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Alternative Isoforms of the *mi* Transcription Factor (MITF) Regulate the Expression of mMCP-6 in the Connective Tissue-Type Mast Cells Cultured with Stem Cell Factor

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mi transcription factor (MITF) is important in regulating the differentiation of mast cells. In particular, MITF regulates the transcription of the mouse mast cell-specific serine protease (mMCP)-6 gene, which is generally expressed by the connective tissue-type of mast cells. In this study, we investigated alternative isoforms of MITF that regulate transcription of the mMCP-6 gene in bone marrow-derived cultured mast cells in mice. The expression of MITF isoforms was examined by RT-PCR. We observed that MITF-A, -E, -H and -Mc were expressed by mucosal-type mast cells cultured in the presence of IL-3, whereas the connective tissue-type mast cells cultured in the presence of stem cell factor (SCF) expressed MITF-A. Overexpression of MITF isoforms increased luciferase activity through the mMCP-6 promoter in NIH-3T3 cells and elevated the level of mMCP-6 expression in the MC/9 mast cell line. Moreover, mMCP-6 expression in mast cells was significantly inhibited by the depletion of MITF. The transcriptional activity and DNA binding of MITF-A was comparable to that of MITF isoforms, including MITF-E, -H, and -Mc. Our results therefore suggest that MITF-A may be an important isoform of MITF in regulating the transcription of mMCP-6 in mouse connective tissue mast cells.

Key words : *mi* transcription factor, MITF-A, mMCP-6, mast cells, stem cell factor

Introduction

Mast cells play a crucial role in the development of inflammation and allergic responses. The multivalent binding of an antigen to FcεRI-bound IgE triggers the activation of mast cells and subsequently leads to the secretion of granules including mouse mast cell-specific serine proteases (mMCPs) and histamine [17,28]. In mice, mast cells express a family of mMCPs such as tryptases and chymases. Members including mMCP-1, -2, -4, -5 and -9 are chymases, while mMCP-6 and mMCP-7 are tryptases [14]. Each family of mMCPs exhibits a tissue-specific distribution. In particular, mMCP-6 is highly expressed in connective tissue mast cells but not in mucosal mast cells [22]. Members of mMCP-1 and -2 are restricted to mucosal mast cells [2,8]. When bone marrow-derived cultured mast cells (CMCs) and peritoneal mast cells were injected into the stomach walls of mast cell-deficient mice, the mMCPs expression pattern of the mast cells in the mucosa and muscularis propria resembled that of mast cells in the stomach of intact mice [12]. These observations suggest that gene expression of mMCPs is regulated by the local environment.

The *mi* locus of mice encodes the *microphthalmia*-associated transcription factor (MITF) gene which is part of the basic/helix-loop-helix/leucine zipper (b-HLH-Zip) transcription factor. The b-HLH-Zip proteins, including MITF, recognize the CANNTG motifs in the promoter region of target genes. To date, eight isoforms of MITF are known; they are MITF-A [1], -C [3], -Mc [26], -H [23], -M [6,27], -E [19], -D [25], -J [5]. These isoforms have common transcription activation and DNA binding domains, but differ in the N-terminal amino acids encoded by the exon 1. For most isoforms, the initial exon is spliced onto the latter part of exon 1 and then on to the common exons 2-9, which encode the functionally important motifs, including b-HLH-Zip, transactivation domains, and various phosphorylation consensus sequences. However, whether these MITF isoforms confer distinct properties on each protein is not known. MITF-A is expressed widely in the retinal pigment epithelium to regulate these formations [29]. MITF-M is restricted to neural crest-derived melanocytes and is absolutely required for the development of these cells [4].

It is known that mMCP-6 may be important in mast cell-mediated inflammatory reactions [13,21]. A recent study has demonstrated that MITF increased the tran-

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scription activity of the mMCP-6 gene through binding to two motifs [15]. mMCP-6 is one of the tryptases that is constitutively present in skin mast cells of mice. We have shown that the ability of MITF to regulate mMCP-6 expression is dependent on the activation of mast cells with stem cell factor (SCF) to induce the differentiation of the connective tissue-type mast cells [8]. However, MITF isoforms expressed by the connective tissue-type mast cells remain unclear. In the present study, we demonstrate the profile of MITF isoforms expressed in cultured mast cells and their ability to regulate the transcription of the mMCP-6 gene.

