

HNF4 $\alpha$ , and PPAR $\alpha$  may also support *Cyp7a* circadian regulation: Multiple regulators thus support the circadian regulation of *Cyp7a*, and intact CLOCK is also necessary for the strong circadian expression of the other sterol-metabolizing enzymes, *Cyp8b* and *Hmgcr*.

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# Transcriptional repression by the basic helix-loop-helix protein Dec2: Multiple mechanisms through E-box elements

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**Abstract.** Dec2, a member of the basic helix-loop-helix (bHLH) superfamily, has been shown to function as a transcriptional repressor and is implicated in cell proliferation and differentiation. In addition, *Dec2* transcripts exhibit a striking circadian oscillation in the suprachiasmatic nucleus. To identify the molecular mechanisms by which Dec2 regulates gene expression, we carried out structure-function analyses. Gel retardation and luciferase assays showed that Dec2, as well as its related protein Dec1, preferentially binds to class B E-box elements (CACGTG) as a homodimer and represses the transcription of target genes in a histone deacetylase (HDAC)-dependent manner. Functional studies with the GAL4-DNA binding domain fusion proteins identified the domain responsible for the repression activity of Dec2 in its C-terminal region, which is also necessary to recruit HDAC1. In addition, the basic and HLH domains of Dec2 were required for DNA binding and homodimerization, respectively. In contrast, Dec proteins repressed a MyoD-activated promoter activity of muscle creatine kinase gene through class A E-box in an HDAC1-independent manner. Dec2 formed a heterodimer with MyoD through the basic and HLH domains. Consistent with this, both the basic and HLH domains were required for the ability of Dec2 to inhibit the transcriptional activity of MyoD. These findings indicate that Dec2 employs multiple mechanisms, including DNA-binding and protein-protein interactions, to achieve E-box-dependent transcriptional repressions.

## Introduction

The basic helix-loop-helix (bHLH) transcription factors make up a large family of proteins that regulate a wide variety of biological processes, including proliferation and differentiation (1). These proteins, forming homodimers or heterodimers, regulate the expression of target genes by binding to the E-box consensus sequence (CANNTG). The basic region has been shown to be necessary for specific DNA binding, whereas the HLH domain mediates dimerization. These transcription factors are functionally classified into two groups, transcriptional activators and transcriptional repressors. Members of the bHLH-Orange (bHLH-O) proteins are known to be repressive transcription factors, and these proteins share a common sequence motif, the Orange domain, located just at the C-terminal to the bHLH domain. Based on their primary structures, the bHLH-O family is subdivided into five classes. The Hairy, Enhancer of Split, Hey (also named HRT, Hesr, CHF, HERP, and Gridlock), Dec (also named Stra13 and Sharp), and Helt subfamilies (2,3). Among the bHLH-O proteins, Hes1 is most strongly characterized. A homodimer of Hes1 binds to the class B E-box (CANGTG), class C E-box (CANGCG), and N-box (CACNAG) and represses the transcription of target genes (2). Hes1 also antagonizes the transcriptional activity of MyoD by direct interaction and inhibits MyoD-induced myogenesis (2).

Dec1 and Dec2 form a subfamily within bHLH-O proteins (4-7). During mouse embryogenesis, these genes are expressed in many tissues, including the brain, heart, liver, and limb bud, suggesting that they play important roles in the development of ectodermal, mesodermal, and endodermal lineages, and overexpression of Dec1 does indeed induce the differentiation of nerve cells and chondrocytes (6,8). Dec1 and Dec2 also appear to function as negative regulators of adipogenesis and myogenesis (9,10), respectively. Furthermore, Dec1-deficient mice exhibit ineffective elimination of activated T and B cells (11). Interestingly, transcripts of *Dec1* and *Dec2* exhibit a striking circadian oscillation in the suprachiasmatic nucleus, suggesting that they function as regulatory proteins for the clockwork system (12). Collectively, these findings demonstrated that Dec family proteins are involved in many biological

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processes. Dec proteins are known to interact with their promoter through the CACGTG E-box, and therefore repress their own transcription (13,14), but the molecular mechanism by which these proteins, particularly Dec2, function has not been examined extensively.

In this study, we found that Dec2 interacts with HDAC1 and SIRT1, and that HDAC1 is involved in the Dec2-mediated direct repression but not in the Dec2-mediated inhibition of MyoD activity. In addition, we performed functional studies on the Dec2 domains.

## Materials and methods

**Plasmids.** The mammalian expression vectors pcDNA-*mDec1* and pcDNA-*mDec2* were described previously (14). The *mDec2* deletion mutants were generated by PCR followed by self-ligation. The following pairs of oligonucleotides were used for PCR: pcDNA-*Dec2Δb*: 5'-GACCG AATTAATGAATGCATT-3' and 5'-CTTGGTATCGTCT CGCTTCAA-3'; pcDNA-*Dec2ΔHLH*: 5'-GCCTTAACTG AGCAGCAGCAT-3' and 5'-TCGTCTCTTCTTTCTAT TAA-3'; pcDNA-*Dec2ΔO*: 5'-CTGACGCCACAGGTGC CCTCC-3' and 5'-ATCCAAGTCGGCCTGGACCGG-3'. pcDNA-*Dec2ΔC(1-265)* was constructed by digestion of pcDNA-*mDec2* with *ApaI*, followed by re-ligation. The expression vectors for *Dec2* that fused with the GAL4 DNA binding domain (DBD) were constructed by subcloning various *Dec2* cDNA fragments into the *Bam*HI and *Xba*I sites of pBIND vector (Promega). For myc-tagged protein, full-length *Dec1* cDNA and various *Dec2* cDNA fragments were subcloned into pcDNA3.1/Myc-His vector (Invitrogen). The flag-tagged *Dec2* construct was generated by subcloning full-length *Dec2* cDNA into the *Bgl*II and *Sal*I sites of pFLAG-CMV vector (Sigma). The bacterial expression vector for *Dec2* that fused with glutathione S-transferase (GST) was constructed by subcloning full-length *Dec2* cDNA into the *Eco*RI and *Hind*III sites of pET-41 vector (Novagen). Other expression constructs were generated by PCR followed by subcloning into pcDNA3.1 or pFLAG-CMV vectors. For muscle creatine kinase (CKM) reporter construct (pCKM-promoter-Luc), a 1.4-kbp 5'-upstream fragment (-1354 to +37, +1 indicates the translation initiation site) of mouse *CKM* gene was amplified by PCR using a forward primer (5'-ACG CGTCAGCTGAGGTGCAAAGGCTCCTGT-3') and a reverse primer (5'-CTCGAGTGAGTGTCTGTCTGTGCTG TGGA-3'), and ligated to the *Mlu*I and *Xho*I sites of pGL3-Basic vector (Promega). For luciferase-reporter constructs containing three copies of the class A E-box, N-box, and class C E-box, the following pairs of oligonucleotides were annealed and cloned upstream of the thymidine kinase (TK) promoter of pTK-Luc vector (14): p3xE-A1-TK-Luc: 5'-CT AGTCCCAACACCTGCTGCCCAACACCTGCTGCCCA AACACCTGCTGCCA-3' and 5'-TCGATGGCAGCAGGTG TTGGGGCAGCAGGTGTTGGGGCAGCAGGTGTTGGG A-3'; p3xN-TK-Luc: 5'-GTTTCACACGAGCCGTTTCGTTT CACACGAGCCGTTTCGTTTTCACACGAGCCGTTTC-3' and 5'-CGCGGAACGGCTCGTGTGAAACGAACGGCTCGT GTGAAACGAACGGCTCGTGTGAAACGTAC-3'; p3xE-C-TK-Luc: 5'-CATCTGCACGCGACATCCCATCTGCAC GCGACATCCCATCTGCACGCGACATCC-3' and 5'-CGC

GGGATGTCGCGTGCAGATGGGATGTCGCGTGCAGA TGGGATGTCGCGTGCAGATGGTAC-3'. p3xE-B2-TK-Luc, which contains three copies of the proximal class B E-box from the mouse *Dec2* promoter, was described previously (14).

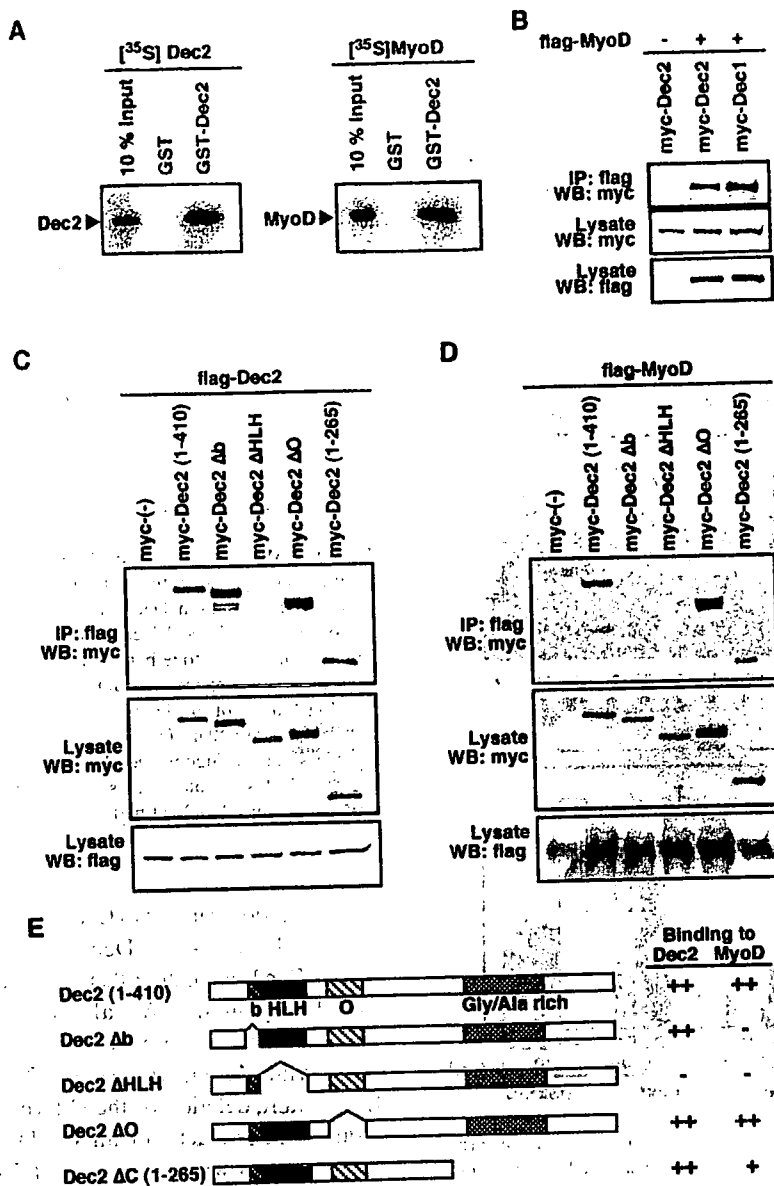
**Luciferase assay.** NIT3T3 and C2C12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (250 ng/ml). Cells were replated on 24-well plates (1x10<sup>4</sup> cells/well), and the next day cells were transfected with the indicated combinations of expression vectors together with 20 ng of a reporter plasmid using PolyFect Transfection Reagent (Qiagen). The pRL-TK vector (2 ng) was cotransfected for normalization, and the total amount of DNA added to cells was adjusted to 0.2 μg/well by addition of pcDNA3.1 vector. Twenty-four hours after transfection, cells were harvested with passive lysis buffer to determine luciferase activity, using Dual-Luciferase reporter assay system (Promega).

