***Gm7325* is MyoD-dependently expressed in activated muscle satellite cells**

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ABSTRACT

The *Gm7325* gene, bioinformatically identified in the mouse genome, encodes a small protein but has not been characterized until recently. Our gene expression analysis revealed that *Gm7325* transcription is remarkably upregulated in injured skeletal muscle tissues. Activated satellite cells and immature myotubes were densely decorated with positive signals for *Gm7325* mRNA in *in situ* hybridization analysis, while no obvious signals were observed in quiescent satellite cells and mature myofibers. In the 5'-flanking regions of mouse *Gm7325* and its human homologue, conserved E-box motifs for helix-loop-helix transcription factors are repeatedly arranged around the putative promoter regions. Reporter gene assays suggested that MyoD, a master transcription factor for myogenesis, binds to the conserved E-box motifs to activate *Gm7325* expression. Therefore, *Gm7325*, as a novel MyoD-target gene, is specifically induced in activated satellite cells, and may have an important role in skeletal myogenesis.

Our database survey found that *Gm7325* expression is enhanced in several muscular dystrophy models, for example, mutant muscles lacking dystrophin and laminin alpha 2 (see NCBI GEO datasets under accession numbers GSE16438 and GSE12049). Based on the observations, we presumed a causal link between muscle damage and *Gm7325* induction. Muscle injured by cardiotoxin (CTX) injection provides a common experimental model in muscle-fiber regeneration studies. In a standard protocol applied to mouse tibialis anterior (TA) muscle (5, 7), vigorous proliferation of activated satellite cells, so-called myoblasts, and their fusion into myotubes are observed on days 3–5 after CTX injection, and the reconstruction of muscle fibers proceeds sequentially for a few weeks afterward. RT-PCR analysis detect-

ed that *Gm7325* expression became active, when CTX damaged muscle and *Gapdh* expression started to decrease (Fig. 1A). *Gm7325* mRNA content was almost undetectable in uninjured muscle on day 0, but increased sharply by approximately three orders of magnitude during days 1–5 after the injection. By performing fluorescence activated cell sorting (FACS) with an optimized antibody set (12), mononucleated cells (MNCs) prepared from CTX-injected mouse hindlimbs can be fractionated into SM/C-2.6⁺ muscle satellite cells (MSCs), CD31⁺/CD45⁺ hematopoietic progenitor cells (HEPs) and SM/C-2.6⁻/CD31⁻/CD45⁻ fibro/adipogenic progenitor cells (FAPs). Between the fractionated preparations, *Gm7325* mRNA was exclusively detected in MSCs (Fig. 1B).

In RNAscope *in situ* hybridization analysis of tissue sections prepared from CTX-injected TA muscle (Fig. 2), dense signals for *Gm7325* mRNA were mainly detected in mononucleated cells clustered between myofibers. These signal-positive cells were

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Fig. 1 *Gm7325* induction in activated satellite cells. **(A)** Upregulated *Gm7325* expression in CTX-injured muscle. Total RNAs were prepared from TA muscles before and after CTX injection, and *Gm7325*, *Myod1*, *Myog* and *Gapdh* mRNA contents were quantified by RT-PCR analysis. Day 0 represents uninjected controls. The cycle threshold (Ct) indicates the cycle number at which the amount of amplified cDNA reached a fixed threshold in each RT-PCR reaction. The data were derived from 4–8 reactions using RNAs from 2–3 mice, and are represented as the mean $\pm$ SEM. **(B)** *Gm7325* expression in MSCs. Mononucleated cells (MNCs) were prepared from injured muscle on day 3 after CTX injection, and MSCs, HEPs and FAPs were FACS-sorted for total RNA preparations. Gene expression levels quantified by RT-PCR analysis were normalized to 18S rRNA content in each cell preparation. *Adgre1* encoding F4/80 protein is a macrophage-specific marker gene, while *Pdgfra* is a mesenchymal progenitor marker gene. The data were derived from three independent preparations, and are represented as the mean $\pm$ SEM.