Materials and Methods

Cell culture

Bone marrow cells derived from C57BL/6 mice (Samtaco animal, Gyunggido, Korea) were cultured in α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA) containing 10% FBS (Hyclone, Logan, UT), 30% WEHI3-cultured condition medium, 50 μM 2-mercaptoethanol and 100 U/ml penicillin-streptomycin (Sigma, St. Louis, MO) for 3 weeks to establish the CMCs. The purity of mast cells by toluidine blue staining was more than 95 %. MC/9 mast cell lines were provided by Dr. Y. Kitamura (Osaka University, Japan). For the experiments, CMCs and MC/9 were grown in α-MEM +10% FBS in the presence of 100 ng/ml recombinant mouse SCF or 50 ng/ml IL-3 (R&D Systems, Minneapolis, MN). NIH-3T3 fibroblasts were maintained in Dulbecco's modification of Eagle's medium (DMEM; Invitrogen) containing 10% FBS and 100 U/ml penicillin-streptomycin.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen). For cDNA synthesis, RNA (2 or 0.2 μg) was reverse-transcribed for 1 hr at 42°C using the superscript One-Step RT-PCR kit (Invitrogen) according the manufacturer's instruction. One microliter of synthesized cDNAs was amplified in a PCR mixture containing Taq DNA polymerase (Invitrogen) and each pair of specific sense and antisense primers (Bioneer, Daejeon, Korea), as described in Table 1. PCR amplification was performed as follows: denaturation (94°C, 30 sec), annealing (58°C, 30 sec), and extension (72°C, 50 sec). The PCR products were electrophoresed in 1.2% agarose gel in 1xTAE buffer and photographed under a UV lamp.

Table 1. The oligonucleotides used for PCR amplification

	Primer Sequence	PCR product
5'-Primers		
MITF-A	GGAAGGCGGGCAAGAGGGAGTCAT	791 bp
MITF-B	GAGTGCCATGCCGTGCCTTGAT	438 bp
MITF-C	TTTTCCCACCAGCTGATTCCTCTA	742 bp
MITF-D	GGGACCTGACAGGCTCTGAA	687 bp
MITF-E	GGAAGATTAAGCCCAGTGAGGTT	744 bp
MITF-H	GATGGAGGCGCTTAGATTTGAGAT	722 bp
MITF-M	CCTTCTTTATGCCGGTCGCTCTTC	555 bp
MITF-Mc	ACACAAGCCCTACCTCAGAACC	689 bp
MITF-J	CCGTGTCTCTGGGCATCTGAAG	745 bp
GAPDH-Fw	TGCACCACCAACTGCTTAG	117 bp
3'-Primers		
MITF-Rv	CGGGTAACGTATTTGCCATTTG	
GAPDH-Rv	GGATGCAGGGATGATGTTC	

Staining of mast cells

Cytospin preparations of cells were fixed with Carnoy's solution containing acetic acid, chloroform and ethanol, and than stained with alcian blue or berberine sulfate (Sigma) for 30 min, as described previously [18]. The alcian blue-positive cells were observed under a light microscope, and the berberine sulfate-positive mast cells were observed under a fluorescence microscope.

Construction of expression and reporter plasmids

cDNA of the MITF isoforms was prepared from the cultured mast cells by RT-PCR and inserted into a TOPO-TA cloning vector (Invitrogen). The plasmids were digested with EcoRI restriction enzymes and then subcloned into pZeoSV2(+) mammalian expression vector (Invitrogen). The sequence of cDNA was identified by sequencing analysis. For the reporter plasmid, the mMCP-6 promoter region between nt -191 and +26 was constructed as described previously [15] and inserted into pGL3-basic vector (Promega, Madison, WI).

Transfection and luciferase activity assay

The expression (0.5 or 2 μg) and reporter plasmids (2 μg) were cotransfected into NIH-3T3 cells (0.5×10^6 in a 6 cm dish) by a transit-LT1 transfection reagent (Mirus, Pittsburgh, PA). Cells were harvested 48 hr after transfection and lysed with a luciferase lysis reagent (Promega). Soluble extracts were assayed for luciferase activity using

a luminometer LB96P (Berthold GmbH, Wilbad, Germany). Luciferase activity was normalized by β-galactosidase and total protein concentration. Each luciferase activity is shown as a relative value calculated with reference to a control expression vector. The sequences of MITF-A siRNA (5′-GCT GCA GGC AGC CCA GTT C-3′) and control siRNA (5′-CTA ACG TAC GGA TCT AAC T-3′) were designed using a BLOCK-iT™ RNAi Designer (Invitrogen) and synthesized by a LineSilence™ complete RNAi kit (Allele Biotech Inc. San Diego, CA).

