Journal of Animal and Veterinary Advances 12 (2): 201-207, 2013
ISSN: 1680-5593

Cloning, Expression and Purification of Nanog Protein from *Capra hircus* in *Escherichia coli*

[1]X.B. Zheng, [1,2]X.M. Cen, [1]L.X. Wang, [1,3]G.Y. Xin, [1]X.H. Fu, [1]P. Liu, [1]G.H. Li, [1]M. Zhang, [1]S.S. Lu and [1]K.H. Lu
[1]Department of Animal Medicine, College of Animal Science and Technology, Guangxi University, No. 100 Daxue Road, 530005 Nanning, Guangxi, China
[2]Qinlian Forest Farm, 536128 Hepu County, Guangxi, China
[3]Department of Life Science and Technology, Yulin Normal College, No. 299 Jiaoyuzhong Road, 537000 Yulin, Guangxi, China

Abstract: Nanog is one of important transcription factors to maintain characteristics of pluripotent stem cells. The present study was to clone Nanog of *Capra hircus* to express His-Nanog protein in *E. coli* BL 21 cells and further to purify it. The total RNA was extracted from primordial genital ridge tissues of a fetal lam and by means of RT-PCR, *Nanog* gene was amplified which was subcloned to pET32a to construct its prokaryotic expression vector. Confirmed by restrictive endonuclease digestion and DNA sequencing, the recombinant plasmid was transformed into *E. coli* BL21(DE3) and His-Nanog fusion protein was expressed by the induction of IPTG and identified with SDS-PAGE analysis. Under denaturing condition, the His-Nanog protein was purified by using Ni-NTA resin and verified by Western blotting assay. The results showed that The Open Reading Frame (ORF) of *Nanog* gene in *Capra hircus* is composed of 903 nucleutide acids, coding 320 amino acids; SDS-PAGE assay showed that His-Nanog fusion protein was efficiently expressed in form of inclusion bodies in *E. coli* BL21 (DE3) inclusion bodies were solubilized in 6 mol L^{-1} GuHCl, His-Nanog fusion protein with higher purity was purified by using Ni-NTA resin; Western blotting assay showed that the purified His-Nanog could bind to anti-His tag antibody specifically indicating the expected immunogenicity. This recombinant protein could be used directly to prepare polyclonal or monoclonal anti-Nanog antibody which will lead to study Nanog's function or characteristics of pluripoten stem cells (such as iPS cells) in *Capra hircus*.

Key words: *Capra hircus*, *Nanog* gene, cloning, prokaryotic expression, protein purification

INTRODUCTION

Being transcribed specifically in pluripotent cells such as Embryonic Stem Cells (ESCs), Embryonic Germ Cells (EGCs) and Embryonic Carcinoma Cells (ECs) in the mouse and human, Nanog has been considered as an important transcription factor (Yamaguchi *et al*., 2005; Huang *et al*., 2007). Since, Nanog enables to prevent human ESCs and ECs differentiation to extraembryonic endoderm and trophectoderm lineages, it works as a gatekeeper of pluripotency (Hyslop *et al*., 2005). Without of feeder cells, overexpressed Nanog in human and primate ESCs leads to proliferation of the cells while whose pluripotency is not compromised (Darr *et al*., 2006). Nanog not only succeeded in reprogramming somatic cells in serum-free medium supplemented with LIF but also in reprogramming epiblast-derived stem cells to naive pluripotency in serum-free medium alone (Theunissen *et al*., 2011a). It has proved that Nanog's expression in NIH3T3 energizes cells to come into the S phase and improves cell propagation (Zhang *et al*., 2005). However, Han *et al*. (2012) silenced endogenous NANOG expression in breast cancer cells by small interference RNA (RNAi) technology and the results denoted that down-regulation of Nanog is not only responsible for the decrease in cell proliferation, colony formation and migration ability but also for the impediment of the cell cycle at the G0/G1 phases. In addition, Nanog plays an important role in the propagation and survival of migrating PGCs of wild-type embryos which was verified by conditional knockdown Nanog (Yamaguchi *et al*., 2009). As reprogramming coctail or Oct-4, Sox-2, c-myc and Klf-4 was sufficient to induce tail-tip fibroblasts to pluripotent cells (induced pluripotent stem cells, iPS cells) in mouse (Takahashi and Yamanaka, 2006). Another report illustrated that Nanog, Oct-4, Sox-2 and Lin28 were

