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Symposium: Animal Nutrition and Metabolism
17th AAAP (Asian-Australasian Association of Animal Production Societies)
Animal Science Congress

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Japanese Society for Animal Nutrition
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2016

Contents

(Vol. 60, No. 2)

会 報 目 次

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平成28年度家畜栄養生理研究会秋季集談会
開催のご案内

開催日時：2016年8月23日（火）

8：30 〜 12：15　シンポジウム・集談会（会場 F：N303）

（ご注意：シンポジウム・集談会に出席には AAAP への
参加登録（registration）が必要となります）

12：30 〜 13：30　評議員会（S304）

会　　場：九州産業大学

（〒813－8503 福岡県福岡市東区松香台2－3－1

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Symposium: Animal Nutrition and Metabolism
17th AAAP (Asian-Australasian Association of Animal Production Societies) Animal Science Congress

Fall meeting of Japanese Society for Animal Nutrition and Metabolism, 2016

8：30 ～ 9：15

Motoi Kikusato（Graduate School of Agricultural Science, Tohoku University）

Roles of mitochondrial oxidative phosphorylation and reactive oxygen species generation in the metabolic modification of avian skeletal muscle

9：15 ～ 10：00

Ryuichi Tatsumi（Graduate School of Agriculture, Kyushu University）

Experiments reveal a novel mechanism to regulate myofiber types and its activation by functional food ingredients

10：00 ～ 10：45

Sang-Gun Roh（Graduate School of Agricultural Science, Tohoku University）

Comparison of the gene expression of rumen epithelium in pre- and post-weaning young cattle

10：45 ～ 11：30

Masayuki Funaba（Graduate School of Agriculture, Kyoto University）

Presence of brown/beige adipocytes in adipose tissues of fattening cattle and diet-related changes in their activity

11：30 ～ 12：15

Ryo Inoue（Graduate School of Life and Environmental Sciences, Kyoto Prefectural University）

Development of the porcine intestine during early postnatal life
－Evaluation focusing on histological, enzymatic and immunological parameters－

Proc Jpn Soc Anim Nutr Metab 60 (2), 2016.

Roles of mitochondrial oxidative phosphorylation and reactive oxygen species generation in the metabolic modification of avian skeletal muscle

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Abstract

Mitochondria are double membrane-bound organelles found in most eukaryotic cells and serve as the centers of intracellular metabolism. One of their major functions is ATP production by oxidative phosphorylation (OXPHOS). OXPHOS is an energy transduction system that is based on coupling NADH/$FADH_2$ oxidation-driven electron transfer with ADP phosphorylation, with the inner membrane potential ($\Delta\Psi$) serving as a mediator. The coupling efficiency of OXPHOS contributes to the growth rate of chickens. The mechanistic understanding of this efficiency is therefore quite important. Herein, we review the general mechanisms underlying OXPHOS, and describe modular kinetic analysis, which is the methodology used for determining OXPHOS efficiency. Moreover, this review describes our experimental adaptation of the kinetic method to avian muscle mitochondria. We show the analysis for the differences in the coupling efficiency between meat- and laying-type chickens and the implication of these differences in determining the growth rate in these birds. The review also discusses the role of mitochondria as a major intracellular reactive oxygen species (ROS) generator and their effects on cellular metabolism. Mitochondria-generated ROS cause oxidative disturbance in cells, and participate in intracellular signal transduction pathways evoking cell death and protein catabolism, in response to several physiological and pathological stimuli. We describe that heat stress (HS) stimulates mitochondrial ROS generation, which may be caused by OXPHOS alterations and the subsequent increase in $\Delta\Psi$. Finally, we suggest that HS-induced mitochondrial ROS generation may play an important role in the induction of ubiquitin-proteasome-dependent protein degradation in avian skeletal muscle, which is partially responsible for growth retardation in the HS-treated birds.

1. Introduction

Mitochondria are double membrane-bound organelles found in most eukaryotic cells. The morphology and functions of this organelle transmute along with intracellular metabolic alterations. A major function of mitochondria is ATP production through oxidative phosphorylation (OXPHOS). In addition to this role, the mitochondria are also central to apoptosis[1], Ca^{2+} regulation[2], adaptive thermogenesis[3-5], and signal transduction via reactive oxygen species (ROS) generation[6,7]. Proper mitochondrial function is therefore critical to the cell[8].

For the past dozen years or so, we have investigated the metabolic role of avian muscle mitochondria in thermogenesis induced by exposure to low temperatures[9,10], as well as their role in ROS generation under heat stress (HS) conditions[11-13] in chickens. In this review, we initially describe the general mechanism of ATP production in OXPHOS, and the methodology used to study this mechanism. Furthermore, we provide

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ニワトリ骨格筋におけるミトコンドリアの酸化的リン酸化および活性酸素産生の代謝調節能
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information on studies on mitochondrial bioenergetic functions, especially regarding the coupling efficiency of OXPHOS and the HS-induced ROS generation. The review describes the effects of the latter on intracellular oxidative disturbance and protein degradation in avian skeletal muscle.

2. Mitochondria are the powerhouses of cells

Mitochondrial OXPHOS is the powerful and highly organized energy production system through which 36 moles of ATP are produced from 1 mole of glucose. Reducing equivalents, NADH and $FADH_2$, are produced by the TCA cycle (tricarboxylic acid cycle, also known as citric acid cycle, or Krebs cycle) or through β-oxidation. NADH and $FADH_2$ are oxidized at the respiratory complexes I (NADH: ubiquinone oxidoreductase, EC 1.6.5.3) and II (succinate dehydrogenase, EC 1.3.5.1), respectively (Fig. 1). The produced electrons are sequentially transferred from complexes I and II to ubiquinone (UQ), complex-III (coenzyme Q: cytochrome *c*-oxidoreductase, EC 1.10.2.2) and cytochrome *c* (Cyt *c*), finally reaching complex-IV (cytochrome *c* oxidase, EC 1.9.3.1) where they react with oxygen molecules. During the substrate oxidation and the consequent electron transports, protons are pumped from the matrix into the intermembrane space through the inner membrane by complexes I, III, and IV, forming a difference in potential ($\Delta\Psi$) across the inner membrane. $\Delta\Psi$ is used by the ATP synthase (ATPase) to generate ATP from ADP and monophosphate (Pi). Thus, mitochondrial OXPHOS is an energy transduction system that is based on the coupled reactions of the substrate oxidation-driven electron transfer and ADP phosphorylation, which are connected with $\Delta\Psi$ serving as an intermediate.

However, not all energy generated by the transfer of electrons is used for the production of ATP. An amount of pumped protons leak back to the matrix due to defects affecting the integrity of the inner membrane, reducing the $\Delta\Psi$. This unavoidable electrical dissipation is called the basal proton leak in mitochondria bioenergetics (Fig. 1); it constitutes one of factors determining the coupling efficiency of ATP production in OXPHOS.

In addition to the unavoidable leaks caused by membrane defects, cells may actually induce proton leaks by expressing proteins that reduce coupling efficiency, and are called UCPs (uncoupling proteins). UCP1, which

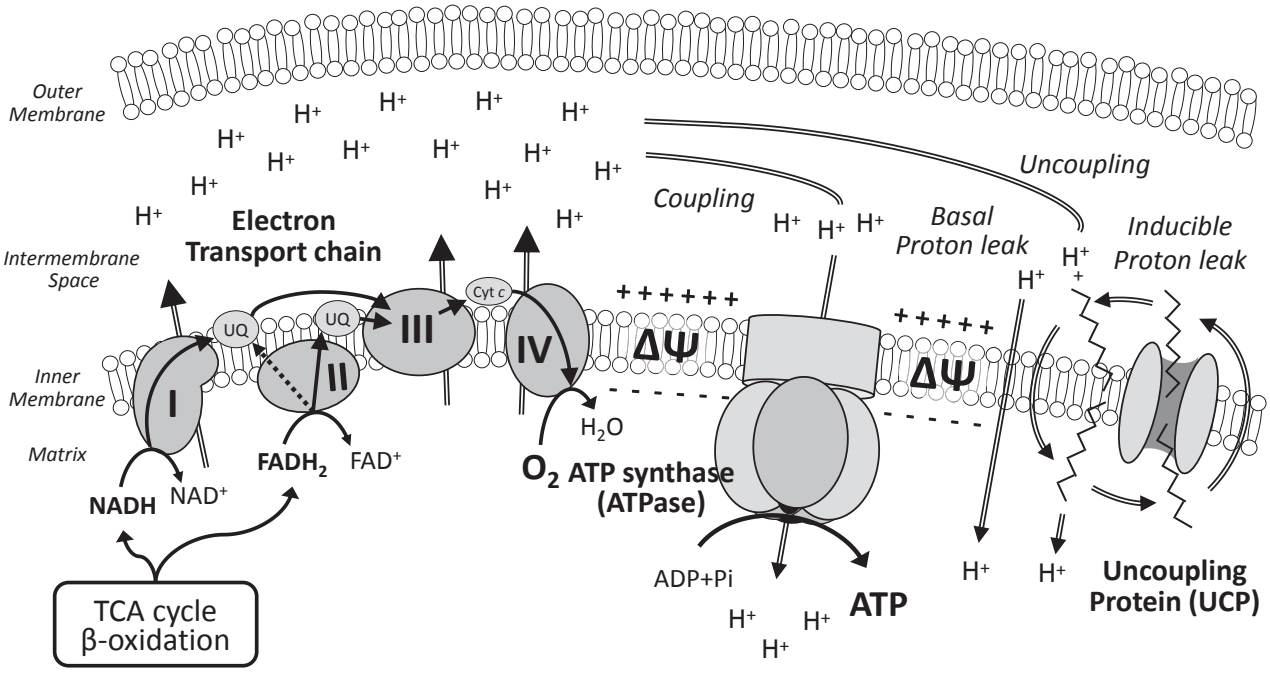

Fig. 1. Schematic representation of mitochondrial oxidative phosphorylation (OXPHOS) and reactive oxygen species (ROS) formation. UQ: Ubiquinone, Cyt *c*: Cytochrome *c*, $\Delta\Psi$: Membrane potential.

is expressed in brown adipose tissue, is known to act as a thermogenic protein in cold environments through the activation of inducible proton conductance. Two UCP1 isoforms, the ubiquitously expressed UCP2, and UCP3 which is predominantly expressed in skeletal muscle tissue[14-17], induce proton leaks after being activated by free fatty acids[18], superoxide[19], lipid hydroperoxides[20] and 4-hydroxy-2-nonenal[21]. An avian uncoupling protein (avUCP), which shares a 71–73% amino acid identity with both mammalian UCP2 and UCP3, was identified in chicken skeletal muscle[10,22].

3. Coupling efficiency of mitochondrial OXPHOS

Mitochondrial OXPHOS is systematically organized as described above, and it is important to estimate the coupling efficiency of the energy transduction system. The coupling efficiency of OXPHOS can be defined as the proportion of the mitochondrial respiratory rate that is used to drive ATP synthesis[23]. To precisely calculate this proportion, Brand and his co-workers established a novel kinetic method, referred to as modular kinetic analysis, that is based on the simultaneous measurement of the mitochondrial inner-membrane $\Delta\Psi$ and of the oxygen consumption rate[24].

Modular kinetic analysis divides OXPHOS into the following three modules (Fig. 2A): (*i*) "substrate oxidation", which includes the $\Delta\Psi$-producing reactions (substrate oxidation followed by electron transfer), (*ii*) "phosphorylation", where a part of the $\Delta\Psi$ is consumed for ATP synthesis, and (*iii*) "proton leak", which includes proton leaks (basal and UCP-mediated) that consume $\Delta\Psi$ without producing ATP[25]. The models representing each kinetic reaction are illustrated in Fig. 2B. The proportion of mitochondrial respiratory rate driving ATP synthesis is calculated by subtracting the portion of non-phosphorylation respiration rate from the overall respiration rate, and then dividing by the overall respiration rate. Respiratory control ratio (RCR), which

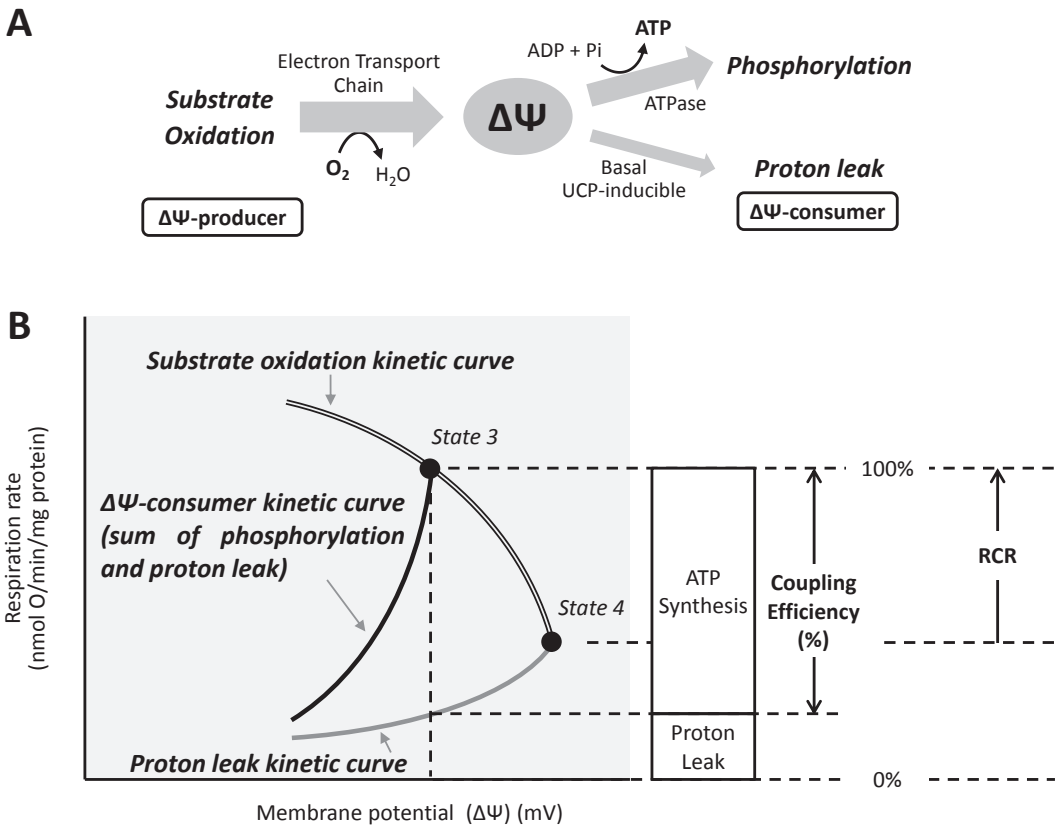

Fig. 2. Schematic representations of the three-branch modular system of mitochondrial oxidative phosphorylation (OXPHOS) (A), and of the kinetic curves comprising the modular kinetic analysis, as well as the values involved in calculating the percentage representing coupling efficiency (B).

is the ratio of the maximal respiration rate consumed for ATP synthesis (state 3) to the non-phosphorylation respiration rate (state 4), is a classical way to evaluate coupling efficiency. However, this approach will slightly underestimate the true coupling efficiency. As proton leak also occurs during phosphorylation, the proton leak-dependent respiration rate is actually smaller than the state 4 respiration rate (Fig. 2B). Modular kinetic analysis can provide data for the respiration rate due to proton leak in ATP synthesis by measuring $\Delta\Psi$.

4. Difference in the coupling efficiency of OXPHOS between meat and laying-type chickens

Meat- and laying-type chickens have undergone extensive selective breeding for maximizing meat and egg production, respectively, resulting in a faster growth rate in meat-type chickens compared with laying-type birds. A lower protein degradation rate has been proposed as one of the mechanisms causing the growth difference between the two types of chickens[26-28]. Mitochondrial basal proton leak is a significant contributor to the standard metabolic rate of the whole animal[29]. In mammals, a negative correlation between the proton leak rate and body mass has been observed[30]. Furthermore, it has been reported that the feed intake versus metabolic body mass index is higher in laying-type chickens than in meat-type chickens, indicating the existence of either a lower metabolic efficiency or a higher energy expenditure in laying-type chickens[31]. Our investigation using modular kinetic analysis demonstrated that skeletal muscle (*M. pectoralis*) mitochondria from laying-type chickens displayed a higher basal proton leak rate, and a lower $\Delta\Psi$ -consuming reaction compared with meat-type chickens, both of which result in a lower coupling efficiency of OXPHOS in laying-type chickens[32]. We also found that the avUCP expression level was higher in laying-type chickens than in meat-type chickens. Our findings suggest that mitochondrial coupling efficiency in skeletal muscle may be a major contributor to determining the body mass of chickens.

5. Mitochondrial ROS generation and heat stress in poultry

ROS are produced continuously as a byproduct of aerobic metabolism, with mitochondria being their primary source. Oxygen free radicals are reactive molecules, but can be converted into hydrogen peroxide (H_2O_2) by the manganese-superoxide dismutase (Mn-SOD, also known as SOD2); hydrogen peroxide is subsequently detoxified in mitochondria by the glutathione peroxidase (GPx). However, this anti-oxidative protection is not perfect; superoxide or H_2O_2 that evade the system can be converted into highly reactive hydroxyl radicals ($^{\bullet}OH$) or hydroperoxyl radicals ($HOO^{\bullet}$) (Fig. 3), which are able to cause irreversible molecular damage, termed as oxidative stress.

Oxidative stress occurs due to several physiological and pathological stimuli, and heat exposure is one of the environmental stressors causing oxidative damage[33,34]. As hyperthermia results in growth retardation[35] and meat quality loss[36,37] in poultry production, the mechanistic understanding of HS-induced oxidative disturbance and its effect on the intracellular metabolism are of great importance for poultry farming. Our previous investigations have revealed that the reduction in growth performance in HS-treated birds is associated with an increase in oxidative damage to skeletal muscle[35], and that overproduction of mitochondrial ROS may play a pivotal role in the induction of oxidative damage in HS-treated birds[11,38].

