Postnatal Expression of Growth/Differentiation Factor-8 (GDF-8) Gene in European and Asian Pigs

C. S. Lin, Y. C. Wu[1], Y. L. Sun[2] and M. C. Huang*,[1]

Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, ROC

ABSTRACT : Myostatin (growth differentiation factor (GDF)-8), is one member of the transforming growth factor β superfamily. Investigations of GDF-8 null mice and double-muscled cattle revealed that GDF-8 has a profound influence upon skeletal muscle growth. Therefore, the GDF-8 effect upon the productive performance of pigs is worth exploring. In the present study, the nucleotide sequences and expression levels of GDF-8 genes in European pigs (Landrace and Duroc) and Asian pigs (Taoyuan and Small-ear) were evaluated. Based upon their genetic background these breeds possess significantly distinct growth rate and muscle production-phenotypes. Our sequence data showed that the nucleotide sequences of European and Asian pigs were 100% similar. Postnatal expression of GDF-8 gene in skeletal muscles, from birth to 12 mo of age, among different breeds was measured. GDF-8 expression levels in the longissimus muscle of neonatal European breed littermates were the highest, however it declined significantly ($p<0.05$) at 1 and 3 mo, and then increased gradually at 6 to 12 mo. The Asian breeds, however, GDF-8 expression level increased markedly at 3 mo and maintained a constant level thereafter. The results indicate that rather than polymorphism within the GDF-8 functional sequence between European and Asia breeds, it was relative to the gene regulation in postnatal muscle growth. *(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 9 : 1244-1249)*

INTRODUCTION

The transforming growth factor β (TGF-β) superfamily encompasses a huge group of secreted growth and differentiation factors, which play significant roles in regulating development and tissue homeostasis (Kingsley, 1994; Griffith et al., 1996; Mcpherron and Lee, 1996). Recently, it has been reported that a member of this family, growth/differentiation (GDF-8) (i.e., myostatin), is expressed predominantly in skeletal muscle (Kambadur et al., 1997; Ji et al., 1998). Furthermore, GDF-8 as a negative regulator of skeletal muscle development and growth in mice was suggested (Mcpherron et al., 1997). The GDF-8 null mice reveal a dramatic increase in skeletal muscle mass. Notably, individual muscles of GDF-8 null mice weighed 2- to 3-fold more than those of wild mice. Furthermore, the double-muscle phenotype of three cattle breeds, Belgian Blue, Piedmontese (Grobet et al., 1997; Kambadur et al., 1997), and Asturiana de los Valles (Dunner et al., 1997), has been linked to GDF-8 coding sequence mutations. These mutations compromise the biological activity of the protein, which leads to results in increased muscle mass hyperplasia and hypertrophy. Accordingly, exploring increased muscle mass production in livestock and poultry employing GDF-8

genotype selection or GDF-8 activity inhibition is meritorious. It is possible that allelic variation of porcine GDF-8 exist, and that this genetic variation affects muscle mass and/or fat deposition significantly (Lee and McPherron, 1999), although no documented cases exist in which swine are analogous to cattle. Therefore, we have characterized both expression level and nucleotide sequence of GDF-8 gene in European and Asian pigs, of which the growth rates and muscle production traits are significantly discrepant.

MATERIALS AND METHODS

Animals

Female Landrace (1 and 3 mo of age, n=10), Duroc (neonatal littermate and 1, 3, 6, and 12 mo, n=36), and Small-ear Lanyu (1 and 3 mo, n=10), and Taoyuan (neonatal littermate and 1, 3, 6, and 12 mo, n=30) pigs were employed in this study. Longissimus muscle tissue between the 10th and 11th ribs was dissected rapidly after pig sacrifice or when pigs were anesthetized, a biopsy sample was extracted Then, for later total RNA extraction, the subjects were immediately frozen in liquid nitrogen and stored at -80°C. The management and treatment of the pigs were conducted according to guidelines established by the National Science Council of Taiwan (1993).

