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Effect of Prime-Boost Immunization Against Myostatin on Antibody Levels, Backfat and Growth in Pigs

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Abstract: The objective was to compare three active immunization schemes using a myostatin P2-P30 recombinant DNA vaccine and myostatin P2-P30 recombinant antigen in two different doses to measure the effect on antibody levels, back fat, body weight up to 84 days and body weight gain. The 36 growing pigs were divided into four groups: a control group without immunization; Group 1 and 2 used a prime-boost immunization system and Group 3 a classic immunization system. The doses of vaccine and antigen were 100 μg for Group 1 and 1000 μg for Group 2 and 3. Antibody levels at doses of 100 μg (0.76 Abs) were not different in unimmunized pigs but were lower and different ($p<0.0001$) compared to 1000 μg doses of vaccine and antigen (1.26 and 1.17 Abs). The dorsal fat in pigs with the 1000 μg dose was lower (16.72 and 15.22 mm) ($p<0.05$) compared with pigs in the 100 μg (19.83 mm) dose. Only the 1000 μg dose of the P2-P30 vaccine or recombinant antigen produces an immune response in pigs with more final weight and body weight gain.

Key words: Myostatin, antibodies, growth, pigs, control group, immunization

INTRODUCTION

Myostatin a member of the family of transforming growth factors-beta was identified by degenerative PCR in 1997 and known as growth and differentiation factor-8 (McPherron *et al*., 1997). Myostatin is a possible determinant of muscle mass in several species (Patel and Amthor, 2005; Moreno *et al*., 2008). It blocks the proliferation and differentiation of myoblasts and keeps satellite cells in a passive state by inhibiting protein synthesis through regulating the size of muscle fibers. In addition, myostatin acts as a signal secreted by the skeletal cells that causes a negative feedback, direct or indirectly in muscle precursor cells, exerting a negative regulation of muscle mass growth (Ramirez, 2013). Myostatin is expressed primarily in skeletal muscle and then in plasma (Gonzalez-Cadavid *et al*., 1998). It has been reported in mice, knockout for the myostatin gene or transgenic mice, expressing various inhibitors of myostatin such as propeptide, follistatin or a dominant negative form of ActRIIB, the presence of hyperplasia and hypertrophy in muscle masses (Whittemore *et al*., 2003; Lee *et al*., 2005; Patel and Amthor, 2005). Because the function of myostatin is highly conserved in many species, seeking alternatives to inhibit its activity may have important applications not only in medicine but also in animal production (Lee *et al*., 2005). Although, the natural inhibitors of myostatin seem to be the undoubted candidates for therapies that block their activity, obtaining and purifying them is complicated (Tang *et al*., 2007). In contrast, vaccines using recombinant antigens seem to be a viable option, since, their construction is relatively cheap they are safe and their production in large quantities is feasible, obtaining high levels of antigen purity (Faurez *et al*., 2010). One type of recombinant vaccine is DNA vaccines which consist of the DNA of a plasmid that expresses the recombinant antigen under the control of an intense and promiscuous promoter such as cytomegalovirus. These vaccines stimulate a broad spectrum of immune responses including B lymphocytes,

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cytotoxic T lymphocytes and have been shown to elicit prolonged immune responses (Coban *et al*., 2008). The use of a myostatin recombinant DNA vaccine which has been modified by fusing the P2-P30 immuno stimulatory epitopes of tetanus toxin showed an increase in muscle masses in immunized mice (Ramirez, 2013). The objective of this study was to evaluate in growing pigs, the effect of immunization schemes with the myostatin P2-P30 DNA vaccine in combination with a recombinant antigen produced in *E. coli*, measuring levels of antibodies, dorsal fat, body weight up to 84 days and body weight gain.