Tryptase activity assay

Cells were sonicated in 10 volumes of 20 mM Na-phosphate buffer (pH 7.4). After centrifuging, the supernatants were used as extract-containing tryptase. The enzyme activity of tryptase was determined using the substrate tosyl-gly-pro-lys-pNA (Sigma Co.) containing 50 μg/ml heparin. Samples were incubated in a reaction buffer mix for 2 hr at 37°C, and then the OD was read at 405 nm in a microplate reader.

Western blot analysis

Cells were lysed in ice-cold lysis buffer (iNtRON Biotech, Korea) for 20 min and centrifuged (12,000 *g*) for 20 min. The protein concentrations in cell extracts were measured using a bicinchoninic acid. Lysates (25 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% skimmed milk in 1×PBS for 1 hr, the membranes were incubated with an anti-MITF polyclonal antibody (N-15, Santa Cruz Biotech., Santa Cruz, CA) at 4°C, washed in TBS containing 0.1% Tween 20, and incubated with HRP-conjugated donkey anti-goat IgG polyclonal antibody for 45 min. Finally, proteins were visualized using an enhanced chemiluminescence WB detection kit (Amersham Pharmacia Biotech).

Electrophoretic mobility gel shift assay (EMSA)

The fusion protein of MITF was produced using the TNT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) according the manufacturer's instruction. Oligonucleotide 5′-CCACCGTGTGACCTGTGG TCATCA was used as a probe. The probe was labeled with α-[^{32}P]-dCTP by Taq polymerase. DNA-binding assays were performed in a 20 μl reaction mixture containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 75 mM KCl, 1 mM dithiothreitol, 4% Ficoll type 400, 50 ng of poly (dI-dC), 25 ng of labeled DNA probe, and 10 μg of MITF fusion protein. After incubation at room temperature for 15 min, the reaction mixture was subjected to electrophoresis in a 5% polyacrylamide gel in 0.25×TBE buffer (pH 8.3). The polyacrylamide gels were dried on Whatman 3MM paper (Whatman, Maidstone, UK) and subjected to autoradiography.

Statistical analysis

Data from the experiments were described to mean ± S.E.M. Statistical significance was determined using the Student's *t*-test to express the difference between groups. All *p*-values <0.05 were considered to reflect a statistically significant difference.

Results

Expression of MITF isoforms in the cultured mast cells derived from bone marrow

We examined the expression of MITF isoforms in the cultured mast cells (CMCs). CMCs were used 3 weeks after the initiation of the culture because the purity of mast cells reached up to 95% at this time judging by alcian blue staining. CMCs were cultured for 4 days in the complete medium containing IL-3 or SCF to obtain the mucosal type or connective tissue type, respectively. To identify the phenotype of mast cells, CMCs were stained with berberine sulfate. RT-PCR was performed to estimate the RNA expression levels of MITF isoforms using specific primers (Table 1). The mucosal-type CMCs expressed the transcripts of various MITF isoforms, such as MITF-A, -E, -H, and -Mc (Fig. 1). However, the connective tissue type CMCs established with SCF showed the expression of MITF-A transcripts, while the transcripts of other isoforms were barely detectable (Fig. 2).

Transactivation of the mMCP-6 gene by MITF-A

It has been reported that MITF is an essential transcription factor for the expression of the mMCP-6 gene [20]. We examined whether MITF-A overexpression in the connective tissue type CMCs affects with the transactivation of the mMCP-6 gene by a luciferase activity assay. To compare the transcriptional activity of MITF-A to that of other isoforms, the expression plasmids of MITF-E, -H or -Mc were cotransfected with a reporter plasmid comprising the mMCP-6 promoter region into NIH-3T3 cells. A significant

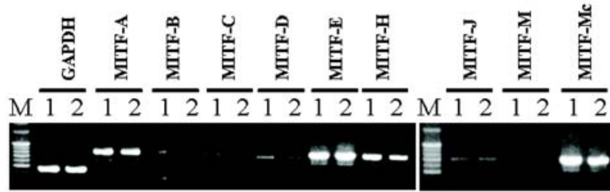

Fig. 1. Expression of MITF isoforms in mucosal-type mast cells cultured with IL-3. Total RNA was extracted from CMCs cultured with IL-3-containing medium for 3 weeks. The RNA expression of MITF isoforms was analyzed by RT-PCR. 1 and 2 indicate the two separate experiments; M, 1kb DNA marker. PCR products were electrophoresed in 1.2% agarose gel.