Fig. 2 *In situ* hybridization analysis using RNAscope probe specific to *Gm7325* mRNA. Representative images from uninjected control and CTX-injected muscles are shown in left and middle panels, respectively. Three higher-magnification images of the boxed regions in the middle panels are also presented as right panels. Red signals were derived from *Gm7325* mRNA reacted with the hybridization probe, while nuclei were blue-stained with hematoxylin. li, inflammatory infiltrate; Mb, myoblast; Mt, myotube. Scale bars, 20 μ m.

reasonably assigned to proliferating satellite cells, because no obvious hybridization signals were detected in uninjured control myofibers maintaining quiescent satellite cells. In addition, the hybridiza-

tion signals were detected within small-sized nascent myotubes but not in inflammatory infiltrates. In longitudinal sections, the positive signals were often distributed around aligned nuclei within na-

Fig. 3 *Gm7325* transcription regulated by MyoD. **(A)** Alignment of 5'-flanking sequences from mouse *Gm7325* and human *LOC101929726*. The mRNA start sites determined are at nucleotide position +1, and the E-box and SP1/3-binding consensus sites are underlined. **(B)** *Gm7325* promoter analysis. A schematic diagram of firefly luciferase (Fluc) reporter gene constructs (left panel). The wild-type *Gm7325* 5'-flanking fragment (WT), point-mutated fragments lacking each E-box ($\Delta E1$, $\Delta E2$, $\Delta E3$ and $\Delta E4$), and mutated fragments lacking all E-boxes (ΔEs) were cloned into pGL4.10 for analyzing promoter activity. To delete E-boxes, the consensus CANNTG sequences were replaced by TANNTG in the mutant fragments. Reporter gene assay in HeLa cells and C2C12 myoblasts (right panel). The cells were transfected with each Fluc construct, and either *Myod1*-expression or empty vector, together with the internal control Renilla luciferase (Rluc) vector, and harvested after incubation for measurements of Fluc and Rluc activities. Representative datasets are shown from three independent experiments providing essentially the same results. The data represent the mean $\pm$ SEM, and statistical differences compared with WT data are marked with an asterisk (* $P < 0.05$ in ANOVA followed by *t*-test with a Bonferroni correction).

scent myotubes, likely following new fusion events.

The results from CTX-injured muscle indicate that *Gm7325* expression is highly induced in proliferating satellite cells/myoblasts, and that its upregulation is maintained during myotube formation. However, *Gm7325* transcription seems to completely turn off in mature myofibers and quiescent satellite cells. This conclusion is further supported by our observations in the mouse C2C12 myoblast cell line. C2C12 cells actively grow in serum-rich medium and then undergo fusion to form primitive myotubes in low-serum medium. In C2C12 cells, *Gm7325* expression was negligible under the growing conditions, but gradually increased as cells began to differentiate into myotubes (Fig. S1). The expression profile of C2C12 cells seems to consistently mimic that of proliferating myoblasts and nascent myotubes derived from satellite cells.

MyoD is a master transcription factor for skeletal myogenesis and binds to E-boxes in target genes, such as the embryonic myosin gene *Myh3*, the skeletal muscle α -actin gene *Acta1*, and the myogenin gene *Myog* (9). During the regeneration of CTX-in-

jected muscle (Fig. 1A) and C2C12 differentiation (Fig. S1), similar temporal characteristics were observed in *Gm7325* and *Myog* induction, suggesting the possibility that *Gm7325* transcription may be controlled by MyoD. To locate the promoter region, the mRNA start sites of *Gm7325* and its human homologue *LOC101929726* were determined by 5'-RACE (rapid amplification of cDNA ends) experiments (Fig. S2A). In 5'-flanking regions thus defined in both the genes, high sequence identity is observed between the ~200-bp segments immediately upstream from the mRNA start sites. A transcription element search (<http://www.cbil.upenn.edu/tess>) found several consensus motifs in the homologous regions, including E-boxes for basic helix-loop-helix (bHLH) proteins, GC-boxes for SP1 and CACCC-boxes for SP3 (Fig. 3A). Especially, four E-boxes (E1–E4) are fully conserved between the mouse and human genes, and fit well with the MyoD-preferred E-box motif CAC/GC/GTG (9, 13).