In mitochondria, superoxide ($^{\bullet}O_2^-$) is generated as the primary ROS via electron leakage followed by the one-electron reduction of oxygen molecules. Eleven distinct mitochondrial sites, associated with substrate oxidation and OXPHOS, have been identified as sites of superoxide production[39]. The electron transport chain complexes I and III are two of these sites[40]. Superoxide production at the two complexes is closely related to the electron flow and the magnitude of $\Delta\Psi$ [41-44]. Forward electron flow toward the complex IV produces a relatively small amount of superoxide at complexes I and III, while a bigger amount is produced at complex I by the reverse

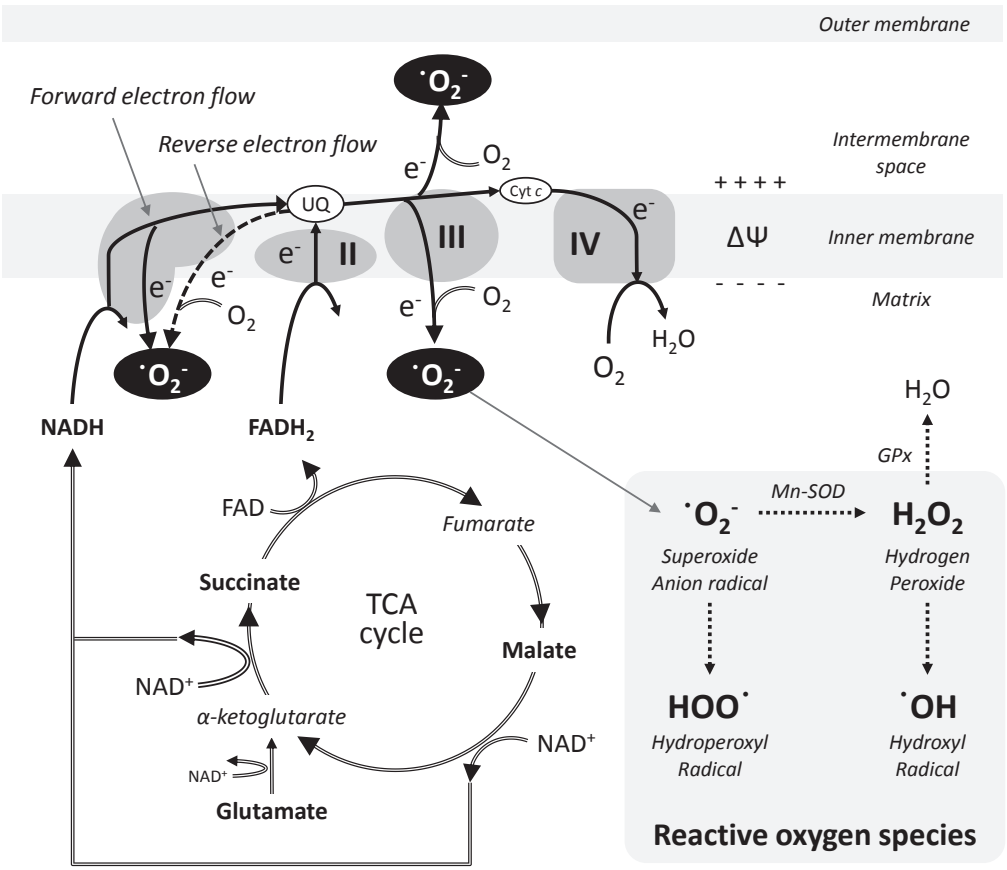

Fig. 3. Mitochondrial superoxide generation and subsequent reactive oxygen species (ROS) formation.

electron flow from complex II (Fig. 3). Both of these amounts increase as ΔΨ increases[45]. Our investigation demonstrated that the mitochondrial ROS production rate and the ΔΨ values in HS-treated birds were higher compared to those of birds kept at a thermoneutral environment[46], which led us to seek the mechanism through how HS-treatment leads to an increase in ΔΨ.

6. The mechanism of ΔΨ increase in response to HS treatment

The magnitude of ΔΨ is tightly regulated, and determined by the balance between the ΔΨ-producer and the two ΔΨ-consumers, as illustrated in Fig. 2A. Our previous investigation showed that the activity of substrate oxidation was markedly increased in response to HS treatment[46]. Furthermore, we found that the expression levels of the ΔΨ-dissipating avUCP were decreased in the muscle mitochondria of HS-treated birds, with respect to both mRNA and protein abundance[47], leading to a down-regulation of the avUCP-induced proton leak. These results suggest that the HS-induced increase in ΔΨ results from both an increase in ΔΨ-producing reactions, as well as from a decrease in ΔΨ-consuming reactions. Regarding the role of muscle avUCP in regulating the HS-induced increase in ΔΨ, we showed that laying-type chickens, whose muscle avUCP expression level is higher than that of meat-type chickens[32], exhibited resistance to HS-induced mitochondrial ROS production and oxidative damage[48]. From these lines of evidence, we postulate that the regulation of the avUCP expression level is an effective means of attenuating HS-induced oxidative disturbance.

7. Nutritional regulation of avUCP to counteract HS-induced oxidative disturbance

Several dietary factors such as theaflavins[49], epigallocatechin gallate[50], fish oil[51], fucoxanthin[52], olive oil[53,54], and oleuropein[55] (which is a phenolic

compound isolated from olive oil), have been reported to upregulate the cellular UCP level through distinct signaling pathways. Our previous studies have shown that meat-type chickens fed an olive oil-supplemented diet exhibited a suppression of HS-induced mitochondrial ROS generation[56], while co-supplementation with olive oil and oleuropein alleviated the ROS production in HS-treated birds via a mechanism dependent on the avUCP expression (Kikusato *et al*., *unpublished data*). The upregulation of avUCP expression due to oleuropein may be mediated by the activation of the peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α)[57], which is a transcriptional co-factor of UCPs[58]. Considering that avUCP is a mitochondrial protein that dissipates energy that would otherwise be used for ATP generation, it can be proposed that the upregulation of this metabolic efficiency-reducing protein may result in a reduction in growth efficiency. We recently obtained data contradicting this hypothesis, as we observed that a low-concentration oleuropein supplementation (<2.5 ppm) did not affect growth performance and feed efficiency, but was able to suppress muscle oxidative damage in normal feeding conditions (Kikusato *et al*., *unpublished data*). Further investigation is required to determine additive amounts that are both effective in suppressing HS-induced oxidative stress, and financially efficient.

8. Involvement of mitochondria-derived ROS in HS-induced muscle protein degradation

A decrease in skeletal muscle mass and the accumulation of oxidative damage are two of the physiological effects taking place under hyperthermic conditions. Taking into account recent findings according to which mitochondria-produced ROS induce atrophy[59,60], apoptosis[61,62], and autophagy[63], it can be assumed that the ROS generated due to HS exposure may function as inducers of protein degradation in avian muscle cells. It is accepted that corticosterone induces the protein degradation observed in HS-treated birds[64], and several studies have demonstrated that treatment with corticosterone or its synthetic analog, dexamethasone, induce muscle protein degradation by upregulating the transcription of muscle specific atrogenes, such as those encoding atrogin-1 and MuRF1[65,66], both of which act as ubiquitin ligases in the ubiquitin-proteasome intracellular protein degradation system (UPS). Contrary to the corticosterone hypothesis, it has been found that hyperthermic treatment of cultured muscle cells results in excess mitochondrial ROS production[67] and enhanced muscle protein degradation, obviously through a mechanism that can't be dependent on corticosterone secretion[68]. Based on this evidence, it is reasonable to assume that mitochondria-derived ROS may induce protein catabolism in skeletal muscle in response to HS treatment. This hypothesis was substantiated by our animal and cell culture studies. In HS-treated birds, circulating levels of corticosterone, muscle UPS-related genes expression, and mitochondrial ROS generation, increase at the early stages of treatment[69]. Thereafter, we evaluated the effects of HS-induced mitochondria-derived ROS and physiologically relevant levels of corticosterone (0 to 30 ng/ml) on the onset of protein degradation in cell cultures. Our results suggest that the mitochondria-derived ROS, rather than corticosterone, play the central role in instigating muscle proteolysis in HS-treated birds[70] (Fig. 4) These studies provide novel findings indicating that mitochondria-produced superoxide in avian HS-treated muscle cells may contribute not only to oxidative damage, but also to intracellular protein degradation, which in turn suggests that the regulation of mitochondrial ROS generation is extremely important to protein homeostasis.

9. Mitochondrial functional specialization in different muscle fiber types

In our aforementioned studies, *M. pectoralis* muscle was the main model used for evaluating avian mitochondrial bioenergetics. Skeletal muscles are composed of three muscle fiber types that are referred to as fast-twitch glycolytic (type IIB), fast-twitch oxidative (type IIA), and slow-twitch oxidative (type I). The three types differ in their mitochondrial content, and this difference in

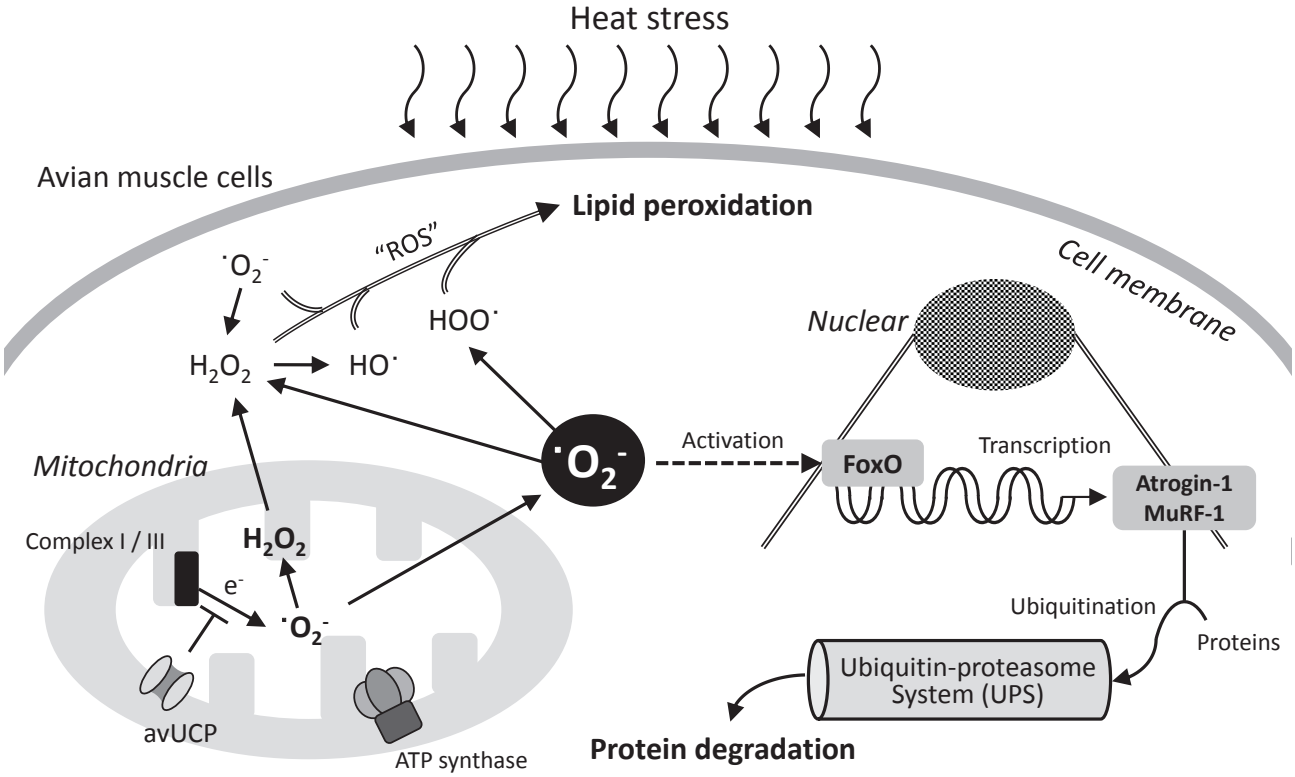

Fig. 4. Involvement of heat stress-induced mitochondrial superoxide generation in inductions of lipid peroxidation and ubiquitin-proteasome protein degradation system in avian muscle cells.

mitochondrial quantity was for a long time considered their major difference. Today, it is known that muscles with different muscle fiber compositions have different bioenergetic characteristics[71]. In regard with the metabolic differences observed in mitochondria isolated from several types of muscle in broiler chickens, we have found that mitochondria isolated from muscles composed of type I (*M. pubo-ischio-femoralis pars medialis*) or type IIA fibers (*M. pubo-ischio-femoralis pars lateralis*) exhibited a higher phosphorylation respiration rate compared with that of muscles made of type IIB fibers (*M. pectoralis*) (Hakamata *et al*., *unpublished data*).

Regarding the HS effects on several types of avian skeletal muscle, we found that hyperthermic treatment induced mitochondrial ROS generation and oxidative damage in *M. pectoralis* (type IIB: 100%,[9]) but these increases did not occur in the *gastrocnemius* muscle (Kikusato *et al*., *unpublished data*), which contains approximately 43% type-IIB fibers[72]. The mRNA levels of avUCP in *M. pectoralis* were downregulated in response to HS treatment, but this decrease was not observed in the *gastrocnemius* muscle. Moreover, the level of Mn-SOD mRNA in the *M. pectoralis* muscle did not change in response to HS treatment, while the level in the *gastrocnemius* muscle was augmented by HS treatment. These results suggest that avian skeletal muscles containing a high percentage of type-IIB fibers could be less tolerant to HS-induced oxidative disturbance than muscles with lower percentages of this type, and that the transcription regulation of the avUCP and Mn-SOD genes in muscles may play an important role in the induction of oxidative disturbance due to HS treatment. This study also demonstrated that the expression levels of avUCP, MnSOD, and PGC-1α (which expresses a co-factor for the transcriptional regulation of MnSOD expression) in the *gastrocnemius* muscle are higher compared to those of *M. pectoralis* under thermoneutral conditions (Kikusato *et al*., *unpublished data*), which might contribute to the observed transcriptional differences in response to HS treatment. In order to elucidate the regulation mechanisms of HS-induced oxidative disturbance, further comparisons of the

molecular machinery governing mRNA expression in the different muscles are required. This may open the way for new approaches, such as line selection method of chicken with avian skeletal muscles containing a lower percentage of type-IIB fibers.

10. Conclusions

Herein, we reviewed the difference in coupling efficiency of mitochondrial OXPHOS between chickens exhibiting different growth rates, and the central role of mitochondrial ROS generation in the induction of oxidative stress and protein degradation in avian skeletal muscles. This review also provides further insight into the molecular machinery that regulates HS-induced oxidative disturbance. Given that skeletal muscle tissue in chickens, as in large mammals including humans, plays an important role in overall bioenergetics and homeostasis, the current results obtained from chicken are likely to find application in research on large mammals and general health. Overall, the findings suggest the importance of mitochondrial functions in avian skeletal muscle; further understanding of mitochondrial bioenergetics has the potential to contribute to the enhancement of poultry production, and gives significance to research in mitochondrial bioenergetics, physiological stress-nutritional factor-gene interaction study, and oxidative stress management.

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Experiments reveal a novel mechanism to regulate myofiber types and its activation by functional food ingredients

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Running title: Slow-myofiber formation by Sema3A

Abstract

Fast/slow fiber-type proportions are responsible for skeletal muscle properties including: contractility (fast- and slow-twitch), metabolism (glycolytic and oxidative), fatigue resistance (low and high), and sensation (differential tasting-component contents, fat deposition, and texture). Therefore the mechanisms that regulate fiber type and their manipulation are hot targets of research for meat-animal production, human sports, and health sciences. Recently, we found that resident muscle stem satellite cells up-regulate a multi-functional secreted protein, semaphorin 3A (Sema3A) exclusively at the early phase of myogenic differentiation in response to muscle injury; however, its physiological significance is still unknown. The current study encouraged a possible mechanism that impacts the formation of slow-twitch fibers through a novel signaling pathway: the Sema3A ligand binds to its cell-membrane receptor (neuropilin2-plexin A3 complex) which activates myogenin/MEF2D expression to result in slow myosin synthesis. This was revealed in both siRNA-transfected cultures and satellite cell-specific Sema3A conditional knockout mice. The *in vitro* knock-down experiments also provided an additional important element that Sema3A-neuropilin1/plexin A1, A2 coupling may enhance slow-fiber formation during myotube formation by activating signals that inhibit fast-myosin heavy chain expression. A subsequent *in vivo* study showed that an 8-week intake of apple polyphenol (APP; prepared from unripe apples) in the diet, significantly improved muscle endurance based on increased proportions of fatigue-resistant fibers in rat leg muscles. Notably, supplementation of apple polyphenols (500 ng/ml) or the major component, chlorogenic acid (10 ng/ml) up-regulated the expression of slow myosin, myogenin, and MEF2D in culture, indicating that chlorogenic acid may be a Sema3A-receptor agonist and activate the Sema3A-dependent pathway. By further understanding this scenario where chlorogenic acid drives Sema3A, we will be able to design new specific ways to target regeneration and fiber-type proportions.

***Keywords*:** skeletal muscle, meat, fiber type, slow fiber, myogenic stem cell, satellite cells, myoblast, secretome, semaphorin 3A (Sema3A), cell-membrane receptor, neuropilin, plexin A, myogenin, MEF2D, slow myosin, agonist, polyphenol, chlorogenic acid, muscle endurance, cell culture, conditional knockout mouse

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筋線維型の新規制御機構の理解と食品機能学的調節
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1. Research background on fiber type regulation

Fiber-type proportions have been thought to be predominantly maintained by the motor-nerve impulse frequency (as demonstrated by pioneered cross-innervation of fast and slow muscles and implantation of electrodes in muscles[1-5]) and also by a transcriptional circuitry centered on PGC1α, PPARδ, and miR208b/499 elements[6-11]. In contrast to these iconic scenarios for the existing un-injured myofibers, in regenerating muscle following traumatic lesions, the regulatory mechanisms remodeling or restoring toward original fiber-type proportions characteristic of individual muscles are not elucidated to any great extent thus far.

In response to muscle injury, new muscle-fibers are formed by self-fusion of myoblast population that is generated by activation, proliferation and differentiation of satellite cells, resident myogenic stem cells normally positioned beneath the basal lamina of mature muscle fibers and hence are major replacements of necrotic muscle fibers seen in damaged muscle[12-17]. Therefore, the spatiotemporal presentation of extracellular ligands from myogenic stem satellite cells is a hypothetical event to impact the fiber-type patterning of generating muscle-fibers (centrally nucleated myotubes yet innervated) in an autonomous manner (see our review paper[18]).

Recently we found that satellite cells up-regulate the expression and secretion of a multi-functional modulator semaphorin 3A (Sema3A; a class 3 vertebrate-secreted semaphorin, also referred to as SemaIII, SemD and collapsin[19-25]). The Sema3A up-regulation occurs exclusively at the early myogenic differentiation phase, in response to *in vivo* muscle injury by crush or cardiotoxin (CTX)-injection and *in vitro* hepatocyte growth factor (HGF) treatment of primary cultures of satellite cells[26-31]. Additionally, emerging evidence showed that satellite cell cultures prepared from adult rat soleus muscle (slow-fiber abundant) secrete more Sema3A than those from the fast-twitch extensor digitorum longus (EDL) muscle upon the growth factor treatment[32], conceiving a hypothesis that the Sema3A secretion burst may signal the slow-fiber formation.

The present study conducted experiments in knock-down cultures and satellite cell-specific Sema3A conditional knockout mice (Pax7CreER^{T2}-Sema3A^{flox}); results encouraged a possible mechanism that the Sema3A ligand impacts the slow fiber formation through a signaling axis composed of a cell-membrane receptor (neuropilin2-plexin A3 complex), myogenin, MEF2D, and slow myosin. Additionally, supplementation of apple polyphenols or the major component chlorogenic acid elevated expression levels of the signaling molecules in differentiation cultures of satellite cells, indicating that chlorogenic acid may function as a Sema3A-receptor agonist to activate the Sema3A-dependent pathway concerned.

