Pakistan J. Zool., vol. 49(1), pp 313-320, 2017. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2017.49.1.313.320

Effect of Prokaryotic Expressed Nucleoplasmin on the Efficiency of Somatic Cell Nuclear Transfer in Banna Mini-pig Inbred Line

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Article Information
Received 01 August 2015
Revised 15 September 2016
Accepted 21 September 2016
Available online 07 January 2017

Authors' Contributions
WC conceived and designed the experiments. WC, WP, YQ, YL, YH, JX performed the experiments. WMC analyzed the data. XQC, YZZ contributed somatic cell. WC and WP wrote the paper. HW helped in preparation of manuscript.

Key words
Banna Mini-pig Inbred Line, Prokaryotic expression, Nucleoplasmin, Polyglutamic acid, Embryo development.

ABSTRACT

Nucleoplasmin (Npm) is the most abundant protein in the nucleus of oocytes which can promote *in vitro* nucleosome formation. In this study, to improve the efficiency of SCNT in Banna Mini-pig Inbred Line (BMI), we obtained the recombinant nucleoplasmin (Npm) by using a prokaryotic expression system and compared the difference on the developmental effects between exogenous Npm and its structural analog, polyglutamic acid (PGA). We chose the pMD18-T vector for Npm expression and transformed *E. coli* BL21 (DE3) cells to construct a prokaryotic expression system. The results showed that the recombinant Npm protein of 32 kDa could be obtained. Recombinant Npm or PGA was injected into porcine oocytes at different concentrations. The results showed that the blastocyst rates in injection 560 ng/μL Npm and 1000 ng/μL PGA groups were higher than those of other groups. Moreover, the blastocyst rate was highest in injection 560 ng/μL Npm group and was lowest in injection 100 ng/μL PGA group. These data suggested that addition of recombinant Npm obtained by prokaryotic expression or PGA could improve the development of SCNT embryos in the BMI.

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is an assisted reproductive technology that can be used in the farm animal production industry, drug production, regenerative medicine and conservation of invaluable genetic resources (Vajta, 2007; Oback, 2008). In addition, SCNT can provide unique and interesting experimental systems for genomic research to learn how the somatic cell genome is reprogrammed into an equivalent state to that of the fertilized oocyte (Gurdon and Wilmut, 2011). Since the birth of Dolly the sheep in 1996 (Campbell *et al*., 1996), many cloned animals have been produced in the cow (Kato *et al*., 1998), mouse (Wakayama *et al*., 1998), goat (Baguishi *et al*., 1999), pig (Polejaeva *et al*., 2000), rabbit (Chesné *et al*., 2002), cat (Shin *et al*., 2002), rat (Zhou *et al*., 2003), horse (Galli *et al*., 2003), mule (Woods *et al*., 2003), dog (Lee *et al*., 2005), ferret (Li *et al*., 2006), buffalo (Shi *et al*., 2007) and the camel (Wani *et al*., 2010).

Banna Mini-pig Inbred Line (BMI) has been bred since the 1980s from full and half siblings. As a unique, highly inbred pig line, BMI can be used in various biomedical studies, including disease models, transgenesis, genomics, and xenotransplantation for medical research (Crabbe *et al*., 2005). Although the cloned piglets of BMI had been obtained (Wei *et al*., 2013), the efficiency of nuclear transfer was between 0–2.5%, *i.e.*, 0-5 live births after transfer of 200 cloned embryos. As with most cloning efforts, developmental defects, including abnormalities in cloned fetuses and placentas, high rates of pregnancy loss and neonatal death have often been encountered (Jiang *et al*., 2007). Reprogramming which is initiated by the oocyte following nuclear transfer (NT), continues during development may often be inefficient (Latham, 2004, 2005). So improving the efficiency of reprogramming may lead to greater cloning success (Blelloch *et al*., 2006).

Nucleoplasmin (Npm), as a molecular chaperone, was found in the eggs of *Xenopus laevis* which facilitated sperm chromatin remodeling at fertilization (Leno *et al*., 1993). It can help load histones onto DNA during nucleosome assembly *in vitro* (Laskey *et al*., 1978). Previous studies showed that Npm could be located in

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0030-9923/2017/0001-0313 $ 9.00/0

bovine oocytes (Fair *et al*., 2001) and porcine embryos (Hyttel *et al*., 2000). In addition, it was suggested that Npm could improve development of nuclear transfer embryos by facilitating reprogramming of the somatic cell nucleus in bovine (Betthauser *et al*., 2006). The acidic polyanion, polyglutamic acid (PGA), can also mediate sperm decondensation and nucleosome assembly *in vitro* (Dean, 1983). However, Npm has not yet been tested to improve developmental competence of BMI nuclear transfer embryos. Moreover, whether PGA enhances the developmental ability of BMI nuclear transfer embryos is presently unknown.

