Krüppel-like factor 5 (Klf5) regulates expression of mouse T1R1 amino acid receptor gene (Tas1r1) in C2C12 myoblast cells

Yuki Hirata1, Takashi Toyono2, Shoichiro Kokabu3, Yui Obikane1, Shinji Kataoka2, Mitsushiro Nakatomi2, Chihiro Masaki1, Ryuji Hosokawa1, and Yuji Seta2

1 Division of Oral Reconstruction and Rehabilitation, Department of Oral Functions, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, Japan; 2 Division of Anatomy, Department of Health Promotion, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, Japan; and 3 Division of Molecular Signaling and Biochemistry, Department of Health Promotion, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, Japan

(Received 12 November 2018; and accepted 31 January 2019)

ABSTRACT

T1R1 and T1R3 are receptors expressed in taste buds that detect L-amino acids. These receptors are also expressed throughout diverse organ systems, such as the digestive system and muscle tissue, and are thought to function as amino acid sensors. The mechanism of transcriptional regulation of the mouse T1R1 gene (Tas1r1) has not been determined; therefore, in this study, we examined the function of Tas1r1 promoter in the mouse myoblast cell line, C2C12. Luciferase reporter assays showed that a 148-bp region upstream of the ATG start codon of Tas1r1 had promoter activity. The GT box in the Tas1r1 promoter was conserved in the dog, human, mouse, and pig. Site-directed mutagenesis of this GT box significantly reduced the promoter activation. The GT box in promoters is a recurring motif for Sp/KLF family members. RNAi-mediated depletion of Sp4 and Klf5 decreased Tas1r1 expression, while overexpression of Klf5, but not Sp4, significantly increased Tas1r1 expression. The ENCODE data of chromatin immunoprecipitation and sequencing (ChIP-seq) showed that Klf5 bound to the GT box during the myogenic differentiation. Furthermore, the Klf5 knockout cell lines led to a considerable decrease in the levels of Tas1r1 expression. Collectively, these results showed that Klf5 binds to the GT box in the Tas1r1 promoter and regulates Tas1r1 expression in C2C12 cells.

T1R1 and T1R3 (encoded by Tas1r1 and Tas1r3) form the amino acid receptor that binds L-amino acids, including L-glutamate (17). Besides T1R1 and T1R3 expression in taste buds, these receptors are also expressed throughout diverse organ systems, including the digestive system and muscle tissue, and are considered as amino acid sensors (4, 11, 32). The heterodimer of T1R1 and T1R3 (T1R1/T1R3) is a direct sensor of the fed state and amino acid availability (32). Knockout of Tas1r3 reduces the capacity of amino acids to signal to the mammalian target of rapamycin C1 (mTORC1) and induces autophagy. T1R1/T1R3 is also expressed at various differentiation periods of C2C12 cells (34). T1R1/T1R3-mediated amino acids regulate extracellular signal-regulated kinase 1 and 2 and mTORC1 through intracellular calcium increase in C2C12 myotubes. Furthermore, T1R1/T1R3 modulates amino acid-induced insulin secretion in pancreatic β-cells (19, 32).

Regulation of Tas1r3 transcription has been investigated. Fushan et al. reported that a novel cis-acting element exists in a distal region of the human TAS1R3 promoter (6), while human TAS1R3 expression is regulated by the CCAAT-enhancer-binding protein β (C/EBPβ) in the cholangiocarcinoma cell
ments were subcloned and sequenced. Plasmids. The upper regions of mouse *Tas1r1* from −886 to +54 bp (Fig. 2A) were amplified by PCR using genomic DNA from a mouse *Tas1r1* BAC (RP-23-37G1; Advanced GenoTechs, Tsukuba, Japan) and the primers listed in Table 1. The PCR fragments were purified from agarose gels after electrophoretic separation, cleaved with *Kpn*I, and then ligated into *Kpn*I/*EcoRV*-linearized pGL4.10 (Promega, Madison, WI, USA) to form pGL−886/+54, pGL−803/+54, pGL−450/+54, pGL−94/+54, and pGL−37/+54. These plasmids were sequenced to check the correct sequences and orientations.

Human *Sp4* cDNA (40034749; Open Biosystems) was cleaved with *Kpn*I/*Xba*I, and ligated into *Kpn*I/*Xba*I-linearized pCMV-SPORT6 to form pCS6-hSp4. Human *KLF5* cDNA (BC042131; Open Biosystems) was cleaved with *EcoRI/*Xho*I and inserted into *EcoRI/*Xho*I-linearized pCMV-SPORT6 to form pCS6-hKLF5.

