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Effect of maternal myostatin antibody on offspring growth performance and body composition in mice

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Summary

Myostatin (GDF8) is a member of the transforming growth factor beta (TGF-β) superfamily. The finding that animals with a knockout or mutation of the myostatin-encoding gene show increased muscle mass suggests that myostatin negatively regulates muscle growth. The study reported here was designed to investigate the effect of induction of maternal myostatin antibody on the growth performance and body composition of the mouse. Female mice were induced to produce myostatin antibody by immunization with synthetic myostatin peptide prior to mating with male mice. The body masses of offspring were measured weekly and the body compositions of offspring were determined at 8 weeks of age. The results showed that myostatin antibody was detected in both immunized female mice and their 8-week-old offspring. The growth performance of offspring from the myostatin antibody-induced (mstn Ab-induced) group was higher than that from the control group at 8 weeks of age. The body composition of both male and female offspring from the mstn Ab-induced group contained higher crude protein and lower crude fat than those from the control group ($P<0.05$). The litter number from the maternal mstn Ab-induced group was less than that from control mice, while embryo development was normal in both groups. However, the amount of developing follicle in ovaries of the mstn Ab-induced group was lower than that in the control group. It is concluded that induction of maternal mstn Ab enhances the growth performance of offspring and influences the offspring body composition by increasing the crude protein and reducing crude fat.

Key words: myostatin, maternal antibody, growth performance, body composition.

Introduction

In mammals, many growth factors are involved in the processes of muscle cell proliferation, myotube differentiation and muscle protein turnover (Arnold and Winter, 1998; Husmann et al., 1996; Molkentin and Olson, 1996). Growth factors such as fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) and transforming growth factors (TGF-βs) play important roles in promoting myogenesis (Musaro et al., 1999; Semsarian et al., 1999; Lowe and Always, 1999). The TGF-β superfamily contains a large group of secreted growth and differentiation factors that play important roles in regulation of development and tissue homeostasis (McPherron and Lee, 1996). Myostatin, a member of the TGF-β superfamily, also known as growth differentiation factor 8 (GDF8), was first identified in mice by McPherron et al. (McPherron et al., 1997). Myostatin is the genetic factor for determination of skeletal muscle mass and weight in the mouse (McPherron et al., 1997). Furthermore, natural mutations of myostatin caused three breeds of cattle, Belgium Blue, Piedmontese and Asturiana de los Valles, to show a double-muscled phenotype (Dunner et al., 1997; Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). It is concluded that myostatin is a negative regulator of muscle growth. To improve growth performance of animal or livestock, several molecular strategies and traditional breeding techniques were developed to manipulate myostatin expression. Myostatin knockout mice and dominant-negative myostatin mice were reported to enhance growth performance (McPherron et al., 1997; Zhu et al., 2000). Overexpression of myostatin binding protein, follistatin, in transgenic mice exhibited a more dramatic increase in skeletal muscle mass compared with that seen in myostatin-knockout mice (Lee and McPherron, 2001). Injection of anti-myostatin monoclonal blocking antibody increased skeletal muscle mass in mice (Bogdanovich et al., 2002). However, the methods used in all above studies are too

complicated, inefficient and impractical for livestock breeding and production. Thus, finding a convenient way to improve livestock growth performance by regulating myostatin expression becomes an important issue.

Immunological memory from the mother plays an important role in regulation of prenatal and postnatal health. During fetal development, the immune system is relatively incompetent. Therefore, transferable maternal immunological memory is essential for the survival of the fetus, newborn and infant. Maternal adaptive immunity has a strong influence on immune responses in the fetus and offspring. These antibodies provide passive protection against infection but also actively shape childhood immunity and tolerance induction by providing the fetus and infant with maternal immunological experience (Zinkernagel, 2001; Uthoff et al., 2003).