The aim of this study was (i) to construct prokaryotic expression vector and obtain efficiently expressed Npm, and (ii) to examine the difference on the developmental effect between exogenous Npm and PGA.

MATERIALS AND METHODS

Materials

Escherichia coli strains DH5α, pCold II vector and LB medium were prepared at Invitrogen Shanghai Co. Ltd. (Shanghai, China). The pMD18-T vector was purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). The BL21 (DE3) and pG-TF2 expression strain, platinum *Taq* DNA polymerase high fidelity, all restriction enzymes, DNA molecular mass markers and the PCR product purification kit used in this study were purchased from Life Technologies Corporation (Invitrogen, Carlsbad, California, USA). Chemicals and reagents used in oocyte and embryo culture, unless otherwise stated, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Synthesis of the Npm2 gene

The full-length cDNA (GenBank accession No. NM_001195362.1) of *Npm2* gene contains 660 nucleotides, which encodes a protein of 219 amino acid residues. Oligos of *Npm2* gene were synthesized and assembled by PCR, containing *Nde* I and *Hind* III restriction sites. The reaction was carried out using the following reaction cycles: initial denaturation at 94°C for 5 min followed by 28 consecutive cycles of denaturation at 94°C for 20 sec, annealing for 30 sec at 55°C, extension at 72°C for 1 min, then final extension at 72°C for 10 min. The amplified *Npm2* gene was gel-purified by highly pure PCR product purification kit. The purified product was inserted into the corresponding region of pMD18-T expression vector according to operation manual, transferred into the bacterium DH5α. After replication, recombinant plasmids were extracted, digested by the restriction enzyme (*Nde* I and *Hind* III) and sequenced. The correct recombinant prokaryotic expression vector was named as pMD18-T-*Npm2*.

Recombinant protein expression and purification

Expression of recombinant protein in *E.coli* BL 21 (DE3) and pG-TF2 expression strains followed with the transformation of the recombinant plasmid, pCold II-*Npm2*. The transformants were cultured in 3 mL of LB medium containing 50 μg/mL ampicillin and grew overnight at 37°C and centrifuged at 3.5×g until the optical density (OD600) of the cultured cells reached 0.5-0.6. Expression of the recombinant protein was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 3 h or 0.1 mM IPTG at 16°C overnight, and identified on 15% SDS-PAGE.

The transformants of the recombinant plasmid, pCold II -*Npm2* were cultured in 100 mL of LB medium containing 50 μg/mL ampicillin and grown overnight at 37°C. In the next step, it was transferred to 1 L XY1 medium containing 50 μg/mL ampicillin, and incubated until the optical density (OD600) of the cultured cells reached 2.0. Expression of the recombinant protein was induced with 0.5 mM IPTG at 37°C for 3 h and harvest bacteria liquid.

A chelating SFF Ni column was equilibrated with buffer medium containing 50 mM Tris, 500 mM NaCl (pH8.0), gradient eluted with medium containing 50 mM Tris, 500 mM NaCl, 500 mM Imidazole (pH8.0) and dialyzed with 20 mM PB, 500mM NaCl (pH7.4) after elution. Recombinant protein was concentrated and quantified.

Preparation of donor cells

Fetuses (47 days old) isolated from the 22nd generation in the 133 families of BMI pigs were washed three times with phosphate-buffered saline. After removing the head, limbs, and viscera, the fetuses were minced and digested in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 20% fetal bovine serum (FBS; Hyclone), 1% penicillin-streptomycin, and 1 mg/mL collagenase IV for 4 h at 37°C. The cells were centrifuged at 30×g for 5 min, suspended in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, and then cultured in a flask until grown to 90% confluence. The cells were then passaged and frozen in DMEM containing 20% FBS and 10% dimethylsulfoxide for future use.

In vitro *maturation of oocytes*

Porcine ovaries were collected from a local slaughter house (Chenggong Ruide Food Co., Ltd, Kunming, Yunnan Province, China) with the permission to use animal parts for this study. The ovaries were transported to the laboratory at 25-30°C in 0.9 % (w/v) NaCl solution. The oocytes were obtained and cultured using the same method previously described (Wei *et al*., 2013).

Porcine follicular fluid (pFF) was aspirated from 3- to 6-mm follicles of ovaries. The fluid was collected under sterile conditions and stored at -20°C. Prior to use, the follicular fluid was centrifuged at 1500×g for 30 min at 4°C, as described previously (Abeydeera *et al*., 1998).

