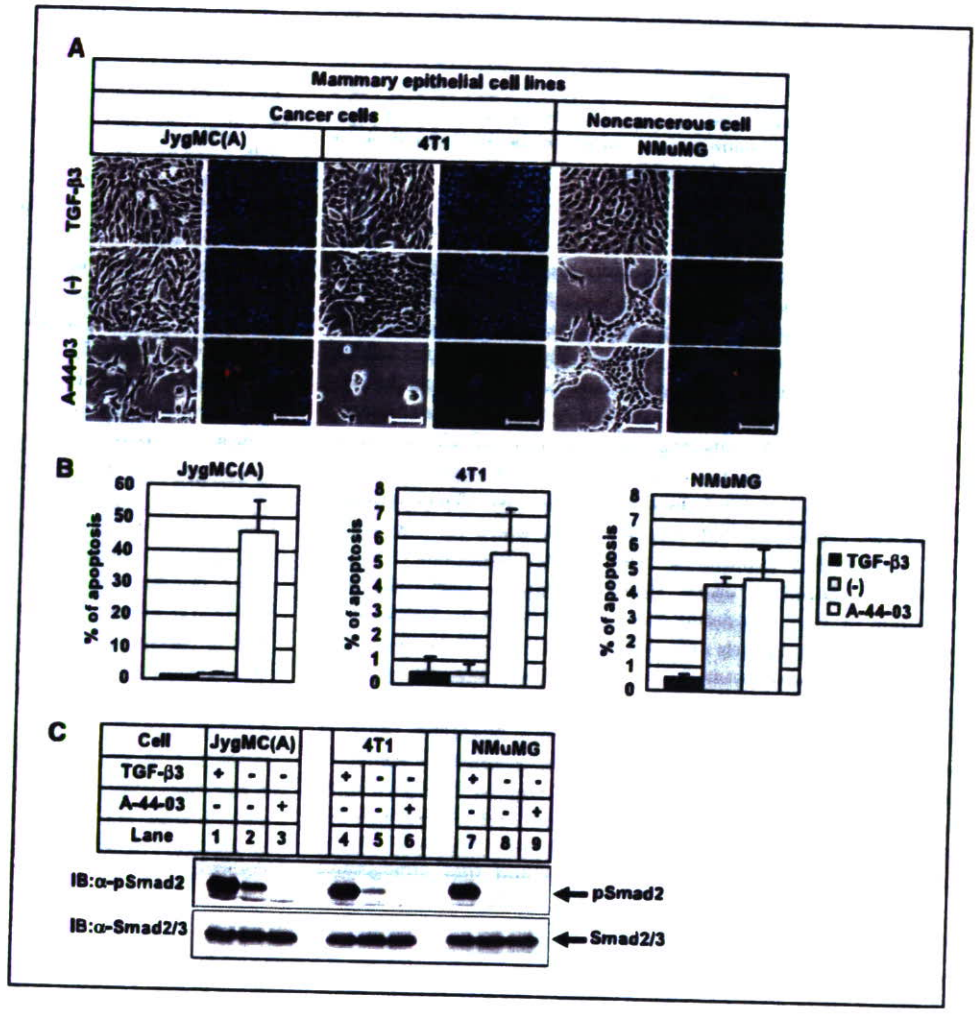


Figure 2. TGF-β–mediated survival of mouse mammary epithelial cells. *A*, mammary cancer cells [JygMC(A) cells and 4T1 cells] and normal mammary epithelial cells (NMuMG cells) were treated with TGF-β3 (1 ng/mL) or A-44-03 (1 μmol/L) in the absence of FBS for 48 h. Cells were observed under phase-contrast microscopy (*left*) and subjected to TUNEL staining (*right*). *Red*, TUNEL; *blue*, DAPI. *Bar*, 100 μm. *B*, the percentage of TUNEL-positive cells among DAPI-positive cells was determined. *Columns*, mean of triplicate determinations; *bars*, SD. *C*, mammary cancer cells [JygMC(A) cells and 4T1 cells] and normal mammary epithelial cells (NMuMG cells) were treated with TGF-β3 (1 ng/mL) or A-44-03 (1 μmol/L) in the absence of FBS for 1 h. Cell lysates were subjected to immunoblotting (*IB*) with anti–phospho-Smad2 (*pSmad2*) antibody (*top*) and anti-Smad2/3 antibody (*bottom*).

was induced by TGF-β and suppressed by A-44-03 (Supplementary Fig. S2*A*).

Because it has been reported that TGF-β induces cell survival by activation of PI3K-Akt signaling (8), we examined whether an inhibitor of PDGF receptor signaling (STI571) or one of Akt signaling (LY294002) inhibits the promotion of cell survival by TGF-β under serum-free conditions. Although STI571 and LY294002 induced apoptosis of JygMC(A) cells in the absence of TGF-β, neither of them was able to induce apoptosis of JygMC(A) cells in the presence of exogenous TGF-β (Supplementary Fig. S2*B* and *C*). Although the combination of STI571 and LY294002 strongly induced apoptosis of JygMC(A) cells in the absence of TGF-β, it induced their apoptosis only weakly in the presence of TGF-β (Supplementary Fig. S2*B* and *C*).

Promotion of the survival of JygMC(A) cells by TGF-β is mediated by DEC1. Because PDGF receptor and PI3K-Akt signaling may not be the major signaling pathways involved in TGF-β–mediated cell survival of JygMC(A) cells, we further analyzed the genes regulated by TGF-β in JygMC(A) cells. Among the 59 genes regulated by TGF-β, we found that mRNA for *Dec1* (also termed *basic helix-loop-helix domain containing, class B2*) was strongly suppressed by Smad7 in the presence of serum (Supplementary Table S1). We confirmed the effects of TGF-β and A-44-03 on the transcription of DEC1 mRNA in the presence or absence of 10% serum (Fig. 3*A*). Quantitative real-time RT-PCR analysis revealed that A-44-03 suppressed the transcription of DEC1 mRNA in JygMC(A) cells in the presence and absence of 10% serum. Although TGF-β induced the transcription of DEC1 mRNA only weakly in the presence of 10% serum, significant increase in DEC1 mRNA by TGF-β was detected in the absence of serum in JygMC(A) cells. SB431542 also suppressed the expression of DEC1 mRNA in serum-free conditions (Supplementary Fig. S1*C*). We also assessed the expression of DEC1 in serum-free conditions using other mammary epithelial cells (Fig. 3*A*). Similar to JygMC(A) cells, 4T1 cells exhibited modest induction of DEC1 mRNA by TGF-β and strong suppression of expression of it by A-44-03. Although TGF-β strongly induced DEC1 mRNA in NMuMG cells, significant suppression of DEC1 mRNA expression by A-44-03 could not be observed because the basal level of expression of DEC1 mRNA in NMuMG cells was very low. It should be noted that these profiles of expression of DEC1 mRNA correlated closely with the pattern of apoptosis induced by A-44-03 treatment, as shown in Fig. 2*B*. TGF-β has also been shown to promote cell survival in human breast cancer MDA-MB-231 cells (22). In agreement with this finding, induction of DEC1 mRNA by TGF-β was also observed in MDA-MB-231 cells (Supplementary Fig. S3).