Site-directed mutagenesis. Site-directed mutagenesis was used to form pGLmEbox and pGLmGT1 using pGL−94/+54 as a template. Site-directed mutagenesis was also used to form pGLmGT2 using pGL−886/+54 as a template. The PrimeSTAR® Mutagenesis Basal Kit (TaKaRa, Otsu, Japan) and the primers listed in Table 2 were used to perform mutagenesis. The core sequence of a putative E box at bases −63 to −58 (shown in bold type) was deleted (CAAC TG to TG). The core sequence of a putative GT box at bases −50 to −41 was mutated (GTCCCACCCC to GTCCA CGAT). After mutagenesis, sequence analyses of the mutated E box and GT box were performed to validate the mutation.

Luciferase assays. For reporter assays, C2C12 cells were plated in 24-well plates. The cells were co-transfected with the firefly luciferase reporter plasmids of line HuCCT1 (30). The myogenic regulatory factors MyoD and myogenin regulate *Tas1r3* promoter activity, and *Tas1r3* expression increases as myoblast C2C12 cells differentiate into skeletal muscle (14). Thus, several mechanisms have been elucidated for transcriptional regulation of *Tas1r3*; however, the mechanisms regulating *Tas1r1* transcription are unknown.

In this study, we investigated the mechanisms regulating the mouse *Tas1r1* gene using reporter assays, quantitative reverse transcription PCR (qRT-PCR), overexpression and RNAi knockdown assays, and chromatin immunoprecipitation sequencing (ChIP-seq) in C2C12 cells.

MATERIALS AND METHODS

Cell culture. The mouse myoblast cell line C2C12 (RCB0987) was provided by the RIKEN BioResource Center through the National BioResource Project of the MEXT, Japan. C2C12 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum at 37°C with 5% CO2. To induce myogenic differentiation, DMEM was replaced with DMEM containing 2% horse serum (Sigma-Aldrich, Saint Louis, MO, USA) when the density of the cells reached 90%. The medium was changed daily.

Rapid amplification of 5′ cDNA ends (5′-RACE) analysis. Total RNA was extracted from C2C12 cells using a GenElute™ mammalian total RNA mini-prep kit (Sigma-Aldrich). Poly(A)-RNA was prepared from the total RNA of C2C12 cells using a GenElute™ mRNA Mini-prep Kit (Sigma-Aldrich). Two hundred and fifty nanograms of poly(A)-RNA was reverse transcribed and amplified using a GeneRacer™ Kit (Invitrogen, Carlsbad, CA, USA) with *Tas1r1* specific primers (Table 1). The PCR fragments were subcloned and sequenced.

Plasmids. The upper regions of mouse *Tas1r1* from −886 to +54 bp (Fig. 2A) were amplified by PCR using genomic DNA from a mouse *Tas1r1* BAC (RP-23-37G1; Advanced GenoTechs, Tsukuba, Japan) and the primers listed in Table 1. The PCR fragments were purified from agarose gels after electrophoretic separation, cleaved with *Kpn*I, and then ligated into *Kpn*I/*EcoRV*-linearized pGL4.10 (Promega, Madison, WI, USA) to form pGL−886/+54, pGL−803/+54, pGL−450/+54, pGL−94/+54, and pGL−37/+54. These plasmids were sequenced to check the correct sequences and orientations.

Human *Sp4* cDNA (40034749; Open Biosystems) was cleaved with *Kpn*I/*Xba*I, and ligated into *Kpn*I/*Xba*I-linearized pCMV-SPORT6 to form pCS6-hSp4. Human *KLF5* cDNA (BC042131; Open Biosystems) was cleaved with *EcoRI/*Xho*I and inserted into *EcoRI/*Xho*I-linearized pCMV-SPORT6 to form pCS6-hKLF5.

**Table 1. Primers used for 5′-RACE and construction of luciferase assay plasmids**

| Project/ primer name | Reverse strand (5′–3′) | Forward strand (5′–3′) |
|----------------------|------------------------|-----------------------|
| 5′-RACE/ GRL         | CAATGAGTGCACCCACCTTTGGAGG | ACAGCGTGTCAGTTATCAGGC |
| Luciferase assay plasmid/ | | |
| −886/+54            | GGGTACGGATCTCAGGACCGGTTCAC | GCTGCCCCAGAGTGCTTCTGTCAGCAT |
| −803/+54            | GGGTACCATCATCTCGGACCTCAGCC | GCTGCCCCAGAGTGCTTCTGTCAGCAT |
| −450/+54            | GGGTACCGGAAAGCTGTTTACAGGTTT | GCTGCCCAGAGTGCTTCTGTCAGCAT |
| −94/+54             | GGGTACCAGCCGGAGGCTGAGTTAAGCA | GCTGCCCAGAGTGCTTCTGTCAGCAT |
| −37/+54             | GGGTACCAGGGGACTGACCTTATGAA | GCTGCCCAGAGTGCTTCTGTCAGCAT |

**Luciferase assays.** For reporter assays, C2C12 cells were plated in 24-well plates. The cells were co-transfected with the firefly luciferase reporter plasmids of...
Klf5 regulates Tas1r1 expression by reverse transcription of 2 μg total RNA using a Superscript® VILO™ cDNA synthesis kit (Invitrogen). The cDNA was amplified by qRT-PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the Eco Real-Time PCR System (IIllumina Inc., San Diego, CA, USA). TaqMan primers and probes (Applied Biosystems) were used for each of the following genes: Tas1r1 (Mm00473433_m1), Tas1r2 (Mm00499716_m1), Tas1r3 (Mm00473459_g1), Klf5 (Mm00456521_m1), myogenin (Mm00446194_m1), and peptidylprolyl isomerase A (Ppia) (Mm02342430_g1). The Ppia primers were used to normalize results obtained with gene-specific primers.