2. Sema3A impacts slow-myosin expression in a myogenin-dependent manner

We started experiments by examining the effect of Sema3A knockdown on myogenic differentiation and the resulting fusion of myoblasts. Differentiation cultures of satellite cell-derived myoblasts were transfected with Sema3A-specific siRNA (Stealth RNAi^{TM} duplexes purchased from Invitrogen) and evaluated for expression level of myogenin, a basic helix-loop-helix (bHLH) muscle-specific transcription factor that is well documented to play crucial roles for differentiation of myogenic cells during neonatal muscle development. RT-qPCR results showed that the Sema3A siRNA treatment decreased expression of Sema3A and myogenin and the down-regulation was further evident by Western blotting of cell lysates (assayed at 24-72 h post-transfection), providing the reliability for Sema3A knockdown experiments to show that Sema3A ligand may mediate myogenin expression *in vitro*. This issue was further examined in the next experiments, in which Sema3A knockdown cultures were evaluated for myotube formation and total myosin heavy chain (MyHC) expression level. Sema3A knockdown cultures looked normal in morphology of generated myotubes as visualized by immunocytochemistry with MF20 anti-MyHC antibody. Total MyHC expression

(MF20 antibody-positive) was also comparable to a level of control siRNA group referenced with β-actin and total protein amount applied to SDS-PAGE. The fusion index actually increased during the 0-72 h post-transfection period followed by a plateau of about 60% (at 72-120 h) equivalent to a level of the control siRNA culture and regular culture without transfection, indicating that myotube formation is independent on Sema3A and the downstream myogenin. These results may provide supportive evidence for recent observations that myogenin-deleted satellite cells can differentiate normally as the wild type[33] and myogenin null mice (conditionally knocked out in postnatal period) have a normal skeletal muscle phenotype[34]. Considering emerging evidence that satellite cells from soleus (typical slow-twitch muscle) have higher myogenin expression than EDL[32,35,36] and that myogenin mRNA expression is up-regulated by recombinant Sema3A addition to satellite cell cultures[32], in the subsequent experiments, we intensely tested a novel concept that the Sema3A-myogenin coupling may promote slow-fiber formation during muscle regeneration.

Sema3A knockdown cultures were evaluated for the expression of fiber-type markers, slow MyHC (type I) and fast MyHC (types IIa, IIx, and IIb), by RT-qPCR and Western blotting. The expression of slow MyHC was significantly decreased in Sema3A knockdown cultures at 48 h (about 60% reduction of the control siRNA culture mean) and similar phenotype was observed at later time-points. In contrast to the slow-MyHC down-regulation, the expression of fast MyHC was alternatively increased with a 24-h delay (detected at 72 h), possibly accounting for the constancy of total MyHC expression level and normal appearance of myotube formation in Sema3A-siRNA cultures. These results were ensured by immunocytochemistry of Sema3A-siRNA cultures, in which slow-MyHC expression was diminished as revealed by the immunofluorescence (at 48-96 h) and by the fusion index measurement to show that myonuclei in slow MyHC-positive myotubes were decreased in percentage with compensatory up-regulation of the fast-myotube myonuclei (assayed at 72-120 h). Importantly, myogenin-knockdown also diminished slow-MyHC expression as in the case of Sema3A knockdown cultures (with null effect on Sema3A levels). Understanding of the Sema3A-myogenin-slow MyHC signaling axis was further extended by observations that each myogenin and Sema3A knockdown culture reduced the expression of myocyte enhancer factor 2D (MEF2D). MEF2D is known to require the cooperation with bHLH factors to activate muscle-specific genes responsible for embryonic muscle development[37-39] and a recent landmark study by Olson and his colleagues[40,41] showed that among MEF2 family (MEF2A, B, C, and D), the skeletal muscle-specific deletion of MEF2C, D induced the reduction in slow fiber numbers in soleus muscle. Overall, the concurrent *in vitro* results demonstrate that the Sema3A-myogenin/MEF2D coupling may impact slow-MyHC-positive myotube generation during the early-differentiation period.

3. Sema3A cell-membrane receptors

A variety of studies have documented that neuropilin1, 2 and plexin A subfamily (A1, A2, A3, and A4) constitute functional composite-receptors for secreted semaphorins in adult neuronal cells and other cells in many tissues/organs, in which neuropilins serve as the ligand-binding component and plexin As generate signals at their intracellular domains[42-44]. However it is still un-known which neuropilin/plexin-A combination mediates the Sema3A-myogenin-slow MyHC pathway. Neuropilin2-siRNA cultures revealed significant decreases in myogenin and slow MyHC expression along with null effect on Sema3A and neuropilin1 levels and similar responses were observed in plexin-A3 siRNA cultures. Notably, it is evident that, in neuropilin2 knockdown cultures (*i.e.* in the presence of endogenous Sema3A secretion), reduced expression of myogenin and slow MyHC was not rescued up to a level comparable to a positive control (control siRNA culture) in response to recombinant Sema3A addition to the differentiation media. Similar results were seen in neuropilin2-Sema3A

double-knockdown cultures with the same strategy of Sema3A addition. These knockdown experiments, therefore, demonstrate that neuropilin2-plexin A3 may be a composite-receptor for Sema3A ligand that activates the myogenin-slow MyHC axis.

In addition to this important signaling pathway, it is worth noting that fast MyHC (IIa, x, b) up-regulation was seen in neuropilin2 and plexin A3 knockdown cultures as in the case of Sema3A knockdown cultures, ensuring an inhibitory fast-MyHC expression pathway to enhance the predominant expression of slow MyHC. In line with this important discussion, neuropilin1 and plexin-A1, 2 knockdown cultures also showed significant increase in fast MyHC expression (types IIx and IIb) at the same assay time-point (72 h). These results hence provide an additional important element that Sema3A-neuropilin1/plexin A1, A2 coupling may enhance slow-myosin-positive fiber formation at the myotube formation period by activating inhibitory signals for fast-MyHC expression. This insight accounts for our recent observation that plexin A2 expression is higher in the early-differentiated satellite cells from soleus muscle than EDL with no significant difference in levels of either plexin A1, A3 or neuropilin1, 2[32]. Nonetheless, the present *in vitro* experiments highlight the positive regulation-pathway centered on Sema3A-neuropilin2/plexin A3-myogenin/MEF2D-slow MyHC signaling axis responsible for slow-myosin-positive fiber formation.

4. *In vivo* demonstration

The physiological significance of the *in vitro* findings was clarified by *in vivo* muscle-injury experiments in satellite cell-specific Sema3A conditional knockout mice (Pax7CreER^{T2} Sema3A^{flox}), which were produced by mating Pax7CreER^{T2} mice (originally developed by Dr. Shahragim Tajbakhsh, Institut Pasteur, France) with Sema3AloxP mice (Dr. Takeshi Yagi, Osaka University, Japan). For conditional activation of Cre recombinase under the control of the Pax7 promoter, adult male mice (8-10 week-old) received an intra-peritoneal (*ip*) injection of tamoxifen (ER^{T2} agonist) once a day for two consecutive days. Subsequently, mice were housed for 2 weeks prior to an intramuscular injection of 10 μM cardiotoxin (CTX; a potent polypeptidic snake-venom; obtained from Sigma-Aldrich) into lateral and medial heads of gastrocnemius muscle (the biggest one of the posterior compartment in the lower hind-limb) under anesthesia. Sema3AloxP mice were served as a control group with the same tamoxifen-*ip* and CTX-injection procedures at the same age and body-weight ranges as Sema3A-conditional knockout mice (Sema3A-cKO). Pax7CreER^{T2} Sema3A^{flox} mice of which Sema3A gene was not truncated even by the tamoxifen treatment were assigned to another control group with the CTX-injection procedure ($n = 3$). Total food-intake and body weight gain were not significantly different between the control and Sema3A-cKO groups. All experiments involving the animals were conducted in strict accordance with the recommendations in the Guidelines for Proper Conduct of Animal Experiments published by the Science Council of Japan and ethics approvals from the Kyushu University Institutional Review Board.

In order to evaluate regenerative phenotypes of fiber-type proportions after CTX-injury, muscle endurance was measured at 0-day (un-injured) and 28-days post-injury (a time-point at which muscle regeneration is expected to be completed). In brief, maximum contraction force of the posterior muscle in the lower hind-limb was generated under anesthesia by electrical stimulation (amplitude 50 V, pulse width 1 ms, duration 196 ms with intervals of 4 ms after each pulse, frequency 200 Hz) via a bipolar hook-shaped electrode on tibial nerve bundles and recorded for 100 s, according to Iwata *et al*. (2010)[45] with some modifications[46]. At day-0 (just before CTX injection), the initial contraction-force generation at the first stimulation (at 0-s) and the time course of the decline (during 0-100 sec) were both comparable between the control ($n = 5$) and Sema3A-cKO groups ($n = 6$), ensuring null difference in the muscle endurance at the starting time-point. Day-28 results showed that while the initial force mean (at 0-s) was significantly higher in Sema3A-cKO mice ($n = 6$) than the two control-groups

($n = 5$ and 3), the resistance to fatigue (force expressed relative to the initial value) was significantly lower in the Sema3A-cKO group as revealed by a faster decline in the force generation (the time course of 0-100 sec period). This is direct evidence that Sema3A deletion significantly diminishes muscle endurance of calf muscle possibly by reduced slow-fiber population after muscle injury. We are now analyzing the fiber-type proportion of gastrocnemius muscles in the Sema3A-cKO mice by immunofluorescence microscopy and SDS-PAGE; the results will be reported soon elsewhere.

In summary, the current *in vitro* and *in vivo* studies demonstrate that Sema3A ligands, which have been secreted from early-differentiated satellite cells (myoblasts) upon muscle injury, impact slow-fiber generation at the stage of centrally-nucleated myotubes (yet innervated) in autocrine and paracrine manners possibly through a signaling pathway composed of neuropilin2-plexin A3 receptor, myogenin (and its cooperating regulators MEF2D), and slow MyHC. Additionally, Sema3A-neuropilin1/plexin A1, 2 association may enhance slow-fiber formation by activating inhibitory signals for fast-MyHC expression. Sema3A secretion occurs in response to HGF secretion burst from anti-inflammatory macrophages (CD206-positive M2) that infiltrate and recruit to collect more myoblasts to the injury site of muscle at 3-7 days post-injury with a delay after the rapid invasion of phagocytotic pro-inflammatory macrophages (CD86-positive M1) as reported previously[26-30,47,48]. Therefore, the above conclusion can be extended to our understanding of the physiological significance of M2 macrophage-satellite cell coupling in regenerative myogenesis including the fiber-type commitment during the early-differentiation phase following injury.

5. Sema3A receptor agonist found in food ingredients

It is worth noting again that the slow MyHC-expression pathway model concerned is activated by the association of Sema3A ligands with the cell-membrane receptor neuropilin2, therefore it may be possible that ligand molecules other than Sema3A alternatively can bind to neuropilin2 to generate the signal. We have already found a promising agonist in food ingredients; briefly, primary differentiation-cultures of rat satellite cells supplemented with 500 ng/ml apple polyphenol (APP; prepared from unripe apples) or 10 ng/ml chlorogenic acid (a major component of APP) revealed up-regulation of myogenin, MEF2D and slow MyHC expression along with unchanged Sema3A level, indicating that the chlorogenic acid may mimic the Sema3A response in culture. Physiological adaptability of these *in vitro* observations was supported by our *in vivo* study, in which the 8-week pair-feeding of 5% (w/w) APP significantly improved the endurance (fatigue resistance) of calf muscles of young-adult Fischer F344 rats, as evidenced by a slower decline in the maximum isometric planter-flexion torque generated by a 100-s train of electrical stimulation of the tibial nerve (see Fig. 1). Similar trend based on significant increase in fatigue-resistant fiber proportions and myoglobin expression was seen after 0.5% APP-feeding to 12-week-old Sprague-Dawley rats (F (98, 1372) = 1.246, $P = 0.0574$, analyzed by the 2-way ANOVA test with repeated measures). Importantly, there was no significant difference in the animal body-phenotypes or locomotor activity shown as total moving distance in light and dark periods between the APP and control groups, indicating that the shift in MyHC isoform proportions from fast to slow did not include a bias due to greater exercise behavior by the treated rats[46,49].

We are now designing additional *in vivo*/*ex vivo* experiments for satellite cell-specific Sema3A-cKO mice fed with APP or chlorogenic acid during muscle regeneration and post-natal muscle growth periods. Results are expected to encourage our Sema3A story concerned in the current study and hopefully contribute to developing novel strategies that promote preferable slow/fast fiber compositions in meat-animal production and the human sports and health sciences and also in aging sciences to combat loss of the fiber-type balance that is often seen in age-related atrophy.

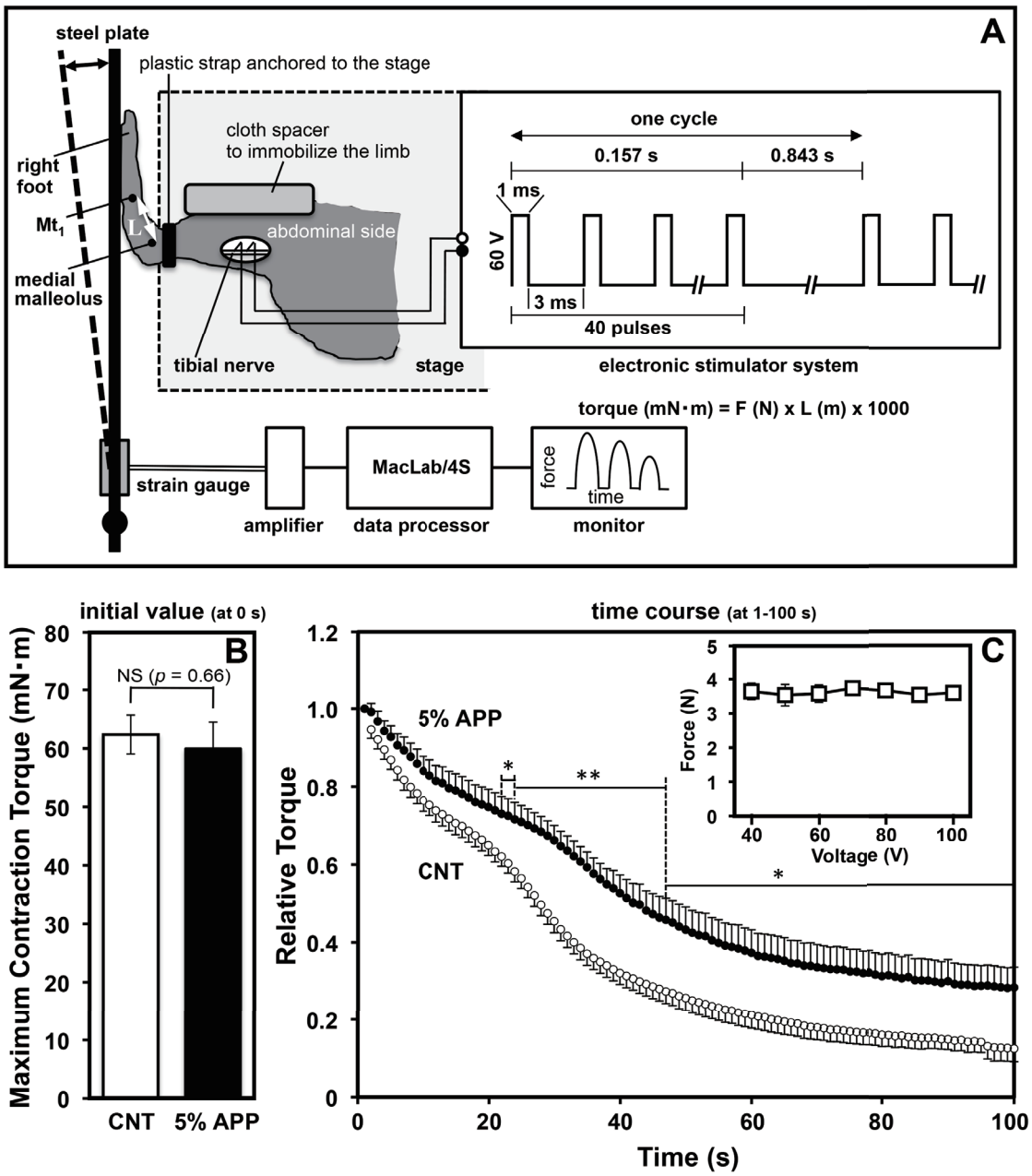

Fig. 1. Improvement of muscle endurance by 5% APP feeding.

Maximum isometric planter-flexion force torque was measured under anesthesia after 8-wk feeding of the control or 5%-APP diets. Schematic configuration of equipment is shown in panel A. Maximum-contraction forces measured were converted to torque (mN · m) by multiplying by the length (L) between the medial malleolus and the head of the first metatarsal bone (Mt1); the initial value at the first stimulation (at 0-s) and the time-course change in response to 100-s successive stimulation (expressed relative to the initial value) are shown in panels B and C, respectively. Data points and bars depict the mean ± SE for nine rats in each group and significant differences from control at $P < 0.05$ and $P < 0.01$ are indicated by (*) and (**), respectively. NS, no significant difference. Reproduced from PLoS ONE article, Mizunoya *et al*. (2015) [46].

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Comparison of the gene expression of rumen epithelium in pre- and post-weaning young cattle

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Abstract

In ruminants, development of the rumen is caused by weaning. After weaning, the length and density of the rumen papillae begins increasing, and these significant developments increase the intraruminal surface area and the efficiency of producing and absorbing volatile fatty acid. Although there are reports about the factors involved in rumen development, the molecular mechanisms in the rumen epithelial tissue during weaning have not been investigated in detail. In this study, we investigated the factors involved in the development of the rumen epithelium between pre- and post-weaning in ruminants using digital differential display (DDD) *in silico* and RNA-seq analysis. The DDD method was used in order to compare the number of assignments of bovine expressed sequence tag libraries from the rumen, reticulum, omasum, abomasum, and a pooled sample of 27 tissues. A total of 110 genes with high-transcript frequency in the rumen epithelium were selected as candidate genes related to rumen development. The expression patterns of 11 genes showed nearly the same results as in DDD. The expression of three genes (HMGCS2, AKR1C1, and FABP3) was significantly changed between pre- and post-weaning in the rumen epithelium. RNA-seq analysis showed that 871 genes with more than 2-fold changes were screened as differentially expressed between pre- and post-weaning. As a result of canonical pathway analysis, "atherosclerosis" was identified as the most related pathway. Moreover, "tretinoin", a derivative of vitamin A, was identified as the most active regulator during weaning. The expression of genes involved in the atherosclerosis pathway and downstream genes of tretinoin were regulated via protein kinase B. In conclusion, the genes and regulating factors screened by DDD and RNA-seq analysis in the rumen epithelium may be affected by weaning and/or aging as well as by energy production and harmonization of intraruminal conditions.

1. Introduction

The rumen has several important physiological functions, including absorption, transport, metabolic activity, and protection[1,2]. In particular, the rumen plays important roles in volatile fatty acid (VFA) production by rumen bacteria and VFA absorption through the rumen epithelium. After VFA is absorbed, it is utilized for the maintenance and growth of ruminants as well as for milk or meat production. A total of 60-80% of feed requirements are supplied as VFA in ruminants[3,4]. Three important VFAs are acetate, butyrate, and propionate, with generation ratios in the rumen of approximately 70%, 20%, and 10%, respectively[3]. Acetate and butyrate are utilized as precursors for lipid synthesis, while propionate is utilized for gluconeogenesis[5]. Therefore, in terms of the maintenance of life and growth, rumen and VFA are very important for ruminants[6].