PCR primers designation

Based upon available data of porcine GDF-8 (GenBank accession number AF019623) or glyceraldehyde-3-phosphate dehydrogenase (GADPH; AF017079) cDNA

* Address reprint request to M. C. Huang. Tel: +886-4-2840203, Fax: +886-4-2850223, E-mail: mchuang@mail.nchu.edu.tw
[1] Department of Animal Science, National Chung-Hsing University, Taichung, Taiwan, ROC.
[2] Division of Biotechnology, Animal Technology Institute Taiwan, ROC.

sequence, three sets of primers for the PCR were designed and synthesized. The first set was used to amplify the entire coding region of the porcine GDF-8 gene. The forward primer (GDF-F, 5'-ATGCAAAAACTGCAAA-TCTATGTTTATATTTACCTG-3') was designed based upon the region between nucleotides 1 and 36 and the reverse primer (GDF-R, 5'-TCATGAGCACCCACAGCG-ATCTACTACCAT-3') was based upon the region between 1,099 and 1,128 of the AF019623 sequence. The second amplified the 3'-region of porcine GDF-8 gene, which quantified the gene expression, the forward primer (GDF-698F, 5'-ATGGTCATGATCTTGCTGTAACC-3') corresponded with nucleotides 698 and 721 and the reverse primer (GDF-1128R, 5'-TCATGAGCACCCACAGCGAT-CTAC-3') corresponded to nucleotides within 1,105 and 1,128. According to the GAPDH gene to amplify the partial sequence as internal control of GDF-8 expression, the third was synthesized. The forward primer (GADPH-1F, 5'-ATGGTGAAGGTCGGAGTGA-3') corresponded to nucleotides 1 and 19 and the reverse primer (GADPH-613R, 5'-TGATGTTCTGGAGAGCCC-3') corresponded to nucleotides between 586 and 613 of the AF017079 sequence.

RT-PCR

According to the manufacture's protocol, total RNA was extracted from muscle tissue (0.5 g) using Trizol (GIBCO, BRL). Notably, RNA yield ranged from 0.4 to 0.6 µg/mg of tissue whereas the $OD_{260/280}$ was from 1.9 to 2.1. Five µg of total RNA were reverse transcribed applying 2.5 µM oligo dT primers (Promega, USA), 1 mM of each dNTP, 20 U ribonuclease inhibitor (HT Biotechnology), and 5 U reverse transcriptase (RT) (HT Biotechnology) in an RT buffer (25 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM DTT, and 5 mM $MgCl_2$) in a total reactive volume of 20 µl, at 39°C for 60 min. Each PCR reaction, 3 µl of RT product were added to a final volume of 50 µl containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 0.001% gelatin, 200 µM of each dNTP, 0.5 U Taq DNA polymerase (HT Biotechnology), and 0.2 µM of each primer pair. PCR was carried out with 30s denaturation at 94°C, 45s annealing at 60°C, and 45s extension at 72°C for 30 cycles in a Perkin-Elmer/Cetus DNA thermal cycler, for gene expression assay. To amplify the full length of the GDF-8 coding gene, PCR was performed with 60s denaturation at 94°C, 60s annealing at 65°C, and 80s extension at 72°C for 40 cycles in a Perkin-Elmer/Cetus DNA thermal cycler.

Analysis of RT-PCR data

DNA products were resolved on a 2% agarose gel with ethidium bromide using the GDF698F/GDF-1128R and GADPH-1F/GADPH-613R primer sets, respectively, after RT-PCR was performed. GDF-8 and GAPDH relative abundance were scanned with a laser densitometer (Molecular Dynamics) and integrated with the Image Quant™ densitometer software. Also, the GDF-8/GAPDH ratio was measured as a relative quantification of GDF-8 gene expression.

The RT-PCR condition was set followed serious pilot studies. The production of PCR product of GDF-8 and GADPH genes logarithmically correlated to number of PCR cycles ranging from 28 to 34 cycles and from 24 to 32 cycles in our condition, respectively. Therefore, it is reliable using RT-PCR method to estimate the gene expression of GDF-8.

Sequencing

Initially, the PCR products obtained from the above individual primer-set were constructed into pCRII-TOPO (Invitrogen, USA) and then sequenced for identification. Applying the PRISM ready reaction dye terminator cycle sequencing kit (Applied Biosystems), DNA clones were sequenced on a 377A automated DNA sequencer (Applied Biosystems).

Phylogenetic sequence analysis

Sequence verification and nucleotide and amino acid sequence comparisons were evaluated by the a GCG program package (Devereux et al., 1984) as well as DNASTAR software. Furthermore, PAUP generated phylogenetic trees, which reflected evolutionary relationships, and GenBank deposited the GDF-8 sequence of livestock and poultry as follows: pig (nucleotide sequence, AF19623; amino acid sequence, AAB866901), cattle (AF019620; AAB86687), sheep (AF019622; AAB86689), chicken (AF019021; AAB86688), and turkey (AF019625, AAB86692).