Immunoblot analysis. Transfected C2C12 cells or myogenically differentiated C2C12 cells were washed with PBS and lysed in sodium dodecyl sulfate sample buffer containing a protease inhibitor cocktail. The lysate was forced through a 20-gauge needle twenty times to reduce the viscosity of the solution. A 1/10 vol. 0.2 M dithiothreitol was added to each sample and heated at 99°C for 3 min prior to gel application. The MISSION predesigned siRNAs were: MISSION siRNA Universal Negative Control (UNC; SIC001), mouse Sp1 siRNA (SASI_Mm01_00145222), mouse Sp3 siRNA (SASI_Mm01_00149247), mouse Sp4 siRNA (SASI_Mm01_00067676), mouse Klf2 siRNA (SASI_Mm02_00313949), mouse Klf4 siRNA (SASI_Mm01_00104981), and mouse Klf5 siRNA (SASI_Mm02_00316973).

**Table 2** Primers used for site-directed mutagenesis and CRISPR-Cas9 mediated knockout study

| Project/primer name | Reverse strand (5’–3’) | Forward strand (5’–3’) |
|---------------------|------------------------|------------------------|
| Site-directed mutagenesis/ |                        |                        |
| Ebox del.            | CCTTCCCATGGGCTCCAGTCCCACCGGCAAGCCCATGGGAAGGCTGTTGGCCT | GAGCCCATGGGAAAGCTTGTGGCCT |
| GT mut.              | AGTCCCAACCTTCAGGGGACCTGACG | GAAAGGTTTGGGACTGGAGCCCAGTT |
| CRISPR-Cas9 mediated knockout study/ |                        |                        |
| Klf5KO1              | CACCGTGCTCTGAAATTCGGGAC | AAAAGTTCGGTAATTTTCAGAGCAC |
| CH1                  | CCTTCCCTAGTACGTCGGTTAGATATACGGT | AGGTGAGTGTACGGAGGAGGAACGCTTC |
| Klf5KO2              | CACCGGAGGGGCGCTCGACTGCTC | AAAAGCAAGCGTGAGCCGCCCTCC |
| CH2                  | CCTGACATCACCTCACTTCGAGACTGGCCTC | GATATGGTCACCTCCGTGGTGCGGCGTGGT |

RNAsi-based gene silencing. We carried out two sequential steps of siRNA transfection to deplete Sp1, Sp3, Sp4, Klf2, Klf4, and Klf5 (29). Trypsinized C2C12 cell suspensions (2 × 10⁵ cells) were transfected with 25 nM of each specific MISSION® siRNA (Sigma Genosys, Ishikari, Japan) using Lipofectamine RNAi MAX (Invitrogen). The transfected cells were seeded in 3.5-cm dishes. After being cultured for 16 h, the culture medium was replaced with fresh culture medium and the cells were incubated for 8 h. Re-transfection of siRNA was performed per abovementioned conditions. Total protein and total RNA were prepared 48 h after the second transfection.

The MISSION predesigned siRNAs were: MISSION siRNA Universal Negative Control (UNC; SIC001), mouse Sp1 siRNA (SASI_Mm01_00145222), mouse Sp3 siRNA (SASI_Mm01_00149247), mouse Sp4 siRNA (SASI_Mm01_00067676), mouse Klf2 siRNA (SASI_Mm02_00313949), mouse Klf4 siRNA (SASI_Mm01_00104981), and mouse Klf5 siRNA (SASI_Mm02_00316973).

Transfection and qRT-PCR. The expression plasmids of Sp4, Klf5 or the empty vector pCMV-SPORT6 were transfected to C2C12 cells using Lipofectamine 3000 (Invitrogen) in 6-well plates. Total RNA was prepared 24 h after the transfection. Following incubation with TURBO™ DNase (Ambion, Austin, TX, USA) to remove any contaminating genomic DNA, first-strand cDNA synthesis was performed by reverse transcription of 2 μg total RNA using a Superscript® VILO™ cDNA synthesis kit (Invitrogen). The cDNA was amplified by qRT-PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the Eco Real-Time PCR System (IIllumina Inc., San Diego, CA, USA). TaqMan primers and probes (Applied Biosystems) were used for each of the following genes: Tas1r1 (Mm00473433_m1), Tas1r2 (Mm00499716_m1), Tas1r3 (Mm00473459_g1), Klf5 (Mm00456521_m1), myogenin (Mm00446194_m1), and peptidylprolyl isomerase A (Ppia) (Mm002342430_g1). The Ppia primers were used to normalize results obtained with gene-specific primers.