After birth the rumen epithelium in calf is morphologically and functionally developed with both the age of the animals and the elevated intake of solid feed. Although many studies have examined the factors related to rumen development, the detailed molecular

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離乳前後の仔牛のルーメン絨毛上皮組織における遺伝子発現量の比較
盧　尚建[1]・加藤大地[1]・鈴木　裕[1]・芳賀　聡[2]
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mechanisms remain unclear. We previously analyzed the expression patterns of several genes affected by weaning and age in the rumen and abomasum of Holstein cattle using a differential display method (DDD)[1]. In neonatal Holstein calves, a grain-based diet and orchard grass hay significantly induced the length and density of the rumen papillae, while less development of these structures was seen when feeding only milk[7]. Moreover, some studies have evaluated the effect of VFA feeding. VFA, mainly butyrate, induced significant differential morphological development and promoted increases in the length and density of the rumen epithelium[8-10]. Recently, however, reports of genes related to alterations in gene expression in the rumen epithelium have increased because of the use of *in silico* analysis involving comprehensive analysis tools or software to evaluate gene expression. A previous study identified the genes for which the expression level changed while converting from liquid feed to solid feed in Holstein calves using microarray and Ingenuity Pathway Analysis[7]. The influence of butyrate infusion on gene expression in the Holstein rumen epithelium was investigated using RNA-seq technology, GO analysis, and ARACNE (an algorithm for reconstructing gene regulatory networks)[11]. While changes in the expression of genes related to rumen papillae development between pre-weaning and weaning have been examined, the details remain unclear. There have been few reports of gene expression related to rumen epithelial tissue development before and after weaning.

In this review, we introduce our recent work on the expression pattern of genes that may contribute to the development of the rumen epithelium during weaning and aging.

2. Identification of candidate genes related to rumen development by DDD analysis

DDD was used to screen for candidate genes associated with rumen development between before and after weaning in Japanese Black cattle[12]. Although the library of bovine genes is currently insufficient, DDD may be useful for searching candidate genes related to rumen development in UniGene database of the National Center for Biotechnology Information (NCBI). To identify the candidate genes involved in bovine rumen development, DDD analysis was conducted to compare the number of assignments of bovine expressed sequence tag (EST) libraries from the rumen, reticulum, omasum, abomasum, and 27 pooled tissue samples[12]. The rumen (4 libraries), reticulum (2 libraries), omasum (1 library), abomasum (3 libraries), and pooled tissues (127 libraries) were selected for comparison. DDD comparisons were designed systematically to reveal the relative abundance of ESTs among the contrasting libraries of digitally 'pooled' contracts from the UniGene Database[13].

A total of 110 candidate genes showing high expression in the rumen epithelium were derived from a library of all tissues[12]. The genes with differential frequencies were classified into three groups: (1) genes with high-transcript frequency in the rumen and no expression in the abomasum, (2) genes with high-transcript frequency in the rumen compared to that in other tissues, and (3) genes with high-transcript frequency in the rumen alone. Among the 110 genes in the three groups, 11 with high-transcript frequency in the rumen were selected and further analyzed in the rumen, reticulum, omasum, and abomasum by quantitative reverse transcriptase-PCR (Q-RT-PCR) to validate the gene expression.

Fig. 1 shows the expression levels of the 11 genes in rumen, reticulum, omasum, and abomasum tissues of pre-weaning and weaned calves by Q-RT-PCR. S100 calcium binding protein A12 (S100A12), BPI fold containing family A, member 2C (BPIFA2C), carbonic anhydrase 1-like (CA1), serpin peptidase inhibitor, clade B-like (SERPINB), S100 calcium binding protein A8 (S100A8), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), and aldo-keto reductase family 1, member C1-like (AKR1C1) were expressed in the rumen, reticulum, and omasum, but not in the abomasum[12]. Compared to in the abomasum, fatty acid binding protein 5 was highly expressed in the rumen, reticulum, and omasum. Among the 11 genes, only HMGCS2, AKR1C1, and fatty acid binding protein 3 (FABP3) showed differential

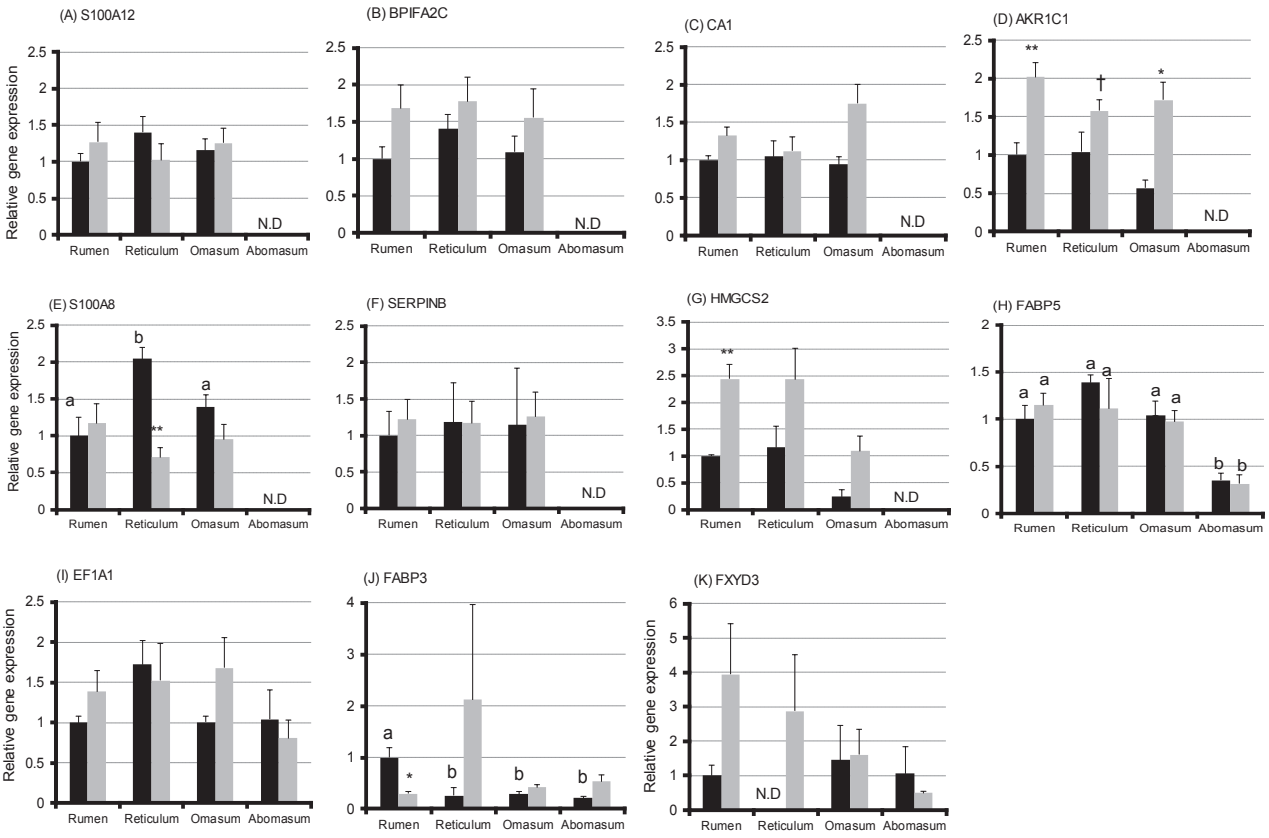

Fig. 1. The expression levels of (A) S100 calcium binding protein A12 (S100A12); (B) BPI fold containing family A, member 2C (BPIFA2C); (C) carbonic anhydrase 1-like (CA1); (D) aldo-keto reductase family 1, member C1-like (AKR1C1); (E) S100 calcium binding protein A8 (S100A8); (F) serpin peptidase inhibitor, clade B-like (SERPINB); (G) 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2); (H) fatty acid binding protein 5 (FABP5); (I) eukaryotic translation elongation factor 1 α 1 (EEF1A1); (J) fatty acid binding protein 3; and (FABP3) (K) FXYD domain-containing ion transport regulator 3 (FXYD3) genes in the rumen, reticulum, omasum, and abomasum tissues of pre-weaning (black bar) and weaned (grey bar) Japanese Black calves. Q-RT-PCR analysis was performed on total RNA extracted from the isolated epithelium of tissues in pre-weaning (n = 3) and weaned (n = 6) calves. 18S ribosomal RNA was used as an internal standard. All data were normalized using 18S ribosomal gene and expressed as fold (n-fold) over the value obtained from the rumen of pre-weaning calves. The data are presented as the mean ± SEM. N.D: Not detected.

[ab]Mean values with different superscripts were significantly different in the rumen, reticulum, omasum, and abomasum tissues ($P < 0.05$).

Values significantly different between pre-weaning and weaned calves in each tissue are marked with asterisks above the column: † : $P < 0.10$, * : $P < 0.05$, ** : $P < 0.01$

(modified from Kato et al. (2016)[12] with permission from publisher)

expression in the rumen between pre-weaning and post-weaning. The expression of HMGCS2 in the rumen was significantly higher at post-weaning than at pre-weaning. The expression of AKR1C1 in the forestomach was significantly higher at post-weaning than at pre-weaning. FABP3 expression in the rumen was significantly lower at post-weaning than at pre-weaning; however, its expression at pre-weaning was higher in the rumen than in other tissues.

HMGCS2 plays an important role in ketogenesis in the rumen epithelium of sheep during development and is upregulated in the rumen epithelium of weaned Holstein calves compared to in non-weaned calves[7, 14]. Moreover, HMGCS2 is a downstream target of peroxisome proliferator-activated receptor-α. Increased production of VFA induced by intake of solid feed during weaning may promote ketogenesis in rumen epithelial cells by the activation of HMGCS2, which is mediated by peroxisome

proliferator-activated receptor-α to promote papillary development[7]. Acetoacetyl-CoA thiolase and HMGCS convert acetyl-CoA to HMG-CoA, a central metabolite in the rumen epithelium involved in the conversion of short-chain fatty acids to acetyl-CoA[15, 16]. In the mitochondria, long-chain fatty acids from feed are converted to acetyl-CoA by β-oxidation. Moreover, acetyl-CoA and VFA (mainly butyrate) produced during fermentation by rumen microorganisms are substrates of ketone bodies such as β-hydroxybutyric acid (BHBA), and ketone bodies can be used to produce energy in various organs. This suggests that HMGCS2 regulates efficient energy expenditure in response to post-weaning increases in VFA and long-chain fatty acid as a result of the introduction of solid feed into the rumen.

AKR1C1 is a member of the aldo-keto reductase superfamily, which catalyzes the reduction of aldehydes, ketones, monosaccharides, ketosteroids and prostaglandins. Solid feed promotes increases in the levels of VFA and conversion rate of ketone bodies, and decreases pH in the rumen. Thus, AKR1C1 may play a role in regulating intra-ruminal pH levels by reducing the production of ketone bodies.

These results indicate that DDD analysis *in silico* is useful for screening candidate genes related to rumen development, and that the changes in expression levels of two genes (HMGCS2 and AKR1C1) in the rumen epithelial tissues may be caused by weaning, aging, or both.

3. Analysis of genes related to rumen development in Japanese black calves using RNA-seq

Comprehensive transcriptome analysis using the RNA-seq method was carried out for further gene expression profiling in the rumen epithelial tissue of Japanese Black cattle during weaning. Moreover, the analysis of canonical pathways, upstream regulators, biofunctions, and networks were performed using Ingenuity Pathway Analysis software. As a result of canonical pathway analysis, the genes associated with atherosclerosis pathway induced between pre- and post-weaning in the rumen epithelium. Because one factor important for atherosclerosis is a high cholesterol level, the conversion to a high-grain diet during weaning may stimulate this pathway. The expression levels of several genes related to atherosclerosis were decreased, and thus their expression can be induced in order to improve the rumen conditions. During weaning, immune response and inflammation reaction induced by the genes which contributes to diseases such as atherosclerosis, may occur in rumen epithelium. In addition, “tretinoin” was identified as an upstream regulator of genes altered during weaning in the rumen epithelium of Japanese Black cattle. Tretinoin is a derivative of vitamin A known as all-trans retinoic acid. Some reports have shown that vitamin A or its derivatives are related to epithelium function, and thus tretinoin may be an important factor that mediates the development of the rumen epithelium physiology during weaning. Moreover, because protein kinase B (PKB) was identified and enriched by the most activated network during weaning, PKB is related to rumen development predominantly between pre- and post-weaning.

4. Culture of rumen epithelial cells *in vitro*

As described above, the morphological and functional development of the rumen epithelial tissue is induced by the consumption and physical stimulation of solid feed and chemical stimulation of VFA. However, knowledge regarding rumen function and changes caused by these stimuli was obtained through *in vivo* experiments. Several studies have analyzed the morphological changes of the rumen when the cattle consume different types of feed; however, experiments involving primary epithelial cell culture are not sufficient. In 1980, Galfi et al. first reported the preparation of rumen epithelial cells using common isolation methods from general media. We also attempted to isolate rumen epithelial cells from adult cattle and sheep[17]. Other groups have isolated rumen epithelial cells for functional evaluation as rumen epithelial cells. Baldwin et al., in 1991, developed an improved method for preparing rumen epithelial cells, based on Galfi`s method. The concentration of the

synthesized BHBA was determined following treatment with butyrate, acetate, and/or propionate[18]. This report showed that the synthesis of BHBA by butyrate is unique to rumen epithelial cells. Upregulation of the HMGCS2 gene in rumen epithelial tissues after weaning results from increased ketone body synthesis. This suggests that the synthesis of BHBA by butyrate is important for evaluating rumen epithelial cells.

5. Conclusion

We profiled gene expression changes between pre- and post-weaning cattle mainly through DDD *in silico* and RNA-seq analyses to clarify the molecular mechanism of rumen development. We have reported candidate genes (AKR1C1 and HMGCS2) related to weaning and aging. However, the 1) morphological characteristics of rumen epithelium, 2) evaluation of rumen epithelial cell function, 3) establishment of long-term culture techniques for rumen epithelial cells *in vitro*, and 4) elucidation of the molecular mechanism of rumen development in ruminants should be conducted. These studies will reveal new information to increase the understanding of rumen development.

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Presence of brown/beige adipocytes in adipose tissues of fattening cattle and diet-related changes in their activity

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Abstract

Brown/beige adipocytes dissipate energy as heat; in mice brown adipocyte constitutes brown adipose tissue, whereas beige adipocytes are sparsely distributed within the white adipose tissue (WAT). Thermogenesis in brown/beige adipocytes is elicited through expression of uncoupling protein 1 (Ucp1); Ucp1 is specifically expressed in brown/beige adipocytes in mammals. Presence of brown/beige adipocytes is not preferable in beef cattle, because it potentially decreases fattening efficiency. At the time when we started to study, it has been believed no brown/beige adipocytes in adult cattle. We first explored Ucp1 expression in WAT of Japanese Black fattening cattle aged 31 months. RT-PCR analyses indicated the significant expression of Ucp1, and immunohistochemical analyses revealed sparse localization of Ucp1-positive adipocytes in WAT. We also explored effect of diet on expression of brown/beige adipocyte-related genes including Ucp1 in WAT of fattening cattle. In the subcutaneous WAT of fattening cattle, expression levels of Ucp1 and the other brown/beige adipocyte-related genes were basically higher in cattle fed the concentrate diet (high concentrate diet) than in those fed the roughage diet (high roughage diet). Feeding vitamin A-deficient diet did not affect Ucp1 expression, but expression levels of the other brown/beige adipocyte-related genes in the mesenteric WAT were generally higher in cattle fed the vitamin A-deficient diet than in those fed the control diet. It has been suggested involvement of the BMP pathway in beige adipocyte differentiation. The diet-related changes in expression level of brown/beige adipocyte-related genes were basically consistent with those in expression level of components to elicit BMP signaling. A series of our studies indicates that 1) beige adipocytes are present also in fattening cattle, 2) diet modulates expression levels of brown/beige adipocyte-related genes, and 3) diet-related changes in the BMP pathway potentially regulate expression level of brown/beige adipocyte-related genes in a WAT location-dependent manner.

1. Introduction

There are three kinds of adipocytes in mammals: white, brown and beige (Fig. 1). White adipocytes constitute white adipose tissue (WAT), which is dispersed throughout the body[1)], are specialized for the storage of excess energy. White adipocytes contain all of the enzymatic machinery necessary to produce triacylglycerols from fatty acids, either those synthesized *de novo* or imported from circulating lipoproteins. In addition, white adipocytes play a central role in the regulation of energy balance through the synthesis and secretion of molecules called adipokines, including leptin[2,3)]. In contrast, brown/beige adipocytes are specialized to dissipate chemical energy in the form of heat in response to cold or excess feeding[4)]; in mice, brown adipocytes constitute brown adipose tissue (BAT),

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肥育牛の脂肪組織中に褐色／ベージュ脂肪細胞が存在することの証明ならびに飼料条件の変化に伴う活性の変化
舟場正幸[1]・山田知哉[2]・橋本　統[3]・村上　賢[4]・金森燿平[1]・松井　徹[1]
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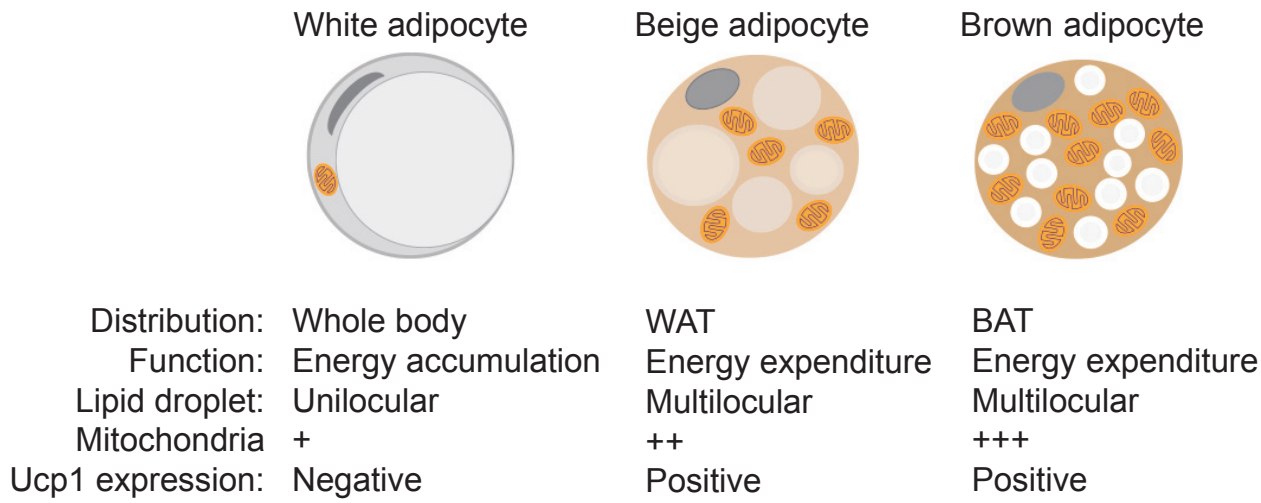

Fig. 1. Three kinds of murine adipocytes with distinct functions and distributions.

which located in the interscapular region[5], whereas beige adipocytes are induced and distributed in WAT. Molecular signature of brown adipocytes is different from that of beige adipocytes; beige adipocytes are differential-lineage cells from brown adipocytes, irrespective of the closely related functions[4]. The thermogenic function of brown/beige adipocytes results from the expression of a series of genes related to high mitochondrial content, as well as elevated cellular respiration largely uncoupled from ATP synthesis. The uncoupling occurs through expression of uncoupling protein 1 (Ucp1), a mitochondrial protein that promotes proton leak across the inner mitochondrial membrane[1,6].