**GST pull-down assay.** The BL21 strain of *Escherichia coli* was transformed with plasmid pET-41-*Dec2* for expression of GST-*Dec2* fusion protein or, as a control, pET-41 for expression of GST. GST pull-down assay was performed using purified GST and GST-*Dec2* fusion proteins as described previously (15). [<sup>35</sup>S]methionine-labeled proteins were synthesized *in vitro* using the TNT Quick coupled transcription/translation system (Promega).

**Immunoprecipitation.** COS-7 cells were transfected with expression vectors using PolyFect transfection reagent. After 48 h, cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors (Roche). Lysates were incubated with anti-FLAG M2-Agarose affinity gel (Sigma) for 4 h at 4°C. Immunoprecipitates were washed 3 times with buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and then were eluted in SDS sample buffer. The immunoprecipitated proteins were analyzed by Western blotting.

**Western blot analysis.** After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (Millipore). Immunoblotting was performed with the appropriate antibodies according to standard protocols and detected using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system. The following primary antibodies were used: Anti-myc-alkaline phosphatase (AP)-conjugated antibody (1:2000 dilution, Invitrogen), anti-FLAG M2 monoclonal antibody (1:5000 dilution, Sigma), and rabbit polyclonal antibody anti-*Dec2* (1:2000 dilution). The *Dec2*-specific antibody was generated in rabbit against a synthetic peptide (Cys-Lys-Pro-Lys-Arg-Ser-Met-Lys-Arg-Asp-Asp-Thr-Lys-Asp) corresponding to a region in the N-terminal region of the protein. The antibody was purified by affinity column chromatography. AP-conjugated secondary antibodies were used at a 1:2000 dilution.

**Electrophoretic mobility shift assay (EMSA).** EMSA was performed as described previously (14). For preparation of the probes, oligonucleotides were annealed and then end-labeled



**Figure 1.** Homodimer and heterodimer formations of Dec proteins. (A) *In vitro* interaction of Dec2 with Dec2 and MyoD. GST and GST-Dec2 fusion proteins were purified from *Escherichia coli* and incubated with  $[^{35}\text{S}]\text{methionine}$ -labeled *in vitro* translated Dec2 or MyoD. Bound proteins were visualized by SDS-PAGE and autoradiography. (B) Interaction of MyoD with Dec2 and Dec1 in mammalian cells. COS-7 cells were transfected with flag-tagged MyoD expression vector together with myc-tagged Dec2 or myc-tagged Dec1 expression vector. Cell lysates were immunoprecipitated with anti-flag antibodies and immunoblotted with anti-myc antibodies. To confirm expression of Dec1, Dec2, and MyoD proteins, aliquots of total cell lysates were immunoblotted with the anti-flag or anti-myc antibodies. (C and D) Mapping of the Dec2 domains required for dimerization. COS-7 cells were transfected with the indicated myc-tagged Dec2 expression vectors together with flag-tagged Dec2 (C) or flag-tagged MyoD (D) expression vector. Immunoprecipitation experiments were performed as described in panel B. (E) Schematic representation of full-length Dec2 (1-410) and its deletion mutants. b, basic region; HLH, helix-loop-helix domain; O, orange domain. Numbers indicate the positions of amino acids in the Dec2 protein.

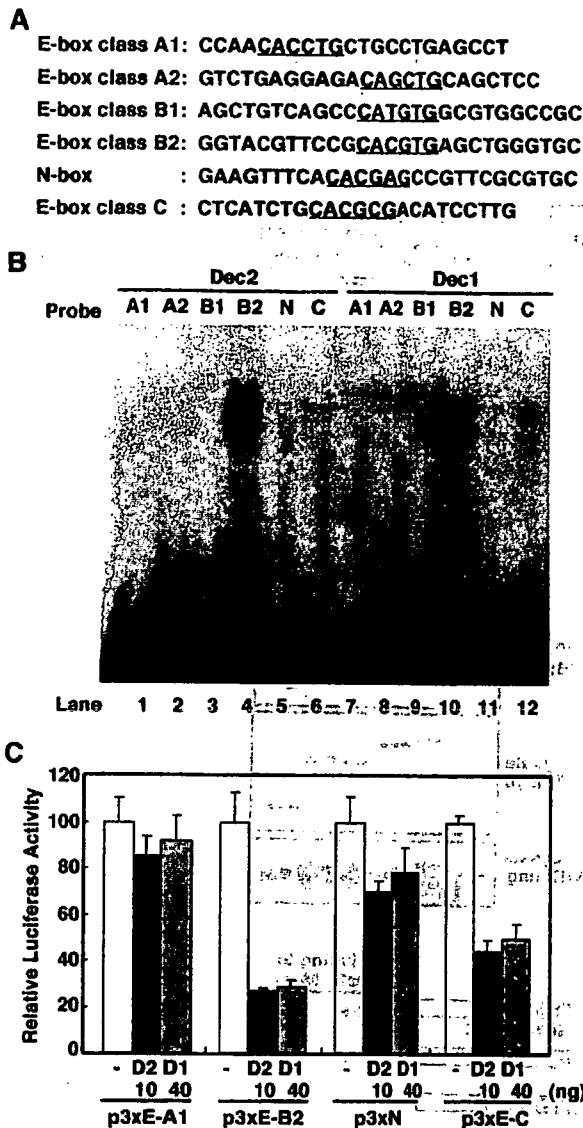
with  $[^{32}\text{P}]\text{dCTP}$  using DNA polymerase I Klenow fragment (Takara).

**Real-time quantitative PCR.** C2C12 cells plated on 6-well plates were transiently transfected as described above with 0.5  $\mu\text{g}$  each of the expression vector pcDNA-MyoD, pcDNA-E47, pcDNA-Dec1, or pcDNA-Dec2 alone or the indicated combination. The total amount of DNA added was adjusted to 1.5  $\mu\text{g}/\text{well}$  by addition of pcDNA3.1 vector. Forty-eight hours after transfection, total RNA was prepared with RNeasy kit (Qiagen) and reverse-transcribed with ReverTra Ace (Toyobo) according to the manufacturer's instructions. Real-time PCR was performed as described previously (16). The

synthesized first-strand cDNA was amplified using specific primers 5'-CATGGCGGCTACAAACCCA-3' and 5'-AGGTCGTCTCCACCCTTGAGGT-3' for mouse CKM. The amplified cDNA was quantified using 6FAM-ACAAGCATAAGACCGACCTCAACCACGAG-TAMRA.

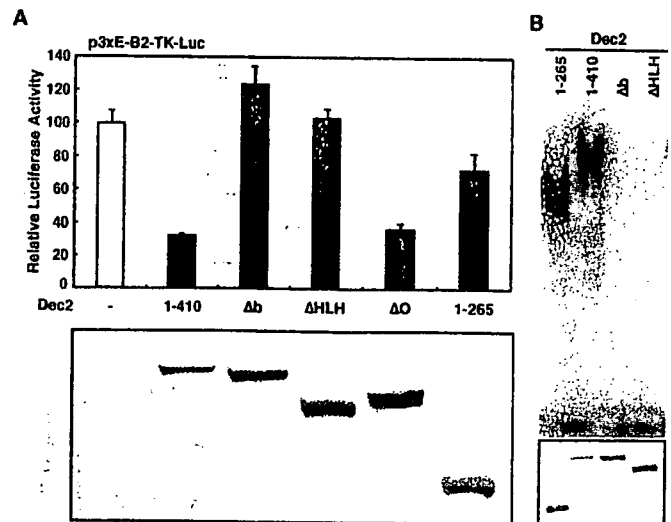
## Results

**Dimerizations of Dec proteins.** We first examined whether Dec2 could form homodimer and heterodimer by GST pull-down assays. Both Dec2 and MyoD bound to GST-Dec2 but not to GST alone, indicating that Dec2 can interact directly with Dec2 and MyoD (Fig. 1A). We further investigated



**Figure 2.** DNA binding and transcriptional repression activity of Dec protein homodimers. (A) Sense-strand sequences of double-stranded oligonucleotides used as probes in EMSA. Each oligonucleotide contains an E-box or N-box element responsive to bHLH transcription factors. The E-box and N-box sequences are underlined. E-boxA1, MyoD binding site from the *CKM* promoter; E-boxA2, MyoD binding site from the *Troponin 1* promoter; E-boxB1, USF1 and USF2 binding site from the *Fatty acid synthase* promoter; E-boxB2, Dec1 and Dec2 binding site from the *Dec2* promoter; N-box, Hes1 binding site from the *Hes1* promoter; E-boxC, Hes1 binding site from the *Acid  $\alpha$ -glucosidase* promoter. (B) These radiolabeled oligonucleotide probes were incubated with *in vitro* translated Dec1 or Dec2 protein and subjected to EMSA. (C) Analysis of transcriptional repression by Dec1 and Dec2. NIH3T3 cells were transfected with the indicated reporter plasmids together with expression vectors for Dec1 (D1) or Dec2 (D2). Twenty-four hours after transfection, cell lysates were prepared and luciferase activity was assayed. The data presented are mean  $\pm$  SD for 4 independent experiments.

the interaction between Dec2 and MyoD in intact cells by co-immunoprecipitation assays. Efficient co-precipitation of Flag-MyoD with Myc-Dec2 was observed, and interaction of Dec1 with MyoD was also observed (Fig. 1B). However, it was not clear which regions of Dec2 were involved in the dimerizations. To address this issue, we generated a series of deletion mutations of Dec2. Deletion of the HLH domain of Dec2 (Dec2 $\Delta$ HLH) resulted in a complete loss of the interaction with full-length Dec2 (Fig. 1C and E). In contrast, all