Corresponding Author: X.B. Zheng, Department of Animal Medicine, College of Animal Science and Technology, Guangxi University, No. 100 Daxue Road, 530005 Nanning, Guangxi, China

also able to reprogram human skin fibroblast cells to iPS cells (Yu *et al*., 2007). However, the ecotopic expression of Oct-4, Sox-2, Klf-4 and c-Myc alone was not capable to inducce bovine adult fibroblasts to stable iPS cells in bovine, unless the addition of Nanog to the reprogramming cocktail (Sumer *et al*., 2011). In the presence of Bmi1, Nanog is sufficient to reprogram mouse fibroblast cells to iPS cells (Moon *et al*., 2013). More interestingly, the capacity of Nanog to establish pluripotency is fully conserved in vertebrates (Theunissen *et al*., 2011b). Taking all factors together, Nanog is a more important factor to maintain the pluripotency of ES cells.

Anti-Nanog antibody is indispensible for studies in Nanog's function and the self-renewing mechanism of stem cells. Li *et al*. (2007) and Ha *et al*. (2009) reported the expression and/or purification of mouse or human Nanog in *E. coli* BL 21 cells. However, the anti-Nanog antibodies commercially available now are produced by immunizing animals with mouse or human Nanog protein as antigens and such kinds of antibodies are not so specific for detecting Nanog expression in tissues and cells of domestic animals.

In this study, *Capra hircus* Nanog was cloned from a fetal lamb primordial genital ridge by means of RT-PCR and prokaryotic expression vector pET32-Nanog was constructed. The recombinant plasmid was transformed into *E. coli* BL21(DE3) and His-Nanog fusion protein was induced by IPTG to express and purified by affinity chromatography under denaturing condition which was subsequently verified by Western blot assay.

MATERIALS AND METHODS

Experimental animals: The use of all animals in the present study was granted by the Committee of Animal Welfare, Guangxi University, China.

Cloning and sequencing of *Nanog* gene: Sense primer 5'GGAAGATCTATGAGTGTGGGCCCAGCTTGTCCC3' and anti-sense primer 5'TCCAAGCTTCA AATCTTC AGGCTGT ATGTTGAG3' (without stop codon) were designed according to the *Nanog* mRNA sequence (AY786437) in GenBank. A fetal lamb at 6 weeks of age was obtained from a local slaughterhouse and primodial genital ridges were removed for RT-PCR. Extracted using Trizol Reagent (Invitrogen, USA), total RNA was reversely transcribed into cDNA with a commercially available kit (Fermentas, Lith.). PCR amplification of Nanog cDNA was initiated in a volume of 25 uL with pre-denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 63°C for 1 min, extension at 72°C for 1 min and ended with extension for 10 min at 72°C. The PCR product was purified from the agarose gel with a purification kit (Wantson, China) and then ligated to vector pMD18-T (Takara, Japan) at 16°C overnight. The ligated product was transformed into competent cells (*E. coli* DH5β) which were subsequently plated on Luria Broth (LB) agar plate supplemented with 100 μg mL^{-1} ampicillin, 40 μg mL^{-1} X-gal (Wolsen, China), 0.5 m M IPTG (Wolsen, China) and incubated at 37°C for 12-16 h. White colonies were picked and cultured in 5 mL LB medium with 100 μg mL^{-1} ampicillin. Plasmids were prepared and then digested by the restriction enzymes BglII and HindIII (Takara, Japan) to verify the putative recombinant plasmids. The desired plasmids were sent to Shanghai Sangon Biotechnology Co. (Shanghai, China) for DNA sequencing.

Prediction of signal peptide: The sequencing-confirmed Nucleutide acid sequence of *Capra hircus* Nanog was translated into amino acid sequence which was submitted to SignalP3.0 server online to predict the presence and location of sigal peptide cleavage.

Construction and identification of expression vector: The sequencing-confirmed recombinant plasmid pMD18-T-Nanog and the vector pET32a (Novagen, USA) were digested respectively by BglII /BamI and HindIII and the Nanog fragment was ligated to the linearised pET32a vector to construct a prokaryotic expression vector. Subsequent procedures (such as transformation, identification of positive clones, etc.) were the same as described earlier with except that the transformed DH5α competent cells grew on LB agar plate without X-gal and IPTG and the resulting plasmids were digested by restrictive endonuclease EcoRV and HindIII because of the fact that BglII and BamI are isocaudarner.

