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Functional Characterization of Human Myostatin C-Domain Recombinant Protein and Production of Myostatin Polyclonal Antibody

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Abstract: Myostatin (GDF-8) is a member of the Transforming Growth Factor-Beta (TGF-β) superfamily that is highly expressed in skeletal muscle and myostatin loss of function leads to doubling of skeletal muscle mass while dysregulated myostatin activity is associated with a number of metabolic disorders including muscle cachexia, obesity and type II diabetes. In this study, to further investigate the application of the myostatin protein, researchers constructed and expressed recombinant myostatin protein and its polyclonal antibody. The C-domain of human myostatin gene was cloned into the prokaryotic expression vector pET-32a-c (+) to express His-tagged myostatin-C protein and was expressed in Origami B DE3) induced by IPTG. After purification, the recombinant protein was used to raise the anti-myostatin polyclonal antibody. In addition, the immunogenicity of the recombinant myostatin-C protein was detected by mice immunized with the protein. The results showed the purity of the recombinant myostatin-C protein is >90%. The final concentration is 3.5 mg mL^{-1}. It reacted with the myostatin antibody with a strong specific reactive band. The titer of anti-myostatin serum was determined to be 1:50,000. Immunization of mice with recombinant protein could significantly increase the body weight of neonatal mice and themselves. These results proved that researchers obtained a high-level expression of the recombinant myostatin-C protein as well as high titer of rabbit polyclonal antibody. It retaining antigen-binding activity can be employed for some therapeutic use. This special polyclonal antibody could provide a good tool for further studying structural and functional characterization of myostatin protein.

Key words: Myostatin, recombinant protein, function characterization, polyclonal antibody, neonatal mice

INTRODUCTION

Myostatin (GDF-8) is most well recognized as a factor that has potent catabolic and anti-anabolic effects on skeletal muscle (Lee, 2004). Myostatin levels are elevated in conditions associated with muscle atrophy such as cancer cachexia (Reisz-Porszasz *et al*., 2003; Zimmers *et al*., 2002) and unloading (Wehling *et al*., 2000; Allen *et al*., 2009) and blocking myostatin function can increase muscle mass and improve muscle regeneration (McPherron *et al*., 1997; Whittemore *et al*., 2003; Sartorelli and Fulco, 2004). Muscle mass, like that of any other tissue, depends on protein turnover and cell turnover (Wagner, 2005). Myostatin is widely recognized as a potent suppressor of muscle growth, development and regeneration. Myostatin has multiple roles during muscle development and regeneration such as regulating myoblast proliferation and differentiation (Hamrick *et al*., 2010). Thus, within this context, myostatin serves as a brake of sorts that guides muscle growth and development to keep muscle fiber size and number within a functionally and metabolically appropriate range. Blocking myostatin using systemic injections of a myostatin inhibitor causes enhanced bone regeneration (McFarlane *et al*., 2011) much smaller functional antibody fragments of myostatin retaining antigen-binding activity can be employed for therapeutic use.

In the present study, the human myostatin-C gene was cloned into pET-32a (+) to yield pET-32a-hM. The His-tagged myostatin protein was then expressed in Origami B (DE3) cells and purified by a Ni-IDA sepharose affinity chromatography under denaturing conditions. Subsequently, a polyclonal antibody was raised against the purified His-tagged myostatin protein in rabbits. Then, the reactivity and specificity of the polyclonal antibody were characterized by indirect Enzyme-Linked Immunosorbent Analysis (ELISA) and Western blotting assays. In addition, researchers explored preliminarily the functional characterization of recombinant myostatin C-domain protein by immunizing Kunming White mice with it.

MATERIALS AND METHODS

The 3 weeks old female and male Kunming White mice were received from Anti-aging Chinese Herbal

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Anhui Engineering Technology Research Center (Fuyang, China) male New Zealand white rabbits (adult, 3 kg) were purchased from Guangdong Medical Experimental Animal Center (Guangzhou, China); pET-32a-c (+) (Novagen, Shanghai, China); Kpn I, Xho I and T4 DNA ligase (Takara, Dalian, China); complete Freund's adjuvant and incomplete Freund's adjuvant (Sigma).

