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Overexpression of *NUDT7*, a candidate quantitative trait locus for pork color, downregulates heme biosynthesis in L6 myoblasts

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ABSTRACT

While testing a quantitative trait locus (QTL) for pork color in a cross population of pigs from the mating of Large White dams to a Japanese wild boar, our laboratory discovered a candidate gene (*NUDT7*) that might affect heme biosynthesis in porcine muscle. Therefore, this experiment was designed to test the effect of *NUDT7* on heme biosynthesis in cultured myoblasts. Rat L6 myoblasts were transfected with a mammalian expression vector for pig *NUDT7* immediately after the induction of cell differentiation, and samples were harvested at 2, 4, 6, and 8 days. Expression of exogenous *NUDT7* mRNA was highest on day 4, when the heme content was substantially lower ($P<0.01$) than that of the control (14.2 vs. 63.9 pmol/10^5 cells). These results suggest that overexpression of pig *NUDT7* may be associated with heme biosynthesis downregulation in skeletal muscle, which may partially explain differences in meat color among breeds of livestock.

1. Introduction

Meat color determines initial acceptance or rejection in the marketplace. To strengthen consumer acceptance, improvement of meat color can be a top priority of the pork industry (Newcom, Stalder, Baas, Goodwin, Parrish and Wiegand, 2004). Thus far, quantitative trait locus (QTL) analyses for pork color have revealed that variation in hematin content, or Minolta measurement a* (redness), in skeletal muscle tissues is at least partly accounted for by a few relevant QTL identified in the pig genome (Nii et al., 2005; Ovilo et al., 2002). In a previous study using F_2 progeny from the mating of a Japanese wild boar (known as red meat) to Large White dams, our laboratory detected a QTL on swine chromosome 6 (SSC6) that explained 9% of phenotypic variance in hematin content, and that the Japanese wild boar allele had a significant additive effect on the redness of pork (Nii et al., 2005). After fine-mapping the QTL region on SSC6 in the same F_2 population and analyzing the genome structure of the corresponding regions in humans, mice, and cattle, a nudix (nucleoside diphosphate linked moiety X)-type motif 7 (*NUDT7*, a member of the nudix hydrolases) gene at the most likely position of the QTL, because *NUDT7* was located within a 10 centi-morgan (cM) region corresponding to the 1.5-LOD (logarithm of odds) confidence interval (Taniguchi et al., 2010). Furthermore, our laboratory identified differential allelic expression of pig *NUDT7*, indicating that the transcription efficiency of the Japanese wild boar allele was less than that of the Large White allele (Taniguchi et al., 2010). Thus, the lower *NUDT7* expression in Japanese wild boar may contribute to the enrichment of meat redness. However, no study has investigated the effect of *NUDT7* on heme biosynthesis in skeletal muscle cells; therefore, the objective of this study was to investigate the relationship between pig *NUDT7* expression and heme content in muscle cells. Rat L6 myoblast cell line was selected as the *in vitro* model, because the cells differentiate into myotubes after confluence and synthesize endogenous heme and myoglobin (Graber & Woodworth, 1986). Furthermore, the association of *NUDT7* expression level and heme content in the process of L6 differentiation was analyzed using a system for the transient expression of exogenous *NUDT7*.

2. Materials and methods

2.1. Cell culture

The rat skeletal muscle cell line L6 (G8.C5) was obtained from the European Collection of Cell Cultures.

2.2. Plasmid vector construction

A gene-specific primer pair for cloning of pig *NUDT7* cDNA was designed on the basis of the pig *NUDT7* gene sequence derived from the Large White allele (GenBank accession: AB473629): forward,

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5′-GGATTCGCTAGCAGGAAAGA-3′ and reverse, 5′-CCTTGTTCTTCTG GGGATCA-3′. A polymerase chain reaction (PCR) fragment, including an intact open reading frame of 714-bp, was amplified from skeletal muscle cDNA and cloned into the pCR2.1-TOPO vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Purified plasmid DNA was digested with the restriction enzymes *Nhe*I and *Eco*RI (Promega, Madison, WI, USA) before the gene was ligated into the pSI mammalian expression vector (Promega), which had been digested with the same restriction enzymes. The pSI-*NUDT7* construct was confirmed by nucleotide sequence analysis.

2.3. L6 differentiation and transient transfection

Cells were cultured under a humidified 5% CO_2 atmosphere at 37 °C in 6-well plates in Dulbecco's modified Eagle's medium (DMEM; high glucose; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). When the last passage was carried out, control cells designated as day 0 were collected. Then, myoblast differentiation was induced by replacing the cell culture medium with one containing 2% FBS to induce cell fusion and differentiation into myotubes as described previously by Kim, Jitrapakdee and Thompson (2007). Immediately after the differentiation induction, cells in each well were transfected with 1.0 μg pSEAP2-*control* vector for monitoring transfection efficiency and 1.0 μg of either pSI mammalian expression vector containing pig *NUDT7* (*NUDT7*-transfected) or pSI mammalian control vector (control-transfected) mixed with 3.0 μL FuGENE HD transfection reagent (Roche Applied Science, Indianapolis, IN, USA) in a total volume of 100 μL made up with serum-free DMEM. Additional L6 cell samples were harvested on day 2 when the cells had reached 100% confluence, as well as on days 4, 6 and 8. Three biological replicates were prepared for each condition. To monitor the transfection efficiency of the plasmid constructs, supernatant from each cell culture medium on day 2 was used for secreted alkaline phosphatase (SEAP) assay in a Great EsCape 3 chemiluminescent reporter system (BD Clontech, Mountain View, CA, USA), and chemiluminescence intensity was measured on a luminometer (CT-9000D; Dia-Iatron, Tokyo, Japan) according to the manufacturer's instructions. The expression level of pig *NUDT7* was normalized against the transfection efficiency calculated from the SEAP activity.