Statistics

All quantitative data were expressed as mean±standard deviation (SD). A Students' t test evaluated the differences between the groups. Furthermore, P values less than 0.05 were statistically significant.

RESULTS AND DISCUSSION

Nucleotide and amino acid sequences of several GDF-8 genes of livestock and poultry, including pig, cattle, sheep, chicken, and turkey, existed within the GenBank, and employed for phylogenetic analyses. Within the nucleotide sequences, the GDF-8 similarities were 80-97% and in the amino acid sequences they were 88-99%. Figure 1 illustrates the phylogenetic relationships based upon nucleotide sequences diversity and amino acid sequences. The result demonstrated that a relationship among pig,
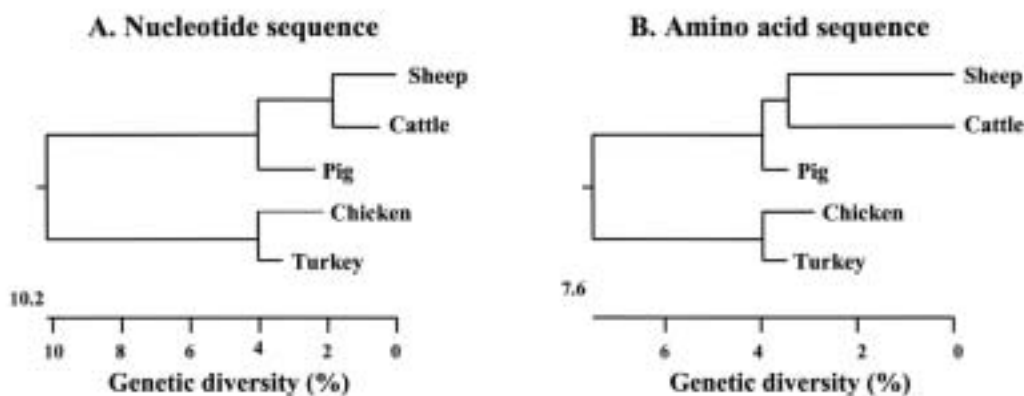
Figure 1. The phylogenetic tree of livestock animals drew from the diversity of nucleotide and amino acid sequences. According to each pair-comparisons, nucleotide sequence (A) and amino acid sequence (B) of pig, cattle, sheep, chicken, and turkey were compared and phylogenetic trees were established and diversity (%) was indicated.

cattle and sheep exists, than does in the other pairs, which were compared. Particularly, the similarity in GDF-8 amino acid sequence between pig and cattle was 96%. Such a high degree of sequence conservation within GDF-8 recommends that GDF-8 functions in pigs are conserved and perform an essential role within muscle growth as in cattle (McPherron and Lee, 1997). Thus, the physiological role of GDF-8 in pig productive performance is worth investigation.

It is well known that European pigs have an increased proficiency for converting feed into lean muscle and produce a higher meat mass (Serra et al., 1992; Young, 1992; Gaur et al., 1998; Hyun et al., 2001), when compared to Asian pigs. Thus, the comparison of nucleotide sequence and postnatal GDF-8 expression level in European and Asian pigs were performed to test if such discrepant phenotypes between pigs are due to varying genotypes and/or expressive levels of GDF-8 gene.

In the present study, using RT-PCR, the entire coding region of porcine GDF-8 gene was amplified from the total RNA and cloned for nucleotide sequencing. Twelve individual sequences from European pigs (Duroc and Landrace) and Asian pigs (Small-ear and Taoyuan) were analyzed. That is, there were 3 sequences from each breed, respectively. All sequences contained 1,128 nucleotides and predictably deduced 375 amino acids. Between European and Asian pigs sequenced herein, the similarities were 100% in both the nucleotide sequence and the amino acid sequence of GDF-8. Notably, our sequence data are consistent with others registered within the GenBank, including Duroc (AF188635), Yorkshire (AF188638), Hampshire (AF188636) and Meishan (AF188637). In addition, our nucleotide and amino acid sequences are the same as those previously reported. Recently, Stratil and Kopecny (1999) reported two polymorphisms of porcine

GDF-8 gene, however, one is localized in the promoter region and the other is localized in the exon 3 region, which fails to result in an amino acid substitution. Due to the limited number of analyzed samples, the latter polymorphism was not observed in our sequence data.