MATERIALS AND METHODS

Animals and treatments: The 36 commercial pigs in the initiation phase with an averageof 45 days of age and 14.24±0.70 kg live weight were used. The pigs were housed in individual pens of 2×2 m with concrete floor and metal railing having an acclimatization period of 7 days and sacrificed 84 days later. The 4 experimental groups were formed: 10 pigs for the control group, 10 pigs for Group 1 and 8 pigs for each of Group 2 and 3. The pigs were fed according to the nutritional requirements with a commercial feed. All the experimental procedures were carried out under the guidelines of the official Mexican Standards NOM-051-ZOO-1995 (humane care of animals), NOM-062-ZOO-1999 (handling of animals in the laboratory), NOM-033-ZOO-1995 (humanitarian sacrifice of domestic animals) and NOM-012-ZOO-1993 (regulation of chemical, pharmaceutical and biological products for use in animals).

The control group received applications of distilled water on days 0, 14 and 28 of the experiment. In the animals of Group 1, three inoculate of 100 μg myostatin P2-P30 DNA vaccine (day 0, 14 and 28) plus a prime boost at day 28 with 100 μg myostatin P2-P30 recombinant antigen were applied. In Group 2, 1000 μg myostatin P2-P30 DNA vaccine was inoculated on day 0 and a prime-boost booster was performed with 1000 μg myostatin P2-P30 recombinant antigen with Freud's complete adjuvant on the day 14. In Group 3, 1000 μg myostatin P2-P30 recombinant antigen with Freud's complete adjuvant were applied on day 0 of the experiment, followed by 3 successive immunizations of inoculum with 1000 μg myostatin P2-P30 recombinant antigen with Freud's incomplete adjuvant on day 14, 28 and 42. Immunizations performed on all animals were subcutaneous at the anterior region of the ear (Anonymous, 1993, 1995, 1999).

Preparation of the myostatin P2-P30 DNA vaccine: The immunogens were elaborated in the Molecular Genetics Laboratory of the Department of Genetics and Biostatistics of the Faculty of Veterinary Medicine and Animal Science of the Universidad Nacional Autonoma de Mexico (UNAM). The DNA vaccine consisted of the pCI-neo plasmid to which a fragment of the myostatin gene obtained by PCR from chicken DNA genome was inserted. It was modified antigenically with the P2 and P30 epitopes of the tetanus toxin in the amino and carboxyl ends, respectively, containing restriction sites for Xbal. The modified myostatin fragment with the epitopes was initially cloned into a T vector and subsequently digested with the Xbal enzyme to obtain the fragment with compatible cohesive ends for the pCI-neo cell expression vector which was previously digested with the Xbal enzyme and dephosphorylated. The digested vector and the modified myostatin fragments were ligated and the product subsequently characterized by PCR to identify the correctly oriented clones. The selected clones were amplified obtaining plasmid DNA free of endotoxins. The recombinant plasmids were recovered using columns of the commercial Kit Quiagen® Endo Free Plasmid Mega Kit; inoculates were prepared with 100 and 1000 μg recombinant plasmid diluted in PBS c.b.p 1 and 2 mL, respectively (Ramirez, 2013).

Preparation of myostatin P2-P30 recombinant antigen: The recombinant antigen was also elaborated in the aforementioned laboratory. The recombinant antigen was done using an expression system in *E. coli* based on the pETII plasmid. The recombinant protein was purified by nickel columns using the QIAexprees® Ni-NTA Kit.

Sampling and measurement of antibodies: In all the experimental groups, 5 mL blood samples were obtained by puncture of the superior vena cava on day 84 of the experiment using Vacutainer® tubes without anticoagulant. The serum obtained was used to evaluate the titers of anti-myostatin antibodies (Absorbance) by means of indirect ELISA tests which were carried out using the following protocol. The myostatin recombinant antigen was bound to the bottom of the ELISA plates (Maxisorb, nunc) by diluting (50 μg/mL) in carbonated buffer (0.1 m sodium carbonate, 0.1 m sodium bicarbonate, pH 9.6) and incubated for 18 h at 4°C. The 3 washes were carried out (10X wash buffer, 1.25 m sodium chloride, 250 mM tris-HCl pH 7.9, 1% Tween-20) before the blocking solution was added (milk 2% skim in Washing Buffer 1 X) to it. It was incubated for 1 h and subsequently, the first antibody (serum of immunized animals) was added, diluting 1/100 and incubated for 1 h at 37°C. Washing was repeated and the second antibody diluted 1/1000 (anti pig IgG coupled to peroxidase) was added. It was incubated for 1 h at 37°C. The wash was repeated and was revealed by placing 100 μL/well of