We next examined the effect of DEC1 on the apoptosis induced by A-44-03 by infection of adenovirus carrying DEC1 cDNA.

Expression of DEC1 protein after adenovirus infection was confirmed by immunoblot analysis (Fig. 3*B*). A-44-03 induced apoptosis of JygMC(A) cells in the absence of serum, and neither control adenovirus carrying the *LacZ* gene nor stimulation by TGF-β significantly affected the apoptosis induced by A-44-03 (Fig. 3*C* and *D*). However, infection of adenovirus carrying DEC1 resulted in potent suppression of apoptosis of the cells induced by A-44-03. These findings suggested that the promotion of the survival of JygMC(A) cells by TGF-β is mediated by DEC1.

Promotion of survival of JygMC(A) cells by TGF-β is mediated in Smad-dependent fashion. To determine whether TGF-β prevents apoptosis of JygMC(A) cells in Smad-dependent fashion and whether the expression of DEC1 is indeed mediated by the TGF-β signaling pathway, we examined the effects of adenoviruses carrying various negative regulators of TGF-β signaling on TGF-β–mediated cell survival. Among the negative regulators of TGF-β signaling, c-Ski binds to Smad complexes and suppresses their transcriptional activity as a transcriptional corepressor, whereas dnTβR-II binds to TGF-β but fails to transduce intracellular signals and acts as a dominant-negative inhibitor of Smad-dependent and non-Smad pathways (20, 23). Smad7 inhibits both TGF-β and BMP signaling, whereas Smad6 inhibits BMP but not TGF-β signaling efficiently (20). As shown in Fig. 4*A*, infection of these adenoviruses resulted in efficient expression of transfected proteins in JygMC(A) cells. Analysis of the expression of a TGF-β target gene, *PAI-1*, revealed that dnTβR-II, Smad7, and c-Ski effectively suppressed TGF-β signaling, whereas Smad6 failed to do so (Fig. 4*B*). Apoptosis of JygMC(A) cells was determined by TUNEL assay in the absence or presence of TGF-β under serum-free conditions (Fig. 4*C*). dnTβR-II, Smad7, and c-Ski, but not Smad6, induced apoptosis of JygMC(A) cells in the presence and absence of TGF-β. Because c-Ski inhibits TGF-β signaling through interaction with Smad2/3 and Smad4, these findings suggest that TGF-β induces survival of JygMC(A) cells in Smad-dependent fashion. We also confirmed that expression of DEC1 is suppressed by dnTβR-II as well as by Smad7 and c-Ski (Fig. 4*D*), indicating that the expression of DEC1 is mediated by TGF-β signaling.

Silencing endogenous DEC1 abolishes the prosurvival effect of TGF-β. Because forced expression of DEC1 rescued the induction of apoptosis of JygMC(A) cells by A-44-03, we further examined the role of endogenous DEC1 in TGF-β–promoted survival of JygMC(A) cells by the siRNA method. As shown in Fig. 5*A*, we efficiently knocked down endogenous DEC1 mRNA in the cells by transfection of siRNA targeting DEC1. Interestingly, reduction of DEC1 mRNA expression resulted in significant increase in the number of apoptotic cells in the absence or presence of TGF-β under serum-free conditions (Fig. 5*B* and *C*). These findings suggest that the promotion of cell survival by TGF-β is dependent on DEC1, which is transcriptionally induced by endogenous TGF-β.

dnDEC1 induces apoptosis of JygMC(A) cells and inhibits their metastasis. Acquisition of resistance to apoptosis is thought to be related to the metastatic phenotype of cancer cells (1). Because DEC1 seemed to act as an important regulator of survival of breast cancer cells, we examined whether the DEC1-mediated survival signal is involved in the process of metastasis. We used a mutant of DEC1 lacking the basic region (dnDEC1), which acts in dominant-negative fashion (24). JygMC(A) cells, which stably express dnDEC1, were established and two stable clones, termed Jyg-dnDEC1#1 and Jyg-dnDEC1#2, were used for further investigations (Fig. 6*A*, *top*). As expected, both Jyg-dnDEC1#1 cells and