Nuclear transfer

SCNT was performed as previously described (Wei *et al*., 2013). After culturing for 38 h to 42 h, oocytes with expanded cumulus cells were briefly treated with 0.1% (w/v) hyaluronidase and cumulus cells were removed using a fine glass capillary pipette. The oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm using a beveled pipette (approximately 20 mm in diameter) in TLH-PVA (Wei *et al*., 2013). Fetal fibroblasts of the fourth to ninth passages were used as nuclear donor cells and injected to the peri-vitelline space of the oocytes. Donor cells were fused with the recipient cytoplasts with a single direct current pulse of 200 V/mm for 20 msec using an embryonic cell fusion system (ET3, Fujihira Industry Co. Ltd., Tokyo, Japan) in fusion medium (Wei *et al*., 2013). The reconstructed embryos were cultured and activated with a single pulse in an activation medium (Wei *et al*., 2013). The reconstructed embryos were equilibrated in PZM-3 supplemented with 5 mg/mL cytochalasin B for 2 h at 38.5°C in humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 (APM-30D, ASTEC, Japan).

Injection of remodeling factors

The SCNT-injection manipulation chamber contained a small drop (10 mL) of injection buffer (IB=70 mM KCl, 20 mM HEPES; pH 7.0) with (Npm or PGA) or without (control) remodeling factor. An injection tip approximately 8 mm diameter at the orifice was placed into the drop containing three different concentrations of Npm and four concentrations of PGA in IB. Approximately 0.4 nL remodeling factor was allowed to flow into the oocyte cytoplasm. Since the concentration of Npm in *Xenopus* egg extract was approximately 500 ng/μL (Mills *et al*., 1980), Npm was injected to an estimated final concentration of 560 ng/μL (1,680 ng/μL stock solution), 250 ng/μL (750 ng/μL stock solution), or 100 ng/μL (300 ng/μL stock solution). PGA was injected to an estimated final concentration of 100 ng/μL (300 ng/μL stock solution injected), 500 ng/μL (1,500 ng/μL stock solution), 1,000 ng/μL (3,000 ng/μL stock solution), or 2,500 ng/μL (7,500 ng/μL stock solution).

Culture of embryos

Reconstructed embryos were cultured in PZM-3 medium and then placed in an incubator supplied with 5% CO_2, 5% O_2, 90% N_2 at 38.5°C in a humidified atmosphere. Cleavage and blastocyst formation were recorded on days 2 and 7, respectively. Cell numbers of blastocyst were carried out after staining with 10 mg/mL Hoechst 33342 under a laser scanning confocal microscope (TCS SP5II, LEICA, Germany).

RESULTS

Recombinant protein

The synthesized nucleotides sequence encoding *Npm2* was amplified by PCR and detected by 2% agarose gel electrophoresis. The location of the resulting DNA fragment is about 660bp on an agarose gel (Fig. 1). Subsequently, the PCR product was ligated into the pMD18-T vector and transformed into the competent *E. coli* DH5α cells. The clone was identified by PCR and restriction analysis, and then a positive clone was sequenced and the result confirmed the *Npm2* gene in frame with C-terminal His_6 tag in the pMD18-T multiple cloning sites.

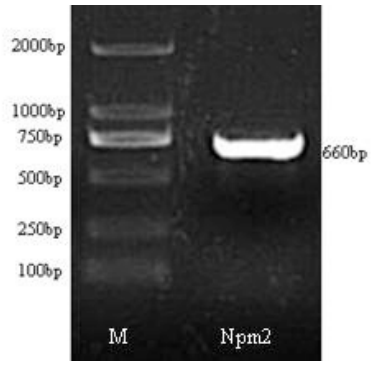

Fig. 1. The PCR results of the Npm. M, marker.

The confirmed recombinant vector was transformed into *E. coli* BL21 (DE3) cells and pG-TF2. In order to make the recombinant protein expressed maximally, we compared the expression conditions as described in "Materials and Methods". SDS-PAGE result showed that the recombinant plasmid pCold II-INPM2 were transformed into BL21 (DE3) expression strains and induced with 0.5 mM IPTG at 37°C for 3 h or 0.1 mM IPTG at 16°C overnight. Correct recombinant protein with molecular weight of 32 kDa was obviously expressed in the transformed *E. coli* BL21 (DE3) cells (Fig. 2A, B) and this protein was almost absent transformed into pG-TF2 expression strains (Fig. 3A, B).

As shown in Figure 4, recombinant protein containing His_6tag was separated and purified by chelating SFF (Ni) column. Recombinant protein product was obtained by dialyzing and concentrating and protein purity was 90% by SDS-PAGE detection (Fig. 5). Finally, the protein

concentration we obtained was 1680 ng/μL.

Statistical analysis

An analysis of variance (ANOVA, PROC GLM) was performed using the SAS statistical package (SAS Institute Inc., Cary, NC, USA). All percentage data were subjected to arcsine transformation before statistical analysis. $P<0.05$ was considered to be statistically significant.