**Table 2** Primers used for site-directed mutagenesis and CRISPR-Cas9 mediated knockout study

| Project/primer name | Reverse strand (5’–3’) | Forward strand (5’–3’) |
|---------------------|------------------------|------------------------|
| Site-directed mutagenesis/ |                        |                        |
| Ebox del.            | CCTTCCCATGGGCTCCAGTCCCACCGGCAAGCCCATGGGAAGGCTGTTGGCCT | GAGCCCATGGGAAAGCTTGTGGCCT |
| GT mut.              | AGTCCCAACCTTCAGGGGACCTGACG | GAAAGGTTTGGGACTGGAGCCCAGTT |
| CRISPR-Cas9 mediated knockout study/ |                        |                        |
| Klf5KO1              | CACCGTGCTCTGAAATTCGGGAC | AAAAGTTCGGTAATTTTCAGAGCAC |
| CH1                  | CCTTCCCTAGTACGTCGGTTAGATATACGGT | AGGTGAGTGTACGGAGGAGGAACGCTTC |
| Klf5KO2              | CACCGGAGGGGCGCTCGACTGCTC | AAAAGCAAGCGTGAGCCGCCCTCC |
| CH2                  | CCTGACATCACCTCACTTCGAGACTGGCCTC | GATATGGTCACCTCCGTGGTGCGGCGTGGT |
Cloning from tongue tissue (Site T; Fig. 1A) (9). Our results indicated that Sites 2 and 4 are the major TSSs in the human and mouse tissues. Sites 2 and 4 are represented in 7 and 8 clones (Fig. 1A). The other two sites (Sites 1 and 3) are represented in 3 clones. Among these sites, Site 4 is indicated as +1.

The downstream promoter element (DPE) lies 30 bp downstream of the TSS. The initiator lies around the TSS. For Site 4, at nucleotide position 54 bp upstream of the ATG in the first exon, the initiator and DPE were found between −2 to +34 (Fig. 1A, B). In the other TSSs (Sites 1–3), the initiator and DPE were not found. The TATA box generally lies ~25–31 bp upstream of the TSS and has a consensus TATA(A/T)A(A/T) sequence. This DNA element was not located around these identified TSSs in the Tas1r1 promoter (Fig. 1A).

The E box has a consensus CANNTG sequence and was located ~58–63 bp upstream of the Site 4 TSS (Fig. 1A). The GT box has a consensus CCACCC sequence and was located ~42–48 bp upstream of the Site 4 TSS (Fig. 1A). These boxes...
Klf5 regulates Tas1r1 expression

5’ truncations of the Tas1r1 promoter driving the expression of a luciferase reporter (Fig. 2). Transfection of C2C12 cells with pGL−803/+54 showed an 18.2-fold higher reporter activity compared with transfection with pGL4.10. Though deletion of the 5’-end from −857 to −94 bp did not change the reporter activity, additional deletion of the 5’-end from −94 bp to −37 bp resulted in an 87.2% decrease in the reporter activity (Fig. 2A, pGL−37/+54). The luciferase reporter assay showed that the 148-bp upstream region of ATG had promoter activity. These results indicated that the two regions (−886 to −803 and −94 to −37 bp) included cis-acting elements that are critically involved in Tas1r1 promoter activity.

The putative E box and GT box were identified in the region −94 to −37 bp (Fig. 1A). To elucidate the cis-acting element from −94 and −37 bp and its effect on modulating promoter activity, additional reporter plasmids were constructed by site-directed mutagenesis of the E box and GT box. Reporter activity was slightly increased by the deletion of the E box (Fig. 2B, pGLmEbox). On the other hand, mutation of the GT box resulted in a 61.9% decrease in reporter activity (Fig. 2B, pGLmGT1), and the mutation of GT box in pGL−886/+54 resulted in a 63.6% decrease in reporter activity (Fig. 2C, pGLmGT2). These reduction data indicated that the GT box may be a binding site for trans-acting factors.

Knockdown of Sp/KLF family members by siRNA represses transactivation of the Tas1r1 promoter

Site-directed mutagenesis analysis showed that trans-acting factors bind the GT box of the Tas1r1 promoter. The Sp/KLF family of transcription factors regulate gene expression by binding to GC or GT boxes in regulatory regions (24). To assess the role of Sp/KLF family members in the regulation of the Tas1r1 promoter, we transfected C2C12 cells with siRNAs targeting Sp1, Sp3, Sp4, Klf2, Klf4, or Klf5. Fig. 3A shows a significant decrease in the protein levels of these factors. Transfection with Sp4 and Klf5 siRNA resulted in a 57.6% and 47% decrease in the level of Tas1r1 expression relative to UNC (Fig. 3B). While transfection with Sp1, Sp3, Klf2, and Klf4 siRNA increased the level of Tas1r1 expression relative to UNC (Fig. 3B).