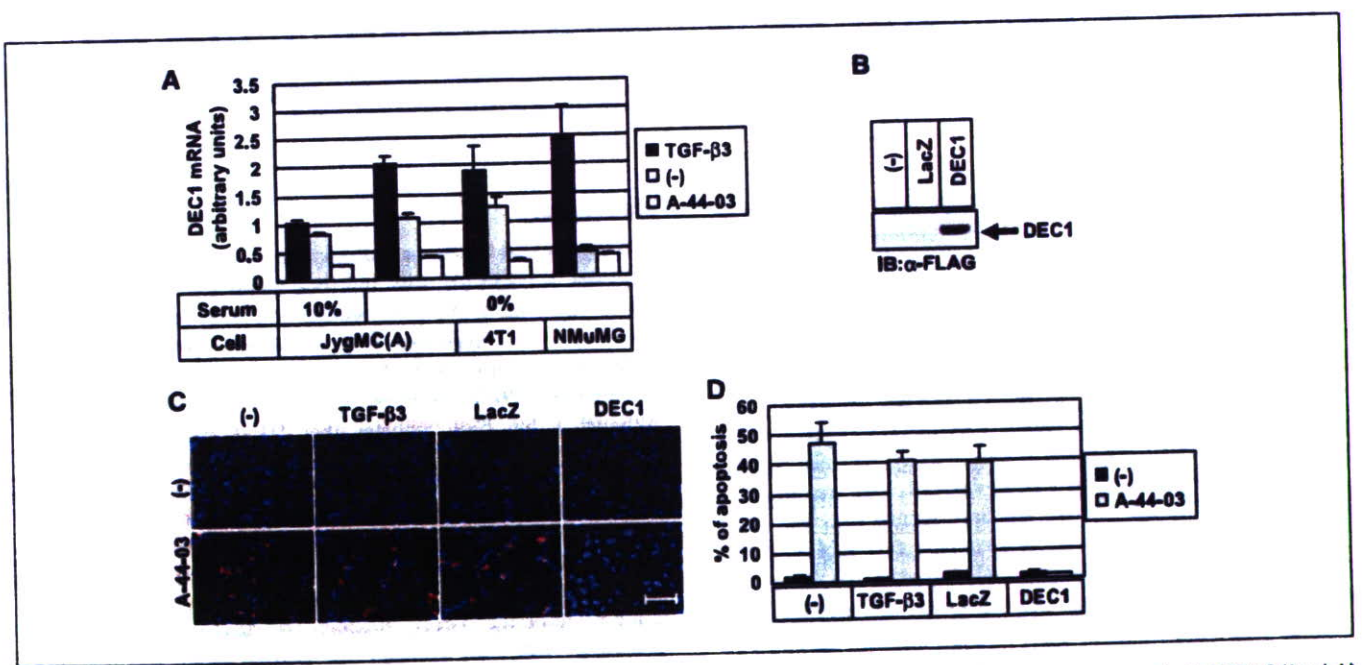


Figure 3. Promotion of survival of JygMC(A) cells by TGF-β is mediated by DEC1. *A*, JygMC(A) cells, 4T1 cells, and NMuMG cells were treated with TGF-β3 (1 ng/mL) or A-44-03 (1 μmol/L) in the presence of the indicated concentrations of FBS for 24 h. Expression of DEC1 mRNA was examined by quantitative real-time RT-PCR. Each value has been normalized to the expression of HPRT1. *Columns*, mean of duplicate determinations; *bars*, SD. *B*, JygMC(A) cells were infected with adenoviruses carrying full-length *LacZ* or DEC1, and expression of DEC1 protein was determined by immunoblotting analysis of the cell lysates 24 h after infection. *C*, JygMC(A) cells were infected with each adenovirus 12 h before A-44-03 treatment and incubated for 48 h with A-44-03 (1 μmol/L). Cells were subjected to TUNEL staining. *Red*, TUNEL; *blue*, DAPI. *Bar*, 100 μm. *D*, the percentage of TUNEL-positive cells among DAPI-positive cells was determined. *Columns*, mean of triplicate determinations; *bars*, SD.