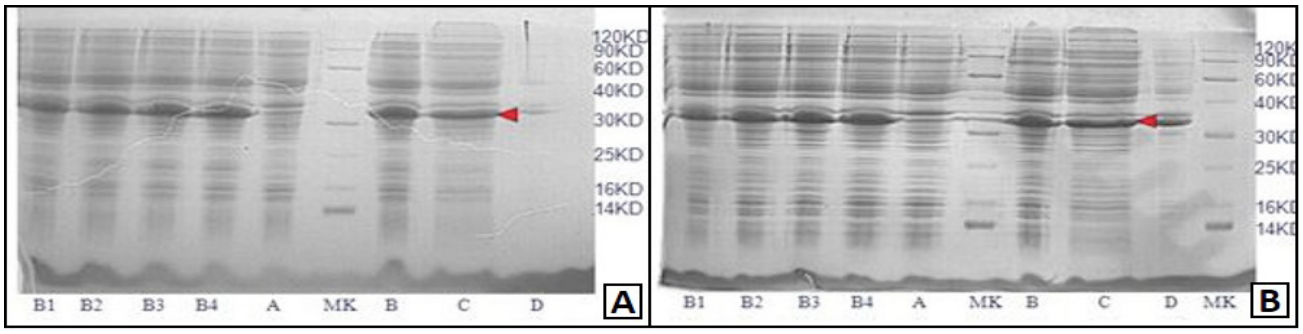

Fig. 2. SDS-PAGE analysis of the pCold II-INPM2 protein expression. **A,** transformed *E. coli* BL21 (DE3) cells and induced with 0.5 mM IPTG; **B**, transformed *E. coli* BL21 (DE3) cells and induced with 0.1mM IPTG. Lane A, non-induced crude; Lane B, Induced crude; LaneB1-B4, Induced crude; Lane C, Supernatant of lysate; Lane D, Precipitation of lysate. MK, Molecular weight marker.

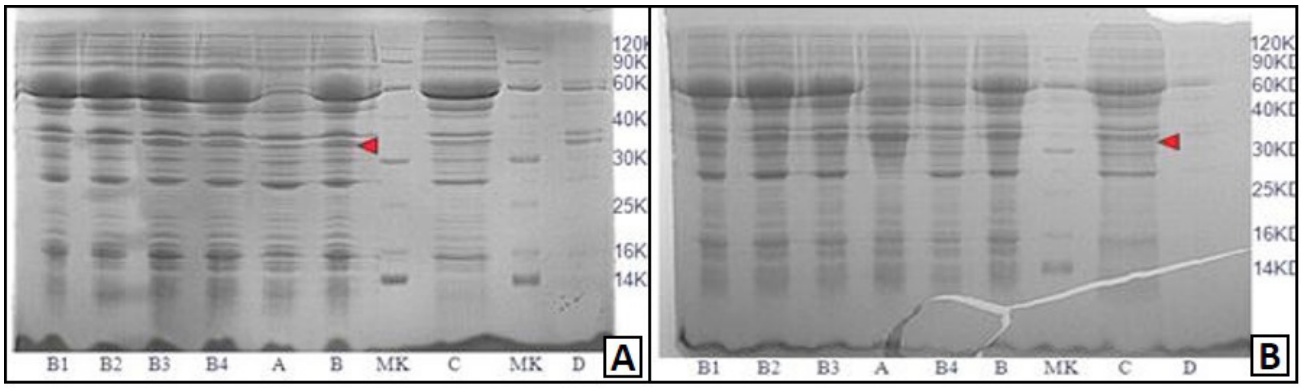

Fig. 3. SDS-PAGE analysis of the pCold II-INPM2 protein expression. **A**, transformed pG-TF2 expression strains and induced with 0.5mM IPTG; **B**, transformed pG-TF2 expression strains and induced with 0.5mM IPTG. Lane A, non-induced crude; Lane B, Induced crude; LaneB1-B4, Induced crude; Lane C, Supernatant of lysate; Lane D, Precipitation of lysate. MK, Molecular weight marker.

Table I.- Effect of different exogenous Npm or PGA on the efficiency of BMI somatic cell nuclear transfer.

Concentration (ng/μL)	Replicate	Oocytes	Cleavage rate (%)	Blastocyst rate (%)	Cell numbers of blastocyst
560 (Npm)	3	103	71.88±9.28^{a}	25.29±6.36^{a}	42.33±2.52^{a}
250 (Npm)	3	108	76.71±7.71^{a}	16.51±6.37^{ab}	34.67±1.15^{ab}
100 (Npm)	3	115	78.08±6.13^{a}	18.82±4.94^{ab}	35.00±2.65^{ab}
2500 (PGA)	3	71	68.71±7.60^{ab}	11.79±4.28^{bc}	29.67±4.04^{b}
1000 (PGA)	3	74	72.97±3.10^{a}	17.41±2.13^{ab}	27.00±7.07^{b}
500 (PGA)	3	72	57.46±4.29^{b}	11.91±4.12^{bc}	29.00±8.19^{b}
100 (PGA)	3	69	56.97±6.08^{b}	6.82±3.10^{c}	30.56±1.90^{b}
Control	6	182	72.06±6.58^{a}	13.84±5.66^{bc}	34.00±7.21^{ab}

Npm, nucleoplasmin; PGA, polyglutamic acid; BMI, Banna Mini-pig Inbred Line.