**Figure 3.** Mapping of functional domains required for Dec2-mediated transcriptional repression through direct DNA-binding. (A) Transcriptional activity of full-length Dec2 and deletion mutants through the CACGTG E-box element. NIH3T3 cells were transfected with the indicated Dec2 expression vectors together with the p3xE-B2-Luc reporter plasmid. The data presented are mean  $\pm$  SD for 4 independent experiments. (B) DNA binding activity of full-length Dec2 and deletion mutants. *In vitro* translated full-length and mutant Dec2 proteins were used in EMSA with radiolabeled double-stranded oligonucleotide E-boxB2. Shown below each lane are the results of Western blot analysis in which the corresponding samples were probed with anti-Dec2 antibody.

other deletion mutants (Dec2 $\Delta$ b, Dec2 $\Delta$ O, and Dec2 $\Delta$ C), as well as the wild-type Dec2, interacted efficiently with full-length Dec2, indicating that Dec2 forms a homodimer through the HLH domain. We also tested the ability of Dec2 to heterodimerize with MyoD (Fig. 1D and E). Dec2 $\Delta$ O interacted with MyoD, whereas Dec2 $\Delta$ b and Dec2 $\Delta$ HLH did not. In addition, deletion of the C-terminal domain of Dec2 partly reduced the interaction with MyoD. These results are consistent with data showing that most bHLH proteins form a homodimer or heterodimer through the HLH domain. In addition, we found that the basic domain in Dec2 was also necessary for the Dec2-MyoD interaction.

**DNA binding specificities of Dec proteins.** We have shown that Dec1 and Dec2 proteins repress their own transcription by binding to CACGTG class B' E-box element (13,14), but bHLH-O proteins are well known to bind to other DNA sequences (2). We thus further examined the specificity of the protein-DNA interaction of Dec proteins. Dec1 and Dec2 proteins bound to the class B2 elements with high affinity (Fig. 2B, lanes 4 and 10), and also bound weakly to the class C elements (Fig. 2B, lanes 6 and 12), while no binding was detected with the A1, A2, B1, and N probes (Fig. 2B, lanes 1-3, 5, 7-9, and 11). To confirm that the Dec binding sites identified by EMSA are correlated with the transcriptional activity, we performed luciferase reporter assays. In transiently transfected NIH3T3 cells, both Dec1 and Dec2 repressed the promoter activity from the reporter containing class B2 elements, and to a lesser extent, class C elements, whereas they did not affect the transcriptional activity of the reporter containing class A1 elements (Fig. 2C). These results were consistent with the DNA binding activity of Dec proteins

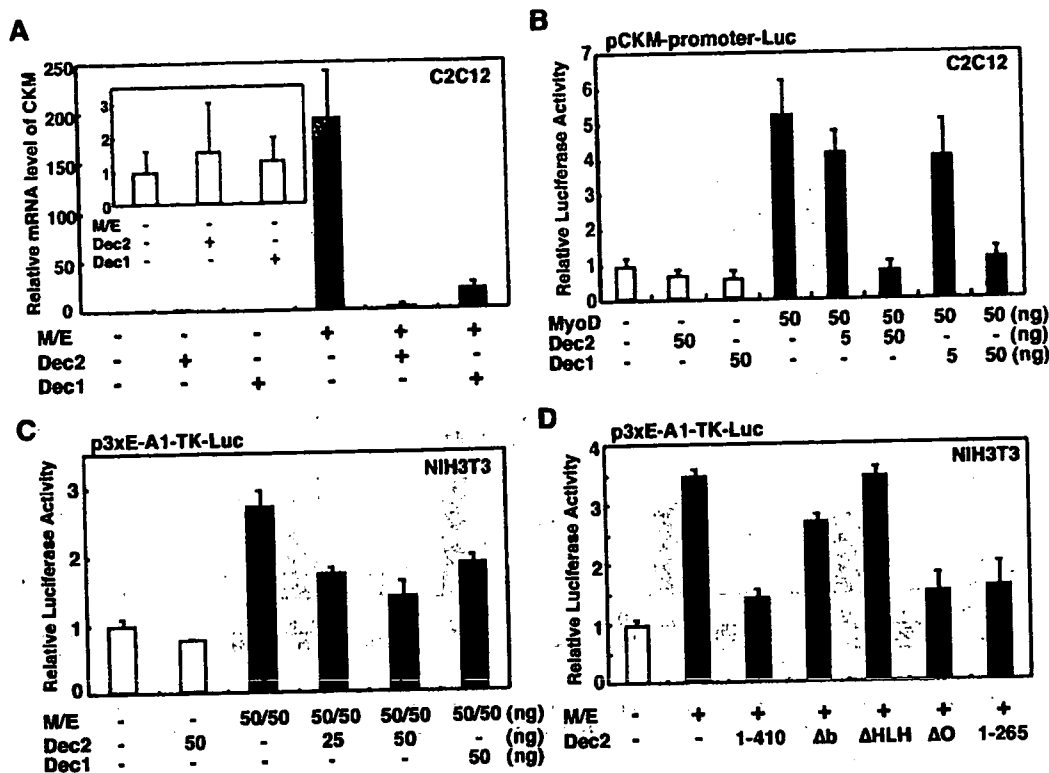


Figure 4. Inhibition of MyoD-mediated transactivation by Dec1 and Dec2. (A) Dec1 and Dec2 negatively regulate MyoD/E47-induced *CKM* mRNA expression. C2C12 cells were transfected with the indicated combinations of expression vectors for *MyoD* (M), *E47* (E), *Dec1*, and *Dec2*. Forty-eight hours after transfection, total RNA was prepared to examine the expression levels of endogenous *CKM* mRNA by real-time PCR analysis. *CKM* expression patterns in the absence of MyoD and E47 are also shown in the inset with an expanded ordinate. (B) Dec1 and Dec2 repress MyoD-induced *CKM* promoter activity. C2C12 cells were transfected with the indicated expression vectors together with the pCKM-promoter-Luc reporter plasmid. (C) Dec1 and Dec2 repress E-box-dependent transactivation activity of MyoD/E47. NIH3T3 cells were transfected with the indicated expression vectors together with the p3xE-A1-Luc reporter plasmid. (D) The bHLH region in Dec2 is required to repress the transactivation of MyoD/E47. NIH3T3 cells were transfected with the indicated combinations of expression vectors for full-length *Dec2*, *Dec2* deletion mutant, *MyoD*, and *E47* together with the p3xE-A1-Luc reporter plasmid. The data presented are mean  $\pm$  SD for 4 independent experiments.

observed in Fig. 2A. Dec1 and Dec2 also repressed promoter activity weakly from the reporter containing N-box element, although no protein-DNA complexes were observed (Fig. 2A). This may be due to the instability of the complex *in vitro* under EMSA conditions.

**Identification of functional domain of Dec2.** To determine the functional domains in Dec2 required for transcriptional repression from CACGTG elements, we examined the effect of a series of deletions on the transcriptional activity of Dec2. Deletion of the Orange domain of Dec2 had little effect on the repression activity, but deletion of the HLH domain completely abolished the repression activity. This mutant did not form a homodimer (Fig. 1C), indicating that homodimer formation is necessary for the repression activity of Dec2 from CACGTG elements. In addition, deletion of the basic domain resulted in the inability of Dec2 to repress transcription, along with an increase in reporter activity over the control levels, suggesting that Dec2 $\Delta$ b acts as a dominant negative mutant to block the endogenous Dec2 function. Since the basic region in bHLH factors has been shown to mediate DNA binding (1), the inability of Dec2 $\Delta$ b mutant to repress the promoter activity may be due to the inactivity of DNA binding. As expected, both Dec2 $\Delta$ b and Dec2 $\Delta$ HLH mutants failed to bind to the E-box sites, suggesting that the basic region and homodimer formation are necessary for the direct DNA binding activity

of Dec2 (Fig. 3B). We also observed that truncation of the C-terminus of Dec2 decreased the repressive activity (Fig. 3A), even though this mutant did bind to the CACGTG E-box elements (Fig. 3B).

**Dec1 and Dec2 repress MyoD-mediated *CKM* promoter activity.** Since Dec1 and Dec2 formed not only a homodimer but also a heterodimer with MyoD (Fig. 1), we examined the effects of Dec proteins on expression of *CKM*, which is a physiological target gene for MyoD during muscle differentiation. Real-time PCR analysis showed that transfection of *MyoD* and *E47* expression vectors induced an ~200-fold increase in *CKM* mRNA expression in C2C12 cells (Fig. 4A). The elevated expression level of *CKM* mRNA was greatly reduced by cotransfection of Dec1 or Dec2, whereas *Dec1* or *Dec2* alone had little effect on *CKM* mRNA expression (Fig. 4A, insert). We also examined the effects of Dec1 and Dec2 expression on the promoter activity of the *CKM* gene by reporter gene assay. MyoD up-regulated transcription of a reporter construct containing 1.3 kb of upstream sequence from the *CKM* gene in C2C12 cells, and this activation was efficiently reduced when *Dec1* or *Dec2* was cotransfected with MyoD (Fig. 4B).