Expression and purification of His-Nanog fusion protein: Confirmed by DNA sequencing, the inducible plasmid pET-Nanog was transformed into BL21 (DE3) competent cells which grew overnight on LB argar plate (LB medium containing 15 g L^{-1} agar). A single colony of BL21 cells was inoculated in a 50 mL tube containing 10 mL of LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 100 mg L^{-1} ampicillin) and cultured overnight at 37°C on a rotary shaker at 220 rpm. The overnight culture was then diluted in 5 mL LB media (1:50) and grew at 37°C on a rotary shaker at 300 rpm till OD600 reached 0.6-0.8. The 1 mL sample was taken immediately as a noninduced control and IPTG was added to a final concentration of 1 mM L^{-1} in the rest of culture to induce the expression of

His-Nanog fusion protein. The culture grew for an additional 4 h at 37°C and 1 mL sample was taken as induced sample. Both of the pellets from noninduced and induced samples were collected and resuspended in 50 μL 1x SDS-PAGE sample buffer (0.09 M Tris·Cl, pH 6.8; 20% glycerol; 2% SDS; 0.02% bromophenol blue; 0.1 M DTT). Subsequently, these sample were heated at 95°C for 5 min then centrifugated at 15,000×g for 1 min, 10 μL of which was respectively loaded on 12% SDS-PAGE gel for electrophoresis which was stained with Coomassie brilliant blue R250.

For purification of His-Nanog fusion protein, 500 mL of LB media was inoculated with 10 mL overnight culture of BL21 (DE3) previously transformed with the pET-Nanog plasmid which was divided in 4 shake flasks and allowed to grow to an optical density (OD600 nm = 0.6-0.8) by incubation at 37°C with vigorous shaking. His-Nanog fusion protein was subsequently induced for 4 h by addition of 1 mM IPTG. Cells were then pelleted, washed once with PBS and resuspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 8.0) or buffer A (100 mM NaH_2PO_4, 10 mM Tris·Cl, 6 M GuHCl, pH 8.0) followed by incubation for 1 h at room temperature with gentle shaking. This suspension was sonicated on ice for 20 min and pelleted for 20 min at 13,000 g. Supernatant was collected and filtered through a 0.45 μm syringe filter. The supernatant and 50% Ni-NTA slurry in a ratio of 1:4 (v/v) were loaded into an empty column with the bottom cap still attached and mixed with gentle shaking for 1 h at room temperature. The bottom cap was removed and the flow-through was collected. Unspecific protein was removed by 3 times of washing with 6 mL buffer C (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 6.3) and buffer D (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 5.9), respectively the recombinant protein was eluted by 4 times with 8 mL buffer E (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 4.3). The flow-through, wash and elution fractions were respectively collected and analyzed by SDS PAGE and visualized by coomassie blue stain.

Western blot assay: The purified His-Nanog protein was separated by 12% PAGE and transferred to Nitrocellulose (NC) membranes afterward. Blocked in 5% nonfat milk in TBST overnight at 4°C and incubated with mouse anti-His tag monoclonal antibody (1:2000, Tiagen, China) for 1 h at room temperature, the membrane was washed for three times (5 min for each) in TBST at room temperature, followed by incubation with HRP-conjugated goat anti-mouse IgG (1:2000) for 1 h at room temperature. Washed another 3 times in TBST at room temperature, the membrane was incubated with pro-light HRP chemiluninescence detection reagent (Tiagen, China) for 5 min and finally exposed to X-ray film from which the specific band was detected after development and fixation treatment. A Page blue protein molecular marker (Fermentas, Lith.) was used to size the specific band.

RESULTS AND DISCUSSION

Cloning of *Capra hircus Nanog* gene: Agarose gel electrophoresis demonstrated that a cDNA fragment was successfully amplified from fetal lamb primodial genital ridges and its length (about 1 kb) was what researchers expected (Fig. 1a). Furthermore, restrictive endonuclesdase digestion indicated that the cDNA fragment inserted in pMD 18-T vector was also nearly 1 kb (Fig. 1b). After DNA sequencing, this putative Nanog sequence was aligned with *Capra hircus* Nanog homolog registered in the GenBank (AY786437) by means of BLAST analysis online. The results showed that the two sequences shared 99.9% identity with only one nucleotide substitution, convincing that the cDNA fragment inserted in pMD 18-T vector was *Capra hircus* Nanog indeed. This DNA sequencing-confirmed plasmid was named pMD18-T-Nanog.