Data are expressed as mean±S.D. $^{a, b, c}$ Values with different superscript letters within a column differ significantly ($P<0.05$).

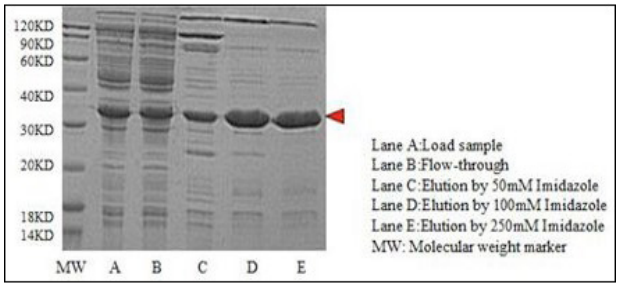

Fig. 4. Protein was concentrated in D and E lanes by chelating SFF (Ni) column separation and purification.

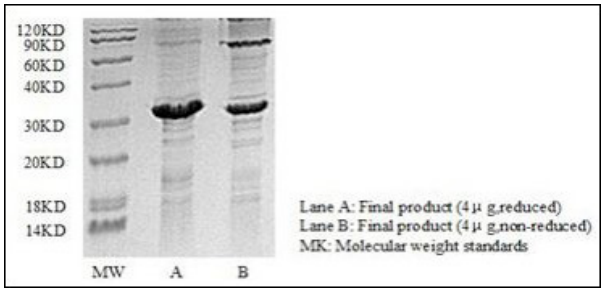

Fig. 5. Protein product. Buffer (20 mM PB, 500 mM NaCl, pH 7.4).

Effect of exogenous Npm or PGA on the developmental competence of somatic cell nuclear transfer embryos in BMI pig

The developmental competence of BMI somatic cell nuclear transfer embryos injected with exogenous Npm or PGA are summarized in Table I.

The results showed that the cleavage rate of embryos injected with 500 ng/μL and 100 ng/μL PGA were significantly lower than those of other groups (P<0.05). No significant differences were observed in other groups (P>0.05). The blastocyst rate in the 560 ng/μL Npm injected group was significantly higher than those injected with 2500 ng/μL, 500 ng/μL, 100 ng/μL PGA groups and control group (P<0.05). Moreover, the blastocyst rate was highest in the 560 ng/μL Npm injected group (25.29±6.36%) and was lowest in the 100 ng/μL PGA injected group (6.82±3.10%). Cell numbers of blastocysts injected with different concentration of PGA groups were significantly lower than those injected with 560 ng/μL Npm group (P<0.05).

DISCUSSION

Nucleoplasmin is the most abundant protein in the nucleus of *Xenopus laevis* oocytes, and its molecular mass is about 30 kD and forms a stable pentamer *in vivo* (Earnshaw *et al*., 1980). Npm could promote *in vitro* nucleosome formation, also participate in the decondensation process that the highly compacted sperm chromatin undergoes after fertilization (Loyola and Almouzni, 2004). Similarly, if somatic cell nuclei are injected into the cytoplasm of an egg, for instance during cloning procedures, they also underwent a remodeling process that results intranscriptional reprogramming of the transplanted nucleus (Wade and Kikyo, 2002). In the mouse, three Npm proteins have been identified. Mammalian NPM2 is crucial for histone deacetylation and heterochromatin formation surrounding nucleoli in oocytes and early zygotes (Burns *et al*., 2003).

Eukaryotic expression systems are frequently employed for the production of recombinant proteins as therapeutics as well as research tools. However, the most appropriate vector and host system must be carefully considered for eukaryotic expression systems. In addition, eukaryotic expression is much more complex and often relies on various feedback mechanisms, developmental processes and environmental factors. Prokaryotic expression systems are widely used because it is simple and effective. In this study, we chose pMD18-T vector to facilitate the following purification. This vector carried a C-terminal His6-Tag, thus, the recombinant protein of Npm should carry a His6 fragment in its C-terminus. The comparatively high level of expression could be achieved with 0.5 mM IPTG at 37°C for 3 h or 0.1mM IPTG at 16°C overnight. At the best expression condition, cells of *E. coli* BL21 transformed with recombinant vector could produce a recombinant protein of 32 kDa. The molecular weight of the resulting protein was inconsistent with the theoretical value (about 30 kDa) calculated from sequence of the recombinant protein. It can be used to latter experiment.