developer solution (0.1 M citric acid pH 4.5 0.1 M sodium citrate pH 4.5). It was incubated for 5 min and then 50 μL/well of the braking solution (42N sulfuric acid) was added and read at 450 nm in a ELISA reader (Crowther, 2010). The 5 mL of 0.1 m citric acid and 5 mL of 0.1 M sodium citrate were mixed per plate and 200 μL of a solution of 5 μg of Ortho-Phenylene Diamine (OPD), 20 μL 30% hydrogen peroxide was added (%).

Response variables: At the height of the tenth rib at the sacrifice of each pig, a fragment of an inch wide which included fat and loin was recovered to measure the back fat using a millimeter vernier. All pigs were weighed at the beginning and at the end of the experiment, the feed was offered ad libitum. The body weight gain of each pig was obtained subtracting the initial weight to the body weight at 84 days of the experiment.

Statistical analysis: A one-way analysis of variance for each response variable was carried out, considering the experimental group as fixed effect. In addition, Pearson correlations of antibody levels with other variables (dorsal fat, final weight at 84 days and final weight gain) were performed, using the SPSS Version 20 for Windows Program (SPSS Inc. Chicago Illinois, USA).

RESULTS AND DISCUSSION

Myostatin antibody levels: The immune response was evaluated by ELISA assays to obtain absorbance. Table 1 shows the antibody levels reached for each treatment. With the dose of 100 μg myostatin P2-P30 DNA vaccine with a booster of 100 μg recombinant antigen (Group 1), the production of antibodies against myostatin was lower compared to the other two immunization groups which used doses of 1000 μg for both immunogens (Group 2) or administering more reinforces of the recombinant antigen (Group 3). No difference was found in the levels of group 1 and control antibodies.

It is reported that myostatin can suppress muscle development (Hayot *et al*., 2011; Lee *et al*., 2011), the Mstn _/_ mouse displays hyperplasia as a consequence of developing an increased number of mono-nucleated muscle cells (Aiello *et al*., 2018). The blocking of myostatin using DNA vaccines or monoclonal antibodies has been shown in some studies to be a potential alternative to promote muscle development in laboratory animals (Bogdanovich *et al*., 2002; Whittemore *et al*., 2003; Kim *et al*., 2006; Tang *et al*., 2007; Ramirez, 2013) and animals of livestock interest (Long *et al*., 2009). In the present study, it was investigated the effects of different active immunization schemes to inhibit the activity of myostatin in pigs, using a recombinant DNA vaccine with myostatin modified with the P2 and P30 epitopes of the tetanus toxin. Regarding the use of DNA vaccines for the blocking of myostatin (Tang *et al*., 2007) immunized mice with a recombinant DNA vaccine anti-myostatin and found that it is possible to produce specific antibodies capable of inhibiting myostatin. In addition, immunized mice showed an increase in body weight of approximately 35% more, compared with the control group. By Long *et al*. (2009) immunized pigs with a recombinant antigen, finding that the immunized pigs had more carcass yield, less intramuscular fat, low expression of the myostatin gene in the Longissimus muscle and low activity of creatine kinase in muscle, compared to the control group. In addition, indirect ELISA analysis of immunized pigs revealed higher titers of antimyostatin antibodies than in the control group. By Ramirez (2013) used different constructions of a recombinant vaccine with the gene coding for myostatin modified with P2 and P30 epitopes of the tetanus toxin, finding that the immunized mice showed no differences in growth as a result of the immunization with the different vaccines but they did found differences in the evaluations of the carcass with a higher yield of the mice immunized with the P2-P30 vaccine. In our study, the dose of 1000 μg vaccine or antigen, proved to be capable of producing antibodies which agrees with the studies of Tang *et al*. (2007) and Long *et al*. (2009). However, the effect of the antibodies did not produce differences in body weight gain which agrees with Ramirez (2013) results where the growth dynamics of the different immunized groups did not show