Expression and purification of recombinant protein: Myostatin-C domain part 110 amino acid sequence (NCBI Reference Sequence: NP_005250.1) after the codon of this corresponding nucleic acid sequence optimized was synthesized by chemical coupling. The product was inserted into pET-32a (+) to yield the recombinant expression plasmid pET-32a-hM. Origami B (DE3) Competent cells was transformed with pET-32a-hM and the recombinant bacteria were inoculated into LB medium containing. Then, the recombinant protein was induced by Isopropyl-β-D-Thiogalactopyranoside (IPTG). All groups have three repeats. The total bacterial lysates were collected and detected by SDS-PAGE electrophoresis with the noninduced recombinant protein as the control. The recombinant myostatin-C protein was purified by Ni-IDA sepharose affinity chromatography.

Production and purification of polyclonal antibodies against myostatin: Five adult male New Zealand white rabbits were immunized and each rabbit was injected subcutaneously with 1 mg purified recombinant myostatin-C protein emulsified with complete Freund's adjuvant (Sigma). Then, each rabbit was booster-immunized with 500 μg purified recombinant myostatin-C protein emulsified in incomplete Freund's adjuvant (Sigma) by subcutaneous injection once every 3 weeks. Before immunization, blood was obtained from each animal to prepare non-immune serum and after four booster immunizations, blood was obtained again. The serum samples were stored at -80°C. Anti-myostatin was purified with caprylic acid-ammonium sulfate precipitation according to reported protocol (Ruan *et al*., 2005; Yang *et al*., 2012).

Western blotting assay of the recombinant myostatin-C protein: Purification of the recombinant myostatin-C protein was detected by Western blotting assays as described previously (Zeng *et al*., 2007). Noninduced *E. coli* Origami B (DE3) transformed by pET-32a-hM plasmid as negative control. The primary antibody used in Western blotting assays was the standard anti-myostatin antibody. Further, the prokaryotic expression recombinant protein was detected with prepared anti-myostatin antibodies by Western blotting.

Determining the immunization efficiency by ELISA: After purification, the titers of the prepared anti-myostatin antibodies were determined using an indirect Enzyme-Linked Immunosorbent Analysis (ELISA) according to a reported protocol (Xue *et al*., 2013). Measurement was repeated at least three times, each time to repeat three holes. P/N≥2.0 is considered positive.

Animal immunization: The mice were randomly assigned to three groups: the first group was the negative control group, the second group for the experimental groups and the third group was the positive control group, each 24 (12 males and 12 females). The mice were housed 4 per cage with continuous access to food and water and a 12 h light/dark cycle. All mice were fed *ad libitum* throughout the experimental period. After 7 days, the experimental groups mice were immunized and each mouse was injected subcutaneously with 50 μg purified myostatin-C protein emulsified with complete Freund's adjuvant (Sigma). Then, each mouse was booster-immunized with 25 μg purified myostatin-C protein emulsified in incomplete Freund's adjuvant (Sigma) by subcutaneous injection once every 3 weeks, 3 times. The positive control group mice were immunized with recombinant myostatin full-length protein (Ma *et al*., 2004) (about 60 kD, stored in the laboratory), the procedure was same as that of the experimental group. The negative control group was injected with physiological saline. Male and female mice were bred mixed after the third immunization 7 days. Body weight of immunized mice and their offspring mice were recorded.

RESULTS AND DISCUSSION

Induced expression and purification of the His-tagged myostatin-C protein: The recombinant plasmid pET-32a-hM was transformed into Origami B (DE3) to be induced for gene expression. After induction with IPTG, Origami B (DE3) harboring pET-32a-hM exhibited a different level of expression. The expressed product was identified by SDS-PAGE (Fig. 1). A distinct band of approximately 26 kDa, corresponding to the expected molecular weight of the His-tagged myostatin-C protein was found only after induction (Fig. 1, lane 4, 6, 8, 10, 12, 14) whereas there was no expression of the myostatin-C protein in Origami B (DE3) harboring pET32a-hM without IPTG induction (Fig. 1, lane 1, 2).

After purified by Ni-IDA sepharose affinity chromatography, researchers collect the solution which washed by 500 mM imidazole for dialysis (dialysis buffer: PBS, 2 mM DTT) using the PEG-20000 to embed the dialyzed protein. The SDS-PAGE result displays that the purity is >90%. The final concentration is 3.5 mg mL^{-1} (Fig. 2).