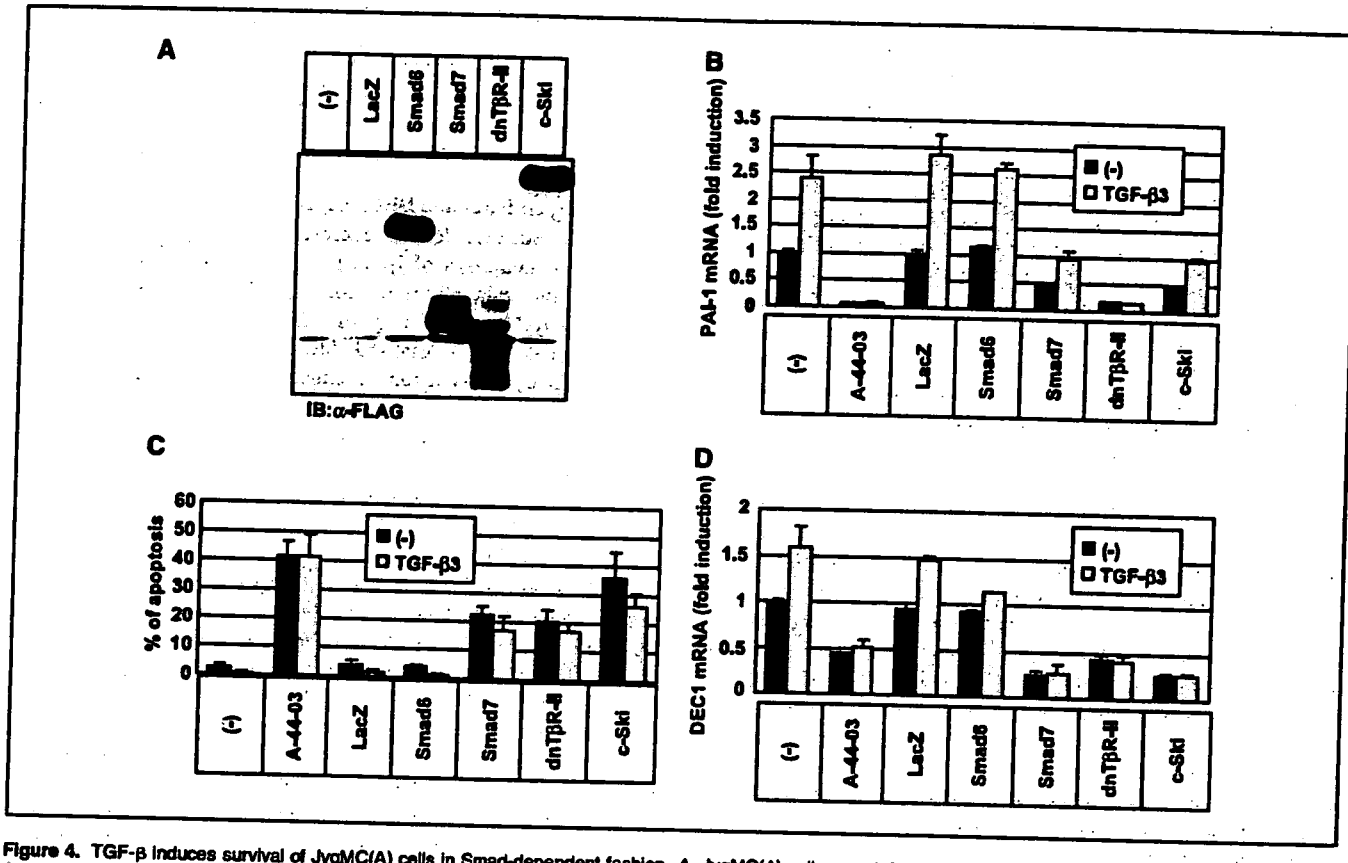


Figure 4. TGF-β induces survival of JygMC(A) cells in Smad-dependent fashion. *A*, JygMC(A) cells were infected with adenoviruses carrying full-length *LacZ*, Smad6, Smad7, dnTβR-II, and c-Ski cDNAs. Expression of recombinant protein was determined by immunoblotting analysis of each cell lysate. *B*, suppression of target genes for TGF-β was measured by quantitative real-time RT-PCR. JygMC(A) cells were infected with each adenovirus 12 h before treatment with or without TGF-β3 (1 ng/mL for 24 h). Total RNAs were extracted, and the levels of expression of *PAI-1* gene were examined by quantitative real-time RT-PCR. Fold changes relative to the uninfected control without TGF-β3 treatment are indicated. Each value has been normalized to the expression of HPRT1. *Columns*, mean of duplicate determinations; *bars*, SD. *C*, the percentage of TUNEL-positive cells among DAPI-positive cells was determined. Twelve hours after adenoviral infection, the cells were treated with or without TGF-β3 (1 ng/mL) under serum-free conditions for 48 h and subjected to TUNEL staining. *Columns*, mean of triplicate determinations; *bars*, SD. *D*, expression of DEC1 mRNA in JygMC(A) cells infected with the adenoviruses described in *B* was examined by quantitative real-time RT-PCR. Fold changes relative to the uninfected control without TGF-β3 treatment are indicated. Each value is presented as in *B*.

Jyg-dnDEC1#2 cells underwent apoptotic cell death in serum-starved conditions (Fig. 6*A*, *bottom*). Furthermore, overexpression of the dnDEC1 mutant induced apoptosis even in the presence of TGF-β, suggesting that dnDEC1 effectively antagonizes TGF-β–mediated cell survival.

Finally, we investigated the growth and metastatic potential of Jyg-dnDEC1 cells in *in vivo* experiments. After s.c. inoculation into nude mice and formation of primary tumors, JygMC(A) cells metastasize to lung and liver within 3 weeks (14). Growth of the primary tumors and metastases to lung and liver in mice bearing parental JygMC(A), Jyg-empty, and Jyg-dnDEC1 cells are shown in Fig. 6*B* to *D*. Growth of the primary tumors did not differ significantly between the mice bearing parental JygMC(A), Jyg-empty, and Jyg-dnDEC1 cells (Fig. 6*B*). However, the lungs and livers in the mice bearing Jyg-dnDEC1#1 or Jyg-dnDEC1#2 weighed less than those in mice bearing parental JygMC(A) or Jyg-empty (Fig. 6*D*, *top*). Intriguingly, stable expression of dnDEC1 in JygMC(A) cells produced few metastatic nodules in the lungs and liver (Fig. 6*C* and *D*, *bottom*). These findings indicate that DEC1 contributes to the metastasis of JygMC(A) cells by promoting cell survival without affecting primary tumor growth *in vivo*.

Discussion

In the present study, we showed that serum starvation induced apoptosis of JygMC(A) cells and that suppression of endogenous TGF-β signaling by the TβR-I kinase inhibitors strongly enhanced this apoptosis, suggesting that autocrine TGF-β signaling in JygMC(A) cells supports their own survival.

Autocrine TGF-β induces survival of breast cancer cells. Although it has been reported that TGF-β causes apoptosis in certain types of cells (8), apoptosis is induced by exogenous TGF-β at much higher concentrations than those required for inhibition of cell growth. Lei et al. (22) reported that disruption of autocrine TGF-β signaling by the ectopic expression of a soluble TGF-β type III receptor induces apoptosis of human breast cancer MDA-MB-231 cells. Their findings indicate that TGF-β, acting in autocrine fashion, supports the survival of cancer cells, consistent with our findings. In normal mammary epithelial NMuMG cells, which exhibited low basal TGF-β signaling activity, a cell survival–promoting effect of TGF-β was observed with the addition of exogenous TGF-β, whereas A-44-03 failed to significantly enhance apoptosis (see Fig. 2*A–C*). By contrast, in breast cancer JygMC(A) cells and 4T1 cells, phosphorylation of Smad2 was observed even in the absence of TGF-β stimulation (see

Fig. 2C), and A-44-03 potently induced apoptosis of these cells. We measured the amounts of TGF-βs but could not detect active forms of TGF-βs in the conditioned medium of these cells.[6] Apparently, latent TGF-β is activated on the surface of cells and immediately binds to the receptors, preventing the detection of active TGF-β in the conditioned medium. Thus, during the progression of cancer, cancer cells might gain the ability to activate TGF-β signaling in autocrine fashion, and this property might protect them from various apoptotic stimuli, enabling their survival.

TGF-β has been reported to act as a prosurvival factor via Akt/protein kinase B signaling (22, 25, 26). Moreover, TGF-β has been reported to induce the expression of PDGF in certain types of cells, which may lead to activation of PI3K-Akt signaling. We reported previously that TGF-β enhances the growth of MG63 osteosarcoma cells through induction of PDGF-A and that this growth-stimulatory activity of TGF-β is abolished by treatment with STI571/Gleevec (18). In addition, TGF-β was shown to stimulate the proliferation of glioblastoma cells via induction of PDGF-B, which may be related to the poor prognosis of human glioma (21). However, in the present study, we showed that pharmacologic inhibition of PDGF receptor signaling by STI571 or that of PI3K-Akt signal by LY294002 failed to induce apoptosis of JygMC(A) cells in the presence of exogenous TGF-β and that the combination of ST1571 and LY294002 only partially antagonized TGF-β–induced cell survival. These findings suggest that survival signals other than PI3K-Akt must be present in these cells.