The data presented in this manuscript suggested that both Npm and PGA could facilitate the *in vitro* development of porcine NT embryos. The ability of nucleoplasmin to decondense sperm is shared by other highly charged polyanionic molecules such as polyglutamic acid, but only nucleoplasmin can cause decondensation at a physiological rate and at a physiological concentration (Philpott *et al*., 1991; Ohsumi and Katagiri, 1991). The physiological concentration of Npm found within cytoplasmic extracts derived from activated *Xenopus* eggs was 500 ng/μL (Philpott and Leno, 1992).The acidic polyanion, PGA, could also mediate sperm decondensation and nucleosome assembly *in vitro* (Dean, 1983). However, in spite of the fact that PGA has a greater overall negative charge than Npm, remodeling of sperm chromatin requires a higher concentration of PGA than Npm (Philpott *et al*., 1991).

Previous studies showed that addition of chromatin remodeling factors to the bovine oocytes may improve development of NT embryos by facilitating reprogramming of the somatic nucleus (Betthauser *et al*., 2006).

In our studies, the results showed that injection of 560 ng/μL exogenous Npm could improve the blastocyst rate of NT embryos in BMI. Its concentration was similar to the physiological concentration of Npm derived from activated *Xenopus laevis* eggs. This may be because of exogenous injection Npm upregulating gene expression related to embryo development and reflecting a global depression of transcription associated with Npm-driven remodeling of somatic chromatin (Betthauser *et al*., 2006). This remodeling might include the removal of somatic histone H1 and other linker-binding proteins from chromatin (Ramos *et al*., 2005), and/or facilitated transcription factor binding concomitant with the removal of nucleosome core particles from the DNA (Chen *et al*., 1994). Compared with injection of Npm, injection of PGA at 1000 ng/μL could improve the developmental ability of BMI pig NT embryos. However, injecting a high concentration of PGA decreased the developmental competence of NT embryos. This result was similar to previous studies (Betthauser *et al*., 2006). This may be due to a remodeling factor-dependent developmental ‘‘threshold’’ and concentrations of Npm above this threshold facilitate blastocyst development, while similar concentrations of PGA inhibit development (Betthauser *et al*., 2006). These results may indicate that Npm and PGA operate through different mechanisms even though Npm contains several acidic tracts within its structure (Dingwall and Laskey, 1990; Prado *et al*., 2004), and PGA can mimic Npm’s decondensation and nucleosome core assembly activities *in vitro* (Dean, 1983). However, Npm-dependent chromatin remodeling does not appear to be mediated through its acidic tracts alone. So, the mechanism of Npm expression, regulation and function need to be further studied.

CONCLUSION

In conclusion, we successfully obtained the recombinant nucleoplasmin by prokaryotic expression system. Moreover, addition of recombinant Npm or PGA could further improve the development of SCNT embryos in the BMI pig. Further studies will be required to understand the mechanism of Npm expression and regulation.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (Grant No. 30800785). These authors are grateful to Professor Peter M Jobst (Virginia Polytechnic Institute and State University) for their critical reading and comments on this manuscript.

Statement of conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Prather, R.S. and Day, B.N., 1998. Presence of b-mercaptoethanol can increase the glutathione content of pig oocytes matured *in vitro* and the rate of blastocyst development after *in vitro* fertilization. *Theriogenology*, **50**: 747-756. https://doi.org/10.1016/S0093-691X(98)00180-0

Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overström, E.W. and Echelard, Y.,1999. Production of goats by somatic cell nuclear transfer. *Nat. Biotechnol.*, **17**: 456-461. https://doi.org/10.1038/8632

Betthauser, J.M., Pfister-Genskow, M., Xu, H., Golueke, P.J., Lacson, J.C., Koppang, R.W., Myers, C., Liu, B., Hoeschele, I., Eilertsen, K.J. and Leno, G.H., 2006. Nucleoplasmin facilitates reprogramming and *in vivo* development of bovine nuclear transfer embryos. *Mol. Reprod. Dev.*, **73**: 977-986. https://doi.org/10.1002/mrd.20493

Blelloch, R., Wang, Z., Meissner, A., Pollard, S., Smith, A. and Jaenisch, R., 2006. Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. *Stem Cells*, **24**: 2007-2013. https://doi.org/10.1634/stemcells.2006-0050

Burns, K.H., Viveiros, M.M., Ren, Y., Wang, P., Demayo, F.J., Frail, D.E., Eppig, J.J. and Matzuk, M.M., 2003. Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. *Science*, **300**: 633-636. https://doi.org/10.1126/science.1081813

Campbell, K.H., McWhir, J., Ritchie, W.A. and Wilmut, I., 1996. Sheep cloned by nuclear transfer from a cultured cell line. *Nature*, **380**: 64-66. https://doi.org/10.1038/380064a0

Chen, H., Li, B. and Workman, J.L., 1994. A histone-binding protein, nucleoplasmin, stimulates transcription factor binding to nucleosomes and factor-induced nucleosome disassembly. *EMBO.*

J., **13**: 380-390.