Transactivation of the mouse Tas1r1 promoter by Sp4 and Klf5

Next, we examined whether Sp4 or Klf5 activates...
ChIP-seq

ChIP-seq peaks for Klf5 around Tas1r1 were identified using the UCSC Genome Browser. Major Klf5 peaks were detected within the upstream region of the Tas1r1 first exon during the myogenic differentiation process (days 0, 2, and 5) (Fig. 4A). However, other peaks were not detected in the Tas1r1 gene without the first exon. The GT box of the Tas1r1 promoter existed in the region of major Klf5 peaks in days 2 and 5 (Fig. 4B). Klf5 bound to the GT box in days 2 and 5, but with slightly different occupancies. Klf5 bound to the GT box more exten-
Klf5 regulates Tas1r1 expression

qRT-PCR and immunoblot analyses of TIR family expression during the myogenic differentiation of C2C12 cells

C2C12 cells can be induced to differentiate into myoblasts by switching them to medium containing 2% horse serum. In this condition, the cells exhibited outstanding morphological changes over the course of 4–9 days, finally fusing into mature multinucleated myotubes (Fig. 5A). One of the myogenic regulatory factors, myogenin, was up-regulated during C2C12 myogenic differentiation. A 76.5-fold increase in myogenin mRNA levels was observed from day 0 to day 3 and a 125.4-fold increase from day 0 to day 9 (Fig. 5C). The morphological changes and the expression profile of myogenin during C2C12 myogenic differentiation were both in agreement with previous observations (10).

The Tas1r1, Tas1r2, and Tas1r3 genes were expressed during C2C12 myogenic differentiation. Tas1r1 and Tas1r2 expression increased from day 0 to day 9 upon differentiation (Fig. 5B). A 23.1-fold increase in Tas1r1 expression was observed from day 0 to day 3 and a 63.0-fold increase from day 0 to day 9 (Fig. 5B). A 1.7-fold increase in Tas1r2 expression was observed from day 0 to day 9 (Fig. 5B).

Klf5 was also expressed during C2C12 myogenic differentiation. Klf5 expression increased from day 0 to day 3, in contrast to Klf5 mRNA expression (Fig. 5D, E).

Knockout of Klf5 in C2C12 cells suppresses Tas1r1 expression at days 0 and 2 upon myogenic differentiation of C2C12 cells

To assess the role of Klf5 in the regulation of the Tas1r1 promoter during myogenic differentiation of C2C12 cells, we generated the Klf5 knockout cell lines (Klf5KO1 and Klf5KO2) in C2C12 cells using the CRISPR-Cas system (Fig. 6A). Fig. 6B shows a significant decrease in the Klf5 protein levels.
Y. Hirata et al.

Differentiation (Fig. 6E, F). These results showed that Klf5 knockout effected on the myogenic differentiation of C2C12.

Immunoblot analysis for Tas1r1 in Klf5KO2 at differentiation days 2 showed that the Tas1r1 protein level was similar in C2C12 cells (Fig. 6G, H). This result showed that the decrease of the level of Tas1r1 expression did not affect the Tas1r1 protein level in Klf5KO2.

DISCUSSION

Promoters can be classified in accordance with the distribution of the TSSs they use (16). Sharp promoters use only one or a few serial nucleotides as the TSSs, and often have a TATA box and an initiator. In contrast, a broad promoter can start transcription over a ~100 bp region, resulting in a population of mRNAs that have diverse sizes. Broad promoters usually regulate more widely expressed genes and are frequently TATA-less and CpG-island-enriched, while sharp promoters tend to regulate tissue-specific genes. The mouse Tas1r1 promoter had four TSSs in a ~100 bp region and Tas1r1 is also expressed in diverse tissues (32); therefore, it should be classified as a broad promoter.

Knockout of Klf5 (Klf5KO1) resulted in a 79.0% and 83.6% decrease in the level of Tas1r1 expression relative to the C2C12 cells at days 0 and 2 upon myogenic differentiation (Fig. 6C). Knockout of Klf5 (Klf5KO2) also resulted in a 51.9% and 69.1% decrease in the level of Tas1r1 expression relative to the C2C12 cells at days 0 and 2 upon myogenic differentiation (Fig. 6C). To confirm that the Klf5 knockout decreases Tas1r1 expression in Klf5KO1 and Klf5KO2, we examined the rescue of Tas1r1 expression by forced expression of Klf5 in these clones (Fig. 6D). Klf5 had a significant activating effect, causing a 2.6- and 2.5-fold increase in Tas1r1 expression levels in Klf5KO1 and Klf5KO2, compared with that of the empty expression vector pCMV-SPORT6. Taken together, these data indicated that Klf5 plays a role in the regulation of the Tas1r1 expression.