The ~250-bp *Gm7325* 5'-flanking DNA fragment was cloned into a reporter vector containing the firefly luciferase (Fluc) gene, and mutant vectors were

also constructed by deleting each or all of the E-box sequences (Fig. 3B). These vectors were subjected to a dual luciferase reporter assay using the Renilla luciferase (Rluc) as an internal control. In HeLa cells derived from human cervical cancer, the promoter activity of the 5'-flanking fragment was significantly enhanced by MyoD coexpression. This MyoD-dependent enhancement was abolished by deleting the E-box motifs from the reporter construct. Moreover, in C2C12 cells, the promoter activity depending upon MyoD coexpression was highly enhanced, as clearly recognized by the augmentation of Fluc/Rluc activity ratio (see the vertical scales). The MyoD-mediated enhancement was obviously attenuated by the loss of E2 or E4 motif from the promoter segment. Therefore, MyoD seems to up-regulate *Gm7325* expression by recognizing both the E-box motifs in the promoter region. Recently, several chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq) experiments using anti-MyoD antibodies have been performed in C2C12 cells from myoblast to myotube stages (1, 3, 6), and these data on genome-wide MyoD-binding were bioinformatically processed and summarized as GEO datasets in the NCBI database (accession number, SRX142534). In the publically-available datasets, specific MyoD binding was observed in the *Gm7325* promoter with intensities comparable to those of well-established MyoD-target genes (Fig. S2B). Taken together, the observations indicate that *Gm7325* promoter is targeted by MyoD in myoblasts and myotubes.

It is worth to note that the MyoD-dependent enhancement of the *Gm7325* reporter expression was fairly potent in C2C12 cells but remained quite limited in HeLa cells. MyoD forms active heterodimers with bHLH E-proteins encoded by the *TCF3*, *4* and *12* genes, and also generates inactive heterodimers lacking DNA-binding activity with ID1–4 proteins (10, 13). The difference between C2C12 and HeLa cells may be explained by cell type-specific expression of MyoD-binding partners. Our RT-PCR analysis implied that *TCF3* and *TCF4* expressions are weaker but *ID1–3* expressions are higher in HeLa cells, compared with C2C12 myoblasts (Fig. S2C). It might be that MyoD expressed in HeLa cells becomes largely inactive under unfavorable cellular conditions, in which insufficient E12/47 (*TCF3* products) and E2-2 (*TCF4* product) contents and relatively high ID1–3 levels are predicted. On the other hand, RT-PCR analysis also detected that *Id1–3* expressions tend to decrease and *Tcf* expressions are roughly constant during myotube formation in C2C12 cells, suggesting the possibility that lowered ID pro-

tein levels take part in the endogenous *Gm7325* induction.

It has been previously reported that *Gm7325* encoding a small protein is expressed in embryonic stem and germ line cells (4). Our observations indicate that *Gm7325* transcription is MyoD-dependently induced in activated satellite cells, and this induction may contribute to skeletal myogenesis. Currently, three independent research groups reported that the *Gm7325* peptide, differentially named Myomixer, Minion or Myomerger, exerts an essential role in myotube formation (2, 8, 14). The *Gm7325* peptide probably contributes to myoblast fusion process, although the detailed molecular mechanisms remain to be clarified.

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Supporting Data

Supporting Methods

Gene expression analysis. All animal experiments in this report were conducted with the approval of the Animal Research Committee at Kyoto University according to the regulations for animal experimentation. CTX-injected TA muscles and hindlimbs were prepared from young-adult C57BL/6J mice, and mononucleated cells were isolated from CTX-injected muscle and fractionated on FACS as described previously (5, 7, 12). Mouse C2C12 myoblasts were maintained in DMEM supplemented with 10% fetal bovine serum, and treated with DMEM containing 2% horse serum for differentiation into myotubes. Total RNAs were extracted from muscle tissues and cell preparations using TRIzol LS (Ambion) or ISOGEN reagent (Nippon Gene Inc, Japan) and used as template for reverse transcription using ReverTra Ace qPCR-RT master mix with gDNA remover (Toyobo Co, Japan). The resulting cDNAs were subjected to quantitative RT-PCR analysis on LightCycler 96 instrument (Roche Molecular Systems) using the primer sets listed in Table SI. The cycle threshold (Ct) was determined in each reaction, and normalized to internal controls using the comparative Ct method when the occasion demands.

