A TRANSCRIPTION MODULATOR, I-MFA (INHIBITOR OF MYOD FAMILY A), IS EXPRESSED IN OSTEOBLAST-LIKE CELLS AND IS UPREGULATED BY 1,25-DIHYDROXYVITAMIN D3.

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[Relevance to Musculoskeletal Conditions] To elucidate the molecular mechanisms of osteoblastic differentiation, we investigated the regulation and function of I-mf (Inhibitor of MyoD family) in osteoblast-like cells.

[Introduction] I-mfa is a transcription modulator that binds to the bHLH domains of MyoD (amino acids 57 to 166). I-mfa transcripts are expressed abundantly in the sclerotome in 11.5 dpc mouse embryos and I-mfa protein has been shown to interact with and to suppress the transcriptional activity of MyoD family members, MyoD, Myf5, and myogenin, suggesting the roles of I-mfa in skeletogenesis via inactivation of myogenic pathway. The aim of this study is to examine the expression and regulation of I-mfa in osteoblasts.

[Materials and Methods] *Cell culture, total RNA preparation, and total cell lysate preparation* - Clonal osteoblastic MC3T3E1 and ROS17/2.8 cells were maintained in alpha MEM and F-12 medium supplemented with 5% fetal bovine serum respectively. To examine the effects of hormones and cytokines on I-mfa expression, the cells were plated $3X10^4$ cells/cm^2 one day prior to the treatment and then the media were replaced next day with fresh ones containing hormones, cytokines or vehicle. Total cellular RNA was extracted according to conventional acid guanidine phenol chloroform method. To prepare the total cell lysate, the cells were washed by PBS, scraped and resuspended in Tris based buffer. The cell suspension was lysed by ultrasonication.

RT-PCR- Total RNA was reverse transcribed and amplified by Titan One Tube RT-PCR kit (Boeringer Mannheim, Germany). PCR condition was determined so that band densities are linearly correlating to RNA amounts and PCR cycle number. The bands were quantitated by densitometory using Bio Profile Version 97 (VILBER LOUMAT, France) and each value was normalized against GAPDH gene expression level. Primers for I-mfa were 5-cagaattcacagcctcaagggaacccc-3 and 5-acagtatgcagtggacgcagca-3.

Northern Blot and Western Blot analysis- Ten micro grams of total RNA was separated in 1% agarose gel and blot onto nylon membrane. Membranes were hybridized with ^{32}P-labeled probes and visualized by BAS2000 Image Analysis System (Fuji Film, Japan). Ten micro gram of cell lysate was separated in 10% SDS-acrylamide gel and transferred onto nitrocellulose membrane. Membranes were incubated in PBS containing rabbit anti mouse I-mfa antiserum and visualized by ECL Plus Western Blot Detection kit (Amersham LIFE SCIENCE, England).

[Results] We found that I-mfa is expressed at a low level in osteoblastic cells by using RT-PCR. I-mfa expression was detectable in a osteoblast-like cell line, MC3T3E1. Among the cytokines and hormones, 1,25-dihydroxyvitamin D3 specifically enhanced the mRNA abundance of I-mfa in MC3T3E1 cells, while BMP2, TGFbeta, retinoic acid and dexamethasone did not affect the I-mfa expression. Upregulation of I-mfa mRNA level by 1,25dihydroxyvitamin D3 was time dependent and was about two fold within 24 hours and was still observed at least up to 96 hours. Enhancement of I-mfa expression by 1,25-dihydroxyvitamin D3 was dose dependent and was observed at 0.1nM and was maximal at 1nM. The effect of 1,25-dihydroxyvitamin D3 was completely blocked by the presence of an RNA polymerase inhibitor, 5,6-dichlorobenzimidasole riboside, but not by a protein synthesis inhibitor, cycloheximide. These observations suggest that 1,25-dihydroxyvitamin D3 upregulates transcription of I-mfa gene without requirement for new protein synthesis. 1,25-dihydroxyvitamin D3 enhancement of I-mfa expression was not altered by the co-treatment with dexamethasone, BMP2, and TGFbeta. Northern and Western blot analysis indicated that 1,25-dihydroxyvitamin D3 increased the I-mfa protein levels several fold in MC3T3E1 cells. I-mfa expression was also observed in ROS17/2.8 cells and primary mouse calvaria cells. 1,25-dihydroxyvitamin D3 enhanced I-mfa expression in these cells similarly to the observation in MC3T3E1 cells. To examine the function of I-mfa in osteoblast like cells, we overexpressed I-mfa or its isoform I-mfc, that cannot efficiently bind to and inhibit the transcriptional activity of MyoD family members, in MC3T3E1 and ROS17/2.8 cells. Expression of type I collagen mRNA was more in I-mfc overexpressed cells compared to I-mfa overexpressed cells.

[Discussion] Here we showed that I-mfa is expressed in osteoblasts and is regulated by calciotrophic hormone, 1,25-dihydroxyvitamine D3. Overexpression of I-mfa in osteoblast-like cells results in downregulation of osteoblastic phenotype related genes such as type I collagen compared to I-mfc, that cannot efficiently inhibit the function of MyoD family. 1,25-dihydroxyvitamin D3 is a potent modulator of differentiation in several types cells including osteoblasts. These data suggested that I-mfa is upregulated and may have a fundamental role in osteoblastic differentiation. I-mfa was identified as a novel type inhibitor of myotome related helix-loop-helix (HLH) type transcription factor, MyoD. Inhibition activity of I-mfa is restricted to MyoD, Myf5, and Myogenin, but slightly to ubiquitous HLH type transcription factor, E12, indicating I-mfa plays specific roles in regulation of myogenesis. Interestingly, I-mfa expression level in E11.5 mouse embryo is more in the screlotome than in the myotome indicating that I-mfa suppresses myogenic differentiation of undifferentiated mesenchymal cells existed in the screlotome. At the same time in mouse development, recently identified runt type transcription factor, Cbfa1/Pebp2aA, which is important for terminal differentiation of osteoblasts, is expressed in the screlotome. I-mfa and Cbfa1/Pebp2aA may co-operatively regulate the fate of undifferentiated mesenchymal cells in the screlotome to skeletal cell lineage. Another possible explanation is that I-mfa may interact with other unknown screlotome specific HLH type transcription factors, such as screlaxis, and play some roles in skeletogenesis by inhibiting the functions of these factors.

[Conclusion] I-mfa is a novel modulator of phenotypic expression in osteoblasts and its level is under the control of the calciotrophic hormone, 1,25(OH)2 vitamin D3.

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