Ventura and coworkers demonstrated that anti-Dengue virus Ab existed both in Dengue virus infected pregnant women and their infants (Ventura et al., 1975). The active transplacental transfer of immunoglobulin G (IgG) begins at 6 months of gestation and increases sharply thereafter. At the end of gestation, IgG concentrations in fetal serum exceed maternal levels by a ratio of 1.2:1 to 1.8:1 (Sato et al., 1979). Baker et al. immunized pregnant women with the polysaccharide vaccine of group B *Streptococcus* (Baker et al., 1988). The vaccine-induced IgG was presented in the mother and the IgG readily crossed the placenta. This evidence indicates that the maternal antibodies pass freely across the placenta to offspring. Female mice were immunized with *Dermatophagoides pteronyssinus* prior to mating with male mice, and the anti-*D. pteronyssinus* antibody was found in both mother and offspring (Victor et al., 2003).

To improve the growth performance of animals or livestock, maternal adaptive immunity may be a potential alternative protocol to inhibit myostatin activity in fetus and offspring. The objective of this study was to investigate the influence of maternal myostatin antibody on the growth performance and body composition of offspring in mice.

Materials and methods

Materials

Mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan, ROC). Complete and incomplete Freund's adjuvant, glutaraldehyde, Tween-20 and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), hydrogen peroxide and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG were from Bio-Rad Laboratories (Hercules, CA, USA). Nitrocellulose membrane was purchased from Schleicher and Scheull (Keene, NH, USA). ECL detection system and Hyperfilm-ECL were obtained from Amersham (Arlington Heights, IL, USA). M199 medium and penicillin/streptomycin solution were from GIBCO (Grand Island, NY, USA). Fetal bovine serum (FBS) was ordered from HyClone (Logan, UT, USA).

Animals

Specific pathogen-free, 4–5-week-old C57BL/6J mice were used in this study. Mice were maintained under 23±1°C and 60±5% humidity with a 12 h:12 h light:dark cycle. Mice were allowed to feed on commercial mouse chow from a local supplier and to drink *ad libitum*.

Immunization of mice

The mouse myostatin amino acid sequence was from GenBank (Access No. NP_034964), and the myostatin antigen peptide was designed by PC Gene (Barioch, 1995). The peptide sequence (N′-ECEFVFLQKYP-C′) was based upon the hydrophilic region between 313 and 323 residues near the active site of the myostatin sequence. Female mice were subcutaneously immunized twice, with a 1-week interval between immunizations, with 50 μl of an emulsion containing 10 μg of keyhole limpet hemocyanin (KLH)-conjugated myostatin peptide as antigen in complete Freund's adjuvant. After 7 days, the mice were immunized twice, with a 1-week interval between immunizations, with 50 μl of an emulsion containing 10 μg of KLH-conjugated myostatin peptide in incomplete Freund's adjuvant. Seven days after the final immunization, dot blotting assay and ELISA were used to determine the titer of myostatin Ab. The F1 mice were sacrificed at eight weeks old by cervical dislocation. The flushed body was weighed and stored at –20°C for body composition analysis.

Determination of antibody titer by ELISA and dot blotting analysis

Purified recombinant myostatin (Liang et al., 2002) was diluted in 0.05 mol l^{-1} sodium carbonate buffer (pH 9.6) containing 100 μg ml^{-1} recombinant myostatin and coated on a 96-well microtiter plate at 4°C overnight. The plates were blocked with 200 μl of phosphate-buffered saline (PBS) containing 2% BSA for 1 h at room temperature and then washed three times with PBS containing 0.05% Tween 20 (PBS-T). Diluted mouse sera were added and the plates were incubated for 2 h at room temperature. The horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (dilution 1:2000) was added and then the plates were incubated for 1 h at room temperature. Following three washes with PBS-T, the ABTS substrate solution was added into each well. The color changes were monitored using a microplate reader (Bio-Rad Model 680, Richmond, CA, USA) at a wavelength of 415 nm.