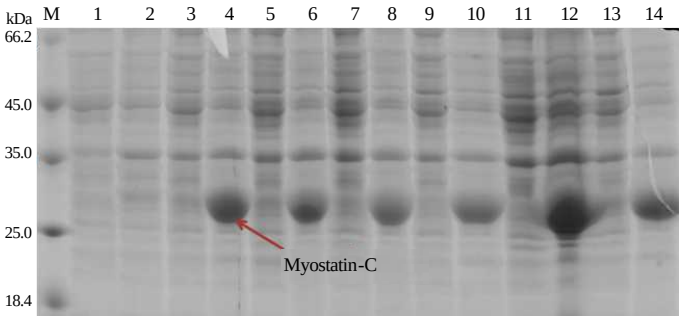

Fig. 1: Expression analysis of expressed His-tagged myostatin-C protein and optimization of the IPTG concentration and induction temperature. Lane M is protein marker with the molecular weight of each standard listed in kDa on the left; Lane 1 is the supernatant from the ammonium sulfate precipitation no IPTG; Lane 2 is the precipitant from the ammonium sulfate precipitation no IPTG; Lane 3 is the supernatant from the ammonium sulfate precipitation with 0.1 mM IPTG 18°C overnight; Lane 4 is the precipitant from the ammonium sulfate precipitation with 0.1 mM IPTG 18°C overnight; Lane 5 is the supernatant from the ammonium sulfate precipitation with 0.2 mM IPTG 18°C overnight; Lane 6 is the precipitant from the ammonium sulfate precipitation with 0.2 mM IPTG 18°C overnight; Lane 7 is the supernatant from the ammonium sulfate precipitation with 0.1 mM IPTG 25°C overnight; Lane 8 is the precipitant from the ammonium sulfate precipitation with 0.1 mM IPTG 25°C overnight; Lane 9 is the supernatant from the ammonium sulfate precipitation with 0.2 mM IPTG 25°C overnight; Lane 10 is the precipitant from the ammonium sulfate precipitation with 0.2 mM IPTG 25°C overnight; Lane 11 is the supernatant from the ammonium sulfate precipitation with 0.1 mM IPTG 30°C overnight; Lane 12 is the precipitant from the ammonium sulfate precipitation with 0.1 mM IPTG 30°C overnight; Lane 13 is the supernatant from the ammonium sulfate precipitation with 0.2 mM IPTG 30°C overnight; Lane 14 is the precipitant from the ammonium sulfate precipitation with 0.2 mM IPTG 30°C overnight; arrowheads indicate the position of the recombinant myostatin-C protein

Western blotting assay of the recombinant myostatin-C protein: The purified target protein was determined by Western blotting which reacted with the standard myostatin antibody with a strong specific reactive band (Fig. 3a, lane 2). Anti-myostatin antibodies from rabbits can also clearly detected the purified recombinant protein with the predicted molecular weight on a Western blotting (Fig. 3b, lane 2).

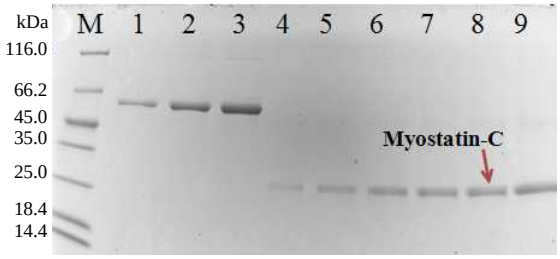

Fig. 2: SDS-PAGE electrophoresis results of the purified recombinant protein; Lane M is protein molecular weight marker with the molecular weight of each standard listed in kDa on the left; Lane 1-3 is recombinant myostatin full-length protein (about 60 kD, stored in the laboratory) derived in the same way as the recombinant myostatin-C protein which was used for the positive control to immunity mice in present study; Lane 4-9 is purified recombinant myostatin-C protein by using the PEG-20000 to embed the dialyzed protein; arrowheads indicate the position of the myostatin-C protein

Determining the immunization efficiency of myostatin antisera by ELISA: Rabbits were immunized with purified myostatin-C protein to generate polyclonal antibodies against myostatin. The purification of anti-myostatin antibodies was carried out by affinity chromatography. Positive serum containing anti-myostatin antibodies and negative serum (five rabbits serum) collected before immunization were diluted (from 1:10,000 to 1:50,000) and their reactivity with myostatin was determined by ELISA. The result showed that the titer of anti-myostatin serum was determined to be 1:50,000 (Fig. 4).