The *CKM* promoter contains a functional E-box element, which is essential for MyoD-induced gene expression in muscle differentiation (17). Therefore, we examined the effects

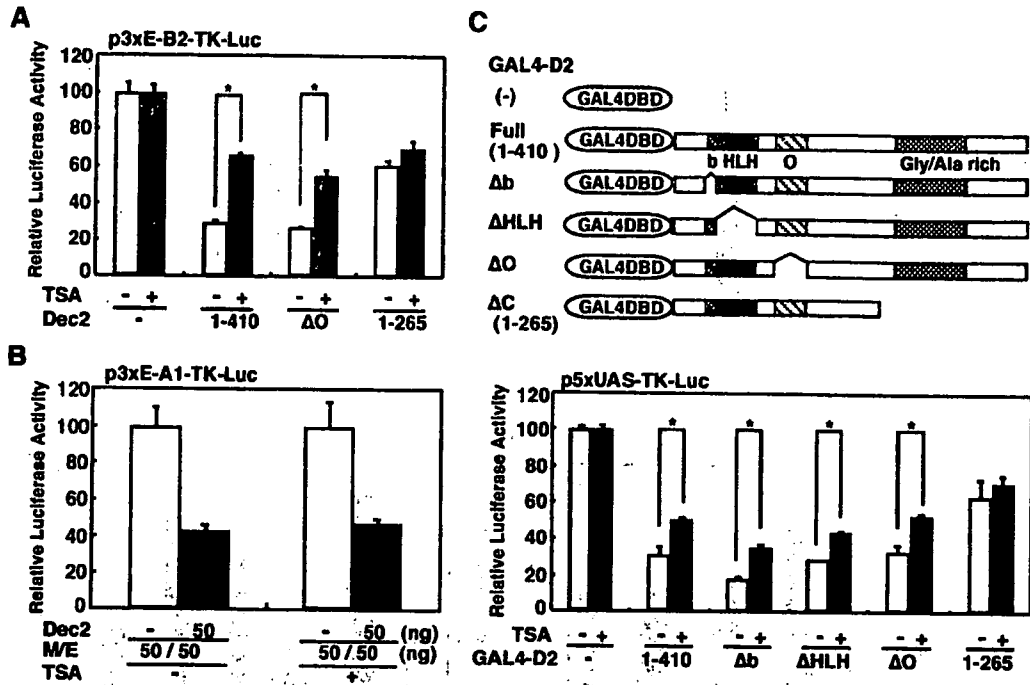


Figure 5. Effects of HDAC inhibitor on Dec2-mediated transcriptional repressions. NIH3T3 cells were transfected with the indicated combinations of expression vectors for *Dec2*, *MyoD* (M), and *E47* (E) together with the p3xE-B2-Luc (A) or p3xE-A1-Luc reporter plasmid (B). TSA (100 nM) was added 3 h after transfection, and then incubation was continued for 21 h. Luciferase activity is expressed as percent in the absence of Dec2. (C) NIH3T3 cells were transfected with the indicated GAL4DBD-Dec2 expression vectors (GAL4-D2) together with the luciferase reporter plasmid containing the 5x GAL4 binding site (p5xUAS-TK-Luc). TSA (100 nM) was added 3 h after transfection, and then incubation was continued for 21 h. Luciferase activity is expressed as percent in the absence of Dec2. The data presented are mean ± SD for 4 independent experiments. \*P<0.02.

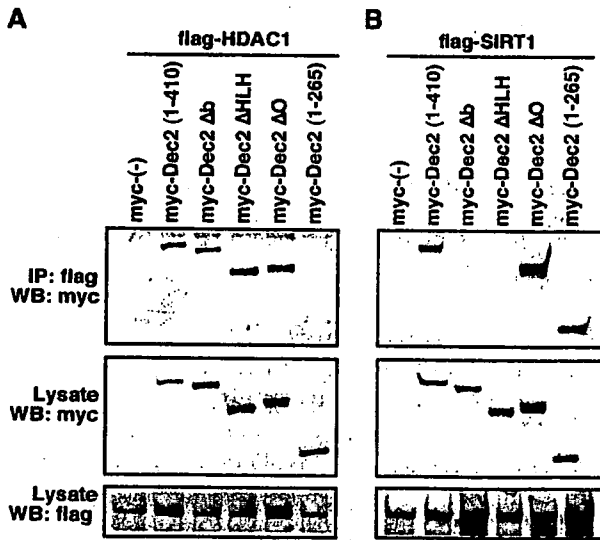


Figure 6. Interaction of Dec2 with HDACs. COS-7 cells were transfected with the indicated myc-tagged *Dec2* expression vectors together with flag-tagged *HDAC1* (A) or flag-tagged *SIRT1* (B) expression vector. Cell lysates were immunoprecipitated with anti-flag antibodies and immunoblotted with anti-myc antibodies. To confirm expression of Dec2, HDAC1, and SIRT1 proteins, aliquots of total cell lysates were immunoblotted with the anti-flag or anti-myc antibodies.

and Dec2 repressed the MyoD-mediated transactivation, although each alone had little effect on the transcriptional activity (Fig. 4C). Deletion of the HLH domain completely abolished the repression activity, and deletion of the basic domain resulted in a 65% reduction in repression activity (Fig. 4D). Taken together with the interaction data (Fig. 1D), this suggests that interaction of the bHLH domain in Dec2 with MyoD is crucial for the ability of Dec2 to repress MyoD-mediated transactivation.

*Effects of trichostatin A (TSA) on Dec2-mediated transcriptional repressions.* Numerous transcriptional repressors associate with histone deacetylase (HDAC) and exert repression by recruitment of HDAC complexes to the appropriate promoter sites. Treatment of NIH3T3 cells with 100 nM TSA, an inhibitor of class I and II HDACs, partially relieved the Dec2-mediated transcriptional repression from CACGTG elements. In addition, transcriptional repression by Dec2ΔO mutant was also inhibited by the TSA treatment, but transcriptional repression by Dec2ΔC mutant was not affected by the TSA treatment (Fig. 5A). Therefore, Dec2-mediated repression of CACGTG E-box containing promoter is probably partially dependent on the activity of TSA-sensitive HDACs, and the C-terminus of Dec2 is required for the HDAC-dependent pathway. However, treatment with TSA had little effect on the ability of Dec2 to repress MyoD-mediated transactivation from CACCTG elements (Fig. 5B). The difference in sensitivity to TSA was not due to a difference in the amount of transfected Dec2 expression construct, because TSA inhibited Dec2-mediated transcriptional repression from CACGTG elements when the same amount of *Dec2* expression

of Dec1 and Dec2 on E-box-dependent transactivation activity of MyoD. Cotransfection of MyoD with E47 activated transcription of the reporter gene p3xE-A1-TK-Luc containing three repeats of the E-box in the *CKM* enhancer. Both Dec1



vector (50 ng) was transfected (data not shown). The findings suggest that Dec2 represses MyoD-mediated transactivation class I and II HDACs-independently.

Using a GAL4 fusion system, we further examined whether binding of Dec2 to a particular promoter would inhibit transcription through the recruitment of HDAC activity (Fig. 5C). As expected, Gal4-Dec2 full-length (1-410) showed a strong transcriptional repression activity, and the repression was partially relieved by TSA treatment. Similarly, several Gal4-fused Dec2 mutants (Gal4-D2Δb, Gal4-D2ΔHLH, Gal4-D2ΔO) also repressed transcription and were sensitive to TSA. In contrast, truncation of the C-terminus reduced Dec2 transcriptional repression activity by 50%, and the repression mediated by Gal4-D2 (1-265) was insensitive to TSA, indicating the presence of a repressor domain within the C-terminal region, which may involve recruitment of HDACs.

**Interaction of Dec2 with HDAC1 and SIRT1.** To confirm the involvement of HDACs in Dec2-mediated transcriptional repression, we investigated the interaction of Dec2 with HDAC1 and SIRT1, which are class I and III HDACs, respectively. Immunoprecipitation analysis revealed that full-length Dec2, Dec2Δb, Dec2ΔHLH, and Dec2ΔO interact with HDAC1, whereas Dec2ΔC did not (Fig. 6A). These results indicate that Dec2 associates with HDAC1 through its C-terminal region *in vivo*. In addition, Dec2 interacted with SIRT1 through its bHLH region (Fig. 6B).

## Discussion

The present study demonstrated that Dec1 and Dec2 function as transcriptional repressors through two different mechanisms. i) Homodimers of Dec proteins bind directly to specific DNA sites and repress transcription of target genes. The repression is partly mediated by the recruitment of corepressor components including HDAC1. ii) Dec can form heterodimers with MyoD and inhibit MyoD-dependent transactivation, probably by preventing MyoD/E-protein from binding to DNA. TSA had no effect on the repression activity of Dec2; therefore, it is likely that HDAC1 is not involved in the ability of Dec2 to repress MyoD-mediated transactivation. Deletion analysis showed that both the basic and HLH domains in Dec2 are essential for the two repression mechanisms, and our results also revealed that Dec1 and Dec2 have similar DNA binding specificities and transcriptional repression activities, which suggests some functional redundancy between these family members.

Previous studies with bHLH-O proteins demonstrated that each subfamily possesses intrinsic transcriptional repressor domains and employs a different mechanism to repress transcription. Hes1 represses transcription by recruiting a TLE/Groucho corepressor through the WRPW motif in the C-terminus (2), which is conserved among the Hes family. Hey1 and Hey2, members of the Hey family, share similar motifs, YRPW and YQPW, respectively, in the C-terminus. However, a recent study demonstrated that these motifs are not essential for their transcriptional repression (18). The repression activity of Hey1 is mediated by the bHLH region, which is involved in the recruitment of the complex containing mSin3A, N-CoR, and HDAC1. On the other hand, Dec and Helt family members

lack the WRPW motif, and both the Orange domain and the C-terminal region in Helt are required for their repression activity (3). The Orange domain of Hes1 is also required for its repressor activity. However, our mutagenesis studies showed that the C-terminal region but not the Orange domain of Dec2 is essential for its repression activity. Thus, the mechanism of Dec-mediated active transcriptional repression differs from those of other bHLH-O family members.

MyoD activity is negatively regulated by several transcriptional regulators, including Id, MyoR, Mist1 and Dermo-1, through protein-protein interactions (19). Many of these proteins form inactive heterodimers with MyoD and/or E-protein and inhibit their function. Recently, Dec2 has been reported to inhibit MyoD-induced muscle differentiation. Dec2 can associate with MyoD and attenuate the ability of the MyoD/E47 heterodimer to bind DNA (10). Our present study extended these findings, and showed that both the basic and HLH domains in Dec2 are required for interaction with MyoD and inhibition of MyoD transcriptional activity. We also showed that similar to Dec2, Dec1 represses the transcriptional activity of MyoD. Furthermore, we found that the bHLH in Dec2 were also required for interaction with Sirt1. It has been reported that SIRT1 deacetylates MyoD and represses its activity. Overexpression of SIRT1 inhibited muscle gene expression and differentiation of myoblasts into multinucleated myocytes, and its deacetylase activity is essential for this inhibition (20). SIRT1 may be involved in Dec2-mediated repression for MyoD activity.

In conclusion, Dec1 and Dec2 negatively regulated transcriptional activity not only by direct binding to target genes but also by inhibiting MyoD activity. Dec1 also interacts with other bHLH proteins, such as USF and Bmal1, and inhibits their activities (15,21). Thus, Dec proteins form multiple dimer combinations with other bHLH proteins and mediate various gene expressions by modulating their transcriptional activities.