For dot blotting analysis, purified recombinant myostatin was spotted onto nitrocellulose membrane. The blot was incubated for 1 h in 5% skimmed milk in PBS-T at room temperature. Various dilutions of mouse sera were applied to the nitrocellulose membrane followed by 1 h incubation with HRP-conjugated goat anti-mouse IgG. The blot was then washed three times in PBS-T. Specific antibody binding was detected by an ECL detection system.

Analysis of body composition

The carcasses of F1 mice were lyophilized for 48 h. The

dried body mass ($M_{b,dry}$) of each mouse was recorded and body water content was determined by subtracting $M_{b,dry}$ from flushed body mass. The carcasses were ground using a Sorvall Omni-Mixer (Sorvall, Newtown, CT, USA) (Eisen and Leatherwood, 1976). Fat content was determined using the ether extraction method, and protein content was measured using a Kjeltec Auto Analyzer (FOSS, Laurel, MD, USA) (Jones, 1984). The water, fat and protein contents were expressed as a percentage of flushed body mass.

Embryo collection and culture

For embryo collection, the female mice were caged individually with males and checked for copulation plugs the next morning. Mice were sacrificed by cervical dislocation and the embryos were flushed from the oviduct. Embryos were collected and cultured in M199 supplemented with 10% FBS. After culturing overnight, the development of the embryo was observed by microscopic examination.

Histological analysis and follicle counting of ovary

The follicle numbers from mouse ovary were determined by histological analysis according to the method of Lee et al. (Lee et al., 2004). Ovaries were fixed in 10% neutral-buffered formalin solution for 24 h at 4°C, dehydrated, paraffin-embedded and serially sectioned at 5 μm thickness. The sections were mounted on glass slides and stained with Mayer's hematoxylin (Sigma Chemical Co.) and eosin. The fifth section of each ovary was used for follicle counting. Only follicles with a visible nucleus in the oocyte were counted to avoid duplicate counting of a follicle.

Statistical analysis

Values are presented as means ± standard deviation (s.d.). A Student's *t*-test was used to evaluate the differences between the groups. A significance level of 5% was adopted for the analysis.

Results

To determine the induction of myostatin Ab in female mice, sera were collected 7 days after final immunization with myostatin peptide, and the titer of myostatin Ab was determined by ELISA and dot blotting assay. The results showed that myostatin Ab was induced by KLH-conjugated synthetic peptide (11 amino acids). No myostatin Ab was detected in the control group (Fig. 1).

The female mice from the mstn Ab-induced and control groups were individual caged with male mice and checked the next morning for a vaginal plug. There was no difference in gestation duration for either group (mean, 20 days). However, the mean litter size in the mstn Ab-induced group was smaller than that in the control group (P<0.05; Table 1). The gender ratio of offspring showed no significant difference between mstn Ab-induced and control groups (Table 1).

To investigate the effect of maternal myostatin Ab on embryo development, the embryos were collected after mating

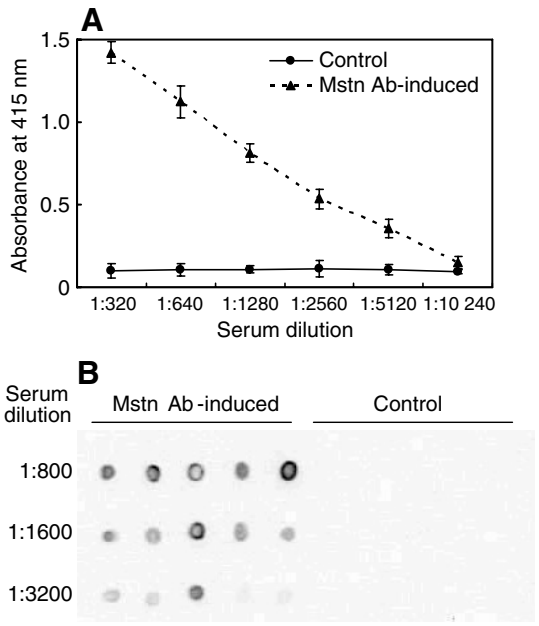

Fig. 1. Determination of maternal myostatin antibody titer. (A) Absorbance at 415 nm by myostatin-ELISA with a twofold series dilution of serum from 1:320. Each data point represents the mean of five mice. (B) Dot blotting analysis of maternal myostatin Ab with two-fold series serum dilution from 1:800.