Chesné, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L. and Renard, J.P., 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat. Biotechnol.*, **20**: 366-369. https://doi.org/10.1038/nbt0402-366

Crabbe, J.C., Metten, P., Cameron, A.J. and Wahlsten, D., 2005. An analysis of the genetics of alcohol intoxication in inbred mice. *Neurosci. Biobehav. Rev.*, **28**: 785-802. https://doi.org/10.1016/j.neubiorev.2004.08.002

Dean, J., 1983. Decondensation of mouse sperm chromatin and reassembly into nucleosomes mediated by polyglutamic acid *in vitro*. *Devel. Biol.*, **99**: 210-216. https://doi.org/10.1016/0012-1606(83)90269-5

Dingwall, C. and Laskey, R.A., 1990. Nucleoplasmin: The archetypal molecular chaperone. *Semin. Cell Biol.*, **1**: 11-17.

Earnshaw, W.C., Honda, B.W., Laskey, R.A. and Thomas, J.O., 1980. Assembly of nucleosomes: the reaction involving *X. laevis* nucleoplasmin. *Cell*, **21**: 373-383. https://doi.org/10.1016/0092-8674(80)90474-2

Fair, T., Hyttel, P., Lonergan, P. and Boland, M.P., 2001. Immunolocalization of nucleolar proteins during bovine oocyte growth, meiotic maturation, and fertilization. *Biol. Reprod.*, **64**: 1516-1525. https://doi.org/10.1095/biolreprod64.5.1516

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. and Lazzari, G., 2003. Pregnancy: a cloned horse born to its dam twin. *Nature*, **424**: 635. https://doi.org/10.1038/424635a

Gurdon, J.B. and Wilmut, I., 2011. Nuclear transfer to eggs and oocytes. *Cold Spring Harb. Perspect. Biol.*, **3**: a002659. https://doi.org/10.1101/cshperspect.a002659

Hyttel, P., Laurincik, J., Rosenkranz, C.H., Rath, D., Niemann, H., Ochs, R.L. and Schellander, K., 2000. Nucleolar proteins and ultrastructure in preimplantation porcine embryos developed *in vivo*. *Biol. Reprod.*, **63**: 1848-1856. https://doi.org/10.1095/biolreprod63.6.1848

Jiang, L,, Jobst, P., Lai, L., Samuel, M., Avares, D., Prather, R.S. and Tian, X.C., 2007. Expression levels of growth-regulating imprinted genes in cloned piglets. *Cloning Stem Cells*, **9**: 97-106. https://doi.org/10.1089/clo.2006.0041

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. and Tsunoda, Y., 1998. Eight calves cloned from somatic cells of single adult. *Science*, **282**: 2095-2098. https://doi.org/10.1126/science.282.5396.2095

Laskey, R.A., Honda, B.M., Mills, A.D. and Finch, J.T., 1978. Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature*, **275**: 416-420. https://doi.org/10.1038/275416a0

Latham, K.E., 2004. Cloning: Questions answered and unsolved. *Differentiation*, **72**: 11-22. https://doi.org/10.1111/j.1432-0436.2004.07201003.x

Latham, K.E., 2005. Early and delayed aspects of nuclear reprogramming during cloning. *Biol. Cell*, **97**: 119-132. https://doi.org/10.1042/BC20040068

Lee, B.C., Kim, M.K., Jang, G., Oh, H.J., Yuda, F., Kim, H.J., Hossein, M.S., Kim, J.J., Kang, S.K., Schatten, G. and Hwang, W.S., 2005. Dogs cloned from adult somatic cells. *Nature*, **436**: 641. https://doi.org/10.1038/436641a

Leno, G.H., Philpott, A. and Laskey, R.A., 1993. Chromatin remodeling by nucleoplasmin. In: Flavell JSH-HaRB, editor. John Innes Symposium: "*The Chromosome*". Bios Scientific; Oxford, UK, pp. 135-147.

