

Expression Profiling of *MYOZ1* Gene in Porcine Tissue and C2C12 Cells

Yin Liu, Ke Zhou, Xin-Yun Li, Shu-Hong Zhao and Jian-Hua Cao
Key Laboratory of Agricultural Animal Genetics,
Breeding and Reproduction of Ministry of Education of China,
College of Animal Science and Technology,
Huazhong Agricultural University, 430070 Wuhan, Hubei, P.R. China

Abstract: The structure and function of Z-discs were related strongly to the normal physiological phenotype and function of skeletal muscle. The protein Myozenin (MYOZ), an α -actinin and γ -filamin-binding Z-disc globular protein expressed predominantly in skeletal muscle was possibly involved in the confirmation and function of Z-discs. Here we described *MYOZ1* gene which has a significantly higher expression level in skeletal muscle than several other tissues in porcine. Furthermore, *MYOZ1* transcripts in three porcine development stages (embryonic 33 days, embryonic 65 days and adult) of skeletal muscle shown stepwise increasingly expression pattern by using quantitative PCR analysis. Functional assays were conducted using C2C12 cells and the results implied *MYOZ1* was not involved in both of cellular proliferation and differentiation.

Key words: Myozenin-1 (*MYOZ1*), expression profiling, porcine, C2C12 cells, skeletal, adult, tissue

INTRODUCTION

The Z-discs of skeletal muscle which partition sarcomeres and cross-link myofilaments into highly ordered three-dimensional structure, play an important role in the contraction regulation and structure formation of skeletal muscle (Luther, 2009; Gontier *et al.*, 2005; Faulkner *et al.*, 2000). The structure disorder of Z-disc can be correlated with muscular pathological procedures associated with injuries or atrophies (Osio *et al.*, 2007; Posch *et al.*, 2007). Moreover, the functions of Z-disc as a key region for reception, transduction and transmission of mechanical and biochemical signals involved development and differentiation of muscle fibers (Pyle and Solaro, 2004).

Two group protein components of skeletal muscle Z-disc were identified based on their locations. The α -actinin and γ -filamin which belong to the second group components appeared to be entirely located in the Z-disc play a central role by cross-linking the actin molecules (Takada *et al.*, 2001). Myozenin (MYOZ) is a α -actinin and γ -filamin-binding Z-disc protein expressed predominantly in skeletal and cardiac muscle. It has been suggested that *MYOZ1* family members may contribute to the formation and maintenance of the Z-disc as well as in cell signaling by binding calcineurin. Myozenin-1 (*MYOZ1*) and Myozenin-3 (*MYOZ3*) are highly expressed in skeletal

muscle fast-twitch fibers whereas myozenin-2 is highly expressed in cardiac muscle slow twitch fibers (Von Nandelstadh *et al.*, 2009).

Although, studies involving porcine skeletal muscle largely converged functional characterization and regulatory networks (Wang *et al.*, 2006; Huang *et al.*, 2008), unlike the extensive studies in human and mouse, the researchs involving MYOZ family members in porcine remain to be investigated. In the present study researchers undertook an extensive expression profiling of *MYOZ1* gene in eight porcine tissues and three stages of skeletal muscle development. The results demonstrated that *MYOZ1* expressed at a very high level in porcine adult skeletal muscle. The functional characterization of *MYOZ1* in myoblast mouse C2C12 cells shown that *MYOZ1* involved neither cellular proliferation nor differentiation. Thus, on the basis of the information known about its binding partners, *MYOZ1* as a skeletal muscle Z-disc protein may participate the confirmation and structural stability of porcine muscle Z-discs. The study provides a new insight into understanding the functions or modulation mechanisms of *MYOZ1* in porcine skeletal muscle. Also, the results will provide more clues and candidate gene for further research on muscular dystrophy as well as shedding light on the normal muscle functions and pathological disorder.

