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RESEARCH ARTICLE

Muscle hypertrophy in transgenic mice due to over-expression of porcine myostatin mutated at its cleavage site

QIAN Li-li[1], MA De-zun[2], GAO Peng-fei[2], JIANG Sheng-wang[2], WANG Qing-qing[2], CAI Chun-bo[1], XIAO Gao-jun[2], AN Xiao-rong[1*], CUI Wen-tao[2*]

[1] *State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100193, P.R.China*

[2] *State Key Laboratory for Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, P.R.China*

Abstract

Myostatin, a member of the transforming growth factor beta (TGF-β) superfamily, is a dominant inhibitor that acts to limit skeletal muscle growth and development. In this study, we generated transgenic mice that express porcine myostatin containg mutations at its cleavage site (RSRR) to evaluate its effect on muscle mass. Results showed that the weight of four skeletal muscles including gastrocnemius, rectus femoris, tibialis anterior, and pectoralis increased by 17.83 and 28.39%, 21.76 and 28.70%, 34.31 and 41.62%, 53.21 and 27.54% in transgenic male and female mice, respectively, compared to their corresponding non-transgenic control mice. Measurement of muscle fiber size and number indicated that the mean myofiber size increased by 50.73 and 61.30% in transgenic male and female mice respectively compared to the non-transgenic controls. However, there was no difference in the number of myofiber between transgenic and non-transgenic male mice. These results clearly demonstrated that the increase in skeletal muscle mass in transgenic mice is caused by hypertrophy instead of hyperplasia.

Keywords: porcine myostatin, muscle mass, transgenic mice, mutation, hypertrophy, hyperplasia

1. Introduction

Myostatin, a member of transforming growth factor beta (TGF-β) superfamily, is a negative regulator of muscle development and growth (Lee 2004). The *myostatin* null mice show a dramatic and widespread increase in skeletal muscle mass (McPherron *et al.* 1997). The natural inactivating mutations in myostatin gene also result in an increase in muscling in cattle (Grobet *et al.* 1997; Kambadur *et al.* 1997; McPherron and Lee 1997; Grobet *et al.* 1998), sheep (Clop *et al.* 2006), dog (Mosher *et al.* 2007) and even human (Schuelke *et al.* 2004). Increase in muscle mass has been also observed in animals that myostatin gene was knocked out. Our lab recently demonstrated that loss-of-function mutation in porcine *myostatin* by Zinc finger nuclease (ZFN)-editing method resulted in a significant increase in muscle mass in pigs (Qian *et al.* 2015).

Simultaneously, we also generated transgenic mice expressing porcine propeptide and its mutant (D76A), and

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QIAN Li-li, E-mail: lili.qian@yahoo.com; Correspondence CUI Wen-tao, Tel/Fax: +86-10-62819480, E-mail: cuiwentao@caas.cn; AN Xiao-rong, Tel/Fax: +86-10-62733355, E-mail: xra@cau.edu.cn

[*] These authors contributed equally to this study.

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very similar results (i.e., an increase in muscle mass) were obtained (Ma *et al.* 2015a, b). All our previous findings suggested that it is practicable to promote muscle growth and development in livestock animals by genetically manipulating *myostatin* expression and regulating myostatin's biological activity.

It is well-known that myostatin, similar to other TGF-β family member, is synthesized as a precursor protein and then undergoes specific cleavage at a dibasic site containing amino acid sequence RSRR to generate an N-terminal propeptide and a C-terminal growth factor dimer. The C-terminal dimer is the biologically active form of myostatin, and it remains bound to the N-terminal propeptide to form the active latent complex (Lee and McPherron 2001; Thies *et al.* 2001; Wolfman *et al.* 2003). Therefore, the native cleavage site containing the RSRR motif plays a critical role in regulating the myostatin activation process *in vivo*. It had been reported that transgenic mice that express murine myostatin containing mutations at RSRR cleavage site (where RSRR is replaced by GLDG) exhibited an increase in skeletal mass (Zhu *et al.* 2000), indicating that mutations of specific cleavage site in myostatin affects muscle development and growth in a dominant negative manner.