To examine the effects on the myogenic differentiation of C2C12 by Klf5 knockout, we examined the expression profiles of a myogenic regulatory factor, myogenin, at days 0 and 2 upon myogenic differentiation of Klf5KO1 by immunoblot analysis. Knockout of Klf5 resulted in a 30.1% and 53.6% decrease in the protein level of myogenin relative to the C2C12 cells at days 0 and 2 upon myogenic differentiation (Fig. 6E, F). These results showed that Klf5 knockout effected on the myogenic differentiation of C2C12.

Immunoblot analysis for Tas1r1 in Klf5KO2 at differentiation days 2 showed that the Tas1r1 protein level was similar in C2C12 cells (Fig. 6G, H). This result showed that the decrease of the level of Tas1r1 expression did not affect the Tas1r1 protein level in Klf5KO2.
The luciferase reporter assay showed that the promoter region of mouse *Tas1r1* was located between −94 and +54 bp in C2C12 cells. The core promoter comprises elements that can elongate 35 bp upstream and/or downstream of the TSS (5, 26). Most core promoter elements seem to interplay directly with modules of the basal transcription machinery. The core promoter elements, an initiator, and a DPE but not the TATA box, were located in the *Tas1r1* promoter. Initiators can function independently of the TATA box. However in TATA-containing promoters, the initiator acts synergistically to enhance the efficiency of transcription initiation (1). In contrast to the TATA box, the DPE needs an initiator. Therefore, the initiator may function in collaboration with the DPE in the *Tas1r1* promoter.

The Sp/KLF family of transcription factors currently has 26 members (18). This family is charac-
terized by a highly conserved DNA binding domain near the C-terminus, which recognizes GC (consensus sequence: GGGGCGGGG) as well as GT/CACC (GGTGTGGGG) boxes. GC and GT boxes are important regulatory elements in the promoter region of many genes (20, 33). The Tas1r1 promoter has a GT box located −48 to −42 bp upstream of the TSS. Important transcriptional regulatory elements often show conservation between species (15). Sequence alignment revealed conservation of the GT box in identical positions in the dog, human, mouse, and pig species. These results suggested that involvement of the GT box in Tas1r1 transcription has been evolutionarily conserved.

Site-directed mutagenesis analysis showed that the trans-acting factors bound to the GT box of Tas1r1 promoter. However, it was unclear which members of the Sp/KLF family bound to the GT

**Fig. 6** Knockout of Klf5 in C2C12 cells suppresses Tas1r1 expression at days 0 and 2 of myogenic differentiation of C2C12 cells. (A) Sequencing data demonstrating the patterns of genomic editing in the Klf5 knockout clones (Klf5KO1 and Klf5KO2). WT shows untreated C2C12 cells. Protospacer adjacent motif (PAM) underlined; the deletions are indicated by a hyphen (-). (B) The protein levels of the WT (C2C12 cells), Klf5KO1 and Klf5KO2 were analyzed by immunoblot analysis with the indicated antibodies. The level of Klf5 protein was significantly reduced in Klf5KO1 and Klf5KO2. As a control, expression of β-actin is shown. (C) The expression levels of Tas1r1 were measured at days 0 and 2 of myogenic differentiation of WT (C2C12 cells), Klf5KO1, and Klf5KO2 using qRT-PCR. The expression levels were normalized to Ppia expression. The results are expressed as mean ± S.D. for three different experiments. **P < 0.01 when compared with the wild-type clone. (D) Overexpression of Klf5 upregulates Tas1r1 expression in Klf5KO1 and Klf5KO2. The expression levels of Tas1r1 were quantified by qRT-PCR. The expression levels were normalized to Ppia expression. The results are expressed as mean ± S.D. for three different experiments. **P < 0.01 when compared with the control vectors. (E) The protein levels of myogenin were analyzed at days 0 and 2 of myogenic differentiation of WT (C2C12 cells) and Klf5KO1 by immunoblot analysis with the indicated antibodies. (F) Immunoblot quantification of myogenin levels. The expression levels of myogenin were normalized to β-actin. The results are expressed as mean ± S.D. for three different experiments. **P < 0.01 when compared with the wild-type clone. (G) The protein levels of the WT (C2C12 cells) and Klf5KO2 were analyzed by immunoblot analysis with the indicated antibodies. (H) Immunoblot quantification of Tas1r1 levels. The expression levels of Tas1r1 were normalized to β-actin. The results are expressed as mean ± S.D. for three different experiments.
Klf5 regulates Tas1r1 expression