Table 1: Means and standard errors by different schemes of myostatin P2-P30 DNA vaccine and recombinant myostatin P2-P30 antigen for some economical important traits

Variables	Control	Group 1	Group 2	Group 3
Initial weight	14.72±2.82^{a}	16.00±3.33^{a}	14.51±4.45^{a}	14.28±3.36^{a}
Antibody levels (Absorbance)***	0.64±0.05^{b}	0.76±0.06^{b}	1.26±0.06^{a}	1.17±0.06^{a}
Dorsal fat (mm)*	20.00±1.44^{a}	19.83±1.31^{a}	16.72±1.13^{ab}	15.22±1.13^{b}
Final weight at 84 days (kg)*	66.33±3.28^{b}	71.31±3.47^{ab}	79.35±3.47^{a}	76.52±3.47^{ab}
Body weight gain (kg)**	51.31±2.61^{b}	58.33±3.01^{ab}	64.83±2.61^{a}	62.24±2.61^{a}

Group 1: Three doses of 100 μg P2-P30 vaccine plus a boost of recombinant antigen; Group 2: A dose of 1000 μg P2-P3 vaccine plus a boost of recombinant antigen; Group 3: Four doses of 1000 μg recombinant antigen. Different letters by row, indicate significant difference between groups using Duncan test; ***p<0.0001, **p<0.01, *p<0.05

significant differences in mice. Although, here, there was not difference in body weight gain there was a positive correlation between the levels of antibodies with body weight and the body weight gain which suggest an effect of the antibodies in blocking the myostatin. The results here found and those of Ramirez (2013), indicate that the effect of P2-P3 vaccine on blocking myostatin was not as marked as in other studies where the blocking of myostatin produced an evident increase in muscle mass and body weight (Bogdanovich *et al*., 2002; Whittemore *et al*., 2003; Zhu *et al*., 2004; Tang *et al*., 2007; Haidet *et al*., 2008; Lawlor *et al*., 2011; Murphy *et al*., 2011; Tang *et al*., 2014) this probably due to the low dose here used which may have not been sufficient to produce a perceptible effect in the immunized pigs.

In the case of the immunization schemes used in this study, an attempt was made to compare a classical immunization scheme against a prime-boost scheme which enhances the immune response. Prime-boost immunization involves first priming the immune system to a target antigen emitted by a vector and then selectively boosting this immunity by re-administration of a recombinant antigen or a different vector. With the first prime-boost strategy, the effects on immunization were merely additive while with some strategies (involving poxvirus or adenovirus) powerful synergistic effects have been observed (Woodland, 2004). This improved synergy of immunity to the target antigen is reflected in an increase in the number of antigen-specific T cells, selective enrichment of high avidity T cells and increased efficacy against the challenge of the pathogen (McShane, 2002; Estcourt *et al*., 2002). In addition, activation of both $CD8^+$ and $CD4^+$T lymphocytes has been demonstrated (Tanghe *et al*., 2001). The results obtained in the pigs of Group 2 which were immunized under a prime-boost scheme, showed slightly elevated antibody levels compared with Group 3 where a traditional immunization scheme was used but without significant differences.

Back fat: The animals in Group 3, treated with doses of 1000 μg antigen had lower dorsal fat thickness ($p<0.05$) than the control and Group 1 (Table 1). There was not difference between Group 2 and 3.

Body weight: The animals in the groups treated with dose of 1000 μg of both recombinant antigen or DNA vaccine (Group 2 and 3) were heavier compared to those of the control group, however, no difference in weight was found between control and Group 2 (Table 1).

Body weight gain: No significant differences were found for body weight between the different doses and vaccination schemes used. Group control and Group 2 was different ($p<0.05$) (Table 1).

Correlation of antibody levels with the measured variables: The correlations between antibody levels with dorsal fat, final weight and weight gain were -0.26 ($p<0.20$), 0.53 ($p<0.01$) and 0.36 ($p<0.05$), respectively.