Thermogenesis in brown/beige adipocytes has been considered to occur in a limited number of animal species, including small rodents, and under the limited (patho)physiological status such as newborn humans and patients with phenochromocytoma[1,6]. Several years ago, a significant amount of a functional brown adipocyte depot was identified in adult humans by integrated positron emission tomography-computed tomography (PET-CT) studies using an ^{18}F-labeled fluorodeoxyglucose, a glucose analogue, as a tracer, and by the immunohistochemical analyses to detect Ucp1[7-10]. In view of ability of brown/beige adipocytes to promote energy expenditure, activation of brown/beige adipocytes has been a topic of considerable interest as the therapeutic potential in obesity and obesity-related diseases in humans[4,11]. Thus, activation of brown/beige adipocytes is desirable in adult humans.

In contrast to preferential role of brown/beige adipocytes in human health, presence of functional brown/beige adipocytes are not favorable in beef cattle. They are raised as industrial animals, and fattening efficiency is one of the determining factors of the economy of beef production; activation of brown/beige adipocytes potentially leads to decrease in fattening efficiency. Thus, activation of brown/beige adipocytes is undesirable in beef cattle.

In view of evidence in adult humans, presence of brown/beige adipocytes in fattening cattle was expected, but it had not been verified. Thus, we started the project to identify 1) presence of brown/beige adipocytes in beef cattle, 2) endogenous factors affecting brown adipogenesis, and 3) dietary factors affecting expression of genes related to brown/beige adipocyte activity.

2. Presence of brown/beige adipocytes in adipose tissues of fattening cattle[12]

At present, Ucp1 expression is believed to be limited to brown/beige adipocytes in mammals[1,13]; Ucp1 is a marker of molecule of brown/beige adipocytes. We first explored Ucp1 expression by RT-PCR in WAT of fattening cattle. Using the primer set to detect Ucp1 specifically, we explore Ucp1 expression by RT-PCR in perirenal WAT of fattening cattle aged 31 months; a

band with the expected size was reproducibly detected only in the sample treated with reverse transcriptase (Fig. 2). Next, we evaluated localization of Ucp1 in WAT; Ucp1-positive small adipocytes were scatteredly located between white adipocytes in the subcutaneous WAT of fattening cattle (Fig. 3). These results indicated that beige adipocytes are also present in WAT depots of fattening cattle as well as mice.

Previous studies showed that Ucp1 expression was detected in the subcutaneous WAT of fetal calves[14, 15]. However, the expression level was decreased to detection limit at birth, and Ucp1 expression could not be detected in the subcutaneous, perirenal and intermuscular WAT of mature cattle[14, 16, 17]. The discrepant results may result from different methods to evaluate Ucp1 expression or differences of breed of cattle or both; in the previous studies, Ucp1 expression was examined by Northern blot or slot blot analyses; these analyses are generally insensitive methods.

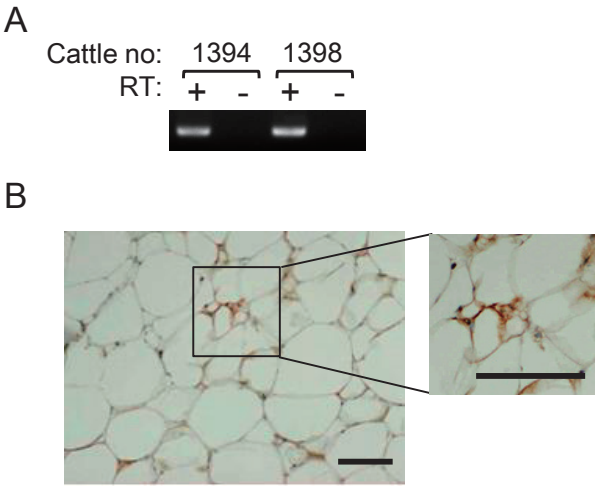

Fig. 2. Expression of Ucp1 in WAT depot of fattening cattle.

(A) Ucp1 expression in the perirenal WAT depot of fattening cattle aged 31 months was examined by RT-PCR. The cDNA was prepared by treatment with (RT+) or without (RT-) reverse transcriptase. (B) Localization of Ucp1 in the subcutaneous WAT of fattening cattle was examined by immunohistochemical analyses. Bar: 100 μm. Reproduced with modification from reference[12] with permission.

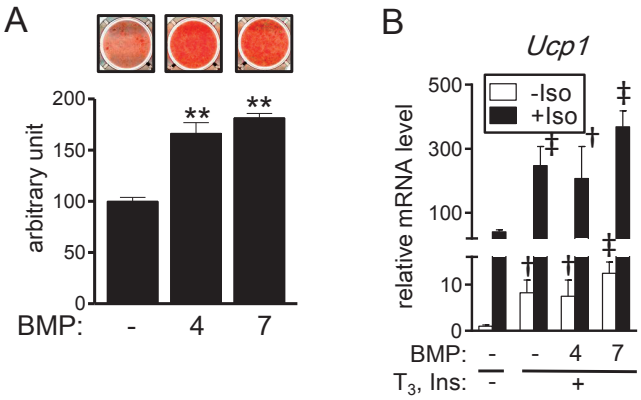

Fig. 3. BMP4 and BMP7 during brown adipogenesis stimulate lipid accumulation but not increase Ucp1 expression in brown adipocytes.

Effects of exogenous Bmp on lipid accumulation (A) and expression of Ucp1 (B) in HB2 cells. BMP4 or BMP7 was treated during brown adipogenesis induced by T_3 and insulin (Ins). (A) Lipid accumulation was examined on day 8 by Oil Red O staining (*upper panel*), and the dye was quantified (*lower panel*). * *: $P < 0.01$. (B) HB2 cells on day 8 were treated with or without isoproterenol (Iso) for 4 h. The gene transcript level of Ucp1 was measured by RT-qPCR. † and ‡ : $P < 0.05$ and $P < 0.01$, respectively, *vs.* cells treated without BMP, T_3 and insulin. Reproduced with modification from reference[18] with permission.

3. Role of the TGF-β family in brown adipogenesis[18]

Total activity of adipocytes in whole body is determined by the number of adipocytes and the activity in an adipocyte; differentiation of preadipocytes to adipocytes, i.e., adipogenesis, is a determinant of the adipocyte number[3]. In contrast to the information about regulation of white adipogenesis[3], knowledge of factors affecting brown/beige adipogenesis is limited. The transforming growth factor-β (TGF-β) family consisting of TGF-β, activin and BMP subgroups potently regulates commitment, differentiation and maturation of mesenchymal cells[19]. This family also participates in the process of the development and function of white adipocytes[20]. Despite the differences in the developmental origins of brown and white adipocytes[21], the transcriptional cascade is shared between the development of brown adipocytes and white adipocytes[22]. Thus, the roles of the TGF-β family in brown adipogenesis were explored. We used HB2 brown

preadipocytes isolated from the interscapular brown adipose tissue of p53-knockout mice[23].

At first we explored whether BMP4 or BMP7 has an ability to modulate brown adipogenesis. Treatment with BMP4 or BMP7 during brown adipogenesis significantly increased lipid accumulation on day 8 (Fig. 3A). Ucp1 expression is enhanced by β-adrenergic receptor activation[6]. Consistent with this, isoproterenol, a β-receptor agonist, increased expression of Ucp1, but neither BMP7 nor BMP4 affected the basal or the induced levels of Ucp1 (Fig. 3B). Ucp1 expression level basically reflects activity of brown/beige adipocytes[4,6]. Thus, it is suggested that BMP4 and BMP7 do not affect main activity of brown adipocytes.

Next, we evaluated the role of TGF-β and activin A in brown adipogenesis; TGF-β1 or activin A inhibited lipid accumulation (Fig. 4A), basal Ucp1 expression (Fig. 4B), and expression of the other brown adipocyte-selective genes such as Pgc1α, Cidea and Cox7a (Fig. 4B). The expression of Pparγ2, C/ebpα and Fabp4, genes involved in adipogenesis itself and expressed in mature adipocytes[3], was also decreased by TGF-β1 or activin A (Fig. 4C). Furthermore, the isoproterenol-induced Ucp1 expression was blunted by TGF-β1 or activin A, which was verified at the mRNA level (Fig. 5A) and at the protein level (Fig. 5B).

The role of BMP in brown adipogenesis shown here is distinct from that in a different cell line of brown preadipocytes prepared from murine brown adipose tissue; BMP7 stimulated brown adipogenesis[24,25]. Currently, the reason of the discrepant results is not clear, but it may relate to the differences in the stage of brown preadipocytes (i.e., the extent of commitment as preadipocytes). BMP4 potentiated the commitment of pluripotent mesenchymal cells to adipocyte-lineage cells[26,27]. It is possible that the brown preadipocytes used in previous studies are relatively closer to mesenchymal cells than to HB2 cells. In fact, Schulz et al.[25] showed

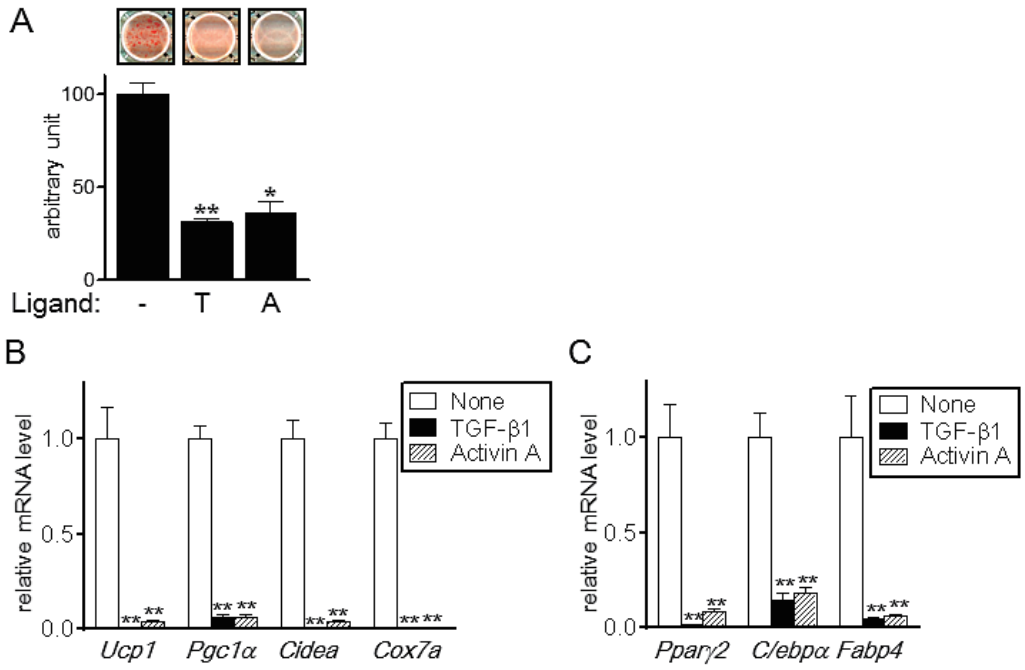

Fig. 4. TGF-β1 and activin A inhibit brown adipogenesis.

Effects of TGF-β1 and activin A on insulin-mediated lipid accumulation (A) and expression of genes related to brown adipocyte differentiation (B and C). TGF-β1 or activin A was treated during brown adipogenesis. (A) Lipid accumulation was examined on day 8 by Oil Red O staining (*upper panel*), and the dye was quantified (*lower panel*). * and ** : $P < 0.05$ and $P < 0.01$, respectively. (B and C) The gene transcript levels of Ucp1, Pgc1α, Cidea and Cox7a (B), and Pparγ2, C/ebpα and Fabp4 (C) on day 8 were measured by RT-qPCR. ** : $P < 0.01$. Reproduced with modification from reference[18] with permission.

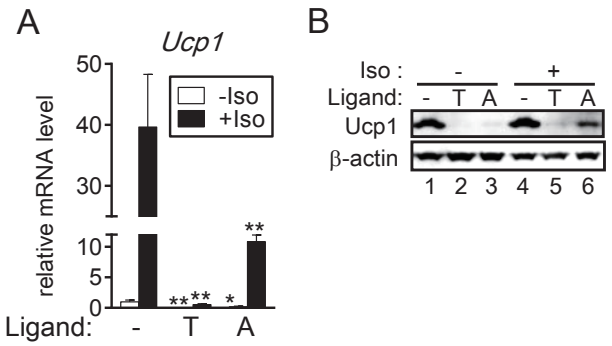

Fig. 5. TGF-β1 and activin A blunt responsiveness to β3 adrenergic receptor activation.

Effects of TGF-β1 and activin A on brown adipocyte differentiation. TGF-β1 or activin A was treated during brown adipogenesis. HB2 cells on day 8 were treated with or without isoproterenol (Iso) for 4 h. (A) The gene transcript level of Ucp1 was measured by RT-qPCR. * and ** : $P<0.05$ and $P<0.01$, respectively, *vs*. respective cells treated with or without isoproterenol and treated without TGF-β1 and activin A. (B) Ucp1 expression (*upper panel*) was examined by Western blotting, followed by re-blotting with an anti-β-actin antibody (*lower panel*). Reproduced with modification from reference[18] with permission.

that BMP7 treatment in Sca1$^+$/CD45$^-$/Mac1$^-$ stem cells promoted brown adipogenesis. Furthermore, the BMP pathway stimulates beige adipogenesis rather than brown adipogenesis more strongly; BMP4 and BMP7 induced differentiation of stromal vascular cells from WAT or mesenchymal stem cells to beige adipocytes[28,29].

Previous studies revealed that TGF-β and activin A inhibited differentiation of white preadipocytes through inhibition of transcriptional activity of the C/ebpβ, or the expression of Pparγ[30,31]. The present results also revealed the down-regulation of Pparγ expression in HB2 brown (pre)adipocytes treated with TGF-β1 or activin A. It is possible that these growth factors negatively regulates common pathway in white adipogenesis and brown adipogenesis, resulting in inhibition of adipogenesis itself.

4. Diet-related changes in expression of brown/beige adipocyte-related genes

4.1. Dietary roughage to concentrate ratio affects Ucp1 expression in brown/beige adipocytes in subcutaneous WAT of fattening cattle[12]

We next explored dietary factors affecting expression of brown/beige adipocyte-related genes in WAT of fattening cattle. At first, effects of dietary ratio of roughage to concentrate were examined in WAT of fattening cattle. Japanese Black steers aged 10 months were allotted to one of two groups: the roughage diet group or the concentrate diet group. The roughage diet consisted of 35% roughage and 65% concentrate mixture, whereas the concentrate diet contained 10% roughage and 90% concentrate mixture on a TDN basis[32]. To eliminate the influence of the total TDN intake between groups, the steers were pair-fed for 20 months. At 30 months of age, WAT samples were collected from five types of WAT (subcutaneous, mesenteric, perirenal, intermuscular, and intramuscular), and expression levels of brown/beige adipocyte-related genes including Ucp1 were examined. Expression of Ucp1 in the subcutaneous WAT was significantly higher in cattle fed the concentrate diet than in those fed the roughage diet (Fig. 6A). In contrast, the expression in the other regions of WAT depots was not different between the groups, although it tended to be higher in the concentrate diet group in the intramuscular WAT.

Expression levels of the other brown/beige adipocyte-related genes, Pgc1α, Cidea, Dio2, Cox1, Cox7a and Cox8b, were also examined (Fig. 6B); these genes are involved in up-regulating Ucp1 expression and in activating brown/beige adipocytes[4,33]. Consistent with increased expression of Ucp1, expression levels of the brown/beige adipocyte-related genes in the subcutaneous WAT were generally higher in the concentrate diet group than in the roughage diet group. Specifically, expression levels of Dio2, Cox1 and Cox8b were significantly higher in the concentrate diet group. In contrast, expression levels of the brown/beige adipocyte-related genes were comparable between the groups in the mesenteric WAT,

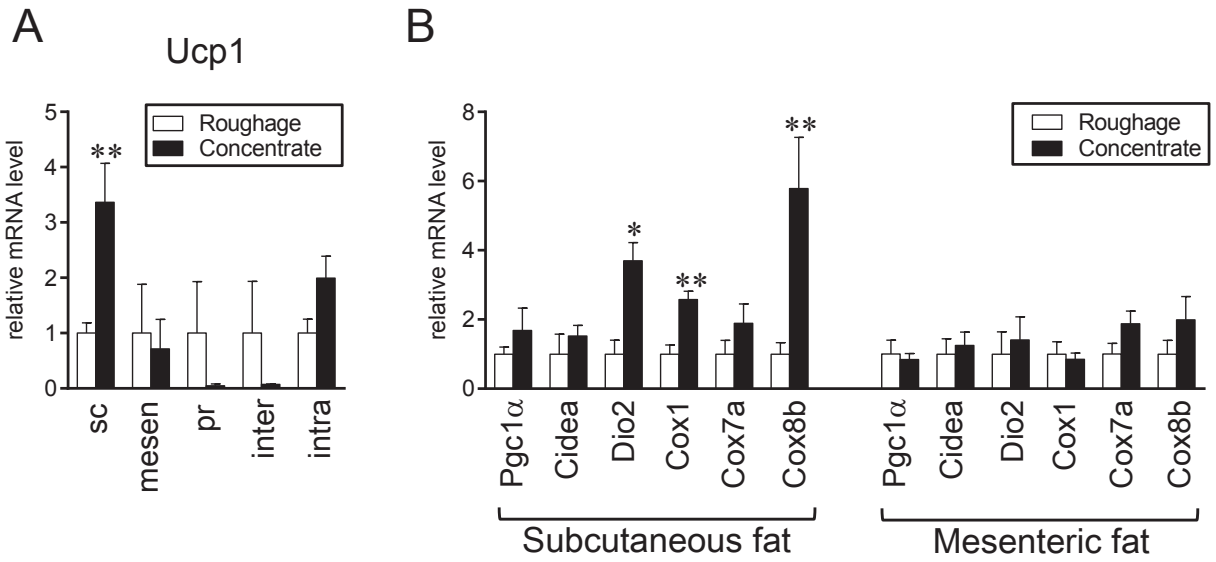

Fig. 6. Relative expression of brown/beige adipocyte-related genes in WAT of fattening cattle fed the roughage diet or the concentrate diet.

Fattening cattle were fed either the roughage diet or the concentrate diet for 20 months. At 30 months of age, (A) expression of Ucp1 in the subcutaneous (sc), mesenteric (mesen), perirenal (pr), intermuscular (inter) and intramuscular (intra) WAT and (B) expression of brown/beige adipocyte-related genes in the subcutaneous and mesenteric WAT were examined by RT-qPCR. The expression of Ucp1 in the control diet group in each WAT (A) and the expression in the control diet group in each WAT (B) were set to 1. * and ** : $P < 0.05$ and $P < 0.01$, respectively. Reproduced with modification from reference[12] with permission.

intermuscular WAT and intramuscular WAT, and the expression in the perirenal WAT was numerically higher in the roughage diet group (Fig. 6B, data not shown). The results that expression level of brown/beige adipocyte-related genes in the subcutaneous WAT was increased by feeding the concentrate diet may reflect the increase in the number of functional brown/beige adipocytes.

4.2. Vitamin A restriction affects expression of brown/beige adipocyte-related in mesenteric WAT of fattening cattle[34]

Retinoic acid, which is metabolized from the β-carotene (provitamin A), is known to stimulate the transcription of murine Ucp1[35]. In addition, dietary vitamin A affected the Ucp1 expression in murine BAT; dietary supplementation with vitamin A increased the expression of Ucp1[36,37], whereas a vitamin A deficiency suppressed it[38]. Thus, the vitamin A status may affect expression of brown/beige adipocyte-related genes also in WAT of fattening cattle.