Identification of prokaryotic expression vector of *Capra hircus* Nanog: Since, Signal P3.0 predicted the

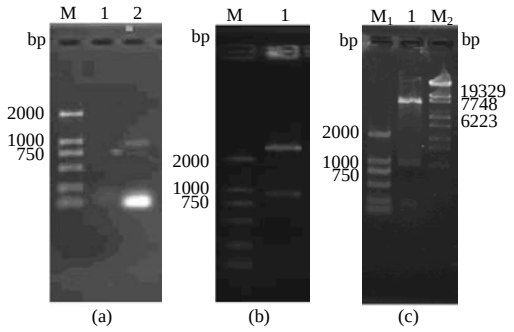

Fig. 1: a) Nanog cDNA was cloned by RT-PCR. Lane M: DL2000 DNA marker; Lane 1: Negative control (water was used as template); Lane 2: PCR product of Nanog cDNA (about 1 kb) from RNA of primodial genital ridges; b) Nanog cDNA was ligated to pMD18-T vector by TA cloning. Double digestion of the recombinant plasmid by restriction endonuclease showed that Nanog cDNA was inserted into pMD18-T vector. Lane M: DL 2000 DNA marker; Lane 1: pMD18-T-Nanog digested by BglII and HindIII; c) The prokaryotic expression vector-pET-Nanog was succsessfully constructed. Lane M1: DL 2000 DNA marker; Lane 1: pET-Nanog digested by EcoRV and HindIII; Lane M2: III-EcoT14I digest DNA marker

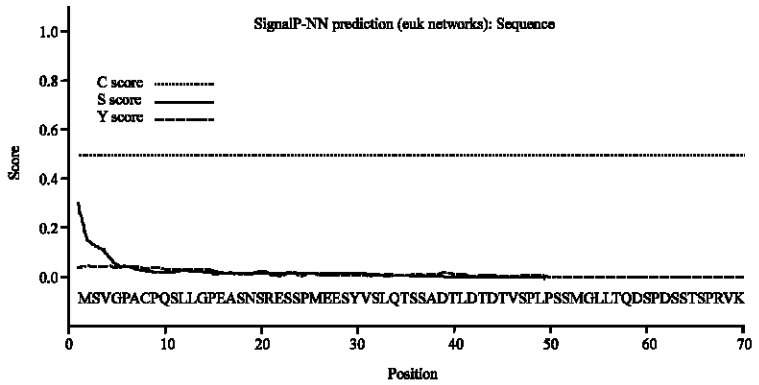

Fig. 2: The results of signal peptide prediction. Nucleotide sequences of *Capra hircus* were translated into amino acid sequences and subsequently submitted to online server SignalP3.0 to predict the presence of signal peptide. The results showed that there was no signal peptide at N-terminus of amino acids of *Capra hircus* Nanog

absence of signal peptide cleavage in the N-terminus of amino acids of *Capra hircus* Nanog (Fig. 2), the whole length of Nanog's ORF (Open Reading Frame) could be used to construct prokaryotic expression vector. Removed from pMD18-T-Nanog, Nanog cDNA fragment was subcloned into the Multi-Clone Sites (MCS) of the pET32a vector. Restrictive endonuclease digestion illustrated that the inserted fragment was a single one (about 1 kb) as showed in Fig. 1c, DNA sequencing also indicated that the Nanog cDNA has been successfully inserted in the pET32a vector. This plasmid was named pET-Nanog.