2.4. Gene expression assays

The number of cells used for total RNA extraction was adjusted to 1.0×10^5 in each sample. Total RNA was extracted with an RNeasy Protect Mini Kit (Qiagen, Valencia, CA, USA), then cDNA was synthesized with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan), according to the manufacturers' instructions.

Table 1
Nucleotide sequences used for RT-PCR assays.

Gene symbol	Nucleotide sequence (5′–3′)	Size (bp)	GenBank accession No.
Myog	Fw: CACTCCCTTACGTCCATCGT Rv: CAGGGCACTCATGTCTCTCA	448	NM_017115
Mb	Fw: GACCCTGGAAAAGTTCGACA Rv: ATGTCATTCCGGAACAGCTC	312	NM_021588
Nudt7	Fw: GGCTCGCTTGAAAAAGTTTG Rv: AAAGGCACGAGGAACACATC	387	NM_001108450
Gapdh	Fw: AACTTTGGCATTGTGGAAGG Rv: TGTGAGGGAGATGCTCAGTG	599	XR_006741.1
NUDT7[a]	Fw: CCATCTAGCAAATATTCCATCCTTTT Rv: CCGGAGGGTGAGCAACA Probe: CCACTGCTGGCTAAA	75	AB473629

[a] Primers and probe for TaqMan qPCR were specifically designed for pig *NUDT7* (Taniguchi et al., 2010).

Expression of rat *myogenin* (*Myog*), *myoglobin* (*Mb*), *Nudt7*, and *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) was confirmed by reverse-transcription PCR (RT-PCR) at 95 °C for 10 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min (PCR primers listed in Table 1).

The abundance of *NUDT7* mRNA was measured by absolute gene expression assay using TaqMan quantitative real-time PCR (qPCR) as previously described by Taniguchi et al. (2010). Briefly, a standard curve was drawn from serial cDNA dilutions of pig *NUDT7* ranging from 1.25×10^{-11} to 1.00×10^{-7} μg. The mRNA copy number was taken from the standard curve (Pfaffl, Georgieva, Georgiev, et al., 2002). The TaqMan system was used with the ABI PRISM 7500 sequence detection system and SDS software (version 1.4; Applied Biosystems, Foster City, CA, USA). Four technical replicates were prepared for each sample. Nucleotide sequences of the primers and gene-specific probe used for each PCR assay are listed in Table 1.

2.5. Quantification of heme

The highly sensitive heme assay of Masuda and Takahashi (2006) was used to quantify heme content in myoblast cultures. The principle of the method is based on the ability of horseradish peroxidase (HRP) apo-enzyme to spontaneously reconstitute with heme to form an active holo-enzyme; therefore, HRP activity can be used for quantification of heme content. Detection of chemiluminescence emitted by HRP-catalyzed luminol oxidation allowed heme to be measured.

The number of cells used in the heme assay was adjusted to 2.5×10^5 in each sample. Cell pellets were homogenized in 100 μL acidified acetone solvent with glass beads in a vortex-type homogenizer (Shakeman 2; Bio Medical Science, Tokyo, Japan) and centrifuged at 10,000 *g* at 4 °C for 5 min (Hornsey, 1956). A stock solution of HRP apo-enzyme (Biozyme, Blaenavon, Wales, UK) was prepared at 2.5 mM in water. A stock solution of heme (Nakalai Tesque, Kyoto, Japan) was prepared in dimethyl sulfoxide (DMSO), and working solutions were freshly prepared in 10 mM KOH. A reaction mixture containing final concentrations of 2.5 nM HRP apo-enzyme, 100 mM Tris·HCl (pH 8.4) and heme (10 μL, range of 20 to 800 pM in serial dilution for calibration curve) or L6-extracted heme, and an acidified acetone (10 μL, diluted 1:800 in 100 mM Tris·HCl, pH 8.4) was used to bring the final volume of each sample to 100 μL. Samples were then incubated for 30 min at ambient temperature for reconstitution of active HRP, and peroxidase activity was assayed with ECL Plus Western blotting detection reagents (GE Healthcare Bioscience, Piscataway, NJ, USA) on the basis of the catalytic luminol oxidation. A 100-μL assay mixture containing detection solutions A and B in a ratio of 40:1 was added to the reaction mixture, and, after a 15-min incubation at ambient

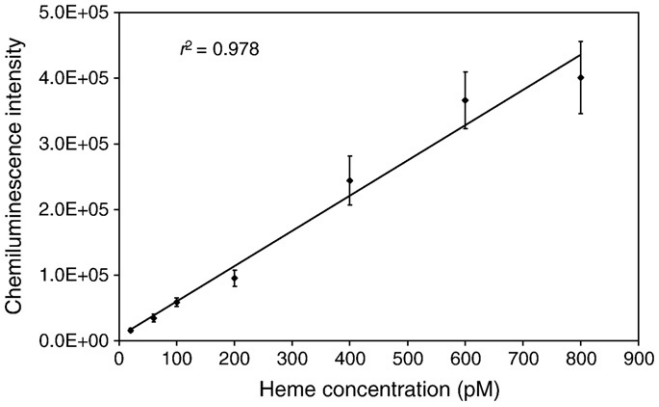

Fig. 1. Calibration curve for heme content determination. Correlation between chemiluminescence intensity and heme content was examined by regression analysis ($r^2=0.978$, $P<0.0001$).