from females in the mstn Ab-induced and control groups. The results showed that the mstn Ab-induced group had significantly fewer embryos than did the control group (P<0.05) (Fig. 2A). After culturing overnight, all embryos from both groups developed normally from the one-cell stage to the two-cell stage (Fig. 2A–E).

The effect of myostatin Ab on the development of the follicle in the ovary was determined by Mayer's hematoxylin and eosin histological staining. Primordial follicles were defined as those containing flat pregranulosa cells. Developing follicles including primary and secondary follicles were the follicles beyond the primordial stage. Primary follicles were those with one layer of cuboidal follicle cells and secondary follicles were those with two or more layers of cuboidal cells (Lee et al., 2004). The results showed that induction of myostatin Ab increased primordial follicle number and decreased developing follicle number in ovary (P<0.05) (Fig. 3).

The sera from offspring were collected at 8 weeks of age for determination of maternal myostatin Ab level. ELISA assay showed that maternal myostatin Ab was detectable in the mstn Ab-induced group. However, no maternal myostatin Ab was detected in the control group (Fig. 4).

To investigate the effect of maternal myostatin Ab on growth performance of offspring, body mass was measured during the 8-week developmental period. In the first week, the male offspring from the mstn Ab-induced group were heavier than those from the control group; however, no significant

Table 1. *Effect of maternal myostatin antibody on first generation litter size*

Maternal treatment	No. of mice	Total pups (average)	Offspring gender (%)	
			Male	Female
Mstn Ab-induced	9	27 (3)[a]	46±9	54±9
Control	9	62 (7)[b]	50±12	49±12

Percentages are means ± s.d. Values in the same column that are followed by a different superscript letter are significantly different at $P<0.05$.

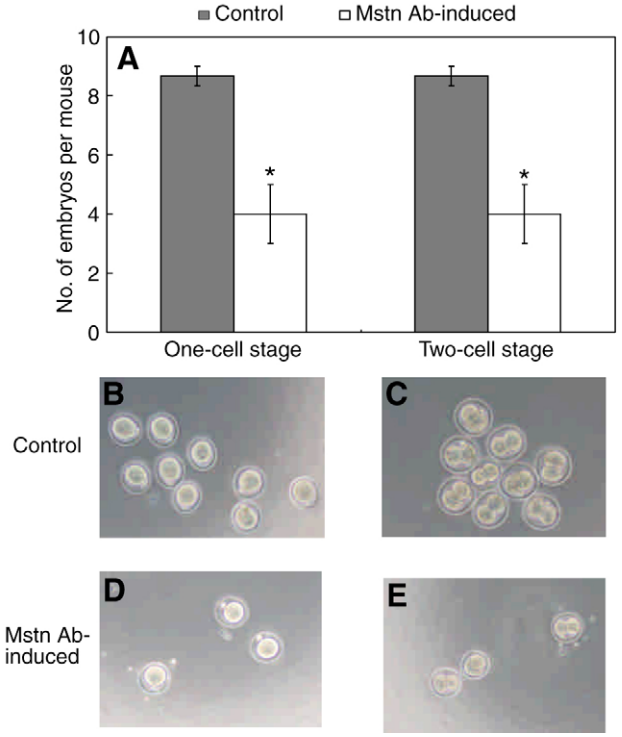

Fig. 2. Effect of maternal myostatin antibody on embryo development in mice. (A) Amount of collected embryo between mstn Ab-induced and control groups. Values are means ± s.d. (N=6). Asterisks indicate a significant difference compared with control ($P<0.05$). (B) Collected embryo from control group and (C) embryo after overnight culture. (D) Collected embryo from mstn Ab-induced mice and (E) after overnight culture.