Corresponding Author: Jian-Hua Cao, Key Laboratory of Agricultural Animal Genetics,
Breeding and Reproduction of Ministry of Education of China, College of Animal Science and Technology,
Huazhong Agricultural University, Wuhan 430070, Hubei, P.R. China

MATERIALS AND METHODS

Materials and Plasmids: Lipofectamine 2000 and TRIzol reagents were purchased from Invitrogen (Carlsbad, CA, USA). Fetal Bovine Serum (FBS), culture media and other solutions used for cell culture were from HyClone Technologies, Inc. (Beijing, China). Restriction enzymes and first strand cDNA synthesis kit were from Fermentas (Shenzhen, China). Fluorescence SYBR Green I Master kit for LightCycler 480 machine were from Roche (Indianapolis, IN, USA). Cell Counting Kit-8 was purchased from Dojindo molecular technologies, Inc. (Rockville, MD, USA). Primary rabbit anti-Flag antibody and secondary goat anti-rabbit antibody coupled to horseradish peroxidase were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Supersignal ELISA femto maximum sensitivity substrate was purchased from Pierce (Rockford, IL, USA). All other reagents used were of analytical grade where appropriate. The pcDNA3.1+plasmid encoding the wild-type MYOZ1 and enhanced green fluorescent protein epitope tagged at their N-terminal ends under the control of a cytomegalovirus promoter namely pcDNA-Flag-MYOZ1 and pcDNA-Flag-GFP have been constructed according to standard molecular cloning protocol.

Cell culture and transfection: Mouse myoblast C2C12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and were conditionally differentiated by replacing 10% FBS to 2% horse serum. About 90% confluent cells in 6 well microplates were transfected by Lipofectamine 2000 reagent as described the manufacturer's instructions. Two million cells were transfected with plasmids DNA containing pcDNA-Flag-MYOZ1 (0.6 μg) and made up to a total amount of 1 μg plasmid DNA with empty pcDNA3.1+vector.

ELISA quantification for cell expression of flag-tagged MYOZ1: Flag-tagged MYOZ1 was detected with a monoclonal rabbit anti-Flag primary antibody at 0.5 $\mu\text{g mL}^{-1}$ and goat anti-rabbit secondary antibodies coupled to horseradish peroxidase at 0.8 $\mu\text{g mL}^{-1}$. After washing 5 times with PBS, secondary antibody was detected and quantified instantaneously by chemiluminescence using Supersignal ELISA substrate and a PerkinElmer Victor luminescence counter.

Reverse transcription PCR and fluorescence quantitative PCR: Total RNAs from porcine skeletal muscle tissue and C2C12 cells were isolated by using TRIzol reagent and the first strand cDNA synthesis was conducted by using

reverse transcription kit. Relative quantification of gene expression was performed using fluorescence SYBR Green I Master kit on Roche Light Cycler 480 machine.

Measurements of cell proliferation and differentiation: The C2C12 cells transfected with pcDNA-Flag-MYOZ1 plasmid in 96 well microplates were incubated overnight and 10 μL cell counting solution for each well was added before incubating another 4 h. Absorbance at 450 nm for each well as proliferation measurement was recorded by using automated spectrophotometer. The Myogenin (MYOG) was selected as marker gene for cell differentiation and quantitative PCR was conducted to identify cell differentiation levels.

RESULTS AND DISCUSSION

MYOZ1 gene highly expressed in porcine skeletal muscle. *MYOZ1* was related to muscle structure and functions which remains unknown in porcine. Tissue profiling PCR of *MYOZ1* gene was performed by using eight tissues (heart, liver, spleen, lung, kidney, small intestine, skeletal muscle and fat) of Landrace pig and Ribosomal Protein L32 (RPL32) as internal control were scaled the profile as well. The result shown that *MYOZ1* was significant highly expressed at porcine skeletal muscle which implied its conservative expression pattern at different species (Fig. 1).

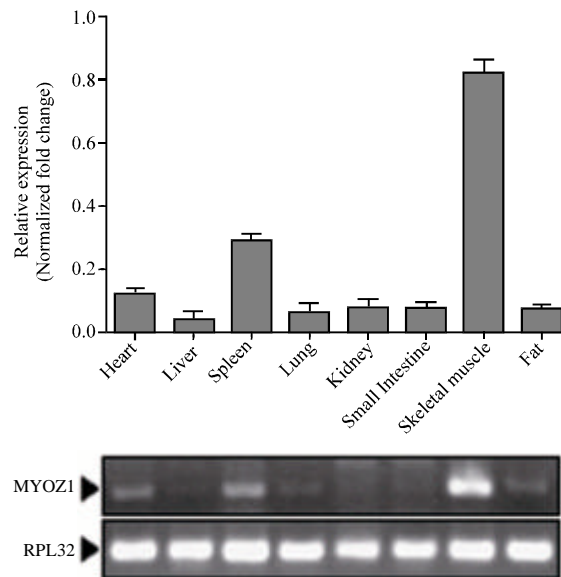


Fig. 1: Tissue profiling PCR of *MYOZ1* gene. Eight tissues of Landrace pig, heart, liver, spleen, lung, kidney, small intestine, skeletal muscle and fat were listed from left to right, respectively. Ribosomal Protein L32 (RPL32) as internal control were scaled the profile as well