Dietary vitamin A is frequently restricted in fattening beef cattle to improve beef marbling. Yamada et al.[39] reported that the plasma concentration of retinol decreased with fattening in steers fed the vitamin A-restricted diet consisting of orchard grass hay and concentrate (0.1 mg/kg of β-carotene), reached approximately 20 IU/dl at 24 months of age and was thereafter maintained at approximately 40 IU/dl. In contrast, the concentration of retinol was maintained above 60 IU/dl in the plasma of fattening cattle fed a fermented total mixed ration (TMR) with more β-carotene (7-16 mg/kg of β-carotene[39]). We evaluated expression levels of brown/beige adipocyte-related genes in WAT of these fattening cattle; Japanese Black steers aged 10 months were fed either the control diet consisting of the TMR or the vitamin A-deficient diet consisting of orchard grass hay and concentrate for 20 months.

No significant differences were observed in the expression of Ucp1 in all examined WAT between the control and vitamin A-deficient groups (Fig. 7A). Although it was not statistically significant because of limited number of cattle (n = 4 in a group), the expression

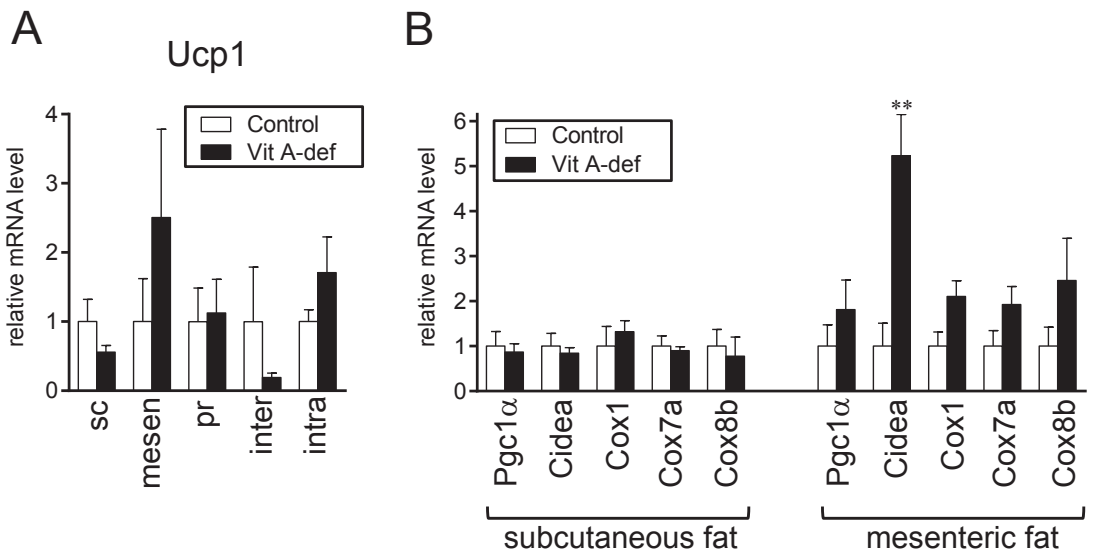

Fig. 7. Relative expression of brown/beige adipocyte-related genes in WAT of fattening cattle fed the vitamin A-deficient diet.

Fattening cattle were fed either the control diet or vitamin A-deficient (VitA-def) diet for 20 months. At 30 months of age, (A) expression of Ucp1 in the subcutaneous (sc), mesenteric (mesen), perirenal (pr), intermuscular (inter) and intramuscular (intra) WAT and (B) expression of brown/beige adipocyte-related genes in the subcutaneous and mesenteric WAT were examined by RT-qPCR. The expression of Ucp1 in the control diet group in each WAT (A) and the expression in the control diet group in each WAT (B) were set to 1. ** : $P < 0.01$. Reproduced with modification from reference[34].

of the other brown/beige adipocyte-related genes, except for Cidea, in the mesenteric WAT was numerically higher in the vitamin A-deficient group than in the control group; Cidea expression was significantly higher in the vitamin A-deficient group (Fig. 7B). In contrast, differences of expression levels between the dietary groups were relatively small in the subcutaneous WAT. As shown above, the expression of brown/beige adipocyte-related genes in the subcutaneous WAT but not in the mesenteric WAT were higher in cattle fed the concentrate diet than in those fed the roughage diet. These results suggest that fat depots, in which potential brown/beige adipocytes emerge, vary depending on the manipulated nutrients.

As compared with brown adipocytes, the basal expression of Ucp1 in beige adipocytes is relatively low, but is enhanced in response to the activation of β adrenergic receptors[40]. Thus, vitamin A deficiency may potentially stimulate the emergence of beige adipocytes in the mesenteric WAT of fattening cattle, and in response to sympathetic nerve activation Ucp1 expression is accelerated.

We expected the expression of Ucp1 to be down-regulated in fattening cattle fed the vitamin A-deficient diet based on the retinoic acid-induced transcription of murine Ucp1[35]. However, the vitamin A deficiency did not affect the expression of Ucp1, and the precise reason for this is currently unknown. A species-dependent effect of retinoic acid-mediated Ucp1 expression has been reported previously; retinoic acid up-regulated the expression of Ucp1 in murine adipocytes, but not in human adipocytes[35,41,42]. Future studies are needed to clarify the reason on species-dependent response of Ucp1 expression to retinoic acid.

The study shown here to evaluate effect of vitamin A status compared TMR diet as the control diet with conventional high-concentrate diet with vitamin A restriction as the vitamin A-deficient diet. However, not only β-carotene content but also content of the other nutrient clearly differs between these two diets, because dietary components are different[34]. Future studies are needed to evaluate difference in content of β-carotene (vitamin A) alone in diet.

4.3. Expression level of components of the BMP pathway changes with diets[43)]

As shown above, the cell culture study revealed that the TGF-β family regulates brown adipogenesis; TGF-β/activin negatively regulate brown adipogenesis. In contrast, BMP does not have a profound effect on differentiation of brown preadipocytes irrespective of stimulatory effects on lipid accumulation. However, the previous studies suggested that BMP stimulates differentiation to brown/beige adipocytes from mesenchymal stem cells[24, 25, 28, 29)]. Thus, we expected that diet-related changes in expression of brown/beige adipocyte-related genes in WAT of fattening cattle are achieved through modulation of TGF-β family signaling. The induction of ligands and their signaling components often occurs to transmit their signals[44)]. Therefore, we examined the expression levels of possible molecules to elicit TGF-β family signaling in WAT of fattening cattle fed the roughage diet or the concentrate diet, and the control or the vitamin A-deficient diet as described above. We speculated that expression of TGF-β or activin A exhibits negative relation to that of brown/beige adipocyte-related genes, or that the BMP expression is positively related to brown/beige adipocyte-related gene expression.

In the subcutaneous WAT, BMP4 expression tended to be higher in the concentrate diet group ($P = 0.06$, Fig. 8A). By contrast, no differences between the dietary groups were detected in the mesenteric WAT. One cattle fed the roughage diet exhibited extremely lower expression of TGF-β1 in the subcutaneous WAT; as the result, TGF-β1 expression in the subcutaneous WAT seemed to be higher in the concentrate diet group. The vitamin A-deficient diet did not affect expression levels of the TGF-β family in subcutaneous WAT, but expression of activin βA and BMP4 was significantly increased by feeding the vitamin A-deficient diet in the mesenteric WAT (Fig. 8B). Expression level of BMP7 was 20-fold higher in the vitamin A-deficient diet group than in the control diet group, although it was not statistically significant because of the large variation in the vitamin A-deficient diet group. It is not clear the reason of large individual differences on BMP7 expression. In fetal mice, loss of retinoic acid synthesis led to decreased expression of BMP7 but not BMP4[45)]; this relationship between retinoic acid and BMP7 expression seems to be opposite in bovine WAT shown here, but it is possible that vitamin A-deficient diet affects bovine BMP7 expression.

Diet-related changes in BMP4 expression was similar to those in brown/beige adipocyte-related genes; expression levels of these genes were generally higher in the subcutaneous WAT of fattening cattle fed the

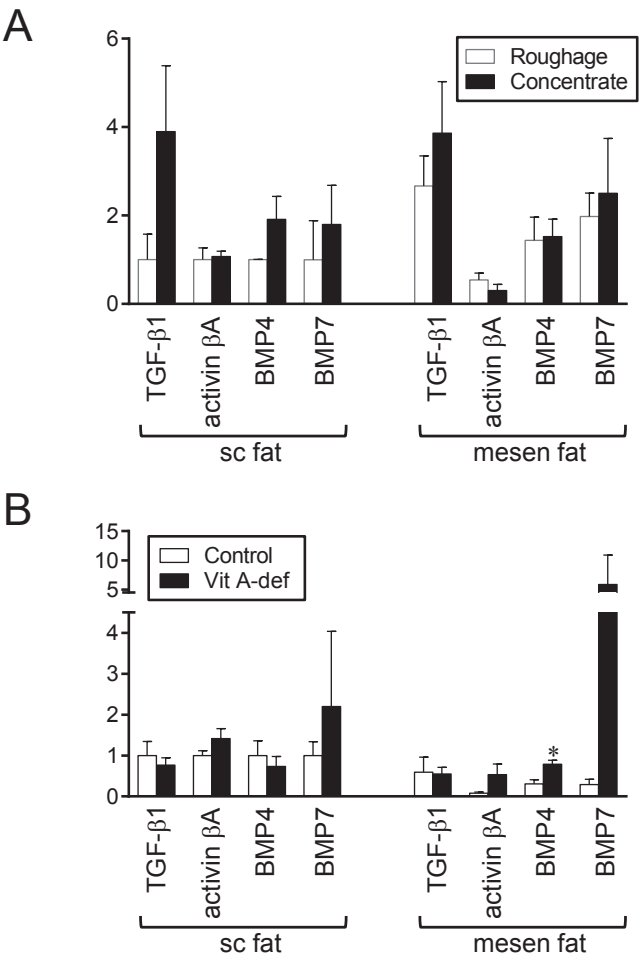

Fig. 8. Expression of TGF-β family in WAT of fattening cattle.

Fattening cattle were fed either the roughage diet or the concentrate diet (A) or the control diet or the vitamin A-deficient diet (B) for 20 months. The subcutaneous and mesenteric WAT were collected, and the mRNA levels of the indicated molecules were examined by RT-qPCR. The expression in each WAT in the roughage diet group (A) or control diet group (B) was set to 1. * : $P < 0.05$. Reproduced with modification from reference[43)] with permission.

concentrate diet and in the mesenteric WAT of those fed the vitamin A-deficient diet. Thus, we next focused to examine expression of components to elicit the BMP signaling. BMP signals through complex formation with the type I receptor and type II receptor[46]; ALK2 and ALK3 are BMP type I receptors, while ActRIIA, ActRIIB and BMPR2 are BMP type II receptors[46]. The expression of ALK2 and ALK3 in the subcutaneous WAT was slightly higher in the concentrate diet group than in the roughage diet group (P = 0.07 and P = 0.09, respectively), whereas no significant differences were observed in expression levels in the mesenteric WAT between the groups (Fig. 9A). In contrast, the vitamin A deficiency did not affect the expression of BMP receptors in the subcutaneous WAT (Fig. 9B). However, the vitamin A-deficient diet significantly or slightly increased the expression of ALK2, ALK3, ActRIIA, and BMPR2 (P = 0.05) in the mesenteric WAT. Significant changes in the expression of ActRIIB were not detected in the WAT of fattening cattle (data not shown). These results suggest that the higher expression of brown/beige adipocyte-related genes in WAT of fattening cattle was achieved by stimulation of the BMP pathway through an enhancement in the expression levels of components involved in BMP signaling.

In humans, expression levels of the molecules involved in the BMP pathway in adipose tissue appear to be related to adiposity; the precise relationship between cause and effect remains unclear. A negative relationship has been reported between BMP4 expression levels in adipose tissue and body mass index[47]. Furthermore, the expression levels of ALK3 and BMPR2 in adipose tissue were found to be higher in obese humans than in lean humans[48,49]. The carcass composition of fattening cattle examined gene expression of BMP signal components was similar between the dietary groups[32,39]. Therefore, this altered expression of genes involved in the BMP pathway may reflect an intrinsic effect of the diet, but not adiposity.

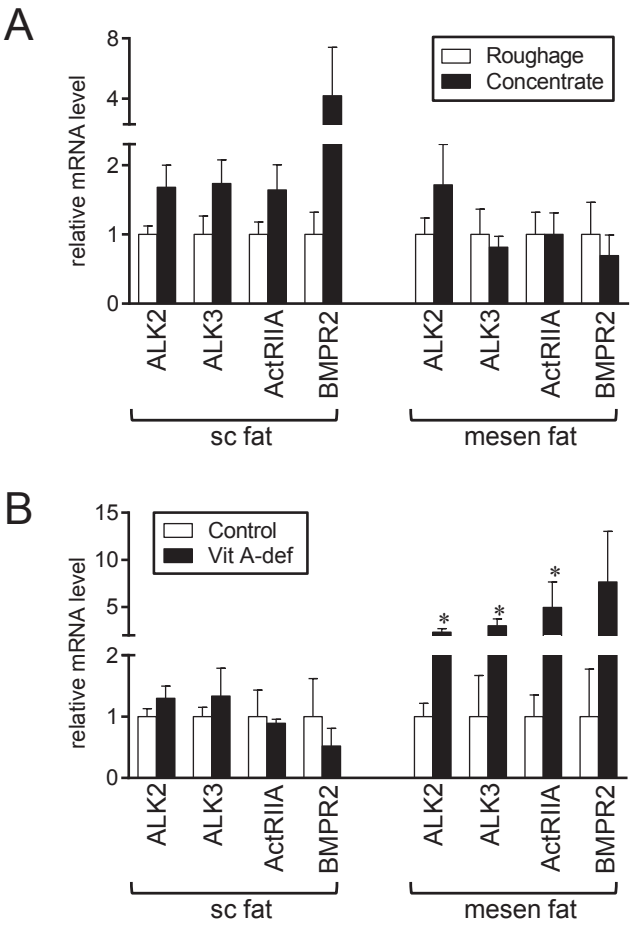

Fig. 9. Expression of receptors transmitting BMP signals in WAT of fattening cattle.
Fattening cattle were fed either the roughage diet or the concentrate diet (A) or the control diet or the vitamin A-deficient diet (B) for 20 months. The subcutaneous and mesenteric WAT were collected, and the mRNA levels of the indicated molecules were examined by RT-qPCR. The expression in each WAT in the roughage diet group (A) or control diet group (B) was set to 1. * : $P < 0.05$. Reproduced with modification from reference[43] with permission.

5. Conclusions and future directions

In adult humans, it has been estimated that the weight of the supraclavicular fat depot consisting of brown/beige adipocytes is estimated to be 63 g, and that full activation of the brown/beige adipocytes accounts for energy expenditure equivalent to 4.1 kg of adipose tissues in a year[10]. In addition, there is another estimation that 50 g of stimulated brown/beige adipocytes account for up to 20% of daily energy expenditure in adult humans[50]. Furthermore, the increased energy expenditure detected during cold exposure can be largely explained by brown/

beige adipocyte activation[51]. We provide the information on the presence of brown/beige adipocytes in adipose tissues of fattening cattle. Immunohistochemical analyses indicated that the number of Ucp1-positive adipocytes is much smaller than that of white adipocytes. Consequently, Ucp1 expression level is relatively lower, when it is expressed as expression level per tissue. However, in view of a large mass of adipose tissue in whole body, it is possible that Ucp1-mediated thermogenesis accounts for significant energy expenditure in fattening cattle.

Obviously, further studies are needed; they should be done from at least three directions. First, it is needed to know whether Ucp1-positive adipocytes in adipose tissues of fattening cattle are functional on dissipation of energy. Beef cattle are raised as industrial animals, and fattening efficiency is one of the determining factors of the economy of beef production. Unlike the case of adult humans, the emergence of beige adipocytes is not preferable in beef cattle. Secondly, Ucp1 expression should be thoroughly examined in whole body of fattening cattle. Even if its expression level is relatively lower in the tissues, Ucp1-mediated energy expenditure may not be able to ignore in view of energy balance of fattening cattle, depending on the tissue mass in the whole body. Thirdly, molecular mechanism underlying regulation of the BMP pathway and dietary factors affecting BMP signaling should be further clarified. As described above, brown/beige adipocyte activity is positively regulated by the BMP pathway, which is controlled by expression levels of components to elicit BMP signal. Thus, clarification of dietary factors affecting BMP pathway may lead to fundamental understanding underlying diet-related changes in expression of brown/beige adipocyte-related genes (Fig. 10). Through these evaluations, more efficient fattening program should be established in fattening cattle.

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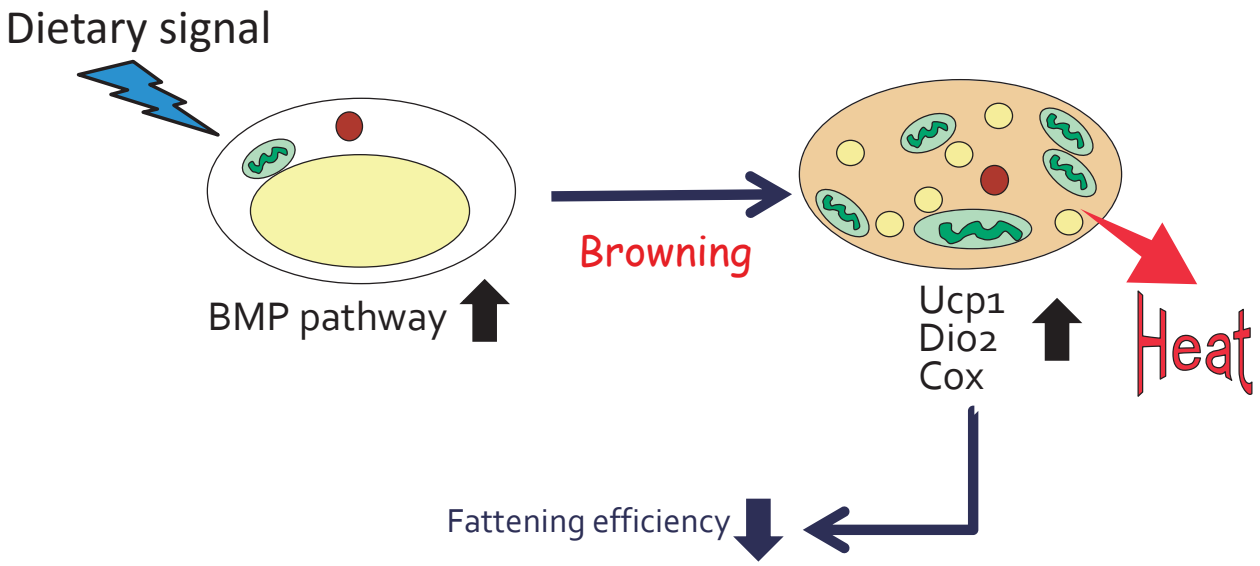

Fig. 10. Possible regulation of beige adipocyte activity by diet-related BMP pathway.

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Development of the porcine intestine during early postnatal life －Evaluation focusing on histological, enzymatic and immunological parameters－

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1. Introduction

Intestinal development of growing piglets during early postnatal days is strongly associated later in life with feeding efficiency and susceptibility to pathogens. For example, a gut with well-developed villous and crypt structure shows an enhanced absorptive capacity that results in a higher feed conversion rate[1)].