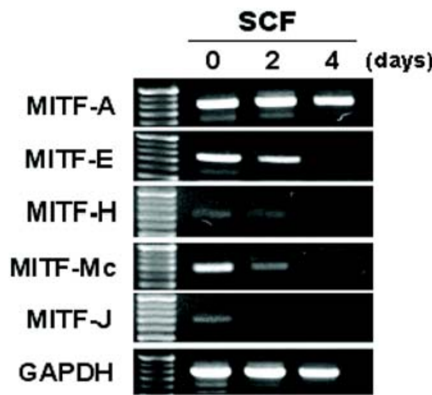

Fig. 2. Expression of MITF isoforms in connective tissue-type mast cells cultured with SCF. CMCs were cultured with SCF for the indicated times before total RNA extraction. The expression of their mRNAs was analyzed by RT-PCR.

increase in luciferase activity was observed in all transfectants overexpressing MITF-A, -E, -H, or -Mc (Fig. 3).

Next, to examine DNA binding activity of MITF-A, we performed EMSA using the oligonucleotide containing GACCTG motif, which represents a part of mMCP-6 promoter region, as a probe. When MITF-A fusion protein was incubated with the probe, DNA-protein complexes were formed in all MITF isoforms. The DNA binding of MITF-A was abolished by the excess amount of the nonlabeled oligonucleotide as a competitor (Fig. 4). However, we observed that a specific DNA/protein complex was barely detectable when nuclear extracts of CMCs were used (data not shown).

The level of tryptase was increased by MITF-A and decreased by introducing MITF siRNA in MC/9 cells

To examine the effect of transcriptional activity of MITF-A on mMCP-6 expression in mast cells cultured with

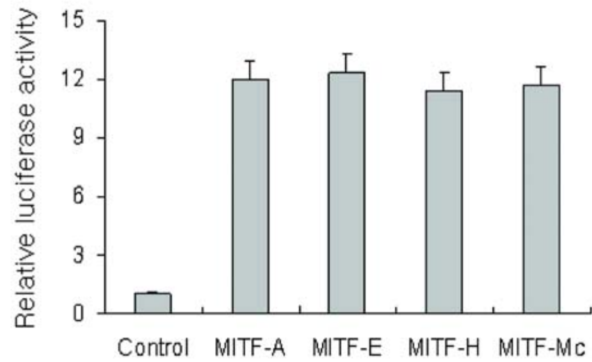

Fig. 3. Transactivation of mMCP-6 promoter by MITF isoforms in NIH-3T3 cells. Expression vectors for MITF-A, -E, -H, and -Mc (2 μg) were used as the effector plasmids. Each effector plasmid was cotransfected with a reporter plasmid containing mMCP-6 promoter into NIH-3T3 cells. The transfectants were incubated and harvested for a luciferase activity assay. Values are presented as relative luciferase activity.

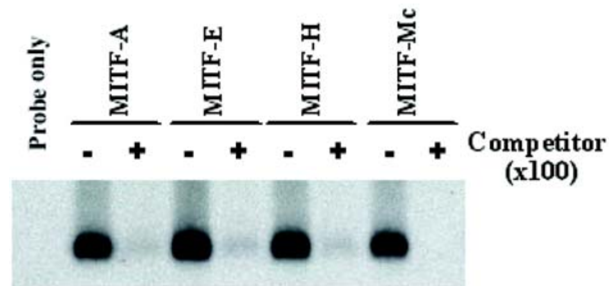

Fig. 4. DNA binding activity of the fusion proteins of MITF isoforms. The synthesized fusion protein of MITF-A, -E, -H, or -Mc was reacted with the labeled oligonucleotide containing the MITF binding motif. A single retarded band was detected by GMSA. An excess amount of nonlabeled oligonucleoride was added as a competitor.