On the other hand, it has been shown that myostatin plays a direct role in the regulation of adipose tissue homeostasis (McPherron *et al*., 1997). Knockout mice for the myostatin gene show detectable levels of myostatin RNA in adipose tissue also show lower serum cholesterol concentration as well as reduced levels of triglycerides (McPherron and Lee, 2002; Lin *et al*., 2002; Guo *et al*., 2009). Knockout mice for the myostatin gene reduce subcutaneous fat levels in the body and leptin concentration levels (Lin *et al*., 2002; McPherron and Lee, 2002). Over expression of the propeptide which is an endogenous inhibitor of myostatin, reduces the activity of myostatin and decreases the subcutaneous adipose tissue (Zhao *et al*., 2005). In addition, myostatin promotes the differentiation of multipotent mesenchymal cells into preadipocytes (Artaza *et al*., 2005) and inhibits the differentiation of brown adipocytes through the ActRIIB Smad 3 pathway *in vitro* (Fournier *et al*., 2012; Kim *et al*., 2012). Over expression of myostatin in transgenic pigs have resulted in a reduction of muscle growth but in a significant increase in intramuscular fat (Yang *et al*., 2009). The results of this study show a reduction of the back fat in the pigs immunized with 1000 μg vaccine compared with the pigs that received a dose of 100 μg in addition, the correlation between the levels of antibodies and the dorsal fat show a negative correlation which would imply an effect of antibodies on adipogenesis. Previous studies on the immunoneutralization of myostatin show an increase in muscle mass without any reduction in abdominal fat in mice (Tang *et al*., 2007) but showed a reduction of intramuscular fat in pigs (Long *et al*., 2009). How myostatin affects adipose tissue it is not clear. One explanation is that the reduction of body fat is the fact that myostatin can act directly on adipocytes promoting proliferation or differentiation in an inverted mechanism to skeletal muscle. Other explanation is that the reduction of body fat may be secondary to the increase in metabolic demand of the significant increase in muscle mass in knockout myostatin gene in mice (Elliott *et al*., 2012).

The results of this study, show a higher body weight increase in the immunized pigs (Group 2 and 3) without achieving a significant separation with the pigs of the

control group with immunized pigs (Group 1 and 2). Similar result was reported by Ma *et al.* (2005) where mice immunized with a plasmid anti-myostatin vector did not show significant differences in body weights compared to the control group. Although, there was no effect of the vaccine on body weight gain, pigs in this study had similar performance than commercial Yorkshire-Landrace male pigs with average body weight gain of 695 g/day (Gondret *et al*., 2005; Lynch *et al*., 2006).

In this study, the characteristics of the carcass, the body proportions in the different segments and the histological changes at the muscle fiber level were not evaluated. Those data could have given more information on the effect of immunization on muscle development.

CONCLUSION

A dose of 100 μg myostatin P2-P30 DNA vaccine did not produce a significant immune response in the parameters of the pig traits evaluated, even in a prime boost vaccination system. The use of 1000 μg vaccine or antigen produced a greater immune response as shown by the levels of antibodies but this response does not produce a differentiated effect on growth performance traits versus 100 μg. Although, the use 1000 μg vaccine or antigen did affect pigs back fat. More studies are need to adjust the adequate dose that effects pig growth.

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REFERENCES

Aiello, D., K. Patel and E. Lasagna, 2018. The myostatin gene: An overview of mechanisms of action and its relevance to livestock animals. Anim. Genet., 49: 505-519.

Anonymous, 1993. [Specifications for the regulation of chemical, pharmaceutical, biological and food products for animal use or consumption by them]. Official Mexican Standard NOM-012-ZOO, La Mexicana, Inc., Mexico, North America. (In Spanish)

Anonymous, 1995. [Humanitarian treatment in the mobilization of animals]. Oficial Mexicana Standard NOM-051-ZOO, La Mexicana, Inc., Mexico, North America (In Spanish).

Anonymous, 1999. [Technical specifications for the production, care and use of laboratory animals]. Oficial Mexicana Standard NOM-062-ZOO, La Mexicana, Inc., Mexico, North America (In Spanish).