Weaning in general can a very stressful event for piglets, but in particular for those raised in commercial pig farms. Indeed, during weaning piglets experience stress brought about by physiological, social, environmental and dietary factors that can cause a serious delay in gut development[2)]. For example, the structure and function of the small intestine is severely interrupted after weaning[3)].

The gut structure and functionality of a young pig are comparable with that of an adult when the animal is around 2 months of age[4,5)]. Nonetheless, during early life and while still suckling, piglets experience intensive development of many parameters associated with the digestive–absorptive and mucosal immune systems[6)].

In the present review, we will discuss data from work on the structural and functional development of the gut of piglets happen from suckling to weaning. The results showed in this review were largely originated from experiments previously conducted by our group[6,7)].

2. Basic information of the slaughtered piglets

The coding system for animal identification and the mean body weight of slaughtered piglets are shown in the slaughter plan described in Table 1. All animals were crossbred (Landrace × Large white × Duroc) piglets and bred in a same farm. Suckling piglets were fed solely breast milk. Weaned piglets received a commercial diet

Table 1. Mean body weight of slaughtered piglets and abbreviations.

	Age						
Stage	1-day	7-day	14-day	21-day	28-day	35-day	42-day
Suckling	S1 1.8 kg	S7 3.7 kg	S14 5.3 kg	S21 7.0 kg	S28 10.4 kg	-	-
14-day-old weaned	-	-	-	W14p7 6.2 kg	W14p14 8.9 kg	-	-
21-day-old weaned	-	-	-	-	W21p7 9.0 kg	W21p14 13.1 kg	
28-day-old weaned	-	-	-	-	-	W28p7 12.4 kg	W28p14 15.9 kg

Four piglets were slaughtered at the respective condition.
This table was originally published in Tsukahara *et al.* 2016[7)].

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新生仔豚の腸管発達　－病理的，酵素的及び免疫的な点での評価－
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for weaning (JustOne Sprout; Toyohashi Feed Mills, Aichi, Japan). Piglets in the suckling groups were slaughtered at 1 (S1), 7 (S7), 14 (S14), 21 (S21) and 28 (S28) days of age. Piglets in the weaning groups were weaned at 14 (W14), 21 (W21) or 28 (W28) days, and slaughtered 7 (p7) or 14 (p14) days after weaning. To understand the experimental coding system, W14p7, for example, denotes piglets that were weaned at 14 days of age and slaughtered 7 days post-weaning. The experimental animals were handled in accordance with the guidelines for animal studies of the Experimental Animal Committee of Kyoto Prefectural University.

Collection and dissection of the small intestine of piglets were as previously described[8]. Small intestines were measured and divided in eight sections of equal length. As shown in the schematics in Fig. 1, sections were sequentially numbered as si-1 for the proximal end to si-8 for the distal end. Furthermore, section si-1 was considered as both the duodenum and proximal jejunum; sections si-2 to si-4, the jejunum; sections si-5 and si-6, both the jejunum and ileum complex; and sections si-7 and si-8, the ileum[8]. Length of the small intestine varies according to the age of slaughtered piglets, as shown in Fig. 2. For example, the length of the small intestine was approximately 400 cm in S1 piglets, but gradual changes in length were observed in intestinal tissues of piglets from days 1 to 28. It can be observed that weaning tended to extend the length of the small intestine (Fig. 2).

Fig. 1. Schematics of the collection and sectioning of the small intestine
Tissues collected from the areas marked with a circle were used for pathologic analysis, and mucosa collected from areas marked with a triangle was used for transcriptome analysis. Mucosa from other areas was used for enzymatic analysis.

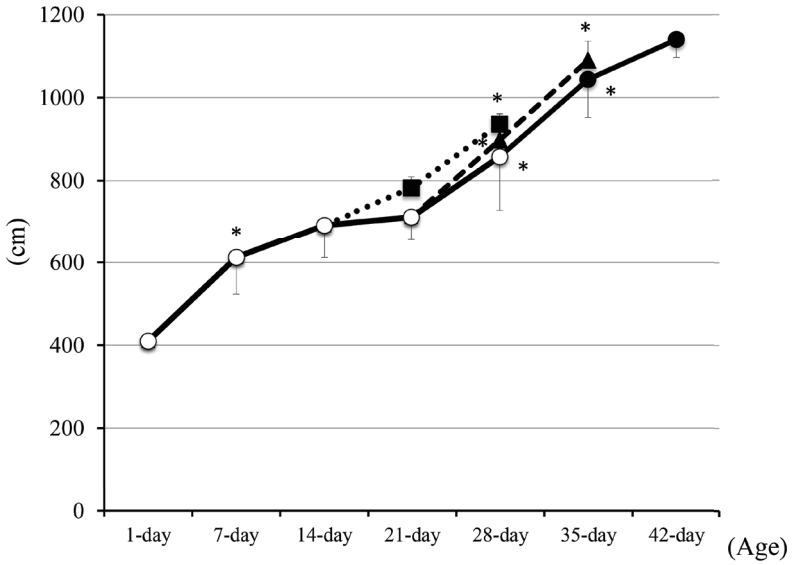

Fig. 2. Length of the small intestine of 1-day to 42-day-old pigs.
Open circles denote the mean values of the length of the small intestine of suckling piglets; closed circles denote those of piglets weaned at 28 days of age; closed triangles denote those of piglets weaned at 21 days of age; and squares denote those of piglets weaned at the age of 14 days. The error bars represent the standard errors. Asterisks denote that differences were significant in comparison with previous sampling points. This figure was originally published in Tsukahara *et al*. 2016[7].

3. Transitional gene expression in the small intestine of neonatal piglets

Transcriptome analysis has been shown to be a useful tool for elucidating gene expression in the intestinal tissues of pigs[9]. Some researchers have previously reported that using microarray analysis-based bioinformatics, they have identified in jejunal tissues of weaned piglets several biological pathways associated with the immune response (e.g., natural killer cell mediated cytotoxicity and chemokine signaling pathway)[10], as well as immune response-related genes affected by foods components, including carbohydrates and fatty acids[11], and amino acids[12]. Moreover, recent bioinformatics work has investigated differential gene expression in the aggregated lymphoid follicles, also known as Peyer's patches (PP), in the jejunum[9,13] and ileum[13,14]. These authors found differential transcriptomic profiles in intestinal tissues, as nutritional pathways were enriched in jejunal PP, but immune pathways were overexpressed in ileal PP. In this chapter, we describe changes that we have previously identified in the transcriptome of ileal mucosa of suckling and weaning piglets[6].

While the number of genes differentially expressed (*t*-test; $p < 0.05$, $-2 \leq$ fold change ≥ 2) between piglets S14 and S21 was 2,124, the number of genes differentially expressed between piglets S21 and S28 was only 60 (Fig. 3). Gene expression in ileal mucosa of piglets was significantly affected by weaning at 21 days of age. For example, the total number of genes differentially expressed between piglets S21 and W21p7, and between piglets S28 and W21p7 was 1,128 and 1,308, respectively (Fig. 3). Biological pathways associated with the differentially expressed genes in tissues of suckling and weaning piglets are shown in Table 2. Remarkable differences were observed between piglets S14 and S21. Among the immune-associated pathways, leukocyte chemotaxis was upregulated in suckling piglets from days 14 to 21. This pathway was also expressed differently between piglets S21 and S28.

On the other hand, weaning also induced drastic transcriptome changes in ileal mucosa. For example, from piglets S21 to W21p7 the immune-associated complement pathways was upregulated, but *histamine H1 receptor signaling* and *PIP3 signaling in B lymphocytes* were downregulated. Interestingly, *antigen presentation by MHC class II* was similarly detected between

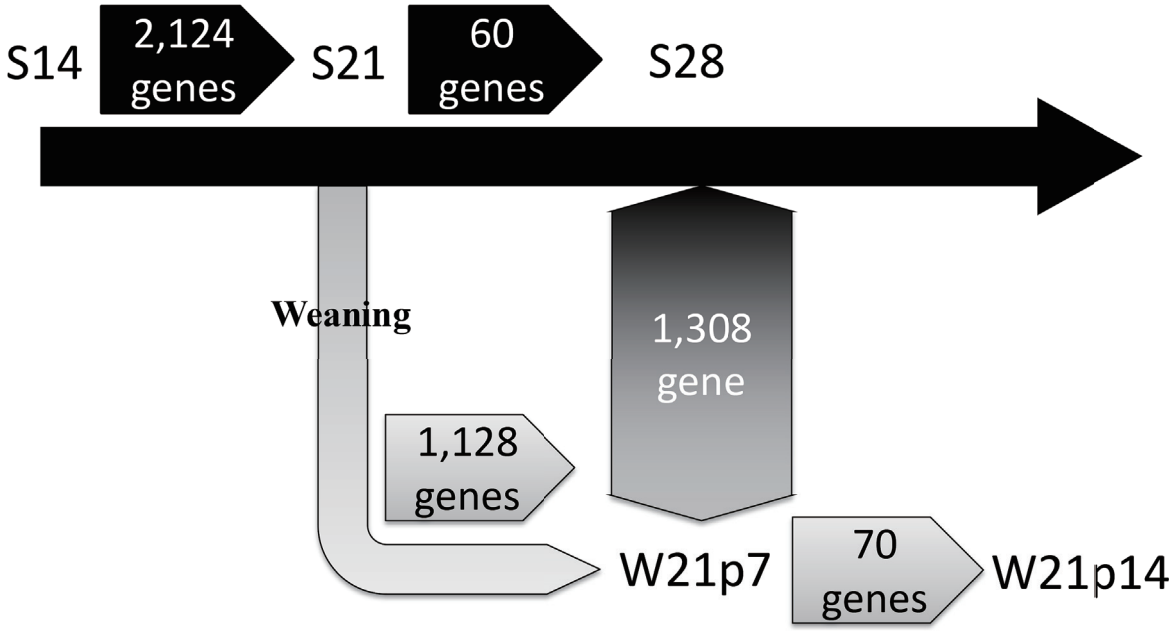

Fig. 3. Differential gene expression in suckling and weaning piglets.
Numbers denote the number of genes differentially expressed ($P < 0.05$, $-2 \leq$ fold change ≥ 2). Student's *t*-test analysis was carried out on the normalized and transformed microarray data, after which the number of significantly affected genes was calculated. This figure was originally published in Inoue *et al*. 2016[6]

Table 2. MetaCore pathway analyses of differentially expressed genes in ileal mucosal tissues.

Pathways	Age (Affected from left to right)	Up-regulation	Down-regulation
Immune system	S14 to S21	Leukocyte chemotaxis Immunological synapse formation CCR3 signaling in eosinophils	—
	S21 to S28	Leukocyte chemotaxis TLR signaling pathways	Antigen presentation by MHC class II
	S21 to W21p7	Alternative complement pathway Antigen presentation by MHC class I Classical complement pathway Lectin induced complement pathway	Leukocyte chemotaxis Antigen presentation by MHC class II Histamine H1 receptor signaling in immune response MIF-mediated glucocorticoid regulation PIP3 signaling in B lymphocytes CCR4-induced leukocyte adhesion
	S28 to W21p7	IFN alpha/beta signaling pathway	Leukocyte chemotaxis CD16 signaling in NK cells Immunological synapse formation PIP3 signaling in B lymphocytes
Cell	S14 to S21	Role of APC in cell cycle regulation DNA damage_Mismatch repair	Cell adhesion_Tight junctions Cell adhesion_Chemokines and adhesion
	S21 to S28	—	WNT signaling pathway
	S21 to W21p7	Apoptosis and survival_Caspase cascade Apoptosis and survival_Granzyme B signaling	VEGF signaling and activation
	S28 to W21p7	Cell adhesion_Tight junctions Apoptosis and survival_Caspase cascade Plasmalogen biosynthesis	Role of APC in cell cycle regulation
Transport	S14 to S21	—	CFTR-dependent regulation of ion channels Development_regulation of epithelial-to-mesenchymal transition
	S21 to S28	Intracellular cholesterol transport in norm	Cholesterol and Sphingolipids transport Regulation of translation initiation
	S21 to W21p7	—	—
	S28 to W21p7	—	—
Metabolism	S14 to S21	—	—
	S21 to S28	Low-density lipoprotein particle clearance Cholesterol storage	Insulin regulation of glycogen metabolism Regulation of lipid metabolism Vitamin K metabolism
	S21 to W21p7	Cholesterol Biosynthesis Bile acids regulation of glucose and lipid metabolism Triacylglycerol metabolism	—
	S28 to W21p7	Bile acids regulation of glucose and lipid metabolism Triacylglycerol metabolism	—
Others	S14 to S21	Biological regulation	NF-AT signaling in Cardiac Hypertrophy
	S21 to S28	—	—
	S21 to W21p7	—	—
	S28 to W21p7	—	—

S14. suckling piglets slaughtered at 14 days of age, S21. suckling piglets slaughter at 21 days of age, S28. suckling piglets slaughtered at 28 days of age, W21p7. piglets weaned at 21 days of age and slaughter 7 days after weaning.
The pathways of significant difference (Student's *t* -test, $p<0.05$) between the groups and 2-fold difference between the mean values are mensioned.
This table was originally published in Inoue *et al*. 2016[6].

piglets S21 and S28 and piglets S21 and W21p7, which may indicate that this pathway changes ontogenetic. Regarding the effects of weaning on the transcriptome, based on a transcriptome comparison between piglets S28 and W21p7, it was found that while the immune-associated pathways such as *IFN alpha/beta signaling* was upregulated, *CCR4-induced leukocyte adhesion*, *leukocyte chemotaxis*, *CD16 signaling in NK cells*, *immunological synapse formation*, *PIP3 signaling in B lymphocytes* were downregulated. The pathways relating to nutrient transports such as pathways for amino acid and saccharide transporters were not affected during suckling periods and by weaning at least in our pathway analyses.

A striking difference was detected in the transcriptome profiles of suckling piglets (Fig. 3). Indeed, a transcriptome comparison showed that the number of genes differentially expressed between piglets S14 and S21 was more than 30-fold higher than those found between piglets S21 and S28. This finding indicated that significant development of ileum took place within a week, between days 14 and 21 of age; however, development was only relatively modest afterwards. Pathways associated with the immune system and gene expression of leukocyte chemotaxis were upregulated continuously during the suckling period. Moreover, while innate immunity such as *TLR like signaling* was being developed, acquired immunity such as *Antigen*

presentation by MHC class II declined from day 21 to day 28 in suckling piglets. Weaning also affected the transcriptome associated the immune system of ileal mucosa. According to our results, the weaning process stimulated the innate immune response, while at the same time attenuated acquired immune system in the ileal mucosa. It is worth noting that when early weaning at 14 days of age is imposed on suckling piglets, aggregation of leukocytes in the gut-associated immune system is insufficient and immunodeficiency is likely to develop after weaning[6].

4. Disaccharidase activity in the small intestine at weaning

The epithelial cells lining the intestinal villi consist of columnar enterocytes, recognized as nutrient absorptive cells. The apical membrane of enterocytes with microvilli known as the brush border membrane provides various channels and nutrient transporters for absorption of nutrients[15]. In addition, digestive enzymes such as disaccharidases and peptidases are secreted from the brush-border membrane to accelerate this absorption of nutrients. Maltase and sucrase are enzymes found among the disaccharidases secreted in the small intestine. While the activity of maltase (MA) and sucrase in the brush border membrane increases with age, lactase (LA) activity attenuates as a result of dietary changes such as those experienced after weaning[16]. In previous studies we demonstrated the effect of age at weaning on disaccharidase (LA and MA) activity detected in all eight segments in which we sectioned the entire porcine small intestine[7].

Lactase activity gradually enhanced in suckling piglets from 1 to 14 days of age in sections si-1 to si-7 (Fig. 4). Further activation was observed in suckling piglets from 14 to 21 days of age in sections si-2, si-3 and si-7. The highest activity was approximately 400 U in the sections of the proximal small intestine, whereas 500–600 U of LA activity was detected in sections si-5 and si-6. Different dynamics of LA activity was observed in section si-8. Section si-8 had the lowest LA activity, but a rapid decline was detected in the other sections (si-1 to si-7) after LA activity peaked. Moreover, LA activity was diminished in all small intestine sections of S28 piglets.

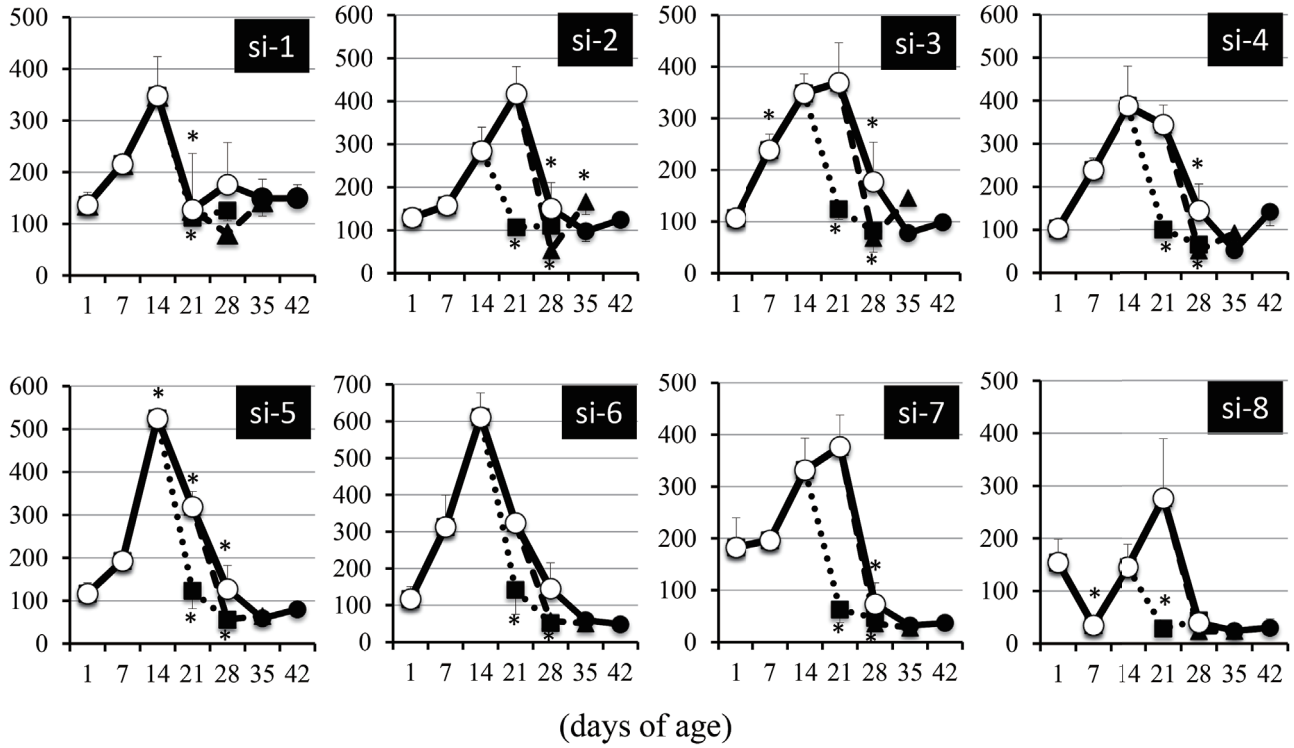

Fig. 4. Lactase activity (U) in the eight sections of the small intestine of 1-day-old to 42-day-old pigs.
This figure was originally published in Tsukahara *et al*. 2016[7].
For further details, please refer to Fig. 2.