In situ hybridization analysis. Formalin-fixed paraffin sections (5 μ m in thickness) were prepared from control and injured TA muscles on day 3 after unilateral CTX injection. The sections were stained using an RNAscope 2.5 HD detection kit (Red) and the RNAscope probe Mm-Gm7325 (Advanced Cell

Diagnostics), and then counterstained with Gill's hematoxylin I (Polysciences, Inc) according to the manufacturer's instructions. Staining images were captured using a digital camera-mounted photomicroscope (DP21-CKX41, Olympus).

Reporter gene assay. To define mRNA start sites in *Gm7325* and *LOC101929726*, total RNAs were prepared from C2C12 cells and CD56⁺/CD82⁺ cells (11) separated from human skeletal muscle myoblasts (Lonza). The 5'-terminal cDNA segments were reverse-transcribed from the RNA preparations and amplified using 5'-full RACE core set (Takara Co, Japan) and the primer sets listed in Table SII. The resulting RACE products were examined by agarose gel electrophoresis, and cloned into a plasmid vector for sequence analysis. The dual-luciferase reporter assay system was applied as instructed by the manufacturer (Promega). The 230-bp *Gm7325* genomic fragment (−222 to +8 in Fig. 3A) was cloned into pGL4.10, and mutated to delete the E-box consensus sequences (see Fig. 3B). The *Myod1* protein-coding region was amplified from a C2C12 cDNA preparation and cloned into pcDNA3.1 (Invitrogen) to yield pMyoD. HeLa and C2C12 cells were transfected with plasmid sets using Lipofectamine LTX (Invitrogen); 25 ng of pGL4.10-based reporter gene plasmid, 470 ng of pMyoD (or empty pcDNA3.1 plasmid), and 5 ng of pGL4.73 per well in 24-well dishes. The cells were harvested 24 h post-transfection and subjected to analysis of Fluc and Rluc activities using Dual-Glo Luciferase Assay System and GloMax 96 Microplate Luminometer (Promega).

Table SI Primers used in RT-PCR analysis

<i>Gm7325</i> mRNA	For	GCTTCAGGTGCAGGTCCTG	<i>Tcf3</i> mRNA	For	GCTACCAGCTGCATGGATCT
	Rev	GGTTAGAACTGGTGCAGGTC		Rev	CTGCTGGCTGTAGTCCCTC
<i>18S rRNA</i>	For	CTCAACACGGGAAACCTCAC	<i>Tcf4</i> mRNA	For	TCCGATGTCCACGTTCCATC
	Rev	AGACAAATCGCTCCACCAAC		Rev	AGCTGTTGTTCTGTGGTCA
<i>Gapdh</i> mRNA	For	TGTGTCCGTCGTGGATCTGA	<i>Tcf12</i> mRNA	For	ACCAGTCTGCCTACTAGCCA
	Rev	TTGCTGTTGAAGTCGCAGGAG		Rev	ACAGGACCGAATGATTGCCA
<i>Pdgfra</i> mRNA	For	GACGAGTGTCTTCGCCAAAGTG	<i>ID1</i> mRNA	For	CGACTACATCAGGGACCTTC
	Rev	CAAAATCCGACCAAGCACGAGG		Rev	TCAGCGACACAAGATGCGAT
<i>Adgre1</i> mRNA	For	AAGCATCCGAGACACACACA	<i>ID2</i> mRNA	For	TATTGTCAGCCTGCATCACC
	Rev	GGCAAGACATAACCAGGGAGA		Rev	AGCCACACAGTGCTTTGCTGTCA
<i>Myog</i> mRNA	For	TCCCAACCCAGGAGATCATTTG	<i>ID3</i> mRNA	For	CACCTTCCCATCCAGACAGC
	Rev	ACAATCTCAGTTGGGCATGG		Rev	GGGCCCATCCCTGCCGTCC
<i>Myod1</i> mRNA	For	CAACTGCTCTGATGGCATGATG	<i>ID4</i> mRNA	For	GAGACGCACCCGGCCCTGCT
	Rev	AGATGCGCTCCACTATGCTG		Rev	TGTCGCCCTGCTTGTTTACC
<i>Ctnna2</i> mRNA	For	TGAGACAATGCGGATTGCCT	<i>TCF3</i> mRNA	For	GCTACCAGCTGCATGGAGCA
	Rev	CCAAGGCCTCCTCGACAATT		Rev	CTGCCAGCTGTGGTCCCTC
<i>Id1</i> mRNA	For	CGACTACATCAGGGACCTGC	<i>TCF4</i> mRNA	For	TCCGATGTCCACTTTCCATC
	Rev	TCAGCGACACAAGATGCGAT		Rev	AGCTGTTGTTAGTGTGATCT
<i>Id2</i> mRNA	For	TATCGTCAGCCTGCATCACC	<i>TCF12</i> mRNA	For	ACCAGTTTGCCTGCTGGTCA
	Rev	AGCCACAGAGTACTTTGCTATCA		Rev	ACAGGACTGAATGATTGCCA
<i>Id3</i> mRNA	For	CATCTCCCGATCCAGACAGC	<i>GAPDH</i> mRNA	For	CGCTCTCTGCTCCTCCTGTT
	Rev	GAAGCTCATCCATGCCCTCA		Rev	CCATGGTGTCTGAGCGATGT
<i>Id4</i> mRNA	For	GAGACTCACCTGCTTTGCT			
	Rev	TGTCACCCTGCTTGTTACAG			