MYOZ1 gene increasingly expressed during porcine skeletal muscle development. In order to determine *MYOZ1* expression pattern during porcine muscle development, three stages, Embryonic 33 days (E33), Embryonic 65 days (E65) and Adult (ADU) of Landrace pig longissimus muscle were chosen to identify the amount transcripts of *MYOZ1* gene. Reverse transcription PCR was conducted under the internal control of RPL32 gene and quantitative PCR was also used to quantify the relative expression level of *MYOZ1* gene. The results demonstrated that *MYOZ1* gene has an increasing expression pattern during porcine muscle development (Fig. 2). Moreover, *MYOZ1* gene significantly high

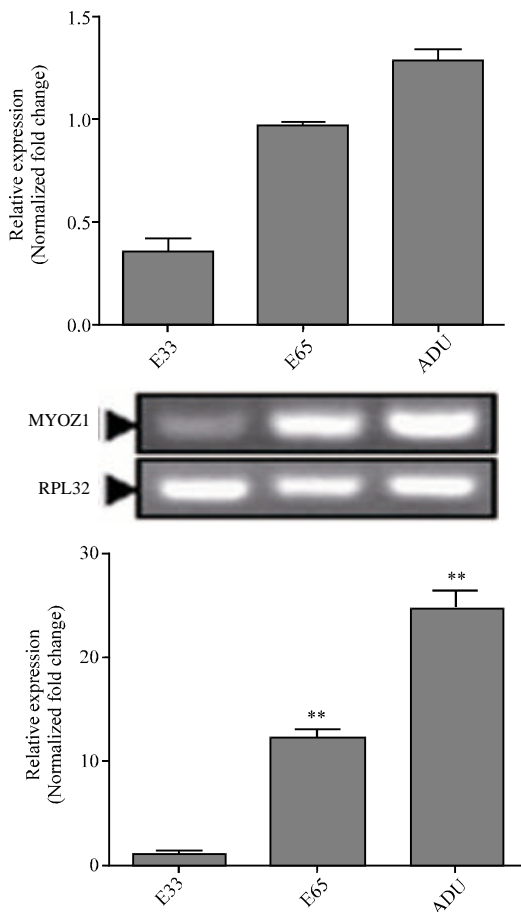


Fig. 2: a) Expression profiling of *MYOZ1* gene at three stages of muscle development. Landrace pig longissimus muscles at Embryonic 33 days (E33), Embryonic 65 day (E65) and Adult (ADU) stages from left to right were determined under the internal control of RPL32; b) *MYOZ1* gene has an increasing expression pattern during porcine muscle development and significantly high expressed at adult stage by using quantitative PCR

expressed at adult stage of porcine muscle development which suggested *MYOZ1* may exert its functions at muscle hypertrophy processing.

MYOZ1 not involved cell proliferation and differentiation. *MYOZ1* as a structural protein may participate the functions of skeletal muscle. In order to test the functions of *MYOZ1*, cell proliferation and differentiation determinations were conducted on mouse myoblast C2C12 cells. Cell proliferation counting and differentiation marker gene (*MYOG*) examining methodologies were performed to C2C12 cells transfected with Flag-tagged *MYOZ1*. The ELSIA result shown that the Flag-tagged *MYOZ1* was effectively over-expressed in C2C12 cells (Fig. 3). However, cell proliferation and differentiation were independent of *MYOZ1* (Fig. 4) which implied the functions of *MYOZ1* did not involve cell proliferation and differentiation.