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## Original Research Report

# Comprehensive Analysis of Chemotactic Factors for Bone Marrow Mesenchymal Stem Cells

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### ABSTRACT

To understand which growth factors/cytokines can affect migration of mesenchymal stem cells (MSCs) to injured tissues, we compared the effects of many (26) growth factors/cytokines on the migration activity of rabbit and human MSCs using a microchemotaxis chamber. Among them, platelet-derived growth factor (PDGF)-BB, PDGF-AB, epidermal growth factor (EGF), HB-EGF, transforming growth factor (TGF- $\alpha$ ), insulin growth factor (IGF-I), hepatocyte growth factor (HGF), fibroblast growth factor (FGF-2), and thrombin consistently enhanced the migration of rabbit and human MSCs at appropriate concentrations. PDGF-BB showed the greatest effect on migration. Various combinations of these factors further enhanced the migration of MSCs, whereas combinations of factors that shared common cell-surface receptors did not induce the additive stimulation. On the other hand, some combinations, including that of FGF-2 or thrombin with PDGF-BB, suppressed the migration activity of MSCs. These findings suggest that combinations of growth factors are important to eliciting the maximal chemotactic effect. The factors that induced the migration of MSCs also enhanced their proliferation, suggesting that migration and proliferation can take place simultaneously. The above factors were also effective in stimulating the migration of fibroblasts, but thrombin alone selectively enhanced the migration of MSCs, suggesting that thrombin is useful to stimulate migration of MSCs without migration of fibroblasts.

### INTRODUCTION

MESENCHYMAL STEM CELLS (MSCs) possess multilineage developmental potential and can differentiate into osteoblasts, chondrocytes, and adipocytes *in vitro* and/or *in vivo* (1–3). MSCs have some therapeutic benefits in comparison with bone or cartilage cells regarding their use in clinical settings, because MSCs can easily be isolated by bone marrow aspiration with minimal pain.

Various routes of administration of MSCs to treat traumatic brain injury (4,5), cerebral ischemia (6,7), bone fracture (8), osteogenesis imperfecta (9,10), and infarcted myocardium (11) have been reported. Intravenously in-

jected MSCs were detected primarily in the lungs and then in the liver and other organs (12). In a previous study, primary MSCs showed highly efficient homing to bone marrow as compared with MSCs cultured for a long time (13), whereas some accumulated in injured tissues (14). On the other hand, locally injected MSCs dispersed in marrow spaces 6 mm away from the original cartilage defect (15). Such migration of MSCs from the implanted site could delay tissue repair. However, factors that may attract or mobilize MSCs have not been fully understood. Wang et al. have shown that monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1), interleukin-8 (IL-8), and ischemic cerebral tis-

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TABLE 1. ABBREVIATIONS AND CONCENTRATIONS OF FACTORS EXAMINED IN THIS MIGRATION ASSAY

Factors		Concentrations (ng/ml)
PDGF-AA	Platelet derived growth factor-AA	0.1–100
PDGF-AB	Platelet derived growth factor-AB	0.1–100
PDGF-BB	Platelet derived growth factor-BB	0.01–50
HB-EGF	Heparin binding epidermal growth factor like growth factor	0.01–50
EGF	Epidermal growth factor	0.01–50
TGF- $\alpha$	Transforming growth factor- $\alpha$	0.01–50
FGF-2	Fibroblast growth factor-2	0.01–50
IGF-I	Insulin-like growth factor-I	0.1–300
HGF	Hepatocyte growth factor	0.1–100
TGF- $\beta$ 1	Transforming growth factor- $\beta$ 1	0.01–100
TGF- $\beta$ 3	Transforming growth factor- $\beta$ 3	0.01–100
VEGF	Vascular endothelial growth factor	0.1–100
BMP-2	Bone morphogenetic protein-2	0.1–100
SDF-1 $\alpha$	Stromal cell derived factor-1 $\alpha$	0.01–100
SCF	Stem cell factor	0.01–100
SCGF- $\alpha$	Stem cell growth factor- $\alpha$	0.01–100
LIF	Leukemia inhibitory factor	0.1–200
BDNF	Brain-derived neurotrophic factor	0.1–100
NGF- $\beta$	Nerve growth factor- $\beta$	0.1–100
NT-3	Neurotrophin-3	0.1–100
IL-2	Interleukin-2	0.01–500
IL-8	Interleukin-8	0.01–200
MCP-1	Monocyte chemoattractant protein-1	0.1–100
ANP	Atrial natriuretic peptide	0.03–300
Leptin	Leptin	0.001–50
Thrombin	$\alpha$ -Thrombin	0.01–25 (U/ml)

sue enhance MSC migration (6), whereas there have been no comprehensive analyses of chemotactic factors for MSCs.

## MATERIALS AND METHODS

### Materials

Various factors and their abbreviations, along with the concentrations used, are shown in Table 1. Platelet-derived growth factor-AB and -BB (PDGF-AB, PDGF-BB), transforming growth factor- $\alpha$  (TGF- $\alpha$ ), and vascular endothelial growth factor (VEGF) were purchased from R&D System, Inc. (Minneapolis, MN). Insulin-like growth factor-I (IGF-I) was purchased from BD Biosciences (Bedford, MA). IL-8, MCP-1, and leptin were purchased from DIACLONE Research (Cedex, France). PDGF-AA, epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ),

stem cell factor (SCF), stem cell growth factor- $\alpha$  (SCGF- $\alpha$ ), and interleukin-2 (IL-2) were purchased from Pepro-Tech EC Ltd (London, UK). Heparin-binding epidermal growth factor-like growth factor (HB-EGF), transforming growth factor- $\beta$ 3 (TGF- $\beta$ 3), leukemia inhibitory factor (LIF), brain-derived neurotrophic factor (BDNF), nerve growth factor- $\beta$  (NGF- $\beta$ ), neurotrophin-3 (NT-3), and thrombin, were purchased from SIGMA. Atrial natriuretic peptide (ANP) was purchased from Biogenesis Ltd (Poole, UK). Bone morphogenetic protein (BMP-2) was obtained from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Fibroblast growth factor-2 (FGF-2) was obtained from Kaken Seiyaku (Tokyo, Japan).

### Cells

Human MSCs were obtained from Cambrex Bio Science (Walkersville, MD). These cells were identified to be CD105<sup>+</sup>, CD166<sup>+</sup>, CD29<sup>+</sup>, and CD44<sup>+</sup> but CD14<sup>-</sup>, CD34<sup>-</sup>, and CD45<sup>-</sup> by flow cytometric analysis. Their osteogenic, chondrogenic, and adipogenic potential was

## BONE MARROW MSCs

confirmed after incubation in the appropriate medium. MSCs obtained from cultures at the 2nd to 4th passages were used for the experiments.

Rabbit MSCs were isolated as previously reported (16). Briefly, cells flushed from rabbit femur were seeded into each tissue culture flask in Dulbecco's-modified Eagle medium (DMEM) containing antibiotic-antimycotic (100 unit/ml penicillin G, 100  $\mu\text{g/ml}$  streptomycin, and 0.25  $\mu\text{g/ml}$  amphotericin B; GIBCO) (medium A), and the medium was supplemented with 10% fetal bovine serum (FBS) (medium B). Three days after seeding, floating cells were removed and the medium was replaced by fresh medium B. Thereafter, adhered cells (MSCs) were fed every other day with fresh medium B supplemented with 3 ng/ml FGF-2 at 37°C in 5% CO<sub>2</sub>/95% air. Passages were performed when cells were approaching confluence. Adherent cells were collected with trypsin/EDTA, resuspended in fresh medium B, and transferred to new flasks at a density of  $5 \times 10^3$  cells/cm<sup>2</sup>. The rabbit MSCs obtained from cultures at the 2nd to 4th passages were used for the experiments. These MSCs incubated with the appropriate medium differentiated into chondrocytes, osteoblasts, or adipocytes (data not shown), as shown previously (16).

Human fibroblasts were prepared from explants of normal human gingival tissues from extraction of impacted wisdom teeth were approved by ethical authorities at Hiroshima University. The explants were cut into pieces and cultured in medium B in tissue culture flasks. The medium was changed every 3 days until confluent cell monolayers were formed. Passages were performed when fibroblasts were approaching confluence and cultures at the 6th or 7th passage were used for experiments (17).

### *Chemotaxis and chemokinesis assay*

The chemotactic activities of 26 factors toward MSCs or fibroblasts were evaluated using a 96-well microchemotaxis Boyden chamber, as described previously with some modifications (18). MSCs were resuspended in medium A at  $1.0 \times 10^6$  cells/ml. The cells ( $5.0 \times 10^4/50 \mu\text{l}$ ) were added to the upper wells, and test samples in 25  $\mu\text{l}$  of medium A were added to the lower wells. The contents of the upper and lower wells were separated by a polycarbonate filter (8- $\mu\text{m}$  pore size; Neuro Probe Inc., Gaithersburg, MD). In pilot studies, we examined the effects of various matrix macromolecules coated on the filter, and found that fibronectin or type I collagen is an appropriate adhesion molecule to support adhesion and migration of MSCs. Therefore, both surfaces of the filter were coated with 0.01% type I collagen in distilled water (pH 3.0) at 4°C overnight. Cells were incubated in the chamber for 6 h at 37°C in 5% CO<sub>2</sub>/95% air. After incubation, the migrated cells on the lower side of the filter were fixed and stained with Diff-Quik (International

Reagents, Kobe, Japan). Cells on the upper side of the filter were mechanically removed using soft paper. Stained cell numbers on the lower side were counted using a microscope at five random fields (0.0675 mm<sup>2</sup> each), and the total number of migrated cells per well was calculated. In addition, we directly read the absorbance at 605 nm through the stained filter in a 96-well microplate reader without cell lysis.

The chemotactic index (CI) was determined as previously described with some modifications (19). Briefly, absorbance of the filter in the tested wells was divided by the absorbance of control wells, and the results were normalized by expressing them as a CI. To distinguish between concentration-dependent cell migration (chemotaxis) and random migration (chemokinesis), the chemokinesis index was also determined by adding the same concentration of factors/cytokines to both the upper and lower wells, and the absorbance of filter in the tested wells was divided by the absorbance of control wells.

Anti-human PDGF-BB polyclonal neutralizing antibodies (AB-220-NA; R&D System, Inc.) were used to inhibit the migration activity of MSCs. PDGDF-BB (20 ng/ml) and various concentrations (0.1–10  $\mu\text{g/ml}$ ) of PDGF-BB antibodies were added to the lower wells.

For each experiment, 5–9 replicates were employed.