Mice immunized result: The weight change of male and female mice are shown in Fig. 5, after the first immunization 3 weeks, the mice body weight of three group had almost no difference. However, after the second immunization 3 weeks the mice body weight of experimental group and positive control group were

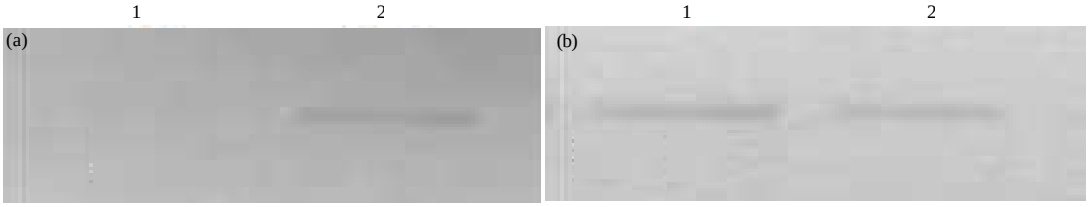

Fig. 3: Identification of His-tagged myostatin-C protein and prepared polyclonal antibody by Western blotting; a) identification of His-tagged myostatin-C protein with standard myostatin antibody; Lane 1 is negative control (Uninduced *E. coli* Origami B (DE3) transformed by pET-32a-hM plasmid); Lane 2 is purified His-tagged myostatin-C protein reacted with the myostatin antibody with a specific reactive band; b) determining purified His-tagged myostatin-C protein with prepared polyclonal antibody. Lane 1 is positive control (standard myostatin antibody), purified His-tagged myostatin-C protein reacted with the standard myostatin antibody with a specific reactive band; Lane 2 is the purified His-tagged myostatin-C protein reacted with the prepared polyclonal antibody with a specific reactive band

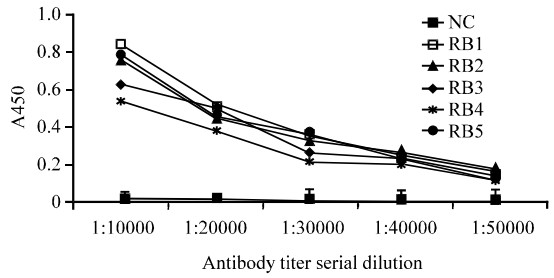

Fig. 4: Indirect ELISA analysis of prepared polyclonal antibody; RB1-RB5 are the titers of anti-myostatin serum of five rabbits immunized with purified myostatin-C protein, NC is negative serum (the titer mean of five rabbits serum collected before immunization)

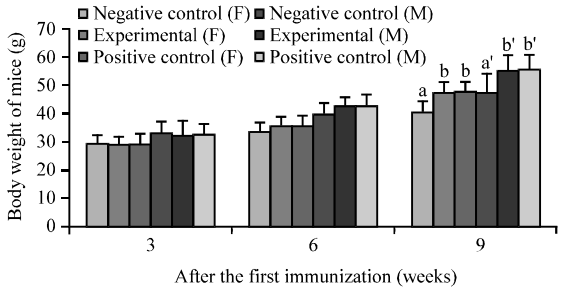

Fig. 5: The weight change of male and female mice after immunization; experimental (F) is the weight change of female mice immunized with purified recombinant myostatin-C protein (n = 12/group); positive control (F) is the weight change of female mice immunized with recombinant myostatin full-length protein (n = 12/group); negative control (F) is the weight change of female mice unimmunized (n = 12/group); experimental (M) is the weight change of male mice immunized with purified recombinant myostatin-C protein (n = 12/group); positive control (M) is the weight change of male mice immunized with recombinant myostatin full-length protein (n = 12/group); negative control (M) is the weight change of male mice unimmunized (n = 12/group); all mice body weights were measured at 3, 6 and 9 weeks after the first immunization; two immunized female mice groups gained more weight at 9 weeks after the first immunization (b: $p<0.05$ vs. a), the same as two immunized male mice groups (b': $p<0.05$ vs. a'); the results are mean values±SD and the statistical significance values were computed using a t-test

higher than that of negative control group but the difference was not significant. After the third immunization 3 weeks the increase of body weight of experiment group and positive control group exceeded significantly that in negative control group ($p<0.05$). There was almost no difference between experiment group and positive control group.

Neonatal mouse body weight of negative control group displayed very significant difference ($p<0.01$) compared with positive control group and experimental group. Offspring mice were weighed weekly after birth (1-8 weeks) as can be seen from (Fig. 6) average weight of experimental group and positive control group offspring mice were very significantly higher than that of negative control group offspring mice during 5 weeks. Then, the differences decreased gradually. After 7 weeks, average weight had no significant differences among three group. There was almost no difference between experiment group and positive control group offspring mice.

Progress in genetic and antibody engineering has made it possible to produce various antibody fragments carrying desirable properties (Winter and Milstein, 1991).