The sequence comparison result concluded that significantly discrepant growth rates and meat production phenotypes between European and Asian pigs were not due to polymorphism in the coding region of GDF-8 gene. However, timing and GDF-8 expression levels within the prenatal and postnatal period, mutations in the promoter region, untranslated regions, and introns remain to be evaluated. Certainly, not all double-muscled cattle possess a deletion or mutation sequence within the GDF-8 gene. For example, double-muscled Maine-Anjou is homozygous for the wild-type allele of the GDF-8 gene. Therefore, the most probable explanation for this finding would be the allelic heterogeneity of the gene (Grobet et al., 1997). Additionally, it could not be excluded that mutations occur in alternate sequences of growth factors or receptors.

It is reported that the physiological role of GDF-8 is largely associated with the prenatal muscle growth (Ji et al., 1998). However, the GDF-8 role in postnatal muscle growth of pigs remains unclear. Therefore, we determined that postnatal muscle growth occurs with detectable changes in GDF-8 expression. Using the ethidium-bromide-stained RT-PCR method, GDF-8 expression levels in the longissimus samples of European and Asian pigs were quantified. Muscle tissue generated a 613 bp PCR product of a partial GADPH gene and a 431 bp of the 3'-terminal GDF-8 gene (Figure 2A).

The GDF-8/GAPDH relative abundance ratio, which responded to GDF-8 expression level in the longissimus samples of Duroc (n=36) and Small-ear (n=30) obtained at each of birth, 1, 3, 6, and 12 mo of age were measured and
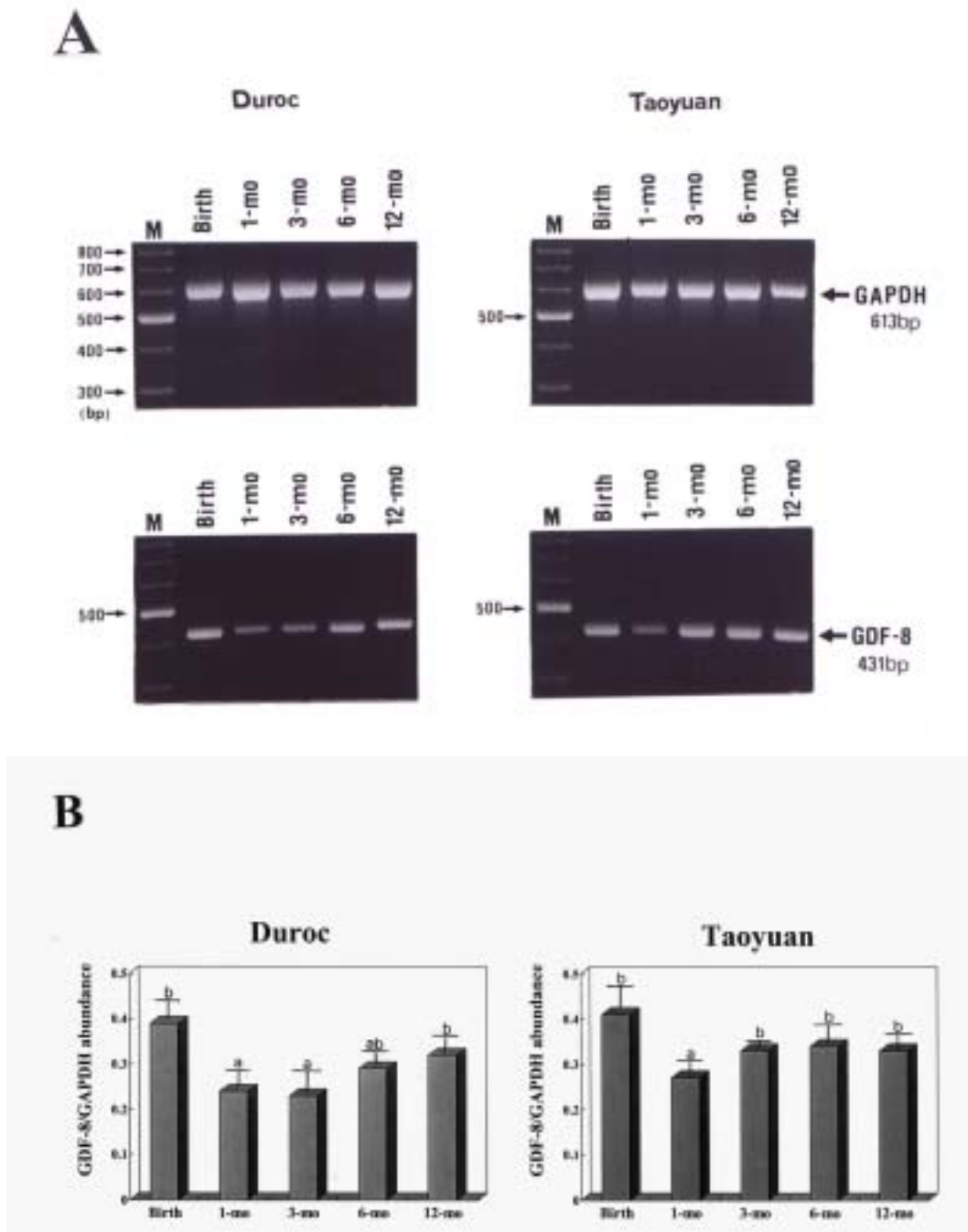
Figure 2. GDF-8 gene expression in longissimus muscle of postnatal Duroc and Taoyuan. A, the total RNA was extracted from the longissimus muscles of Duroc and Taoyuan and applied to quantify the GDF-8 expression with ethidium-bromide-stained RT-PCR. GADPH expression is an internal control. Agarose gel electrophoresis of the 431-bp PCR products of GDF-8 gene and 613-bp PCR products of GAPDH gene were obtained from longissimus muscle cDNA of the Duroc and Taoyuan at birth and 1, 3, 6, and 12 mo of age, respectively. M indicates the DNA marker and the sizes, as indicated. B, measurements of the ratio of GDF-8/GAPDH relative abundance responding to GDF-8 expression level in the longissimus sample of postnatal Duroc and Taoyuan. Thirty-six and thirty total RNAs were extracted from the longissimus muscles of Duroc and Taoyuan, respectively. The Duroc samples include neonatal littermate (n=6) and those at 1 mo (n=8), 3 mo (n=8), 6 mo (n=8), and 12 mo (n=6); The Taoyuan samples include neonatal littermate (n=4) and those at 1 mo (n=8), 3 mo (n=8), 6 mo (n=6), and 12 mo (n=4). SD is shown and bars without a common letter are different (p<0.05).