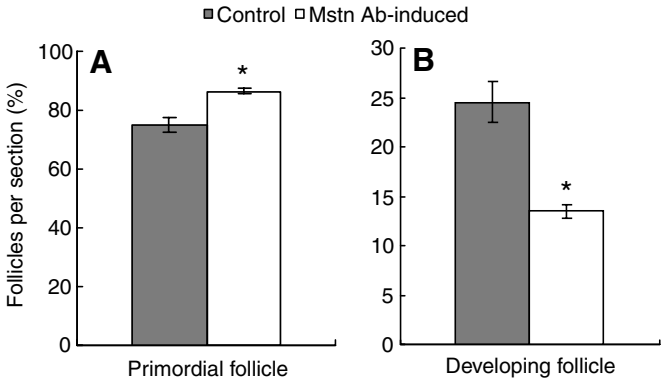

Fig. 3. Histological stain of mouse ovary from control group and mstn Ab-induced group. The number of (A) primordial and (B) developing follicles was determined in each fifth section of a series of 5 mm sections as described in Materials and methods. Values are means ± s.d. (N=6). Asterisks indicate a significant difference compared with control ($P<0.05$).

difference was found in female offspring. The growth performances of both male and female offspring from the mtsn Ab-induced group were higher than those from the control group during the 8-week period ($P<0.05$) (Fig. 5).

To investigate the effect of maternal myostatin Ab on body composition of offspring, crude protein, crude fat and water content were determined. The results showed that both male and female offspring from the mstn Ab-induced group contained higher crude protein and lower crude fat ($P<0.05$) (Fig. 6A,B). There was no significant difference in water content of offspring between the mstn Ab-induced and control groups (Fig. 6C).

Discussion

Increased skeletal muscle mass in animals can be regulated not only by positive effectors of muscle growth such as IGF-1 (Barton et al., 2002; Lynch et al., 2001) but also by negative growth factors such as myostatin (Bogdanovich et al., 2002; Wagner et al., 2002). Myostatin is a member of the TGF-β superfamily. Animals with myostatin gene mutation or knockout showed an increase in skeletal muscle mass (Dunner et al., 1997; Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). The increase in muscle mass was contributed by the hyperplasia and hypertrophy of skeletal muscle cells. To improve the growth performance of animals, several molecular strategies have been developed to manipulate myostatin gene expression. Mice with a myostatin gene knockout or expression of a dominant-negative myostatin gene were reported to have enhanced growth performance (McPherron et al., 1997; Zhu et al., 2000). Overexpression of myostatin binding protein, follistatin, in transgenic mice exhibited a more dramatic increase in skeletal muscle mass (Lee and McPherron, 2001). However, these strategies are involved in the alternation of animal genome and are not practical for livestock breeding and production. Bogdanovich et al. reported that the blockage of endogenous myostatin by peritoneal injection of monoclonal antibody for three months resulted in an increase in mice body mass and that the increased

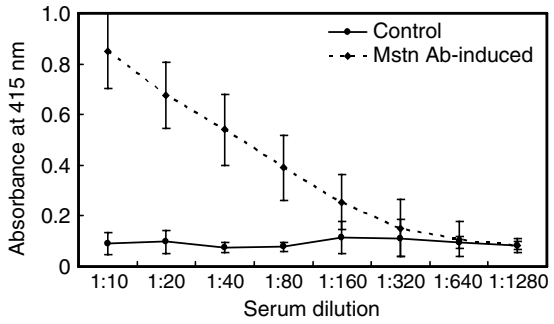

Fig. 4. Titer determination of myostatin Ab in offspring mice by myostatin-ELISA with a twofold series dilution of serum from 1:10. Each data point represents the mean of five mice ± s.d.