TGF-β induces survival of breast cancer cells through DEC1. We further analyzed DNA microarray data to identify the downstream molecule(s) involved in TGF-β–mediated cell survival and found that TGF-β induces the expression of DEC1, which may antagonize serum deprivation–induced apoptosis. DEC1, also known as SHARP2 and Stra13, is widely expressed in most normal tissues (27, 28) and associated with developmental events in many cells and regulation of circadian rhythms (29–31). DEC1 has been suggested to serve as a downstream target of TGF-β (32). Separate from TGF-β signaling, DEC1 is also induced in response to hypoxia (33). DEC1 is overexpressed in various cancers, including breast cancer, colorectal cancer, pancreatic cancer, non–small cell lung cancer, and oligodendroglioma (15, 34–37). Moreover, a significant correlation between DEC1 expression and grade in breast carcinomas has been reported (16). Although DEC1 has been reported to antagonize serum deprivation–induced apoptosis of colon carcinoma (38), the role of DEC1 in TGF-β–induced cell survival has not been elucidated. In the present study, we found that levels of expression of DEC1 in the breast cancer JygMC(A) cells and 4T1 cells were much higher than that in the normal mammary epithelial NMuMG cells and that DEC1 plays a critical role in TGF-β–mediated survival of breast carcinoma cells, strongly suggesting that expression of DEC1 is important for cancer progression.

DEC1 was originally identified as a basic helix-loop-helix transcription factor (39). DEC1 binds to CACGTG E-boxes (24, 32) and represses the transcription of many target genes (30, 40, 41). In the present study, we showed that dnDEC1, which competes with endogenous DEC1 for DNA binding, induces apoptosis of JygMC(A) cells and prevents metastasis, suggesting that TGF-β–induced DEC1 prevents apoptosis of JygMC(A) cells at the transcriptional level. Li et al. (42) reported that the antiapoptotic protein survivin is a target of DEC1 and suggested that survivin is responsible for the survival of cells induced by DEC1. However, our microarray analysis data indicated that survivin is not induced by TGF-β in JygMC(A) cells, and we were unable to identify other well-known apoptosis-related genes. Identification of the transcriptional target(s) of DEC1 responsible for the survival of breast cancer cells is thus required in the future.

DEC1 is involved in the metastasis of breast cancer induced by TGF-β signaling. We have reported previously that systemic gene transfer of Smad7 inhibits the metastasis of JygMC(A) cells through induction of EMT and suppression of cell migration (14). In the present study, we showed that suppression of the DEC1-mediated survival signal caused apoptosis, whereas forced

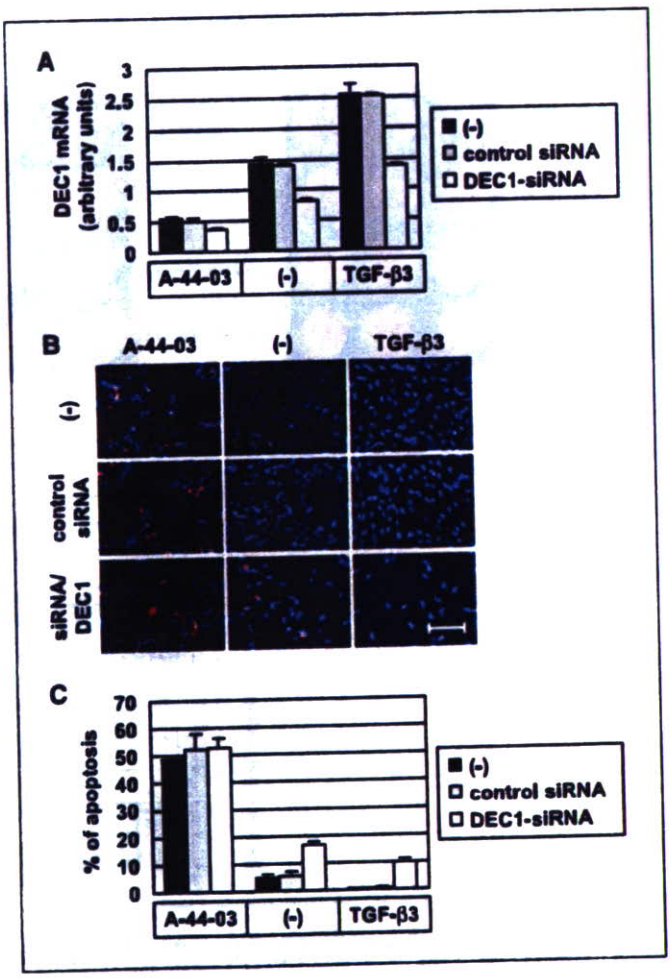
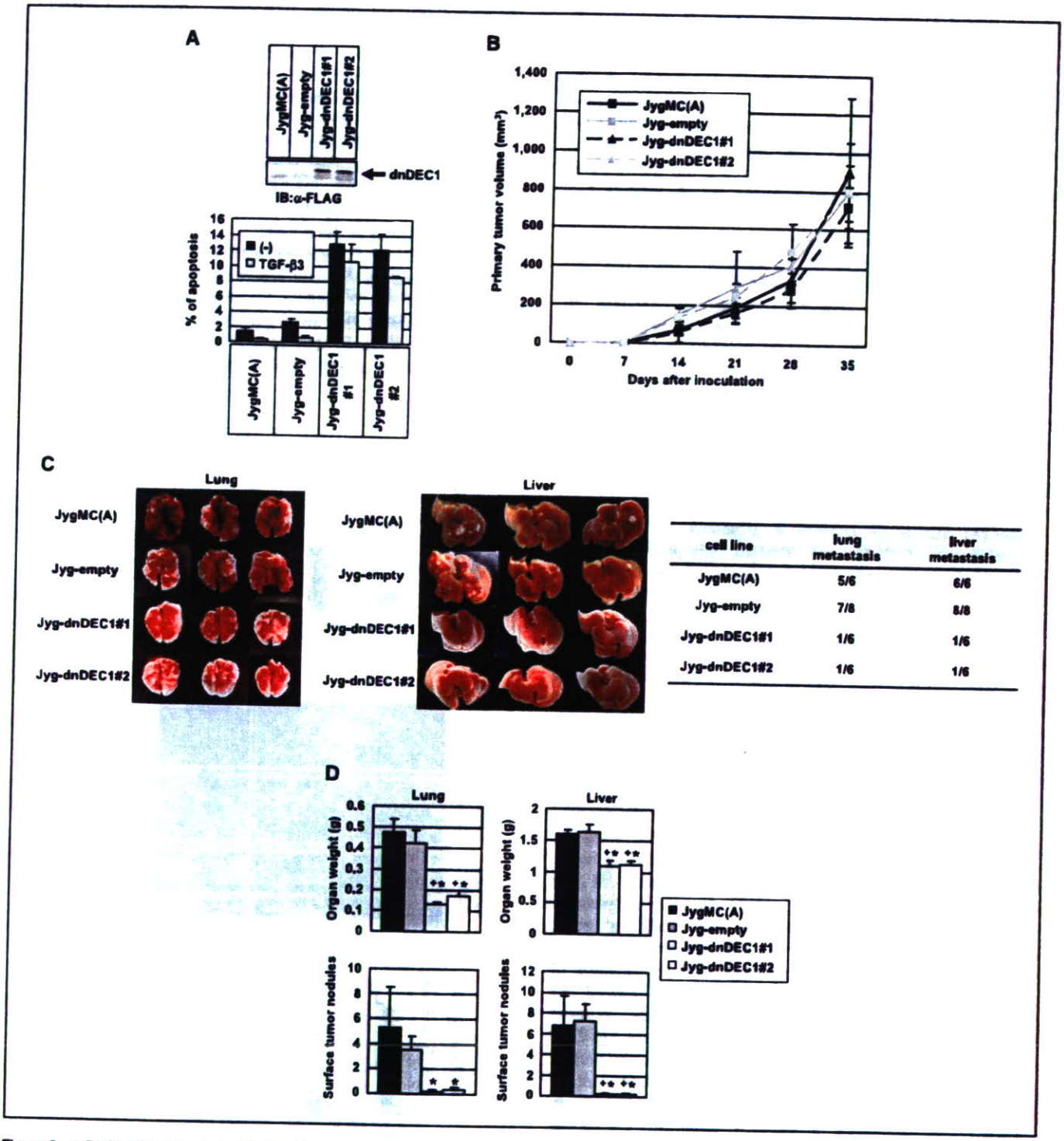