Myostatin gene sequences have been identified in a number of different species including human, mice, rats, pigs, chickens and turkeys. The amino acid sequence of myostatin protein, especially in the C-terminal domain, is highly conserved. Pig is a livestock species that is routinely used as a valuable animal model in biomedical and agricultural research. To further investigate different approaches of regulating myostatin activities *in vivo* and to establish an animal model for muscle biology and agriculture studies, we generated transgenic mice expressing a porcine myostatin containing mutations at its cleavage site by replacing RSRR with GLDG, and evaluated the effect of this mutated porcine myostatin on muscle mass in mice. Data from this study show that porcine myostatin containing mutations at its cleavage site enhances the muscle development and growth in transgenic mice. This finding provides a direct scientific evidence to support future studies to improve the muscle mass and quality in transgenic pig by inhibiting myostatin activation process through the mutations at its cleavage site (RSRR).

2. Materials and methods

2.1. Generation of transgenic mice expressing mutated porcine myostatin at its cleavage site (RSRR)

*Not*I restriction sites were added to both ends of porcine mutated myostatin gene (RSRR→GLDG), and the full cDNA sequence of mutated porcine myostatin was confirmed by sequencing during the process of vector construction. Porcine mutated *myostatin* was then inserted in the presence of T4 ligase into "Sleeping Beauty" transposon (pT2-HB) carrier that was digested with *Not*I restriction endonuclease.

The plasmid was then linearized with *Sca*I restriction endonuclease, agarose gel electrophoresis was used to confirm that plasmid was linearized completely. The linearized plasma was then extracted with phenol/chloroform, purified by ethanol precipitation, and solubilized and diluted in injection buffer (10 mmol L^{-1} Tris-HCl/0.1 mmol L^{-1} EDTA, pH 7.5) 4 μg mL^{-1}. Purified plasmid was mixed with SB100 transposase at 1:1 ratio, then microinjected into the pronuclear of fertilized C57BL/6J mouse eggs. The fertilized eggs were then transferred to oviduct of recipient mice to generate transgenic mice.

All mice used in this study were F_1 offsprings generated by breeding male F_0 transgenic mice with female wild type C57BL/6 J. All F_1 mice were from the same litter with an age of 10 weeks.

2.2. Analysis of porcine myostatin mutant (RSRR→GLDG) expression in transgenic mice

Genomic DNA was extracted from tail of each transgenic mouse at week 4 and detected by PCR. PCR primers are specific for SV40 promotor and MLC1 enhancer, their sequences are 5´-CACTGCATTCTAGTTGTGGTTTGT-3´ and 5´-AAGCATGATGTCTGTGCGGT-3´. PCR was performed under the following conditions: 95°C for 5 min, 35 cycles including 30 s at 95°C, 30 s at 64°C, 40 s at 72°C and 72°C for 10 min. All PCR products were sequenced to confirm the transgene identities.

Detection of transgenic mice by reverse-transcription PCR (RT-PCR): primers were designed to specifically amplify the exogenous porcine myostatin gene only. The primers are: 5´-AGAACAGCGAGCAAAAGGAAA-3´ and 5´-TCCACTTGCATTAGAAGATCAGA-3´. RT-PCR was performed per the following conditions: 95°C for 5 min, 35 cycles including 30 s at 95°C, 30 s at 60°C, 20 s at 72°C and 72°C for 10 min.

The real-time quantitative PCR was also used to measure mRNA levels of murine endogenous and porcine exogenous myostatin mutant in transgenic and wild-type (WT) mice. Total RNA from gastrocnemius was extracted using Trizol (Invitrogen, USA) according to manufacturer's protocol. The cDNA of total RNA was synthesized by using the First Strand cDNA Synthesis Kit (Fermentas, Lithuania), and the real-time quantitative PCR was performed by using SYBR Green Kit (Applied Biosystems, USA) and the ABI-7500 Real-Time PCR System (Applied Biosystems). Gene expression levels were calculated according to the $2^{-\Delta\Delta C_T}$ method. All methods were the same as previously described (Wang

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