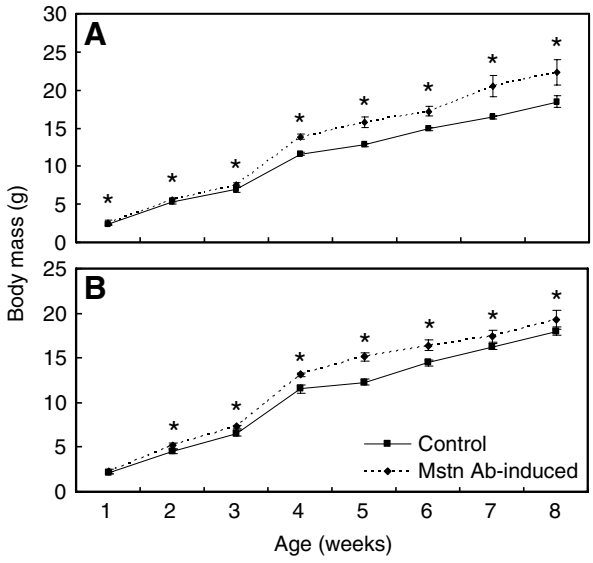

Fig. 5. Effect of maternal myostatin Ab on growth performance in (A) male and (B) female offspring. Values are means ± s.d. Body masses were measured from male control mice (*N*=33), male maternal mstn Ab-induced mice (*N*=13), female control mice (*N*=31) and female maternal mstn Ab-induced mice (*N*=13). Asterisks indicate a significant difference in body mass between mstn Ab-induced and control groups at the same age ($P<0.05$).

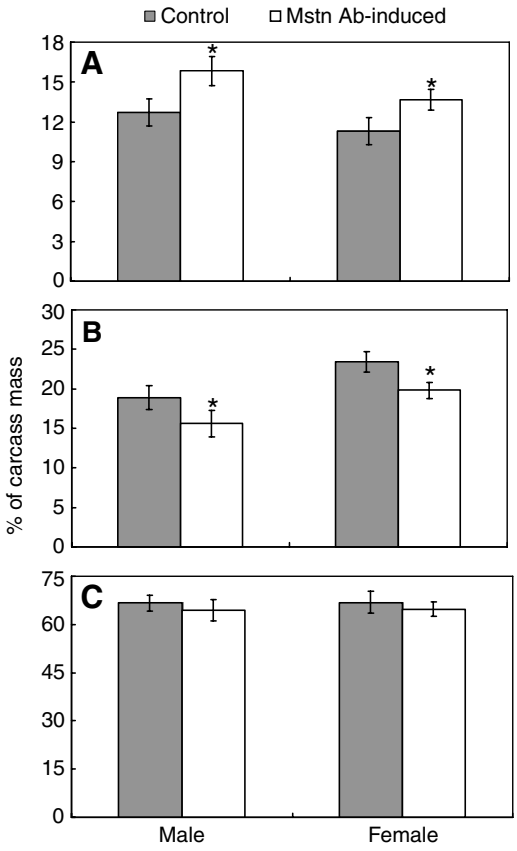

Fig. 6. Effect of maternal myostatin Ab on body composition of offspring. (A) Protein, (B) fat and (C) water were measured from male control mice (*N*=33), male maternal mstn Ab-induced mice (*N*=13), female control mice (*N*=31) and female maternal mstn Ab-induced mice (*N*=13). Data represent means ± s.d. Asterisks indicate a significant difference between mstn Ab-induced and control groups of the same gender ($P<0.05$).

growth performance in animals is due to the hyperplasia and hypertrophy in skeletal muscle (Bogdanovich et al., 2002). It was also reported that recombinant myostatin inhibited the proliferation of C2C12 mouse myoblasts (Thomas et al., 2000; Joulia et al., 2003). During the development of the embryo, expression of myostatin was detected in day 9.5 post-coitus (McPherron et al., 1997). Expression of myostatin in the embryo plays an important role in the proliferation of muscle cells (Deveaux et al., 2001). Therefore, repression of myostatin activity in the embryo is crucial to eliminate the negative effects on animal growth.