Expression and purification of His-Nanog fusion protein: The resulting plasmid pET-Nanog was transformed into BL21 (DE3) cells which were grown in LB medium. The 65 kDa His-Nanog fusion protein was expressed in recombinant BL21 (DE3) at a high-level and fusion protein reach its peak expression level after the induction of 1 mM IPTG for 4 h. SDS-PAGE analysis showed that His-Nanog fusion protein expressed in BL21 (DE3) cells (Fig. 3) but the recombinant protein was insoluble (formed as inclusion bodies). Bacteria were disrupted by incubation with gentle shaking in lysis buffer (buffer B) and subsequently lysed with ultrasonication. SDS-PAGE showed that quite a lot of His-Nanog fusion protein was still in cell lysis precipetates (Fig. 4), resulting in the insufficient yield of purified His-Nanog protein after washes and elutions. However, inclusion bodies were completely solubilized in buffer A after 1 h gentle shaking and 20 min ultrasonication. In addition, nonspecific binding was reduced after three times washes of buffer C and buffer D and the final yield of His-Nanog protein was significantly increased (Fig. 5) after elutions of buffer E. His-Nanog protein was seen as a single band on the

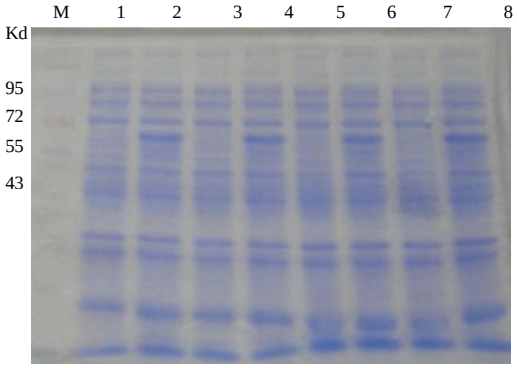

Fig. 3: SDS-PAGE profile of cell lysate obtained from His-Nanog expressed in *E. coli* BL21 cells. Lane M: Molecular weight markers; Lane 1, 3, 5 and 7: nonindueced; Lane 2, 4, 6 and 8: induced with 1 mM IPTG

SDS-PAGE gel (Fig. 5) indicating that only one protein population was produced. Moreover, this band appears around the molecular mass of His-Nanog. Moreover, the His-Nanog protein could specifically bind to anti-His antibody (Fig. 6) indicating the expected immunogenicity.

It is well known that Nanog is an important factor to maintain pluripotency of ES cells. Firstly expressed in the compacted morula and then in the Inner Cell Mass (ICM), mouse Nanog expression is down-regulated since, implantation while it can be detected in germ cells of the genital ridges of E11.5 mouse embryos (Chambers *et al*., 2003). Hambiliki *et al*. (2012) reported that Nanog and another transcript factor-Oct 4 were co-expressed in morula and blastocyst of human embryos and in human ES cells. *In vitro*, Nanog mRNA is highly expressed in ES, EG and EC (Embryonic Carcinoma) cells but not in adult

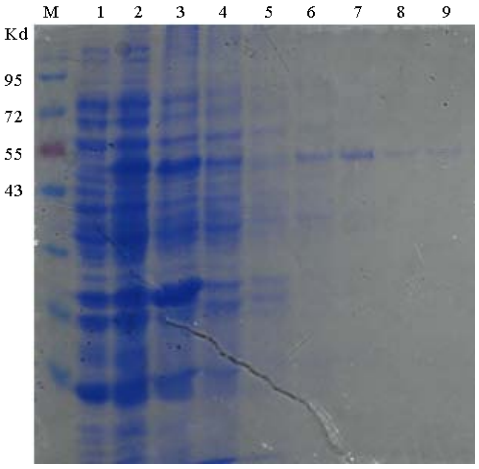

Fig. 4: SDS-PAGE profile of His-Nanog protein isolated from *E. coli* BL21 cell lysate (in buffer B). Lane M: prestained protein marker; Lane 1: noninduced; Lane 2: cleared lysate; Lane 3: precipitats; Lane 4: flow-throgh; Lane 5: the second time of buffer C wash; Lane 6: the second time of buffer D wash; Lane 7-9: buffer E elution of His-Nanog fusion protein