### *Wound migration assay*

The scrape wound migration assay was performed as described previously (20). Briefly, rabbit MSCs were plated in 3.5-cm culture dishes and grown to confluence in medium B. Media were changed with medium A containing 1% fetal bovine serum (FBS), and six wounds per well were made by scraping the cell layers with a plastic pipette tip. Subsequently, media were replaced by fresh medium A, to which various growth factors/cytokines were added. For each experiment, the distances of the cell-free area at six marked sites per well were measured in triplicate wells using inverse phase-contrast microscopy. Wound closure was quantified as a percentage of the starting distance between the wound edges after 24 h. Data are presented as the mean  $\pm$  standard deviation.

### *DNA synthesis*

DNA synthesis was determined by measuring the incorporation of [<sup>3</sup>H]thymidine into 5% trichloroacetic acid-insoluble cell precipitate (21). Rabbit MSCs were seeded at a density of  $3 \times 10^3$  cells/well in 96-well culture plates and incubated with medium B until the cultures became confluent. The cells were preincubated in medium A containing 1% FBS for 10 h, and then incubated in 0.1 ml of medium A (serum-free) supplemented

with various testing factors for 18 h. Ten microliters of medium A supplemented with 50  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]thymidine was added 6 h before the end of incubation. For each experiment, 5–9 replicates were employed.

### Statistical analysis

One-way analysis of variance (ANOVA) was used for comparison of chemotaxis and chemokinesis, and the Student's *t*-test was used in other experiments.

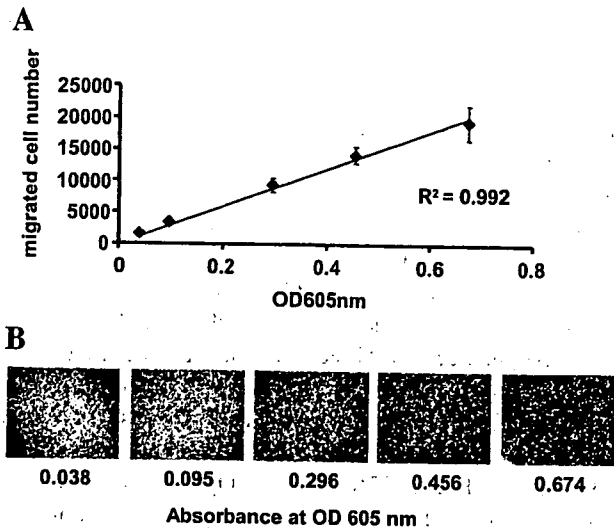
## RESULTS

### Correlation between cell number and the absorbance of stained cells on the filter

First, we seeded various numbers of rabbit MSCs in a 96-well chemotaxis upper chamber and incubated these cells for 6 h in the absence of chemotactic factors. Even in the absence of the factors, some MSCs spontaneously migrated to the lower chamber. The migrated cells on the lower side of the filter were fixed and stained with Diff-Quik. Cells on the upper side of the filter were mechanically removed using soft paper. Thereafter, we counted the migrated cells on the lower side of the filter using a microscope and calculated the total number of migrated cells per well. Next, we directly read the absorbance at 605 nm of the same stained filter that was placed in a 96-well microplate reader without cell lysis. We were able to get a good correlation curve between the absorbance and the migrated cell number on the lower side, so we measured the absorbance of the filter in the following experiments (Fig. 1).

### Effects of various factors on migration of rabbit MSCs

We examined the effects of 26 growth factors/cytokines on the migration of rabbit MSCs (Table 1). Most of those peptides have been reported to stimulate migration of various cells: Some were selected because DNA microarray analysis showed enhanced expression of receptors for thrombin, ANP, and leptin in MSCs as compared with fibroblasts (data not shown) (22). Of the 26 peptides, PDGF-BB, HB-EGF, PDGF-AB, TGF- $\alpha$ , EGF, FGF-2, IGF-I, HGF, and thrombin significantly enhanced migration of rabbit MSC at appropriate concentrations (Fig. 2). The concentration–response curves were bell-shaped, except for PDGF-AB. The other factors had little effect on migration at any concentration, although MCP-1, MIP-1, and IL-8 have been reported to stimulate MSC migration (6). To be sure that the effects of these agents are direct via receptor binding, we examined the effects of neutralizing PDGF-BB antibodies on the mi-



**FIG. 1.** Various numbers of rabbit MSCs were seeded and incubated for 6 h in a 96-well chemotaxis upper chamber. (A) A correlation curve between cell number and absorbance on the lower side of the membrane filter. A good correlation curve between the absorbance and the migrated cell number on the lower side was observed ( $R^2 = 0.992$ ), when cells on the upper side were removed. (B) Photographs of migrated cells on the lower side of the filter.

gration of MSCs. Rabbit MSC migration stimulated by PDGF-BB (20 ng/ml) was completely inhibited by the neutralizing antibodies at  $> 2 \mu\text{g/ml}$  (Fig. 3).

### Comparison of chemokinetic and chemotactic effects

Random cell motility (chemokinesis) and concentration-dependent cell migration (chemotaxis) of MSCs in response to nine factors (PDGF-BB, HB-EGF, PDGF-AB, TGF- $\alpha$ , EGF, FGF-2, IGF-I, HGF, and thrombin) were compared (Fig. 4). All of these factors stimulated both chemokinesis and chemotaxis indexes. However, the chemotaxis indexes were always higher than the chemokinesis indexes. Thus, these factors are essential chemoattractants for MSCs.

### Effects of combinations of growth factors/cytokines on migration

Chemotactic effects of PDGF-AB, HB-EGF, TGF- $\alpha$ , EGF, thrombin, FGF-2, IGF-I, and HGF in the presence of PDGF-BB are shown in Fig. 5A. HB-EGF, TGF- $\alpha$ , EGF, IGF-I, and HGF showed additive effects with PDGF-BB, whereas PDGF-AB, thrombin, and FGF-2 decreased the effects of PDGF-BB. In addition, various combinations of the two growth factors/cytokines showed additive chemotactic effects (Fig. 5B–I). However, TGF- $\alpha$ , EGF, and FGF-2 decreased the chemotactic effects of HB-EGF; HB-EGF, EGF, and FGF-2 decreased these ef-

BONE MARROW MSCs

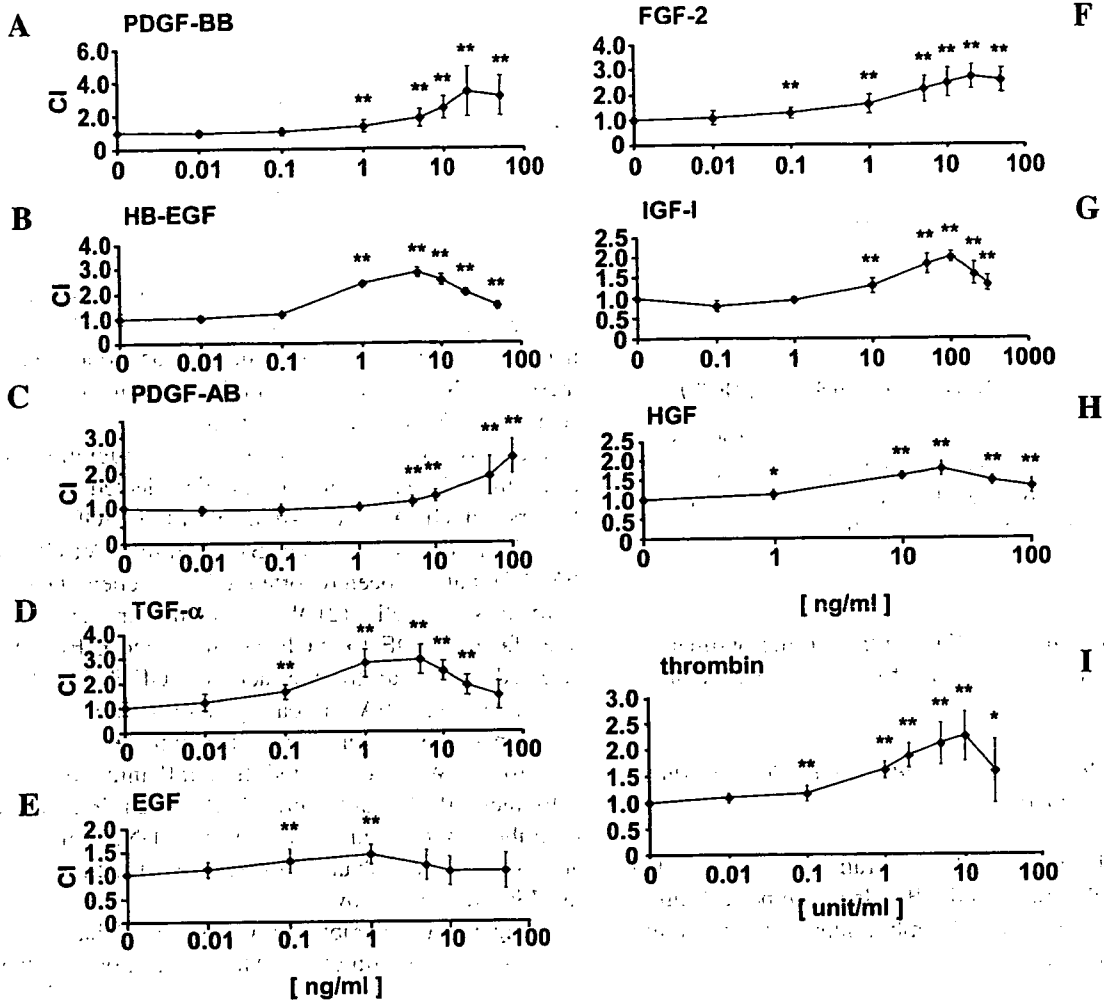


FIG. 2. Effects of increasing concentrations of PDGF-BB, (A) HB-EGF (B), PDGF-AB (C), TGF- $\alpha$  (D), EGF (E), FGF-2 (F), IGF-I (G), HGF (H), and thrombin (I) on migration of rabbit MSCs. Data are represented as mean  $\pm$  SD. (\* $p$  < 0.05, \*\* $p$  < 0.01).

ffects of TGF- $\alpha$ ; TGF- $\alpha$ , HB-EGF and FGF-2 decreased the chemotactic effects of EGF (Fig. 5B,D,E). Furthermore, FGF-2 decreased the chemotactic effects of thrombin, and IGF-I (Fig. 5F,H). These findings indicate that combination of chemotactic factors is critical for the migration of MSCs.