compared, respectively (Figure 2A). In the Duroc, the GDF-8 expression level in longissimus muscle was highest at birth however; it declined significantly (p<0.05) at 1 and 3 mo, and then gradually increased from 6 to 12 mo

(Figure 2B). The result was consistent with those reported by Ji et al. (1998). Furthermore, they also demonstrated that GDF-8 expression in skeletal muscle increased from 2 to 15 wk and then plateaued thereafter (Ji et al., 1998). As the

Duroc, the neonatal littermates of Taoyuan had the similar level of GDF-8 expression and decreased significantly (p<0.05) when pigs were 1 mo old. However, when these piglets were 3 mo of age, there was a marked (p<0.05) increased, which then maintained a constant level thereafter (Figure 2B). Average body weights at birth and 1, 3, 6, and 12 mo of the Duroc were 1.4, 7, 52, 106, and 155 kg, respectively. At the corresponding ages, average body weights of the Taoyuan were 0.8, 5, 25, 55, and 65 kg, respectively. Among the Duroc, the growth rate did not change significantly throughout 1 to 12 mo. While in the Taoyuan, the growth rate declined significantly after 6 mo.

In the Landrace and Small-ear breeds, the GDF-8 expression level in the longissimus muscle was also measured at 1 and 3 mo. The result showed that in the former, the GDF-8/GAPDH relative abundance was 0.25± 0.02 at 1 mo and 0.26±0.04 at 3 mo, and 0.25±0.05 at 1 mo and 0.32±0.03 at 3 mo in the latter (Figure 3). In the Landrace, the tendency of GDF-8 expression from aged 1 to 3 mo was similar to the Duroc. Alternately, Small-ear and Taoyaun possessed similar GDF-8 expression tendencies from 1 to 3 mo of age, i.e., the GDF-8 expression level in longissimus muscle at 3 mo was significantly (p<0.05) larger than that at 1 mo.