Somewhat unexpected was the fact that early weaning (weaning at 14 days of age) induced rapid reduction of LA activity in sections si-2 to si-8. The effect of weaning on piglets weaned at the age of 21 days was limited because LA activity in these animals was also attenuated as it was in S21 piglets. As expected, nonetheless, was the little effect observed in W28 piglets.

Maltase activity gradually increased with age in suckling piglets (Fig. 5). For example, activity enhancement was observed in suckling piglets from day 1 to day 21 of age in sections si-1 to si-7. The highest activity was approximately 400 U in the proximal sections and section si-5, whereas 900 and 500 U were detected in sections si-6 and si-7, respectively. The lowest MA activity was detected in section si-8. Two hundred U or less of MA activity was detected throughout the suckling period. As discussed above, it was found that early weaning (e.g., weaning at 14 days of age) also induced attenuation of MA activity at 21 days of age (7 days post-weaning) in all small intestine sections. At 28 days of age, attenuation of MA activity was still detected in all sections (14 days post-weaning), except for si-1. The effect of weaning was moderate but still detectable in piglets weaned at 21 days of age; ontogenetic decline of MA activity was observed from day 21 to day 28 of age in suckling period. The effect of weaning at 21 days on the MA activity itself seems to be moderate since the reduction of the MA activity in the W21p7 pigs might be due to not only the effect of weaning but an ontogenetic decline. While attenuation of MA activity was observed at 28 days of age (7 days post-weaning), MA activity was detected in the sections of the proximal small intestine (si-1 to si-4). Nonetheless, little effect of weaning was observed on piglets at 28 days of age. Nonetheless, in our work we found that while MA activity temporarily declined during the weaning period, it was reactivated at growing piglets[8]. The changes of MA activities of si-2 and si-4 after days 28 suggested that the activities recovered after a temporal decline. Indeed, we already observed a reactivation of MA activity in growing pigs[8].

After birth, LA activity quickly initiates in the small intestine and is maintained during suckling[5]. In our work, the highest LA activity among suckling piglets was detected in section si-5 and si-6 of S14 piglets.

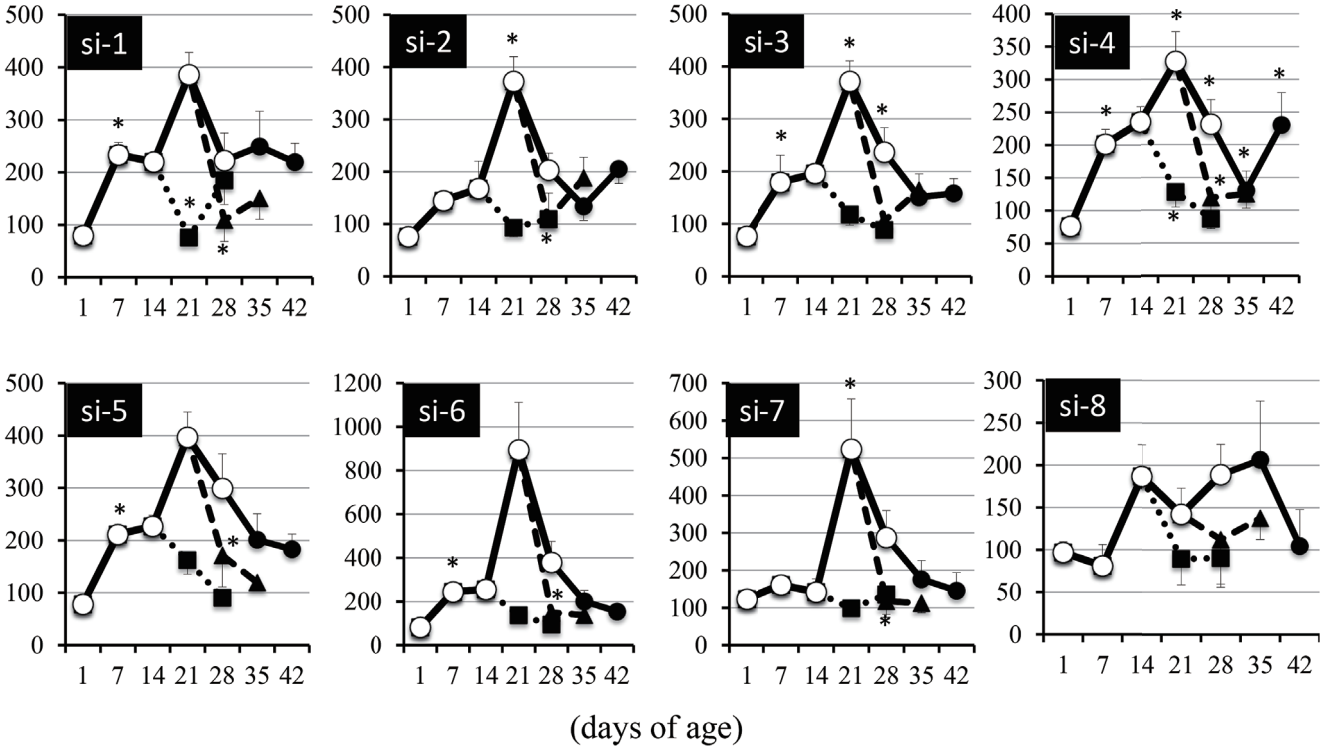

Fig. 5. Maltase activity (U) in the eight sections of the small intestine of 1-day-old to 42-day-old pigs.
This figure was originally published in Tsukahara *et al*. 2016[7].
For further details, please refer to Fig. 2.

These results suggest that the distal small intestine is one of the major sites for lactose digestion during the suckling period. Separately, MA activity was detected in days 1 to 21 of the suckling period, but MA activity declined from days 21 to 28 even though piglets were still suckling. During the suckling period, it has been found that for pigs, lactose is the main component of dietary carbohydrates; MA activity has been rarely reported in the previous study. Nonetheless, in our work we found that while MA activity temporarily declined during the weaning period, it was restored as piglets grew[8]. Therefore, it is likely that the decline of MA activity in S28 piglets found in our work is part of the developmental process of the small intestine.

Reduced LA activity followed by increased MA activity during weaning has been consistently reported[5,17-19]. Thus, existing data seem to indicate that the intestinal tract of pigs necessarily modifies its digestive enzymes in response to dietary changes[20]. However, age also seems to play an important role in the enzymatic activity in the intestinal tract of pigs. For example, only a temporary reduction of MA activity was observed when piglets were weaned at 21 days of age or older, and negligible values were observed by the end of the first week after weaning[21,22]. In contrast, weaning piglets at 14 days of age seemed to seriously affect disaccharidase activity in the small intestine, as no MA activity was detected for at least 2 weeks after weaning. In our work, the highest MA activity was observed in S21 piglets, and an apparent reduction was observed in piglets weaned at 21 days of age. By contrast, MA activity was reduced in suckling piglets from S21 to S28, and the effect of weaning on MA activity of piglets weaned at 28 days of age was only moderate.

5. Microstructure of the small intestine at weaning

The small intestine and the intestinal mucosa lose 20%–30% of their relative weight during the first 2 days after weaning, and redevelopment requires 5–10 days before full recovery is attained[2]. In the small intestine, in particular, early weaning causes structural changes including shortening of villi and crypt proliferation[19,24]. Shortening of villi is assumed to impair the digestive ability of the small intestine, thereby inducing malabsorption and diarrhea in weaned piglets[25,26]. Regarding the intestinal mucosa, its immune system is enhanced during the first 3 weeks of life of piglets, as remarkable accumulation of lymphocytes and enlargement of PP have been observed in the small intestinal lamina propria[27,28]. Later on, maturation of the porcine mucosal immune system with functional lymphoid organs, including PP, provides enhanced protection against pathogens[29,30]. In humans, as much as 46% of PP populates the distal 25 cm of ileum[31]. Peyer's patches are likely found in pigs in similar distribution[32,33]. Thus, it is increasingly evident that the well-being of growing pigs greatly depends on a sound development of the digestive–absorptive and mucosal immune systems in the gut, including structures such as ileal PP. In this section, we will discuss the development of microstructures including intestinal villi and PP in the small intestine of piglets during early postnatal life.

The height of intestinal villi is shown in Fig. 6. In tissues from suckling piglets, we found that at 1 day of life, the height of villi was approximately 1.1–1.2 mm across all sections of the small intestine, but that it gradually shortened from days 1 to 28. Shortening of villi was typically observed in the sections from the distal small intestine, such as si-5, si-6, si-7 and si-8. The height of villi in the distal sections of the small intestine of S28 piglets was approximately half of that measured in the intestinal tissues of S1 piglets. Interestingly, although ageing of suckling piglets seems to inversely affect the height of villi, it gradually but directly influences the elongation of their small intestine. In weaned piglets, the height of villi in sections from their proximal small intestine, i.e., si-1, si-2, si-3 and si-4, were significantly affected by weaning. Shortening of villi was typically observed in piglets weaned at 14 or 21 days of age.

Early weaning such as that imposed on piglets at 14 days of life was found to induce an abnormal development of villi in comparison with that of villi

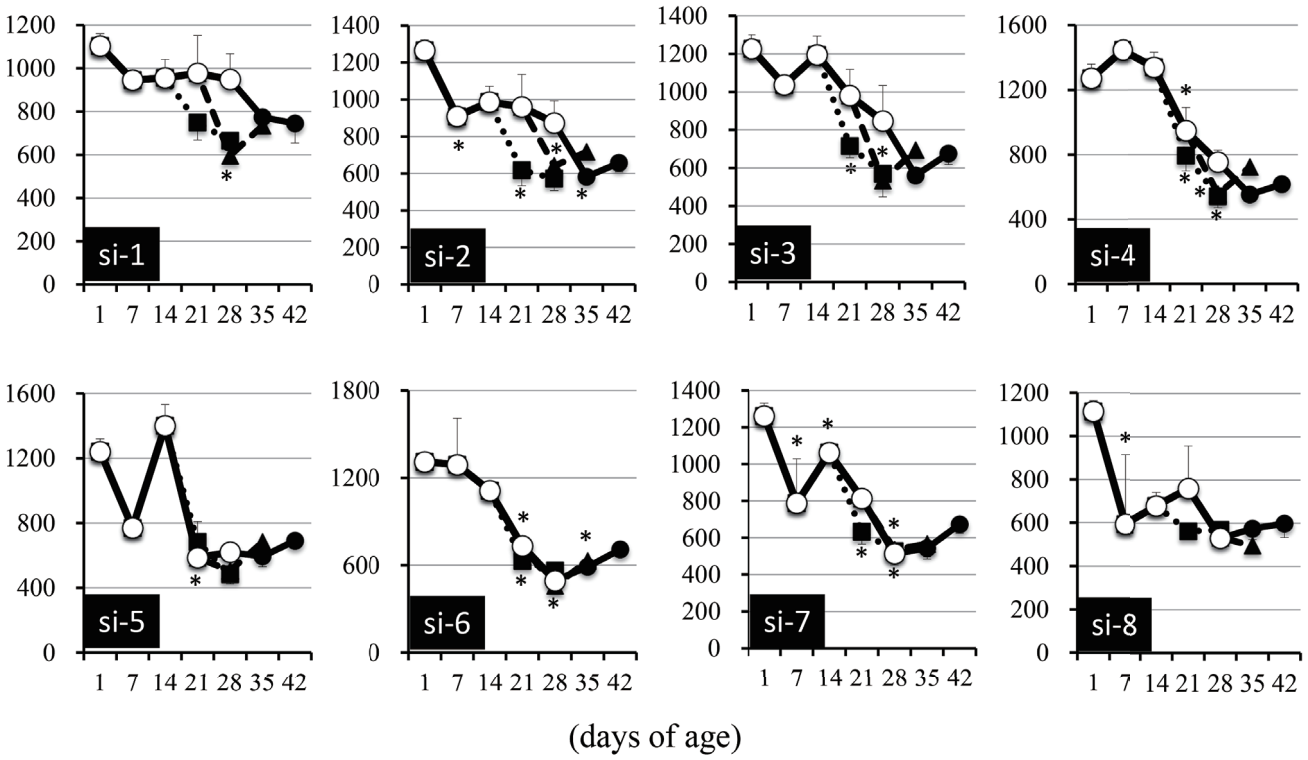

Fig. 6. Height of villi (μm) in the eight sections of the small intestine of 1-day-old to 42-day-old pigs.
This figure was originally published in Tsukahara *et al*. 2016[7].
For other details, please refer to Fig. 2.

observed in intestinal tissues of piglets weaned later on at 28 days of life[19]. In our work, weaning was imposed on piglets at 14, 21 and 28 days after birth. In all cases, a tendency to atrophy was observed in villi at 7 days post-weaning. Furthermore, the recovery time of villi from atrophy was affected by the age at weaning. For example, the recovery from atrophy was not observed in villi of piglets weaned at 14 days of age at least 14 days post-weaning, which indicated that villi still shortened during that period. In contrast, villi elongated from days 7 to 14 after weaning in piglets that were weaned at 21 or 28 days of age. Significant atrophy of villi was observed at early weaning in proximal (si-1 to si-4) and distal (si-6 and si-7) sections. Section-specific atrophy of villi was also observed at weaning in murine[24] and porcine[17] models, and the atrophy reported in those studies was similar to that found in our work.

Peyer's patches populating areas of the ileum of suckling and weaning piglets can be seen in Fig. 7. Ileal PP areas enlarge continuously during suckling period. For example, remarkable enlargement was observed in suckling piglets from days 1 to 7, and from days 14 to 21. By contrast, the weaning process halted PP development in the ileum. From days 14 to 21 and from days 21 to 28 of the suckling period, the dynamics of PP enlargement in suckling piglets had a similar trend to that shown by gene expression such as *leukocyte chemotaxis* upregulation (Table 2). Clearly, weaning induces stagnation of PP

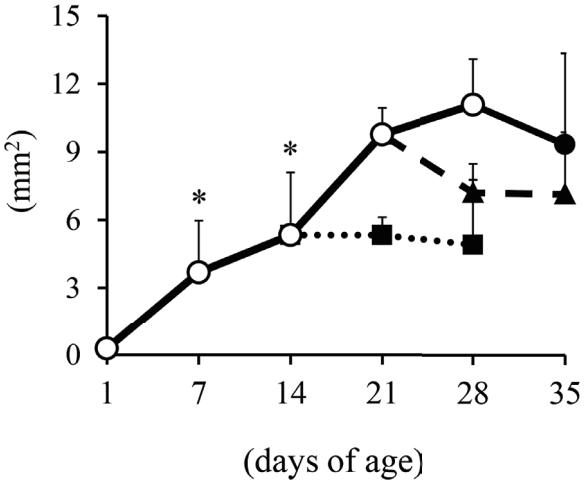

Fig. 7. Areas of the Peyer's patches (mm²) in intestinal section si-8 of 1-day-old to 42-day-old pigs.
This figure was originally published in Inoue *et al*. 2016[6].
For other details, please refer to Fig. 2.

development. These data indicate that weaning before 21 days of age causes a critically adverse effect on PP development of piglets, which leads to an altered immune system in weaned piglets.

6. Conclusion

Within the first 4 weeks after birth, the body weight of piglets increases >5-fold, with gastrointestinal organs growing faster than many of other organs[34]. Body weight gain after weaning is therefore considered highly correlated with the height of villi[22]. This evidence suggests that maintenance of the small intestinal structure and functionality after weaning is important for the growth of piglets. The data from our work demonstrated that weaning imposed on piglets as early as 14 days after birth caused severe adverse effects on young piglets such as atrophy of villi, stagnation of immune system development, and suboptimal maltase activity in the small intestine after weaning. Furthermore, the results from our transcriptome analyses of ileal mucosa showed upregulation of cell cycle pathways and downregulation of cell adhesion pathways in suckling piglets at 14 to 21 days of age (Table 2).

In summary, our histological, enzymatic and immunological evaluation of tissues from the porcine small intestine suggests that crossbred piglets should not be weaned earlier than 21 after birth, and that weaning at 28 days of age is more recommended.

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家畜栄養生理研究会会則

（総　　則）

第一条　本会は家畜栄養生理研究会と称する。

第二条　本会は家畜栄養生理に関する研究及びその成果の応用を促進する事を目的とする。

第三条　前条の目的を達成するため下記の事業を行う。

一．家畜栄養生理の研究に関する討議（春季・秋季集談会）

二．家畜栄養生理に関する研究情報、文献の蒐集配布並びに交換

三．家畜栄養生理に関する共同研究の促進及び研究相互の連絡

四．家畜栄養に関する研究成果及び技術につき必要と認めた場合、印刷物刊行等により普及を図ること

五．その他本会の目的達成のために必要な事業

（会　　則）

第四条　会員は正会員と賛助会員及び名誉会員とする。

第五条　本会の正会員になろうとするものは、会員の推薦により事務局に申し込むものとする。

２．事務局は前項の申し込みに対して可否を決定する。但し、名誉会員は評議員会が推薦し、総会において決定する。評議員は名誉会員に推戴された時点をもってその職を退任することとする。

３．会員は総会及び集談会に参加し発言することができる。但し、非会員も別途定める参加費を納入すれば、集談会に参加し、発言することができる。

４．名誉会員は評議員会に出席し、助言することができる。

第六条　会員は会費を納入するものとする。但し、名誉会員は会費を免除する。

２．正会員の年会費は4,000円とし、賛助会費の年会費は20,000円とする。年会費の徴収方法は事務局が決定する。

第七条　会員は下記の事項に該当する時は会員たる資格を失う。

一．本人の意志による退会

二．長期の会費未納の場合

三．会員として不適当と事務局が認め、総会がこれを承認した場合

但し、第一項により退会しようとする時は、事務局宛に届け出るものとする。

第八条　本会の事業年度は毎年3月1日に始まり翌年2月末日に終わる。

（役　　員）

第九条　本会に役員として会長1名、事務局員若干名、会計監査2名、評議員若干名、及び編集委員若干名を置く。役員の任期は2年とし、重任を妨げない。

第十条　役員は総会において選定する。

第十一条　会長及び事務局員は事務局を、編集委員は編集委員会を組織する。

２．事務局は総会で議決された方針に従って会務を運営する。

３．会長は本会を代表し、事務局員・編集委員の業務を統轄する。

４．事務局員は庶務、会計その他の業務を分担する。

５．編集委員は評議員の推薦による話題提供者の論文等を査読し、編集業務を担当する。

第十二条　評議員は本会の重要な事項につき会長の諮問に応じる。

（会　　議）

第十三条　会議は総会及び評議員会とする。

第十四条　総会を分けて通常総会及び臨時総会とする。

２．通常総会は毎年1回開催する。

３．総会は評議員会の議を得て会長がこれを召集する。

４．総会の議長はその都度選出するものとする。

第十五条　総会は本会の経理、人事及び事業の全般に亘る主要事項を審議決定する。

第十六条　評議員会は役員を以て構成する。

２．評議員会は会長がこれを召集し、その議長となる。

３．評議員会は原則として年2回開催する。

第十七条　評議員会は本会運営の基本方針を審議し、主要事項について総会へ提出し、その審議を求める。

（昭和59年4月6日改正）（平成12年5月13日改正）（平成15年5月10日改正）
（平成17年11月12日一部改正）（平成27年11月14日一部改正）

栄養生理研究会報編集・投稿規程

1. 本会報は、家畜栄養生理に関する論文および研究会資料を掲載する。
2. 掲載論文の著作権は家畜栄養生理研究会に属する。
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