*Chemotactic activity in the wound migration assay*

The chemotactic activities of nine factors were also examined by the wound migration assay. PDGF-BB, HB-EGF, PDGF-AB, and thrombin significantly enhanced migration of MSCs in this assay, whereas the other five factors did not significantly enhance this migration (Fig. 6). Because the wound migration assay does not include the initial stage for cell attachment and a concentration gradient of tested factors, the chemotactic activity may not be fully assessed in this assay. Alternatively, cells in

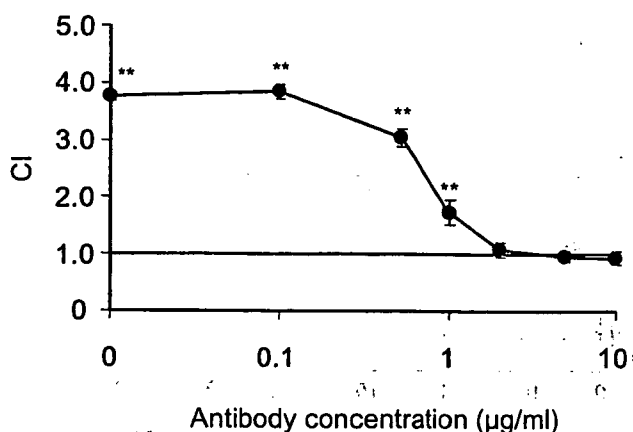
the wound migration assay may have lower sensitivity to chemotactic factors.

*Effects of chemotactic factors on [<sup>3</sup>H]thymidine incorporation*

Of the 26 factors, all nine factors that had chemotactic activity enhanced DNA synthesis in serum-free cultures of rabbit MSCs, whereas the other factors had little effect on DNA synthesis (Table 2 and data not shown).

*Effects of growth factors/cytokines on migration of human MSCs*

Because human and rabbit MSCs may have different responsiveness to chemotactic factors, the Boyden chamber cell migration assay was performed with human MSCs isolated from two different donors. Similar enhancements of migration in response to these factors were



**FIG. 3.** Inhibition of MSC migration by anti-PDGF-BB antibodies. PDGF-BB neutralizing antibodies (0.1–10 µg/ml) and 20 ng/ml PDGF-BB were added to the lower wells. Data are represented as mean ± SD (\*\**p* < 0.01).

observed with human MSCs, although the optimal concentrations were somewhat different between rabbit and human MSCs (Table 3).

*Effects of the identified chemotactic factors on fibroblast migration*

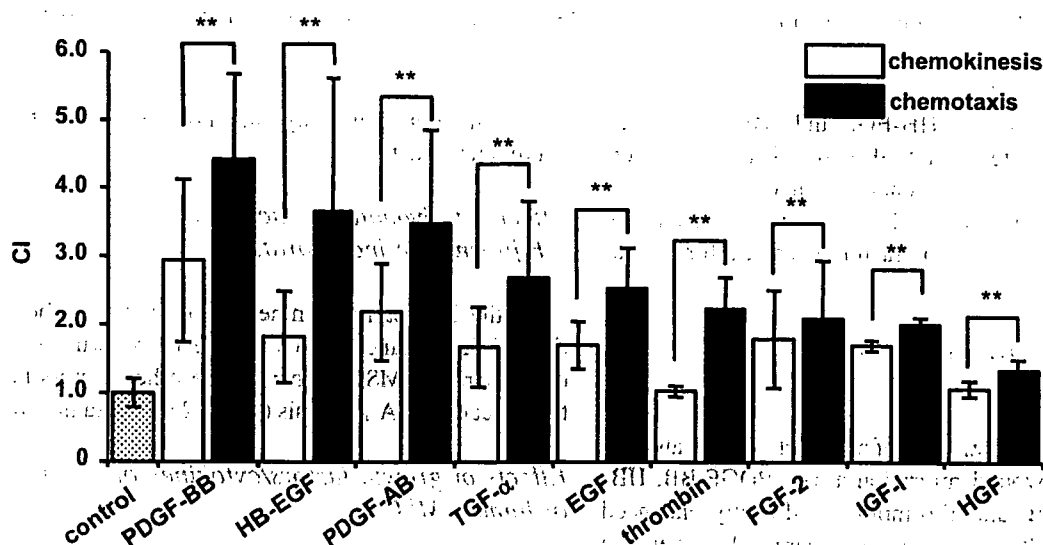
Factors that induced MSC migration also enhanced migration of fibroblasts in the Boyden chamber cell migration assay, except that thrombin suppressed the migration of fibroblasts (Fig. 7).

**DISCUSSION**

The migration assay requires large numbers of MSCs, so it was difficult to test many factors in MSC cultures simultaneously. In addition, it was difficult to distinguish MSCs from fibroblasts because of their similar appearance. However, we used MSCs that were not contaminated by fibroblasts (22). MSCs maintained in culture for a long period may lose their multilineage differentiation potential, but MSCs expanded *ex vivo* in the presence of FGF-2 retained their differentiation potential throughout many mitotic divisions (16). Thus, the data obtained with these MSCs should be useful in bettering our understanding of MSC migration activity.

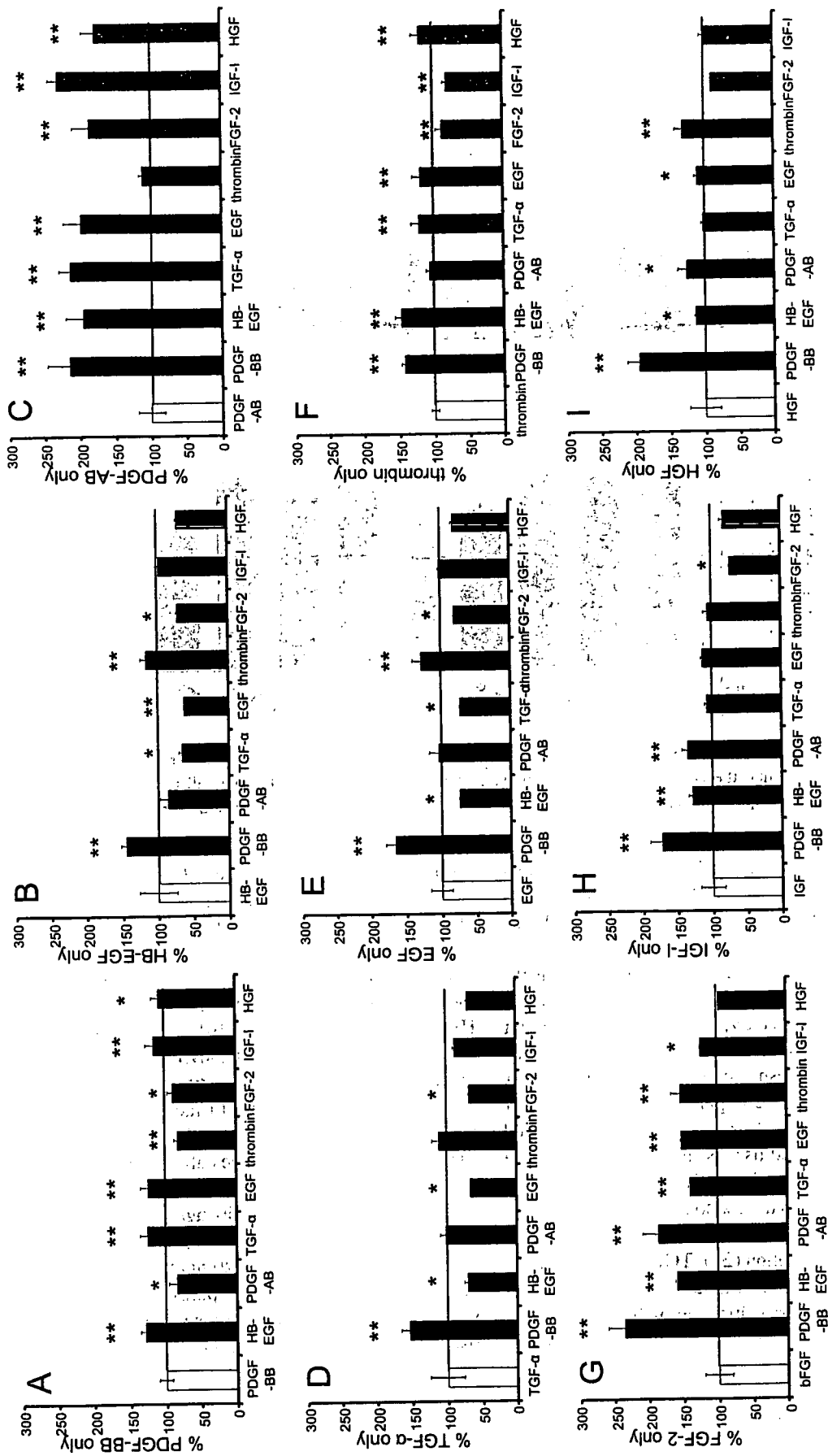
We chose 26 factors/cytokines for the migration assay using the following criteria: (1) We selected 13 growth factors (PDGF-AA,-AB, -BB, HB-EGF, EGF, TGF-α, FGF-2, IGF-I, HGF, TGF-β1, TGF-β3, VEGF, and BMP-2) that had been reported to have a chemotactic activity in some cells. (2) We selected four stem cell-related factors (SDF-1α, SCF, SCGF-α, and LIF). (3) We selected three neurotrophic factors (NGF-β, BDNF, and NT-3) because DNA microarray analysis showed the expression of BDNF in MSCs at a high level (data not shown). (4) We also selected three inflammation-related cytokines (IL-2, IL-8, and MCP-1). (5) We selected thrombin, ANP, and leptin because DNA microarray analysis showed enhanced expression of their receptors in MSC (data not shown).

Using human and rabbit MSCs, we found that nine factors consistently stimulated MSC migration. Of these,



**FIG. 4.** Chemotactic and chemokinetic effects of various growth factors. The effects of PDGF-BB (20 ng/ml), HB-EGF (5 ng/ml), PDGF-AB (50 ng/ml), TGF-α (5 ng/ml), EGF (1 ng/ml), FGF-2 (20 ng/ml), IGF-I (100 ng/ml), HGF (5 ng/ml), and thrombin (10 unit/ml) on rabbit MSC migration were examined using a microchemotaxis Boyden chamber. One-way ANOVA was performed (\*\**p* < 0.01). Data are represented as mean ± SD.





**FIG. 5.** Effects of combinations of various growth factors on migration of rabbit MSCs. The concentrations of growth factors were as described in Fig. 3. Values are the percentage of the CI obtained with the appropriate single factor. Data are represented as mean  $\pm$  SD (\* $p$  < 0.05, \*\* $p$  < 0.01).