Figure 5. Silencing of endogenous DEC1 abolishes TGF-β–induced survival of JygMC(A) cells. *A*, JygMC(A) cells were transfected with control siRNA or DEC1-targeting siRNA, and levels of expression of DEC1 mRNAs were determined by quantitative real-time RT-PCR analysis. Each value has been normalized to the expression of HPRT1. *Columns*, mean of duplicate determinations; *bars*, SD. *B*, 12 h after transfection, cells were cultured in serum-free medium with TGF-β3 (1 ng/mL) or A-44-03 (1 μmol/L) for 48 h. Cells were subjected to TUNEL staining. *Red*, TUNEL; *blue*, DAPI. *Bar*, 100 μm. *C*, the percentage of TUNEL-positive cells among DAPI-positive cells was determined. *Columns*, mean of triplicate determinations; *bars*, SD.

[6] S. Ehata, unpublished data.



cell line	lung metastasis	liver metastasis
JygMC(A)	5/6	6/6
Jyg-empty	7/8	8/8
Jyg-dnDEC1#1	1/6	1/6
Jyg-dnDEC1#2	1/6	1/6

Figure 6. dnDEC1 expression in JygMC(A) cells reduces tumor metastasis of xenografted mice. *A*, establishment of JygMC(A) cells that stably express the dnDEC1. Parental JygMC(A) cells, empty vector-transfected cells (Jyg-empty), and two stable clones of dnDEC1-expressing cells (Jyg-dnDEC1#1 and Jyg-dnDEC1#2) were examined for dnDEC1 expression by immunoblotting using anti-Flag antibody (*top*). Equal amounts of parental JygMC(A) cells, Jyg-empty cells, Jyg-dnDEC1#1, and Jyg-dnDEC1#2 were cultured in serum-free medium with TGF-β3 (1 ng/mL) for 48 h and subjected to TUNEL staining (*bottom*). The percentage of TUNEL-positive cells among DAPI-positive cells was determined. *Columns*, mean of triplicate determinations; *bars*, SD. *B* to *D*, JygMC(A), Jyg-empty, Jyg-dnDEC1#1, or Jyg-dnDEC1#2 cells were injected into the mammary fat pad of BALB/c *nu/nu* mice. All mice were euthanized on day 35 post-transplantation or earlier if they seemed moribund. *B*, the effects of dnDEC1 expression in JygMC(A) cells on primary tumor growth. The longest axis (*a*) and shortest perpendicular axis (*b*) of the primary tumor were measured every 7 d, and tumor volume was calculated using the formula 0.4 ab^2. *Points*, mean; *bars*, SD. *C*, the effects of dnDEC1 expression in JygMC(A) cells on liver and lung metastasis. Representative photographs showing lungs with mediastinum (*top left*) and livers (*top right*) from mice subjected to injection with the indicated cells. Incidences of metastasis in lung and liver are shown as macroscopic metastases/total mice (*bottom*). *D*, quantitative analysis of the weights of tumor-bearing lung and liver (*top*) and the number of visually observable surface tumor nodules in lung and liver (*bottom*). These experiments were repeated twice with similar results. *Columns*, mean of all sacrificed mice; *bars*, SE. +, $P < 0.05$, statistically significant difference compared with JygMC(A); *, $P < 0.05$, statistically significant difference compared with Jyg-empty.

expression of DEC1 did not affect the migration of cells (data not shown). dnDEC1 thus prevented the metastasis of JygMC(A) cells through loss of resistance to apoptosis and not through reduction of cancer cell motility.

Several molecules have been reported to be involved in the metastasis induced by TGF-β. Blockade of TGF-β signaling by dnTβR-II results in decrease in the secretion of parathyroid hormone–related protein and prevention of bone metastasis (43). Multiple secreted and cell surface proteins, including CTGF and interleukin 11, have been shown to coordinately regulate the osteolytic metastasis of breast cancers (44). In addition to these secreted and cell surface molecules, the homeodomain transcription factor CUTL1/CDP/Cux-1 was shown to be induced by TGF-β and to affect the motility and invasion of breast carcinomas (45). Yang et al. (46) also reported that the transcription factor Twist induces EMT and plays an essential role in the metastasis of breast carcinoma. In our microarray experiment (Supplementary Table S1), however, only CTGF, but not other candidate genes, was significantly regulated by TGF-β signaling. Metastases are induced in multiple steps, and different molecules may be involved in organ-specific metastases (47). Our findings suggest that DEC1 may participate in the process of metastasis of breast cancers through induction of cell survival.