Li, Z., Sun, X., Chen, J., Liu, X., Wisely, S.M., Zhou, Q., Renard, J.P., Leno, G.H. and Engelhardt, J.F., 2006. Cloned ferrets produced by somatic cell nuclear transfer. *Devel. Biol.*, **293**: 439-448. https://doi.org/10.1016/j.ydbio.2006.02.016

Loyola, A. and Almouzni, G., 2004. Histone chaperones, a supporting role in the limelight. *Biochim. biophys. Acta*, **1677**: 3-11. https://doi.org/10.1016/j.bbaexp.2003.09.012

Mills, A.D., Laskey, R.A., Black, P. and deRobertis, E.M., 1980. An acidic protein which assembles nucleosomes *in vitro* is the most abundant protein in *Xenopus* oocytes nuclei. *J. mol. Biol.*, **139**: 561-568. https://doi.org/10.1016/0022-2836(80)90148-5

Oback, B., 2008. Climbing mount efficiency-small steps, not giant leaps towards higher cloning success in farm animals. *Reprod. Domest. Anim.*, **43**: 407-416. https://doi.org/10.1111/j.1439-0531.2008.01192.x

Ohsumi, K. and Katagiri, C., 1991. Characterization of the ooplasmic factor inducing decondensation of and protamine removal from toad sperm nuclei: involvement of nucleoplasmin. *Devel. Biol.*, **148**: 295-305. https://doi.org/10.1016/0012-1606(91)90338-4

Philpott, A. and Leno, G.H., 1992. Nucleoplasmin remodels sperm chromatinin *Xenopus* egg extracts.

Cell, **69**: 759-767. https://doi.org/10.1016/0092-8674(92)90288-N

Philpott, A., Leno, G.H. and Laskey, R.A., 1991. Sperm decondensation in *Xenopus* egg cytoplasm is mediated by nucleoplasmin. *Cell*, **65**: 569-578. https://doi.org/10.1016/0092-8674(91)90089-H

Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A. and Campbell, K.H., 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature*, **407**: 86-90. https://doi.org/10.1038/35024082

Prado, A., Ramos, I., Frehlick, L.J., Muga, A. and Ausió, J., 2004. Nucleoplasmin: A nuclear chaperone. *Biochem. Cell Biol.*, **82**: 437-445. https://doi.org/10.1139/o04-042

Ramos, I., Prado, A., Finn, R.M., Muga, A. and Ausió, J., 2005. Nucleoplasmin mediated unfolding of chromatin involves the displacement of linker associated chromatin proteins. *Biochemistry*, **44**: 8274-8281. https://doi.org/10.1021/bi050386w

Shi, D., Lu, F., Wei, Y., Cui, K., Yang, S., Wei, J. and Liu, Q., 2007. Buffalos (*Bubalus bubalis*) cloned by nuclear transfer of somatic cells. *Biol. Reprod.*, **77**: 285-291. https://doi.org/10.1095/biolreprod.107.060210

Shin, T., Kraemer, D., Pryor, J., Liu, L., Rugila, J., Howe, L., Buck, S., Murphy, K., Lyons, L. and Westhusin, M., 2002. A cat cloned by nuclear transplantation. *Nature*, **415**: 859. https://doi.org/10.1038/nature723

Vajta, G.L., 2007. Somatic cell nuclear transfer in its first and second decades: successes, setbacks, paradoxes and perspectives. *Reprod. Biomed. Online*, **15**: 582-590. https://doi.org/10.1016/S1472-6483(10)60391-4

Wade, P.A. and Kikyo, N., 2002. Chromatin remodeling in nuclear cloning. *Eur. J. Biochem.*, **269**: 2284-2287. https://doi.org/10.1046/j.1432-1033.2002.02887.x

Wakayama, T., Perry, A.C., Zuccotti, M., Johnsonk, K.R. and Yanagimachi, R., 1998. Full term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature*, **394**: 369-374. https://doi.org/10.1038/28615

Wani, N.A., Wernery, U., Hassan, F.A., Wernery, R. and Skidmore, J.A., 2010. Production of the first cloned camel by somatic cell nuclear transfer. *Biol. Reprod.*, **82**: 373-379. https://doi.org/10.1095/biolreprod.109.081083

Wei, H.J., Qing, Y.B., Pan, W.R., Zhao, H.Y., Li, H.H., Cheng, W.M., Zhao, L., Xu, C.S., Li, H., Li, S., Ye, L., Wei, T.Y., Li, X.B., Fu, G.W., Li, W.G., Xin, J.G. and Zeng, Y.Z., 2013. Comparison of the efficiency of banna miniature inbred pig somatic cell nuclear transfer among different donor cells. *PLoS One*, **8**: e57728. https://doi.org/10.1371/journal.pone.0057728

Woods, G.L., White, K.L., Vanderwall, D.K., Li, G.P., Aston, K.I., Bunch, T.D., Meerdo, L.N. and Pate, B.J., 2003. A mule cloned from fetal cells by nuclear transfer. *Science*, **301**: 1063. https://doi.org/10.1126/science.1086743

Zhou, Q., Renard, J.P., Le Friec, G., Brochard, V., Beaujean, N., Cherifi, Y., Fraichard, A. and Cozzi, J., 2003. Generation of fertile cloned rats by regulating oocyte activation. *Science*, **302**: 1179. https://doi.org/10.1126/science.1088313