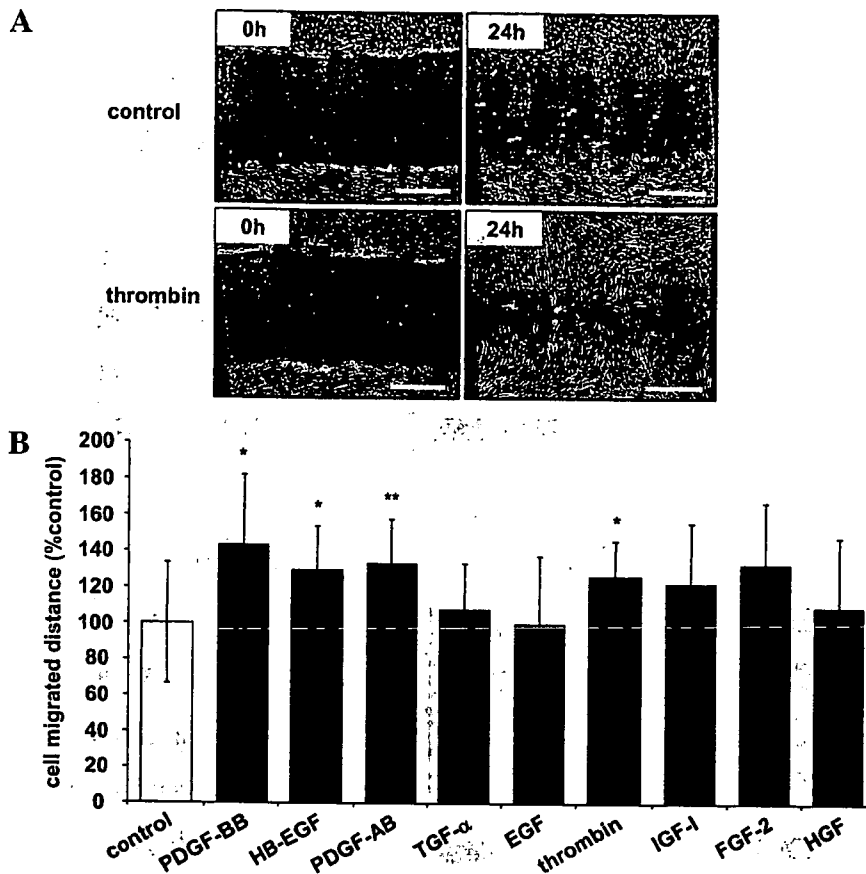


FIG. 6. Wound migration assay of rabbit MSCs. The concentrations of growth factors were as described in Fig. 3. (A) Photographs of cells migrating into the cell-free area. The white scale bar indicates 0.5 mm. (B) The cell migrated distance in each culture was quantified in comparison with that in control plates. Data are represented as mean  $\pm$  SD (\* $p$  < 0.05, \*\* $p$  < 0.01).

PDGF-BB has been reported to be the major chemoattractant for various adherent cells (23,24). PDGF-AB showed less chemotactic action on MSCs, but PDGF-AA did not have chemotactic activity for MSCs, indicating that PDGFR $\beta$  is involved in MSC migration, as in the case of smooth muscle cells (25), fibroblasts, or monocytes (26). In a previous study, mesenchymal progenitor cells also responded to PDGF-BB, whereas osteogenic precursor cells had less responsiveness (27), although this effect of PDGF was not compared with that of other growth factors/cytokines. In any case, PDGF released from platelets and injured tissues may play a major role in migration of MSCs to injured tissues.

The other eight factors have also been reported to have chemotactic activity for some cells. HB-EGF stimulates smooth muscle cell migration (28). TGF- $\alpha$  stimulates migration of osteoblasts (29) and keratocytes (30). EGF enhances migration of bronchial epithelial cells (31), colonic myofibroblasts (32), and keratocytes (30). FGF-2 stimulates migration of myogenic cells (33), bone marrow-derived MSC (34), and keratocytes (30). IGF-1 stimulates migration of osteoblasts (19), colonic myofi-

broblasts (32), and keratocytes (30). HGF stimulates migration of skeletal muscle satellite cells (35) and osteoclasts (36). Thrombin stimulates migration of vascular smooth muscle cells (37).

In this study, various combinations of two growth factors showed additive chemotactic effects. For example, HB-EGF, TGF- $\alpha$ , EGF, IGF-1, or HGF increased the chemotactic effects of PDGF-BB (Fig. 5A), and many other combinations showed additive chemotactic effects. Thus, combinations of growth factors administered into target tissues will be useful to enhance the persistence of MSCs within the target tissues after transplantation or an intravenous injection of MSCs.

In contrast, PDGF-AB (50 ng/ml), which shares the same receptor with PDGF-BB, decreased the chemotactic effects of PDGF-BB (20 ng/ml) (Fig. 5A), perhaps because the concentration-response curve for PDGF-BB was bell-shaped, although this suppression was not statistically significant (Fig. 2A). Similarly, TGF- $\alpha$  and EGF, HB-EGF and EGF, HB-EGF and TGF- $\alpha$ , which share the same receptor, decreased the chemotactic effects of HB-EGF (5 ng/ml), TGF- $\alpha$  (5 ng/ml) and EGF

## BONE MARROW MSCs

TABLE 2. CELL PROLIFERATION ASSAY OF RABBIT MSCs TO VARIOUS FACTORS

Factor	% control of disintegrations per minute (DPM)	± SD
PDGF-BB	2,495	358
HB-EGF	1,389	248
EGF	1,219	181
TGF- $\alpha$	1,124	185
PDGF-AB	1,086	239
FGF-2	809	130
IGF-I	301	132
HGF	276	85
Thrombin	208	70
SCGF- $\alpha$	146	34
BDNF	136	31
NGF- $\beta$	134	29
ANP	109	57
PDGF-AA	101	21
IL-8	98	45
Leptin	93	13
MCP-1	91	24
IL-2	90	39
SDF-1 $\alpha$	86	32
SCF	49	8
BMP-2	43	4
TGF- $\beta$ 3	13	5
TGF- $\beta$ 1	11	6

Values are means for three cultures and the % of control of [<sup>3</sup>H] radioactivity incorporated into DNA are revealed ( $n = 3$ ).

(1 ng/ml), respectively (Fig. 5B,D,E), perhaps because their concentration-response curves were bell-shaped (Fig. 2B,D,E). At higher concentrations than the optimal level, the combinations decreased the migration activity of MSCs. However, the inhibition of migration was not limited in the same growth factor family members. The PDGF-BB-induced MSC migration was also inhibited by FGF-2 or thrombin (Fig. 5A). Previous studies have also shown that FGF-2 decreases the chemotactic and mitogenic effects of PDGF-BB in cultures of rat aorta smooth muscle cells (38).

In the present study, FGF-2 suppressed the migration activity of PDGF-BB, HB-EGF, TGF- $\alpha$ , EGF, thrombin, and IGF-I. Several mechanisms underlying the FGF-2 inhibition of PDGF-BB action have been proposed by Facchiano et al. (38), but the precise mechanism remains unknown. It is also unknown how PDGF-BB-induced MSC migration is suppressed by thrombin. Although thrombin and PDGF-BB bind different receptors, both peptides regulate cell motility via phosphatidylinositol (PI) 3-kinase (24,39,40). At super optimal concentrations, the bell-shaped suppression may occur at a PI 3'-kinase level.

TABLE 3. CELL MIGRATION ASSAY OF HUMAN MSCs TO NINE FACTORS THAT HAVE CHEMOTACTIC ACTIVITY ON RABBIT MSCs

	MSC1	MSC2
PDGF-BB (20 ng/ml)	6.4 (± 2.9)**	6.0 (± 0.8)**
HB-EGF (5 ng/ml)	1.7 (± 0.4)**	1.4 (± 0.1)**
PDGF-AB (100 ng/ml)	8.5 (± 2.1)**	3.7 (± 0.5)**
TGF- $\alpha$ (10 ng/ml)	2.2 (± 0.7)**	2.2 (± 0.4)**
EGF (1 ng/ml)	1.5 (± 0.3)**	1.7 (± 0.2)**
Thrombin (10 unit/ml)	1.3 (± 0.1)*	2.3 (± 0.4)**
IGF-I (100 ng/ml)	1.3 (± 0.1)	1.8 (± 0.4)**
FGF-2	2.0 (± 0.7)**	1.0 (± 0.1)
HGF (1 ng/ml)	15 (± 0.2)**	1.6 (± 0.3)**

Chemotaxis index, values (CI) are revealed at their approximate concentrations.

\* $p < 0.05$ ; \*\* $p < 0.01$ .

In any case, migration of MSCs to injured tissues can be modulated by PDGF, FGF, thrombin, HGF, and EGF family members released from platelets or injured tissues concentration and combination dependently.

It is unknown why thrombin had the opposite effect on migration of fibroblasts and MSCs. However, three types of thrombin receptors on mammalian cells, PAR-1, -3, and -4, have been identified (41), and their expression levels in MSCs were found to differ from those in fibroblasts (data not shown). MSCs and fibroblasts have different gene expression profiles (22) and may have distinct responsiveness to chemotactic factors. This issue should be investigated in the future.

In conclusion, the extensive analysis of chemotactic factors revealed that PDGF-BB, HB-EGF, PDGF-AB, TGF- $\alpha$ , EGF, FGF-2, IGF-I, and HGF modulate migration of rabbit and human MSCs and human fibroblasts

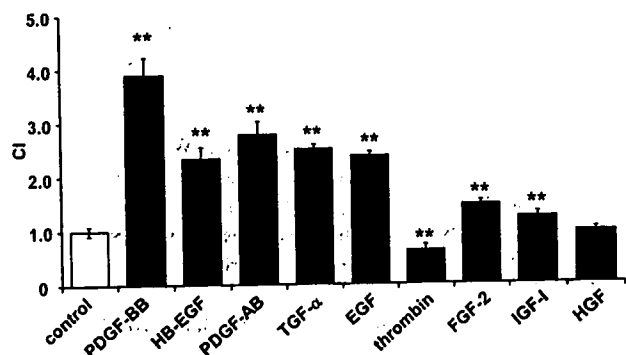


FIG. 7. Chemotactic responses of human fibroblasts to growth factors that had chemotactic activity on MSCs. Data are represented as mean ± SD (\*\* $p < 0.01$ ).

concentration and combination dependently, whereas thrombin selectively enhances MSC migration.

## ACKNOWLEDGMENT

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