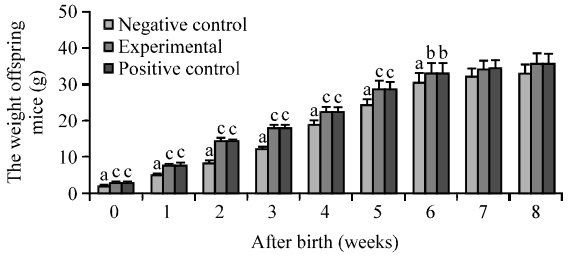

Fig. 6: The body weight change of offspring mice after birth; positive control is the weight change of offspring mice whose parents were immunized with recombinant myostatin full-length protein. Negative control is the weight change of offspring mice whose parents were unimmunized. Experimental group is the weight change of offspring mice whose parents were immunized with purified recombinant myostatin-C protein. All offspring mice body weights were measured at 0, 1, 2, 3, 4, 5, 6, 7 and 8 weeks after birth. The birth weight of positive control and experimental group displayed very significant difference (c: $p<0.01$ vs. a) compared with that of negative control group. This difference remains until 5 weeks after birth. Then, the difference decreased gradually. There was significant difference (b: $p<0.05$ vs. a) at 6 weeks after birth; after 7 weeks, average weight had no significant differences among three group. The results are mean values±SD and the statistical significance values were computed using a t-test

Such recombinant antibody fragments have several advantages. They do not require glycosylation and are well produced in bacteriain functional form, greatly simplifying the access to these molecules (Proba and Riesenberg, 1996; Wlad *et al*., 2001; Arbabi-Ghahroudi *et al*., 2005; Andersen and Reilly, 2004; Rodriguez-Carmona *et al*., 2012). Their smaller size allows improved tissue penetration for therapeutic applications such as cancer therapy (Batra *et al*., 2002; Rothlisberger *et al*., 2005). In this study, researchers constructed and expressed myostatin antigen in Origami B (DE3). Myostatin plays an important role in many aspects. Myostatin signaling is critical for understanding the pathogenesis of muscle wasting since blocking it mitigates muscle losses in rodent models of catabolic diseases including cancer, chronic kidney or heart failure topic of clinical investigation. As noted, myostatin signaling in muscle can be blocked pharmacologically. Researchers are entering an era of treatment and no longer stuck at devising new definitions for the degree of muscle wasting.

In the present study, researchers obtained a high-level expression of the recombinant myostatin-C protein as well as high titers of rabbit polyclonal antibody. Western blot analysis demonstrated that the recombinant myostatin-C protein was specifically recognized by the polyclonal antibody. The studies suggest that the recombinant protein may improve not only immunized juvenile mice muscle growing but also their offspring birth weight and body weight. May be specific anti-myostatin antibodies of parent mice enter into the fetus body through the placenta, blocking its myostatin action pathway by being combined with fetal high levels myostatin there by promoting fetal skeletal muscle development. Therefore, the positive control group and experimental group offspring birth weight was significantly higher than that of negative control group. This suggests that recombinant protein immunized animals can induce the appearance of specific antibodies. These results proved that recombinant myostatin antigen may be used for therapy in muscle growing. This special polyclonal antibody provides a good tool for further studying structural and functional characterization of myostatin protein. The polyclonal antibodies prepared can be used for various biological tests including ELISA and Western blotting assays.

Mechanisms by which myostatin influences muscle mass have been investigated in myostatin knockout mice (Welle *et al*., 2006). But myostatin gene knockout did not change muscle protein degradation. In contrast, the inhibition of myostatin with the peptibody blunted the muscle proteolysis of CKD. Myostatin inhibition can suppress inflammation and ameliorate the muscle wasting in a catabolic condition that is characterized by inflammation, impaired IGF-1 intracellular signaling and excess glucocorticoids (Zhang *et al*., 2011). The difference in these conclusion could reflect the fact that (Welle *et al*., 2006) assessed protein half-lives and did not measure protein degradation. Myostatin inhibition could also prevent muscle atrophy by improving the function of skeletal muscle progenitor or satellite cells (Shortreed *et al*., 2008; McCroskery *et al*., 2003). Myostatin inhibition has elicited beneficial responses in models of muscular dystrophies (Bartoli *et al*., 2007). However, myostatin inhibition did not correct severe spinal muscular atrophy (Sumner *et al*., 2009). Whether the variability in responses obtained in these studies was due to the types of muscle disease or an inability to inhibit myostatin more completely is not known.

CONCLUSION

Researchers have started to investigate the effects of recombinant myostatin antigen in blood parameters, skeletal muscle mass and strength and cell morphology from an immunotherapy perspective. For further research, researchers will work hard to reveal its molecular mechanism and main signal transduction pathways.

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