box of the Tas1r1 promoter. Sp1, Klf2, and Klf4 all play a key role in the fusion process during skeletal muscle differentiation (27). In addition, Klf5 is an important factor in skeletal muscle regeneration and myogenic differentiation (8). In the Sp family, Sp1–4 have a very similar modular domain structure (33). Sp1, Sp3, and Sp4 can bind to GT and GC boxes; however, Sp2 has a weaker binding affinity to the GT box (12, 28). Therefore, we examined whether Sp1, Sp3, Sp4, Klf2, Klf4, or Klf5 activates Tas1r1 transcription in C2C12 cells. RNAi-mediated depletion of Sp4 and Klf5 led to a dramatic decrease in the expression of Tas1r1 during C2C12 myogenic differentiation. The knockout of Klf5 by changing its binding affinity for various co-regulators (3). Therefore, we deduced that the inhibition of C2C12 myogenic differentiation was in agreement with the previous report (8). Therefore, it is possible that the inhibition of C2C12 myogenic differentiation by Klf5 knockout decreases the level of Tas1r1 expression. The Klf5 knockdown by Klf5 siRNA in C2C12 myotubes will be necessary for revealing the exact functional role of Klf5 in the regulation of Tas1r1 expression in a direct or indirect manner.

Klf5 was expressed during C2C12 myogenic differentiation; however, its expression decreased from day 0 to day 3. These results showed that Klf5 expression level was not consistent with that of Tas1r1. It is possible that Tas1r1 expression during C2C12 myogenic differentiation is regulated by other trans-acting factors in addition to Klf5. Klf5 undergoes many post-translational modifications, which can alternatively regulate the transactivation capacity of Klf5 by changing its binding affinity for various co-regulators (3). Therefore, we deduced that the post-translational modifications of Klf5 modulated the regulation of Tas1r1 expression during C2C12 myogenic differentiation.

In conclusion, we have demonstrated that Klf5 binds to the GT box of the Tas1r1 promoter and regulates Tas1r1 expression in C2C12 cells.

qRT-PCR analysis showed that Tas1r1 expression was markedly increased after induction of C2C12 myogenic differentiation. In addition, immunoblot analysis for Tas1r1 showed that protein expression increased from day 0 to day 3 upon differentiation. These expression patterns upon C2C12 myogenic differentiation were in agreement with previous observations (34). T1R1/T1R3 is an amino acid sensor and regulates mTORC1 and autophagy in a wide variety of tissues (32). mTOR is also a key regulator of skeletal myogenesis by governing multiple stages of myogenic differentiation (7). Therefore, the increased expression of Tas1r1 may regulate mTORC1 in multiple stages of myogenic differentiation.

The CREB-binding protein (CBP/p300) is essential for muscle cell terminal differentiation, and CBP/p300 interacts with MyoD (21, 22). In addition, Klf5 and MyoD jointly modulate muscle differentiation by directly targeting muscle-specific genes in mice (8). Therefore, Klf5 may interact with CBP/p300 and MyoD in the activation of the Tas1r1 promoter during C2C12 myogenic differentiation.

Tas1r1 expression was markedly increased during C2C12 myogenic differentiation. The knockout of Klf5 (Klf5KO1 and Klf5KO2) resulted in a substantial decrease in the level of Tas1r1 expression relative to that in C2C12 cells at day 0 and 2 upon myogenic differentiation. In addition, knockout of Klf5 resulted in a significant decrease in the protein level of myogenin relative to the C2C12 cells at days 0 and 2 upon myogenic differentiation. This expression pattern of Klf5 knockout clone upon C2C12 myogenic differentiation was in agreement with the previous report (8). Therefore, it is possible that the inhibition of C2C12 myogenic differentiation by Klf5 knockout decreases the level of Tas1r1 expression. The Klf5 knockdown by Klf5 siRNA in C2C12 myotubes will be necessary for revealing the exact functional role of Klf5 in the regulation of Tas1r1 expression in a direct or indirect manner.

Acknowledgements

This work was supported by research grants from The Society for Research on Umami Taste and the Japan Society for the Promotion of Science, Grant-in-Aid for Scientific Research (C), 16K11455, 2016. We thank Ms. Midori Ishikawa for support during this work.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