Maternal adaptive immunity has a strong influence on the immune responses of the fetus and offspring. Transplacental antibody transport is a selective and active process mediated by the Fc receptor. The diaplacental transfer of IgG might play an important role in offspring immunity (Uthoff et al., 2003). Kaden and Lange reported that maternal antibodies produced after oral vaccination of young female wild boars have a protective effect against classical swine fever for approximately two months following vaccination (Kaden and Lange, 2004). In the current study, the effect of maternal myostatin antibodies on the offspring growth performance was investigated by active immunization of myostatin synthetic peptides in female mice before mating.

Female mice immunized with synthetic myostatin peptides produced a high titer of myostatin Ab (Fig. 1). The body mass of female mice was not different between the mstn Ab-induced and control groups (data not shown). These results imply that neutralization of myostatin by myostatin Ab in mature animals did not affect the further growth of animals. The litter size and the number of embryos collected from mstn Ab-induced mice were less than that from the control group (Fig. 2A). However, the embryos collected from both groups divided normally into the two-cell stage after overnight culture (Fig. 2A,D,E). These

results indicate that induction of myostatin Ab reduced the amount of oocytes released from ovary but did not affect the development of the embryo. Ovary histochemical stain showed that the amount of developing follicle was reduced in the mstn Ab-induced group (Fig. 3). These data implied that myostatin may be involved in the development of follicles in ovary. In addition, cytokines of the TGF-β superfamily sharing similar active sites, such as inhibin and GDF-9 (Pangas and Matuzk, 2004; Juengel and McNatty, 2005), are involved in follicle development (Stock et al., 1997; Carabatsos et al., 1998; Elvin et al., 1999). It is possible that the induction of myostatin Ab may block the action of other members of the TGF-β superfamily. Furthermore, in the current study, synthetic myostatin peptides were conjugated with KLH for amplification of immune response. To rule out the possible influence of KLH Ab on litter size, immunization of mice with KLH alone was conducted. The result indicated that the litter size was similar between KLH immunized and control groups (data not shown).

The growth performances of both male and female offspring from the mstn Ab-induced group were higher than those from the control group during the 8-week period (Fig. 5). Owing to the long half-life of IgG, the 2-month-old offspring of the maternal mstn Ab-induced group maintained higher myostatin Ab titer than did the control group based on the ELISA assay (Fig. 4). Due to the negative effect of myostatin on skeletal muscle growth, reduction of myostatin activity during embryo development and the newborn stage will cause hyperplasia and hypertrophy of skeletal muscle cells. This result implies that neutralization of myostatin by maternal Ab affected the growth performance of offspring. In this study, one week after birth, the body mass of male offspring in the maternal mstn Ab-induced group was higher than that in the control group (Fig. 5A). This result may be attributed to the neutralization of myostatin by maternal myostatin Ab or nutrient limitation of newborn mice in the control group during the first week after birth. In myostatin-null mice or negative-dominant myostatin mice, the body mass was higher than in the wild type (McPherron et al., 1997). Since myostatin is the negative regulator of muscle growth, inhibition of myostatin will enhance muscle mass. The increase of muscle mass was contributed by the increase of crude protein. Offspring from mstn Ab-induced mice have a lower fat content than those in the control group (Fig. 6B). It is reported that myostatin stimulated the adipogenesis of C3H 10T(1/2) mescenchymal multipotent cells (Artaza et al., 2005). Increased fat mass in male mice was also observed by overexpression of myostatin in skeletal muscle (Reisz-Porszasz et al., 2003). In conclusion, induction of maternal myostatin Ab resulted in increased crude protein and decreased fat content in the body composition of offspring. This provides an animal model to improve livestock production without altering the genome of the animal. However, owing to the larger animal size, as well as longer growth and gestation periods, the application of a maternal mstn Ab strategy in farm animals still needs further investigation.

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