MYOZ1 as a novel gene responding for sarcomeric calcineurin-binding proteins (Frey *et al.*, 2000), undertaking the structural maintenance and physiological functions exhibition of skeletal muscle was unveiled its roles in regulation of contraction and interactions with actin molecular. Of interest, a recent reports show that *MYOZ1* could involve higher level functions of skeletal muscle with say, mass weight gain or *MYOZ1* gene knockout mice have reduced body weight and fast-twitch muscle mass without showing muscle atrophy and also reveal a fiber type composition switch toward slow-twitch fibers (Frey *et al.*, 2008) which implied fiber type conversion. The more output of skeletal muscle is of course the most important economical trait of porcine carcass which is primarily constrained by the function

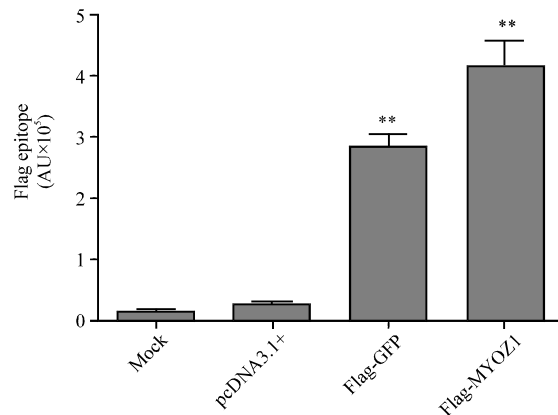


Fig. 3: Flag-tagged *MYOZ1* effectively over-expressed in C2C12 cells. Flag epitope was quantified by ELISA and pcDNA3.1+ and Flag-tagged GFP were conducted as negative and positive controls, respectively

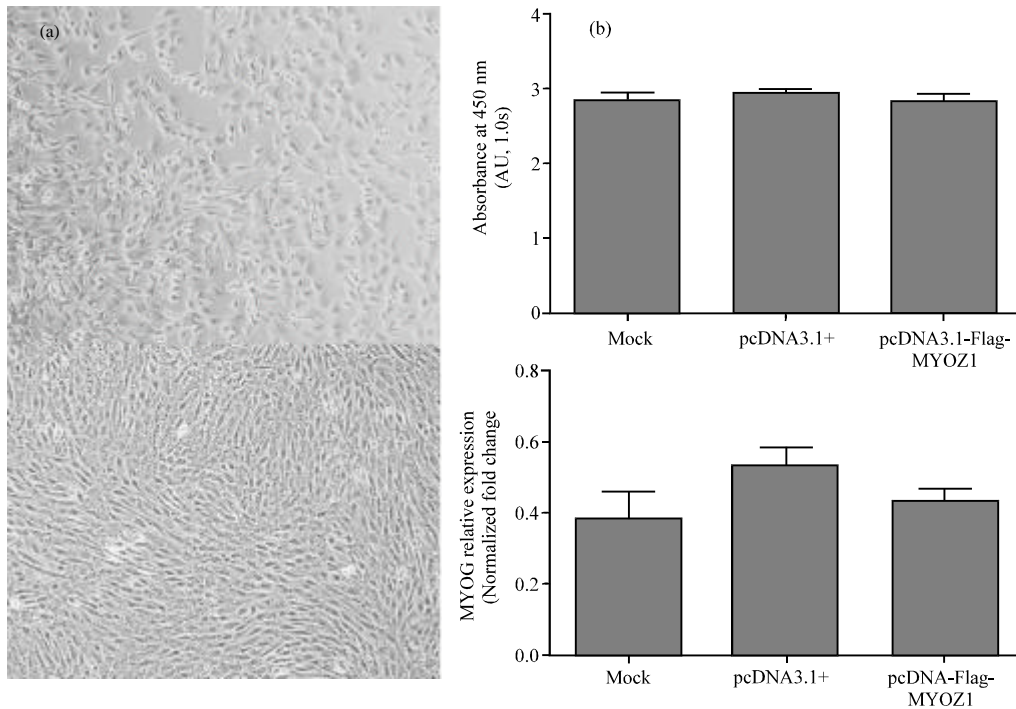


Fig. 4: MYOZ1 was not involved in proliferation and differentiation in C2C12 cells. The proliferation and differentiation stage cells; a) transfected with pcDNA-Flag-MYOZ1 plasmid were significantly independent of cell proliferation and differentiation (b)

characterization and mechanism determination of skeletal muscle. As a member of calsarcin protein family specifically expressed in muscle, MYOZ1 could be particularly useful for disentangling complex molecular mechanism dominating the development of porcine skeletal muscle. However, the expression pattern or regulatory pathway of MYOZ1 remains a major challenge. To dissect the molecular basis underlying MYOZ1, basic expression profiling should firstly be addressed. Consistent with the previous report in human or mouse, the results also proved that MYOZ1 has exclusively high expressed and gradually increased expression pattern in the development stages of porcine skeletal muscle. In addition, MYOZ1 was independent of cell proliferation and differentiation which could imply that its functions may undergo indirect way such as via microRNAs or regulatory networks. Thus, the systematical researches on the functions or regulatory pattern of structural protein MYOZ1 will benefit to elucidate the complex mechanism of the growth and development of porcine skeletal muscle.