SCF, we transfected the expression vector MITF-A into the MC/9 mast cell line. We also transfected the expression vector MITF-E, -H, or -Mc which are expressed in mucosal type CMCs, to compare their transcriptional activity. The overexpression of MITF-A increased the luciferase activity through the mMCP-6 promoter, and the level of its expression by MITF-A was comparable to that of MITF-E, -H, or -Mc (Fig. 5A). Moreover, the tryptase activity in MC/9 cells was increased by overexpression of all isoforms as well (Fig. 5B).

We next examined whether the depletion of MITF-A attenuated the expression of mMCP-6 in MC/9 cells cultured in a medium containing SCF. When the MITF-A siRNA (0.2 or 2 μg) or control siRNA (2 μg) was transfected into

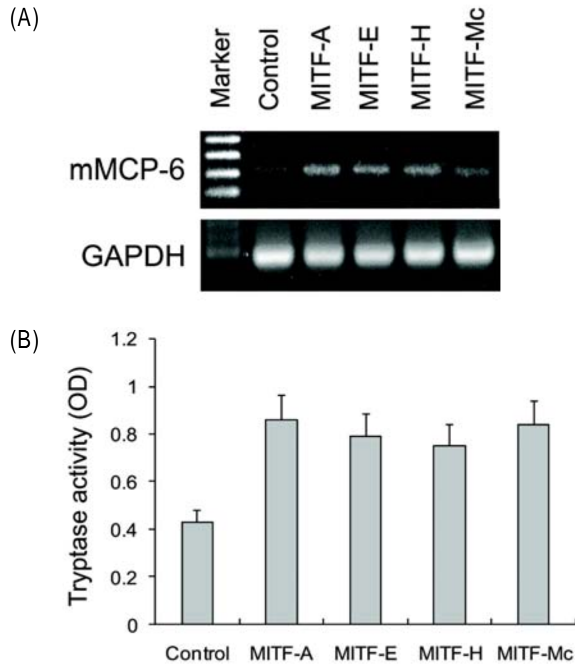

Fig. 5. Upregulation of mMCP-6 expression and tryptase activity by overexpression of MITF isoforms in MC/9 mast cells. (A) The expression plasmids of MITF-A, -E, -H, or -Mc were transfected into MC/9 cells. Empty plasmid was used as negative control. After incubation for 48 hr, total RNAs were extracted to detect the mMCP-6 mRNA by RT-PCR. (B) The transfectants were homogenized to analysis the tryptase activity of mMCP-6. Tryptase activity assay was performed by precision microplate reader and the data represent the mean±SE of three experiments.

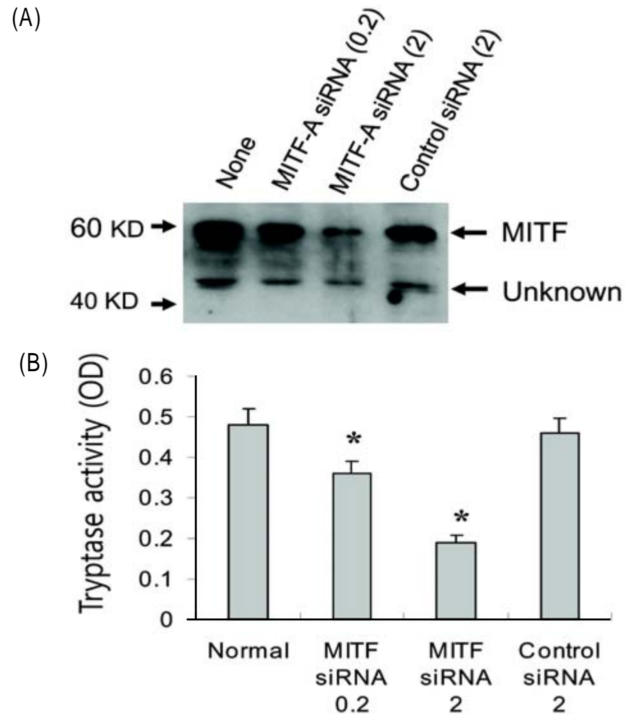

Fig. 6. Downregulation of mMCP-6 expression and tryptase activity by the depletion of MITF in MC/9 mast cells. (A) MITF-A siRNA (0.2 and 2 μg) were transfected into MC/9 cells. Control siRNA (2 μg) was used as a negative control. After incubation for 48 hr, Cells were harvested and lysed to identify the expression of MITF by western blot analysis. (B) The transfectants were homogenized to analyze the tryptase activity of mMCP-6. A tryptase activity assay was performed by a precision microplate reader and the data represent the mean±SE of three experiments. *$p<0.05$.