Our data showed in Figure 2B and Figure 3 present significantly different pattern of GDF-8 expression existed in the muscle tissue of pigs. Based on the result, we proposed that a significant discrepancy in growth rates and their corresponding patterns of growth rate between European and Asian pigs are related to the postnatal change of GDF-8 expression in skeletal muscle. However, the
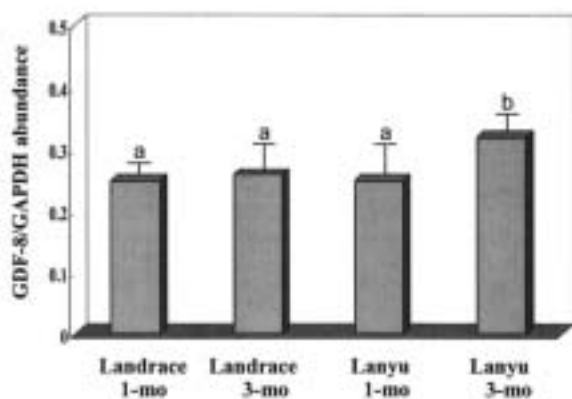
relationships between GDF-8 expression and postnatal muscle growth in both breeds remain to be studied. To our knowledge, neither enhanced nor reduced postnatal muscle growth associated with detectable changes of GDF-8 expression has been reported. Therefore, our findings provide a basis upon which the physiological role of GDF-8 in postnatal muscle growth can be examined.

ACKNOWLEDGEMENTS

REFERENCES

Devereux, J., P. Haeberli and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387-395.

Dunner, S., C. Charlier, F. Farnir, B. Brouwers, J. Canon and M. Georges. 1997. Towards interbreed IBD fine mapping of the mh locus: double-muscling in the Asturiana de los Valles breed involves: the same locus as in Belgian Blue cattle breed. Mamm. Genome 8:430-435.

Gaur, G. K., S. P. S. Ahlawat, A. K. Chhabra and S. Paul. 1998. Genetic trend for growth in a closed Indian herd of Landrace x desi crossbreds. Asian-Aust. J. Anim. Sci. 11:363-367.

Griffith, D. L., P. C. Keck, T. K. Sampath, D. C. Rueger and W. D. Carlson. 1996. Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor-beta superfamily. Proc. Natl. Acad. Sci. USA 93:878-883.

Grobet, L., L. J. R. Martin, D. Poncelet, D. Pirottin, B. Brouwers, J. Riquet, A. Schoeberlein, S. Dunner, F. Menissier, J. Massabanda, R. Fries, R. Hanset and M. Georges. 1997. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. Nat. Genet. 17:71-74.

Hyun, Y., B. F. Wolter and M. Ellis. 2001. Feed intake patterns and growth performance of purebred and crossbred Meishan and Yorkshire pigs. Asian-Aust. J. Anim. Sci. 14:837-843.

Ji, S., R. L. Losinski, S. G. Cornelius, G. R. Frank, G. M. Willis, D. E. Gerrard, F. F. S. Depreux and M. E. Spurlock. 1998. Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. Am. J. Physiol. 44:R1265-1273.

Kambadur, R., M. Sharma, T. P. L. Smith and J. J. Bass. 1997. Mutations in myostatin (GDF-8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res.7:910-916.

Kingsley, D. M. 1994. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. 8:133-146.

Lee, S. J. and A. C. McPherron. 1999. Myostatin and the control of skeletal muscle mass. Curr. Opin. Genet. Dev. 9:604-607.

McPherron, A. C. and S. L. Lee. 1996. In growth factors and cytokines in health and disease, eds. LeRoith, D. and C. Bondy (JAI, Greenwich, CT), Vol. 1B, pp.357-393.

McPherron, A. C. and S. J. Lee. 1997. Double muscling in cattle

Figure 3. GDF-8 gene expression in longissimus muscle of postnatal Landrace and Lanyu. Measurements of the ratio of GDF-8/GAPDH relative abundance responding to GDF-8 expression level in the longissimus sample of postnatal Landrace and Lanyu. The samples include Landrace at 1 mo (n=5) and 3 mo (n=5) age and Lanyu at 1 mo (n=5) and 3 mo (n=5) age. SD is shown and bars without a common letter are different (p<0.05).

due to mutations in the myostatin gene. Proc. Natl. Acad. Sci. USA 94:12457-12461.

McPherron, A. C., A. M. Lawler and S. J. Lee. 1997. Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature 387:83-90.

National Science Council. 1993. Handbook of management and use in laboratory animal. Taipei, Taiwan.

Serra, J. J., M. Ellis and C. S. Haley. 1992. Genetic components of carcass and meat quality traits in Meishan and Large White pigs and their reciprocal crosses. Anim. Prod. 54:117-127.

Stratil, A. and M. Kopecny. 1999. Genomic organization, sequence and polymorphism of the porcine myostatin (GDF8; MSTN) gene. Anim. Genet. 30:462-478.

Young, L. D. 1992. Effects of Duroc, Meishan, Fengjing, and Minzhu boars on productivity of mates and growth of first-cross progeny. J. Anim. Sci. 70:2020-2029.

1250