REFERENCES

1. Allison LA (2007) Transcription in eukaryotes In the Fundamental Molecular Biology, pp. 312–391. Blackwell Publishing, Malden, MA.
2. Consortium EP (2012) An integrated encyclopedia of DNA elements in the human genome. Nature 489, 57–74.
3. Diakiew SM, D’Andrea RJ and Brown AL (2013) The double life of KLF5: Opposing roles in regulation of gene-expression, cellular function, and transformation. IUBMB Life 65, 999–1011.
4. Finger TE and Kinnamon SC (2011) Taste isn’t just for taste buds anymore. F1000 Biol Rep 3, 20.
5. Fukue Y, Sumida N, Nishikawa J and Ohyama T (2004) Core buds anymore. Nature 439, 293–305.
6. Fushan AA, Simons CT, Slack JP, Manichaikul A and Drayna D (2009) Allelic polymorphism within the TASSR3 promoter is associated with human taste sensitivity to sucrose. Curr Biol 19, 1288–1293.
7. Ge Y and Chen J (2012) Mammalian target of rapamycin (mTOR) signaling network in skeletal myogenesis. J Biol Chem 287, 43924–43935.
8. Hayashi S, Manabe I, Suzuki Y, Relaix F and Oishi Y (2016) human genome browser at UCSC. Genome Res 24, 996–1006.
9. Janssen S and Depoortere I (2013) Nutrient sensing in the gut: new roads to therapeutics? Trends Endocrinol Metab 24, 99–111.
10. Joo J, Park K, Lee H, Kim H, Cho S et al. (2013) Analysis of nuclear lamina proteins in myoblast differentiation by functional complementation. Methods Mol Biol 703, 177–194.
11. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH et al. (2002) The human genome browser at UCSC. Genome Res 12, 996–1006.
12. Kozak C, Cook T and Urrutia R (2003) Sp1- and Krüpel-like transcription factors. Curr Biol 13, 996–1006.
13. Kozak C, Cook T and Urrutia R (2003) Sp1- and Krüpel-like transcription factors. Curr Biol 13, 996–1006.
14. Kokabu S, Lowery JW, Toyono T, Seta Y, Hitomi S et al. (2001) CBP/p300 and muscle differentiation: no HAT, no muscle. EMBO J 20, 6816–6825.
15. Kozak C, Cook T and Urrutia R (2003) Sp1- and Krüpel-like transcription factors. Curr Biol 13, 996–1006.
16. Kozak C, Cook T and Urrutia R (2003) Sp1- and Krüpel-like transcription factors. Curr Biol 13, 996–1006.
17. Kozak C, Cook T and Urrutia R (2003) Sp1- and Krüpel-like transcription factors. Curr Biol 13, 996–1006.
18. O’Connor L, Gilmour J and Bonifer C (2016) The role of the ubiquitously expressed transcription factor Sp1 in tissue-specific transcriptional regulation and in disease. Yale J Biol Med 89, 513–525.
19. Oya M, Suzuki H, Watanabe Y, Sato M and Tsuibo T (2011) Amino acid taste receptor regulates insulin secretion in pancreatic β-cell line MIN6 cells. Genes Cells 16, 608–616.
20. Philippsen S and Suske G (1999) A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res 27, 2991–3000.
21. Polesskaya A, Naguibneva I, Fritsch L, Duquet A, Ait-Si-Ali S et al. (2001) CBP/p300 and muscle differentiation: no HAT, no muscle. EMBO J 20, 6816–6825.
22. Riuo P, Bex F and Gazzolo L (2000) The human T cell leukemia/lymphotropic virus type 1 Tax protein represses MyoD-dependent transcription by inhibiting MyoD-binding to the KIX domain of p300. A potential mechanism for Tax-mediated repression of the transcriptional activity of basic helix-loop-helix factors. J Biol Chem 275, 10551–10560.
23. Rosenbloom KR, Dreszer TR, Long JC, Malladi VS, Sloan CA et al. (2012) ENCODE whole-genome data in the UCSC Genome Browser: update 2012. Nucleic Acids Res 40, D912–D917.
24. Simmen RC and Simmen FA (2002) Progesterone receptors and Sp/Krüpel-like family members in the uterine endometrium. Front Biosci 7, d1556–d1565.
25. Simmen RC and Simmen FA (2002) Progesterone receptors and Sp/Krüpel-like family members in the uterine endometrium. Front Biosci 7, d1556–d1565.
26. Sunadome K, Yamamoto T, Ebisuya M, Kondoh K, Sehara-Fujisawa A et al. (2011) ERK5 regulates muscle cell fusion through Klf transcription factors. Dev Cell 20, 192–205.
27. Sunadome K, Yamamoto T, Ebisuya M, Kondoh K, Sehara-Fujisawa A et al. (2011) ERK5 regulates muscle cell fusion through Klf transcription factors. Dev Cell 20, 192–205.
28. Suske G (1999) The Sp-family of transcription factors. Gene 238, 291–300.
29. Tapia O and Gerace L (2016) Analysis of nuclear lamina proteins in myoblast differentiation by functional complementation. Methods Mol Biol 1411, 177–194.
30. Truett ET, Mynatt RL, Truett AA, Walker JA et al. (2000) Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques 29, 52–54.
31. Wauson EM, Zaganjor E, Lee AY, Guerra ML, Ghosh AB et al. (2012) The G protein-coupled taste receptor T1R1/T1R3 regulates mTORC1 and autophagy. Mol Cell 47, 851–862.
32. Zhao C and Meng A (2005) Sp1-like transcription factors are regulators of embryonic development in vertebrates. Dev Growth Differ 47, 201–211.
33. Zhou Y, Ren J, Song T, Peng J and Wei H (2016) Methionine regulates mTORC1 via the TIR1/TIR3-PLCγ-Ca²⁺-ERK1/2 signal transduction process in C2C12 cells. Int J Mol Sci 17, 1684.