In conclusion, the findings of the present study show that activation of endogenous TGF-β signaling in cancer cells is in certain conditions important for their survival and metastasis. TGF-β mediates survival signals through its downstream target DEC1, suggesting that suppression of DEC1 function may be a novel strategy for treatment of lung and liver metastasis of breast cancer.

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特集 骨の形成と破壊

軟骨/骨/脂肪/他組織での転写因子 DEC1/DEC2の役割

加藤幸夫

DEC1(Differentiated Embryonic Chondrocyte expressed gene-1)はヒト軟骨で発現が亢進する遺伝子として，サブトラクションの変法でクローニングしたbHLHとオレンジドメインをもつ転写因子である[1,2]。一方，オルソローグであるマウスSTRA13，ラットSHARP2も独立して報告されたものの，われわれのDEC1の報告が最も早かった。われわれはさらに，bHLHドメインがほとんど同一であるが他領域の配列が異なるDEC2のクローニングにも成功した[3,4]。SHARP1はDEC2と類似しているが，SHARP1はDEC2の短いminor transcriptあるいはクローニング中のアーティファクトであることがわかった[3]。さらにわれわれは命名委員会と協議して，DEC1をBHLB2，DEC2をBHLH3と番号化した[5]。さらにDEC1遺伝子はHES/Hairy遺伝子らとも構造類似性を示した[5]。DEC1/DEC2はヒストン脱アセチル化酵素であるHDACやSirt1などと結合し，したがって一部の標的遺伝子への転写抑制作用はヒストン脱アセチル化酵素阻害剤(TSA)により減少する。

本総説では，軟骨/骨/脂肪/筋肉でのDEC1/DEC2の作用を中心に紹介し，転写因子としての一般的な特徴についても簡潔に述べる。

1 成長板，静止軟骨での軟骨分化に及ぼすDECの作用

ATDC5軟骨細胞培養系および間葉系幹細胞の軟骨分化系で，DEC1の発現レベルは分化を促進するインスリン，TGF-β，BMP2により上昇して，ATDC5の分化を抑制するPTH/PTHrPにより低下した[1]。またDEC1の強制発現は，アグリカン軟骨基質の合成を促進するのみならず，肥大化(アルカリホスファターゼ，X型コラーゲン)と石灰化をも促進した。しかし静止軟骨細胞培養系では，DEC1はアグリカン合成を促進したものの，肥大化と石灰化を誘導しなかった(Iwataら)。つまり，発生過程の成長軟骨や成長板ではDEC1は内軟骨性骨形成の全体を促進するものの，永久軟骨では基質合成のみを促進して異所性に石灰化を誘起しなかった。

軟骨は本来血管が乏しく，代謝が低酸素に順応できる。また低酸素応答転写因子であるHIF-1αは軟骨細胞の分化，生存に重要な役割を果たしている[6-8]。培養系で低酸素はアグリカン合成を促進したが，肥大化を抑制した[8]。この低酸素の作用の一部はsox9が仲介していると推察されているが[9]，一方でHIF-1αはDEC1/DEC2プロモータ上の低酸素応答エレメント(HRE)に結合して，DEC1/DEC2の発現を誘導する[10]。したがって，低酸素による軟骨分化の促進にもDEC1/DEC2が関与していると考えられる(図1)。ただしインスリン存在下では，低酸素はむしろインスリンの軟骨分化促進作用を抑制した。これは両者の軟骨促進作用がARNTを必要として，ARNTに対して競合的に作用するためでないかと示唆されている[8,11]。

Role of DEC1/DEC2 in cartilage, bone, adipose tissue and other tissues
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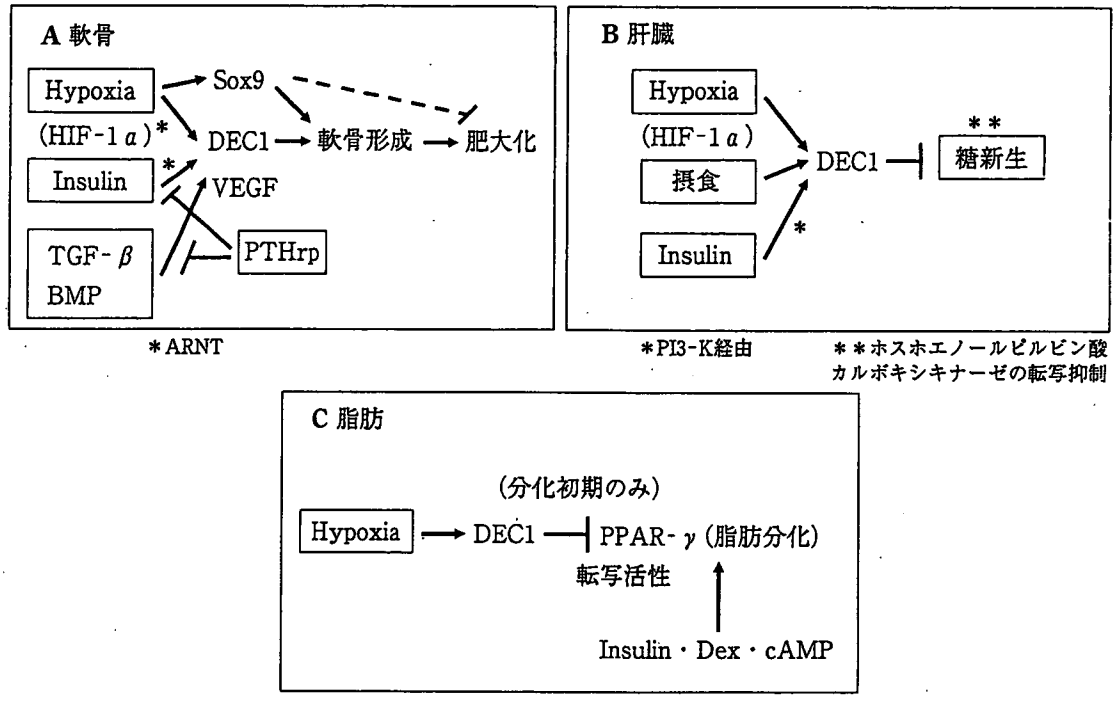