Table SII Primers used in 5'-RACE analysis

	RT	pCCGGACTGTCTTCG		RT	pGCAAGGCATTCTTCC
<i>Gm7325</i>	S1	GTTAGTCCAGGCGTCGGTCC	<i>LOC101929726</i>	S1	GGACAGAAGATATAGTGAGG
5'RACE	S2	ATGGACGGCAGACTGCCCAG	5'RACE	S2	AAAAAGCTTCAGACATGCCTCTCCACTCC
	A1	TGGGCGTTGCTGTTTCCAGG		A1	CTTGTGGCCTCACTTTTGGG
	A2	ACTTCTGGGGGGCCCAATCTC		A2	CTAACCTCTCCCTCCTCTCC

Fig. S1 *Gm7325* expression in C2C12 cells. **(A)** Culture protocol and cellular images for C2C12 myoblast differentiation. Scale bar, 50 μ m. **(B)** *Gm7325* expression profile during C2C12 cell differentiation. The data were derived from 4–8 reactions using three separate RNA preparations, and represent the mean $\pm$ SEM.; error bars fall within the symbols and thus are not shown in most cases.

Fig. S2 Characterization of 5'-flanking regions in *Gm7325*. **(A)** 5'-RACE analysis. (upper panels) To locate the transcription initiation site in mouse *Gm7325*, RACE fragments were examined by agarose gel electrophoresis (gel image, M: size markers; P: RACE products) and subjected to sequencing. Of ten independent RACE clones sequenced, three clones carried the start site (sequence electropherogram) corresponding to the A residue numbered +1 in Fig. 3A, and the rest contained partial cDNAs starting at residues +2~31. (lower panels) The start site of human *LOC101929726* was also located by sequencing RACE fragments (gel image) as indicated by the A residue numbered +1 in Fig. 3A. Of thirteen independent RACE clones sequenced, five clones carried the full-length cDNA (electropherogram) and the rest contained partial cDNAs starting at residues +5~45. **(B)** MyoD-binding profile for *Gm7325* 5'-flanking region in C2C12 cells. At the NCBI GEO datasets under the accession number SRX142534, ChIP-seq indexes around the 5'-flanking regions were downloaded (RPM, reads per million fragments mapped), and are presented together with graphic exon-intron organization images in typical marker genes as well as *Gm7325*. The genomic segments profiled are derived from the skeletal muscle actin $\alpha 1$ (*Acta1*), embryonic myosin heavy chain (*Myh3*), myogenin (*Myog*), cysteine and glycine-rich protein 3 (*Csrp3*), catenin $\alpha 2$ (*Ctnna2*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and aldolase A (*Aldoa*) genes. **(C)** Relative expressions of MyoD-binding partner genes in HeLa cells, C2C12 myoblasts and myotubes. Gene expression levels defined by RT-PCR were normalized to *Gapdh* or *GAPDH* mRNA content in each cell preparation. The data represent the mean $\pm$ SEM, and statistical differences compared with the data from C2C12 myoblasts are marked with asterisks (* $P < 0.05$ and ** $P < 0.01$ in ANOVA followed by *t*-test with a Bonferroni correction).