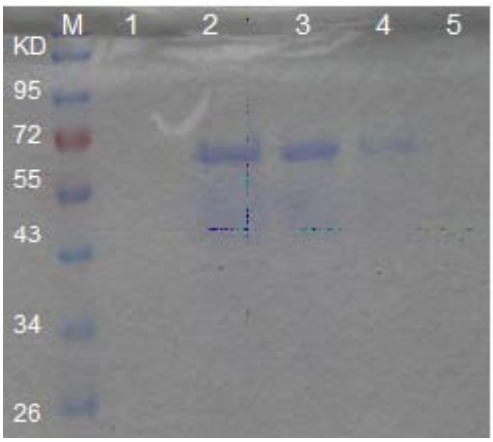

Fig. 5: SDS-PAGE profile of His-Nanog protein isolated from *E. coli* BL21 cell lysate (in buffer A). Lane M: prestained protein marker; Lane 1: the second time of buffer D wash; Lane 2-5: buffer E elution of His-Nanog fusion protein

tissues (Chambers *et al*., 2003; Yamaguchi *et al*., 2005; Huang *et al*., 2007). Based on this knowledge, researchers successfully obtained *Capra hircus* Nanog cDNA by RT-PCR from fetal lamb primordial genital ridges. Afterward, the prokaryotic expression vector pET-Nanog was constructed with DNA sequencing-confirmed pMD18-T-Nanog and vector pET32a.

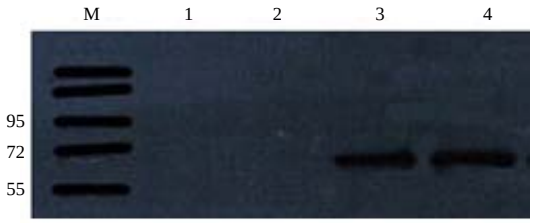

Fig. 6: Western blotting assay showed that the purified His-Nanog could specifically bind to ant-His antibody while negative (Lane 1) and noninduced (Lane 2) controls did not. Lane M: prestained protein marker; Lane 1: intact *E. coli* BL21 cell lysate (in buffer A); Lane 2: noninduced recombinant *E. coli* BL21 cell lysate (in buffer A); Lane 3: the first time elution fraction of His-Nanog protein; Lane 4: the second time elution fraction of His-Nanog protein

The 6xHis affinity tag facilitates binding to Ni-NTA. Using pET32a vector, it can be placed at the C or N-terminus of the protein of interest. It is poorly immunogenic and at pH 8.0 the tag is small, uncharged and therefore does not generally affect secretion, compartmentalization or folding of the fusion protein within the cell. In most cases, the 6xHis tag does not interfere with the structure or function of the purified protein as demonstrated for a wide variety of proteins including enzymes, transcription factors and vaccines. A further advantage of the 6xHis tag is that it allows the immobilization of the protein on metalchelating surfaces such as Ni-NTA HisSorb™ Strips or Plates and therefore simplifies many types of protein interaction studies. In addition, anti·His antibodies can be used indirectly for detection of the protein of interest.

His levels of expression of recombinant proteins in a variety of expression systems can lead to the formation of insoluble aggregates; in *E. coli*, these are known as inclusion bodies. In the present study, the recombinant plasmid pET-Nanog expressed in BL21 competent cells and His-Nanog protein was insoluble, so it was purified under denaturing condition. Strong denaturants such as 6 M GuHCl or 8 M urea completely solubilize inclusion bodies and 6xHis-tagged proteins. Under denaturing conditions, the 6xHis tag on a protein will be fully exposed so that binding to the Ni-NTA matrix will improve and the efficiency of the purification procedure will be maximized by reducing the potential for nonspecific binding. Here, Nanog cDNA (without stop codon) was inserted into sites of BamHI and HindII in MCS of vector pET32a to make sure that both His tags at N terminus and C

terminus would be in the same ORF with the Nanog cDNA which would have more His-tags exposed to bind Ni-NTA matrix thus improving purification efficiency further. On the other hand, some unspecific proteins mixed in the elusion fractions after two times of buffer E elution. Addition of of 20 mM β-Mercaptoethanol (β-ME) to the lysis buffer (buffer A) and 0.6% Triton X-100 to Buffer C overcome the problem because β-ME enables to reduce disulfide bonds that may have formed between contaminating proteins and the 6xHis-tagged protein and these contaminants could be easily removed by washing the Ni-NTA resin under more stringent conditions, by adding low concentrations of a detergent (0.1-1% Triton X-100). Proteins purified under denaturing conditions can then be refolded if necessary before use. However, if proteins are purified under denaturing conditions for use in antibody induction, there is usually no need to renature before injection into the animal. Therefore, the purified His-Nanog protein here can be used directly to prepare polyclonal or monoclonal anti-Nanog antibody which will be used to study Nanog's function or characteristics of pluripoten stem cells (such as iPS cells) in *Capra hircus*.