Artaza, J.N., S. Bhasin, T.R. Magee, S. Reisz-Rorsasaz and R. Shen *et al*., 2005. Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T(1/2) mesenchymal multipotent cells. Endocrinology, 146: 3547-3557.

Bogdanovich, S., T.O. Krag, E.R. Barton, L.D. Morris, L.A. Whittemore, R.S. Ahima and T.S. Khurana, 2002. Functional improvement of dystrophic muscle by myostatin blockade. Nature, 420: 418-421.

Coban, C., S. Koyama, F. Takeshita, S. Akira and K.J. Ishii, 2008. Molecular and cellular mechanisms of DNA vaccines. Hum. Vaccines, 4: 453-457.

Crowther, J.R., 2010. Methods in Molecular Biology (Elisa: Theory and Practice). 2nd Edn., Springer, Berlin, Germany, Pages: 223.

Elliott, B., D. Renshaw, S. Getting and R. Mackenzie, 2012. The central role of myostatin in skeletal muscle and whole body homeostasis. Acta Physiol., 205: 324-340.

Estcourt, M.J., A.J. Ramsay, A. Brooks, S.A. Thomson and C.J. Medveckzy *et al*., 2002. Prime-boost immunization generates a high frequency, high-avidity CD8+ cytotoxic T lymphocyte population. Intl. Immunol., 14: 31-37.

Faurez, F., D. Dory, V. Le Moigne, R. Gravier and A. Jestin, 2010. Biosafety of DNA vaccines: New generation of DNA vectors and current knowledge on the fate of plasmids after injection. Vaccine, 28: 3888-3895.

Fournier, B., B. Murray, S. Gutzwiller, S. Marcaletti and D. Marcellin *et al*., 2012. Blockade of the activin receptor IIb activates functional brown adipogenesis and thermogenesis by inducing mitochondrial oxidative metabolism. Mol. Cell. Boil., 32: 2871-2879.

Gondret, F., L. Lefaucheur, I. Louveau and B. Lebret, 2005. The long-term influences of birth weight on muscle characteristics and eating meat quality in pigs individually reared and fed during fattening. Arch Tierz Dummerstorf, 48: 68-73.

Guo, T., W. Jou, T. Chanturiya, J. Portas and O. Gavrilova *et al*., 2009. Myostatin inhibition in muscle but not adipose tissue, decreases fat mass and improves insulin sensitivity. PloS One, 4: 1-11.

Haidet, A.M., L. Rizo, C. Handy, P. Umapathi and A. Eagle *et al*., 2008. Long-term enhancement of skeletal muscle mass and strength by single gene administration of myostatin inhibitors. Proc. Nat. Acad. Sci., 105: 4318-4322.

Hayot, M., J. Rodriguez, B. Vernus, G. Carnac and E. Jean *et al*., 2011. Myostatin up-regulation is associated with the skeletal muscle response to hypoxic stimuli. Mol. Cell. Endocrinol., 332: 38-47.

Kim, W.K., H.R. Choi, S.G. Park, Y. Ko and K.H. Bae *et al*., 2012. Myostatin inhibits brown adipocyte differentiation via. regulation of Smad3-mediated ß-catenin stabilization. Intl. J. Biochem. Cell Boil., 44: 327-334.

Kim, Y.S., N.K. Bobbili, K.S. Paek and H.J. Jin, 2006. Production of a monoclonal anti-myostatin antibody and the effects of in ovo administration of the antibody on posthatch broiler growth and muscle mass. Poult. Sci., 85: 1062-1071.

Lawlor, M.W., B.P. Read, R. Edelstein, N. Yang and C.R. Pierson *et al*., 2011. Inhibition of activin receptor type IIB increases strength and lifespan in myotubularin-deficient mice. Am. J. Pathol., 178: 784-793.

Lee, J.Y., N.S. Hopkinson and P.R. Kemp, 2011. Myostatin induces autophagy in skeletal muscle *in vitro*. Biochem. Biophys. Res. Commun., 415: 632-636.