MC/9 cells, we observed that the expression of MITF protein was significantly reduced in a dose-dependent manner (Fig. 6A). Moreover, the tryptase activity was subsequently decreased by transfection of MITF siRNA (Fig. 6B).

Discussion

MITF has been reported to regulate the development of mast cells as well as melanocytes. In a recent study, the expression of the mMCP-6 gene was deficient in *mi/mi* mice-derived CMCs expressing mutant MITF [15]. The overexpression of wild type MITF but not mutant MITF was able to transactivate the mMCP-6 promoter through binding to GACCTG and CANNTG motifs. This implies that MITF plays an essential role in the transcription of mMCP-6 [21]. In this study, we demonstrated that MITF-A is a major isoform that regulates mMCP-6 expression in mouse mast cells. The connective tissue-type mast cells present in skin and muscle strongly expressed MITF-A, and the DNA binding activity of MITF was comparable to that of other MITF isoforms. Although all MITF isoforms have transcriptional activity that leads to the expression of the mMCP-6 gene, MITF-A was constitutively expressed in both mucosal and connective tissue-type mast cells. In particular, the overexpression of MITF-A increased mMCP-6 expression and tryptase activity by MC/9 cells cultured with SCF. It suggests that MITF-A is critically involved in the development of mast cells to connective tissue types expressing mMCP-6.

Mast cells are known as effector cells in inflammatory reactions because they secrete various chemical mediators such as histamine, serotonin, chymase, tryptase, and eicosanoids. When the MC is challenged by an external stimulus, it may respond by degranulation [11,24]. In this process, a number of powerful inflammatory mediators are

released. Tryptase is known to regulate allergic and inflammatory responses, including recruitment of neutrophils, Th2 cells, and basophils [12,21]. The mast cells in mouse skin express abundant tryptase mMCP-6. CMCs derived from bone marrow of transgene-insertional *vga9/vga9* mutant mice, which do not express MITF owing to insertion of the gene in the promoter region, showed abnormal phenotypes including deficiencies in expression of mMCP-4, -6, -7, granzyme B, prostaglandin D2, and tryptophan hydroxylase [6,7,9,16,20]. Our finding supports the idea that MITF-A is involved in regulating the function of mast cells.

Our results have shown that MITF-A may be a major isoform in regulating mMCP-6 expression in mouse mast cells. Stimulation of CMCs by SCF elicits the expression of MITF-A and subsequently mMCP-6. However, although the importance of MITF-A in the expression of mMCP-6 has been demonstrated, no one has investigated the transcriptional mechanism generating MITF-A in mast cells. Such identification would promote further analysis of the development of mast cells and would be useful in evaluating the physiological roles of mast cell-derived proteases.

Acknowledgement

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초록 : SCF에서 배양한 결합조직형 비만세포에서 mMCP-6 발현을 조절하는 MITF 이형체

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mi transcription factor (MITF)는 비만세포의 분화를 조절하는 중요한 전사인자이다. 특히 MITF는 결합조직형 비만세포에서 일반적으로 발현하는 비만세포 특이적 세린 단백분해효소의 일종인 mMCP-6 유전자의 전사를 조절한다. 본 연구는 마우스 골수유래 배양비만세포에서 mMCP-6 유전자의 전사를 조절하는 MITF이형체를 규명하였다. MITF 이형체들의 발현은 RT-PCR로 확인하였다. IL-3존재 하에서 배양한 점막형 비만세포들은 MITF-A, -E, -H, -Mc 등이 발현하였다. 반면에 SCF존재 하에서 배양한 결합조직형 비만세포들은 MITF-A가 발현하였다. MITF이형체를 과발현시키면 NIH-3T3 세포에서 mMCP-6 promoter를 통한 luciferase 활성을 증가시키고, MC/9 비만세포주에서는 증가된 mMCP-6발현을 유도하였다. 더불어 비만세포에서의 mMCP-6 발현은 MITF-A 고갈로 인하여 유의적으로 억제되었다. MITF-A의 전사활성과 DNA결합은 MITF-E, -H, -Mc 등의 타 이형체들의 결과와 유사하였다. 따라서 본 연구의 결과들은 MITF-A가 마우스 결합조직형 비만세포에서 발현하여 mMCP-6 전사를 조절하는 중요한 이형체임을 제시한다.