図1 低酸素とインスリンはDEC1を仲介して共通作用を誘導する

マウス[12]，ウサギ，ヒトのほか，ゼブラフィッシュでもDEC1は軟骨/脊索で高レベルに発現しており，脊椎動物の軟骨発生/形成にはDEC1が関与していると推察される。しかしDEC1ノックアウトマウスで骨成長障害が観察されないことから，骨格でのDEC1の作用は，DEC2または他の転写因子と重複している可能性がある。

2 軟骨代謝の概日リズムにおけるDECの役割

われわれは成長板で200以上の遺伝子が概日リズム発現を示すことを明らかにした[13]。これらの遺伝子の多くは，時計エレメントであるCACGTG-E-box，D-box，RREを時計時刻依存性に有している。また，DEC1はこれらの概日リズム遺伝子のCACGTG-E-boxに結合して転写を制御することが，Chipアッセイ系およびレポータアッセイ系などで判明した。つまり，他の時計遺伝子とともにDEC1/DEC2は軟骨細胞の分裂，基質合成，肥大化，石灰化，アポプトーシスの概日リズムに関与していることが明らかになった。

3 脂肪，筋肉分化におけるDECの役割

間葉系幹細胞では，線維芽細胞よりもDEC1/DEC2の発現レベルは5-10倍高い。これは間葉系幹細胞の増殖，老化，分化にDEC1/DEC2が関与していることを示唆している。軟骨，骨分化の過程でDEC1の発現レベルは上昇して，脂肪分化では初期のみ減少[14]，筋肉分化過程では変化しなかった。

軟骨/骨分化誘導培地の存在下で間葉系幹細胞でのDEC1の強制発現は，軟骨分化を顕著に促進するとともに骨分化を助長した。しかし，DEC1の強制発現のみで軟骨，骨分化を誘導しなかった[1,14]。つまりDEC1/DEC2は分化の決定因子ではなく，分化速度の制御因子である。しかし間葉系幹細胞でのDEC1の強制発現は，脂肪分化と筋肉分化を抑制した[14,15]。すなわちDECは脂肪，筋肉分化を抑制することで，軟骨，骨への分化決定を容易にする。

DEC1あるいは低酸素によるPPAR-γ転写活性および脂肪分化の抑制は分化初期のみ観察され，分化後期ではインスリンでDEC1が誘導されると予想されるにもかかわらずDEC1は分化に影響しなかった(図1)。またDEC1の発現レベルは脂肪分化の初期のみ低下した[14]。さらにDEC1

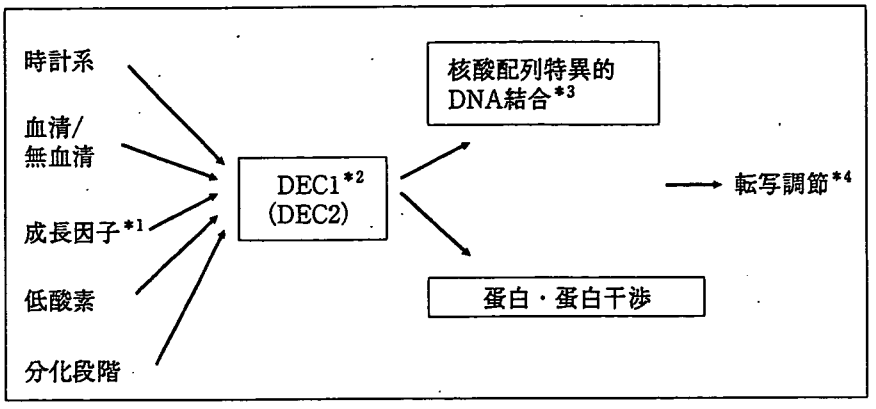

図2　DEC，DEC2の誘導因子と転写調節機構

による脂肪分化の抑制にはHDACへの結合が関与しておらず，DEC1はHAT(ヒストンアセチルトランスフェラーゼ)の補充を抑制するのではないかと推察されている[16]。

一方，DEC1/DEC2はともにMyoDと結合することにより，C2C12細胞でのMyoD/E47のmuscle creatin kinaseプロモータへの結合と活性化および内在性のmuscle creatin kinase mRNAの発現を抑制した[15]。なおこの抑制にもHDACは不必要であった。

4　低酸素応答とDECの作用

低酸素はHIF-1α蛋白を安定化することにより，低酸素応答配列(HRE)を介して，低酸素関連遺伝子(CA9，VEGF，Glut-1など)の発現を促進する。DEC1/DEC2プロモータにもHREが存在して，低酸素により誘導される。しかしヒト各種組織においてDEC1，HIF-1α，VEGF蛋白の発現分布は一致していなかった。がん組織の周囲でもHIF-1α，CA9が壊死組織に隣接する細胞に発現しているのに対して，DEC1は壊死組織から離れた細胞にも発現していた。このように低酸素応答はDEC1発現の要因の一つであるものの，各種組織でのDEC1発現を低酸素応答だけで説明できなかった[17,18]。

表1　Dec1の作用

概日リズム制御	視交差上核，全身の多くの細胞
増殖抑制	NIH3T3，上皮細胞株
分化促進	軟骨，骨，神経
分化抑制	脂肪，筋肉
アポトーシス	マウスT細胞では促進
促進/抑制	(一部はSTAT3との結合，STAT1発現抑制による) (一部はsurvivin発現促進による)
免疫制御	T細胞，B細胞

5　低酸素とインスリン応答

低酸素もインスリンも各組織(軟骨，肝臓など)で初期に類似した遺伝子(Dec1，VEGF，Glut-1など)を誘導する。インスリンはHIF-1αに影響することなくArntと未知の転写因子を介して，HREをもつこれらの低酸素遺伝子群を誘導する。また肝臓で，インスリン，摂食，低酸素はともにDEC1の誘導を介してphosphoenolpyruvate carboxykinaseを抑制して糖新生系を阻止する[19,20]。もっとも最終的にはインスリンと低酸素は反対の作用をする(たとえばインスリンは脂肪分化と脂肪合成の促進，低酸素はそれらの抑制)。つまり，低酸素とインスリンへの共通応答系にのみDEC1が関わっていると推察される。

6　DECの多様な作用とそのメカニズム

表1にDECの主要な作用とその標的細胞を示