CONCLUSION

Capra hircus Nanog was cloned from fetal primodial genital ridges, whose prokaryotic expression vector was transformed into BL21 competent cells and His-Nanog protein expressed in form of inclusion bodies. Purified under denaturing condition, the His-Nanog protein could specifically bind to anti-His antibody indicating the expected immunogenicity. In a word, the purified His-Nanog could be used directly to prepare anti-Nanog antibody.

ACKNOWLEDGEMENTS

This research was supported by grants from the national Natural Science Fund of China (Grant Number: 31260549) and the provincial Natural Science Fund of Guangxi (2011GXNSFD018018). Researchers thank Dr. You-Chuan Wei, Dr. Yun-Lai Chen for their kindly help in experiments.

REFERENCES

Chambers, I., D. Colby, M. Robertson, J. Nichols and S. Lee, 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell., 113: 643-655.

Darr H., Y. Mayshar and N. Benvenisty, 2006. Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features. Development, 133: 1193-1201.

Ha, S.C., J.H. Pereira, J.H. Jeong, J.H. Huh and S.H. Kim, 2009. Purification of human transcription factors Nanog and Sox2, each in complex with Skp, an *Escherichia coli* periplasmic chaperone. Protein Expression Purif., 67: 164-168.

Hambiliki, F., S. Stron, P. Zhang and A. Stavreus-Evers, 2012. Co-localization of NANOG and OCT4 in human pre-implantation embryos and in human embryonic stem cells. J. Assisted Reprod. Genet., 29: 1021-1028.

Han, J., F. Zhang, M. Yu, P. Zhao and W. Ji *et al*., 2012. RNA interference-mediated silencing of NANOG reduces cell proliferation and induces G0/G1 cell cycle arrest in breast cancer cells. Cancer Lett., 321: 80-88.

Huang, B., T.S. Xie, D.S. Shi, T. Li and X.L. Wang *et al*., 2007. Isolation and characterization of EG-like cells from Chinese swamp buffalo (*Bubalus bubalis*). Cell Biol. Int., 31: 1079-1088.

Hyslop, L., M. Stojkovic, L. Armstrong, T. Walter and P. Stojkovic *et al*., 2005. Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. Stem Cells, 23: 1035-1043.

Li, J., C.R. Lv, L. Dou and Z.Y. Dou, 2007. Construction of prokaryotic expression vector of mouse Nanog gene and its expression. Agric. Sci. China, 6: 487-492.

Moon, J.H., W. Yun, J. Kim, S. Hyeon and P.J. Kang *et al*., 2013. Reprogramming of mouse fibroblasts into induced pluripotent stem cells with Nanog. Biochem. Biophys. Res. Commun., 431: 444-449.

Sumer, H., J. Liu, L.F. Malaver-Ortega, M.L. Lim, K. Khodadadi and P.J. Verma, 2011. NANOG is a key factor for induction of pluripotency in bovine adult fibroblasts. J. Anim. Sci., 89: 2708-2716.

Takahashi, K. and S. Yamanaka, 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 126: 663-676.

Theunissen, T.W., A.L. van Oosten, G. Castelo-Branco, J. Hall, A. Smith and J.C.R. Silva, 2011a. Nanog overcomes reprogramming barriers and induces pluripotency in minimal conditions. Curr. Biol., 21: 65-71.

Theunissen, T.W., Y. Costa, A. Radzisheuskaya, A.L. van Oosten and F. Lavial *et al*., 2011b. Reprogramming capacity of NANOG is functionally conserved in vertebrates and resides in a unique homeodomain. Development, 138: 4853-4865.

Yamaguchi, S., H. Kimura, M. Tada, N. Nakatsuji and T. Tada, 2005. Nanog expression in mouse germ cell development. Gene Expression Patterns, 5: 639-646.

Yamaguchi, S., K. Kurimoto, Y. Yabuta, H. Sasaki, N. Nakatsuji, M. Saitou and T. Tada, 2009. Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells. Development, 136: 4011-4020.

Yu, J., M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget and J.L. Frane *et al*., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science, 318: 1917-1920.

Zhang, J., X. Wang, B. Chen, G. Suo, Y. Zhao, Z. Duan and J. Dai, 2005. Expression of Nanog gene promotes NIH3T3 cell proliferation. Biochem. Biophys. Res. Commun., 338: 1098-1102.