CONCLUSION

MYOZ1 as a skeletal muscle Z-disc protein may participate the confirmation and structural stability of

porcine muscle Z-discs. The results will provide more clues and candidate gene for further research on muscular dystrophy as well as shedding light on the normal muscle functions and pathological disorder.

ACKNOWLEDGEMENTS

This research was supported by National Natural Science Foundation of China (Grant 30900792 to JHC), National High Technology Research and Development Program of China (863 project) (Grant 2010AA10A104 to SHZ), NSFC-Guangdong Key Project (Grant U0631005 to SHZ), the Creative Team Project of Education Ministry (Grant IRT-0831).

REFERENCES

- Faulkner, G., A. Pallavicini, A. Comelli, M. Salamon and G. Bortoletto *et al.*, 2000. FATZ, a filamin actinin and telethonin-binding protein of the Z-disc of skeletal muscle. *J. Biol. Chem.*, 275: 41234-41242.
- Frey N., D. Frank, S. Lippl, C. Kuhn and H. Kogler *et al.*, 2008. Calsarcin-2 deficiency increases exercise capacity in mice through calcineurin/NFAT activation. *J. Clin. Invest.*, 118: 3598-3608.

- Frey, N., J.A. Richardson and E.N. Olson, 2000. Calsarcins, a novel family of sarcomeric calcineurin-binding proteins. *Proc. Natl. Acad. Sci. USA.*, 97: 14632-14637.
- Gontier Y., A. Taivainen, L. Fontao, A. Sonnenberg, A. van der Flier and O. Carpen *et al.*, 2005. The Z-disc proteins myotilin and FATZ-1 interact with each other and are connected to the sarcolemma via muscle-specific filamins. *J. Cell. Sci.*, 118: 3739-3749.
- Huang T.H., M.J. Zhu, X.Y. Li and S.H. Zhao, 2008. Discovery of porcine microRNAs and profiling from skeletal muscle tissues during development. *PLoS. One*, 3: e3225-e3225.
- Luther, P.K., 2009. The vertebrate muscle Z-disc: Sarcomere anchor for structure and signalling. *J. Muscle Res. Cell Motil.*, 30: 171-185.
- Osio, A., L. Tan, S.N. Chen, R. Lombardi and S.F. Nagueh *et al.*, 2007. Myozenin 2 is a novel gene for human hypertrophic cardiomyopathy. *Circ. Res.*, 100: 766-768.
- Posch, M.G., A. Perrot, R. Dietz, C. Ozcelik and S. Pankuweit *et al.*, 2007. Mutations in MYOZ1 as well as MYOZ2 encoding the calsarcins are not associated with idiopathic and familial dilated cardiomyopathy. *Mol. Genet. Metab.*, 91: 207-208.
- Pyle, W.G. and R.J. Solaro, 2004. At the crossroads of myocardial signaling: The role of Z-discs in intracellular signaling and cardiac function. *Circ. Res.*, 94: 296-305.
- Takada, F., D.L. van Woude, H.Q. Tong, T.G. Thompson, S.C. Watkins, L.M. Kunkel and A.H. Beggs, 2001. Myozenin: An alpha-actinin- and gamma-filamin-binding protein of skeletal muscle Z lines. *Proc. Natl. Acad. Sci. USA.*, 98: 1595-1600.
- Von Nandelstadh, P., M. Ismail, C. Gardin, H. Suila and I. Zara *et al.*, 2009. A class III PDZ binding motif in the myotilin and FATZ families binds enigma family proteins: A common link for Z-disc myopathies. *Mol. Cell. Biol.*, 29: 822-834.
- Wang, H., Z. Zhu, S. Yang, D. Mo and K. Li, 2006. Characterization of different expression patterns of calsarcin-1 and calsarcin-2 in porcine muscle. *Gene.*, 374: 104-111.