Lee, S.J., L.A. Reed, M.V. Davies, S. Girgenrath and M.E.P. Goad *et al*., 2005. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. Proc. National Acad. Sci., 102: 18117-18122.

Lin, J., H.B. Arnold, M.A. Della-Fera, M.J. Azain and D.L. Hartzell *et al*., 2002. Myostatin knockout in mice increases myogenesis and decreases adipogenesis. Biochem. Biophys. Res. Commun., 291: 701-706.

Long, D.B., K.Y. Zhang, D.W. Chen, X.M. Ding and B. Yu, 2009. Effects of active immunization against myostatin on carcass quality and expression of the myostatin gene in pigs. Anim. Sci. J., 80: 585-590.

Lynch, P.B., A. Cahill, P.G. Lawlor, L. Boyle and J.V. O'Doherty *et al*., 2006. Studies on growth rates in pigs and the effect of birth weight. Master Thesis, Teagasc, Carlow, Ireland.

Ma, X., Y. Cao, D. Shu and Y. Bi, 2005. Cloning and expression of swine myostatin gene and its application in animal immunization trial. Sci. China Ser. C. Life Sci., 48: 368-374.

McPherron, A.C. and S.J. Lee, 2002. Suppression of body fat accumulation in myostatin-deficient mice. J. Clin. Investigat., 109: 595-601.

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

McShane, H., 2002. Prime-boost immunization strategies for infectious diseases. Curr. Opin. Mol. Ther., 4: 23-27.

Moreno, V.R., A.M. Sifuentes, X.F. De La Rosa and B. Pereyra, 2008. [Evaluation of polymorphic regions of the myostatin gene in Beefmaster cattle (In Spanish)]. Arch. Vet. Med., 40: 59-63.

Murphy, K.T., V. Cobani, J.G. Ryall, C. Ibebunjo and G.S. Lynch, 2011. Acute antibody-directed myostatin inhibition attenuates disuse muscle atrophy and weakness in mice. J. Applied Physiol., 110: 1065-1072.

Patel, K. and H. Amthor, 2005. The function of myostatin and strategies of myostatin blockade-new hope for therapies aimed at promoting growth of skeletal muscle. Neuromuscular Disord., 15: 117-126.

Ramirez, A.V., 2013. Construction of a DNA vaccine against myostatin. Master Thesis, National Autonomous University of Mexic City, Mexico.

Tang, L., C.T. Liu, X.D. Wang, K. Luo and A.P. Chi *et al*., 2014. A prepared anti-MSTN polyclonal antibody reverses insulin resistance of diet-induced obese rats via. regulation of PI3K/Akt/mTOR&FoxO1 signal pathways. Biotechnol. Lett., 36: 2417-2423.

Tang, L., Z. Yan, Y. Wan, W. Han and Y. Zhang, 2007. Myostatin DNA vaccine increases skeletal muscle mass and endurance in mice. Muscle Nerve, 36: 342-348.

Tanghe, A., S. D'Souza, V. Rosseels, O. Denis and T.H. Ottenhoff *et al*., 2001. Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. Infect. Immune., 69: 3041-3047.

Whittemore, L.A., K. Song, X. Li, J. Aghajanian adn M. Davies *et al*., 2003. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochem. Biophys. Res. Commun., 300: 965-971.

Woodland, D.L., 2004. Jump-starting the immune system: Prime-boosting comes of age. Trends Immunol., 25: 98-104.

Yang, J., L. Schulman, V.G. Pursel, M.B. Solomon and A.D. Mitchell *et al*., 2009. Expression of porcine myostatin prodomain genomic sequence leads to a decrease in muscle growth but significant intramuscular fat accretion in transgenic pigs. Transgenic Res., 19: 149-149.

Zhao, B., R.J. Wall and J. Yang, 2005. Transgenic expression of myostatin propeptide prevents diet-induced obesity and insulin resistance. Biochem. Biophys. Res. Commun., 337: 248-255.

Zhu, X., S. Topouzis, L.F. Liang and R.L. Stotish, 2004. Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. Cytokine, 26: 262-272.