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Requirement for nuclear autoantigenic sperm protein mRNA expression in bovine preimplantation development

Running title: *NASP* gene knockdown in bovine embryos

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ABSTRACT

Nuclear autoantigenic sperm protein, NASP, is associated with DNA replication, cell proliferation, and cell cycle progression through its specific binding to histones. The aim of this study was to examine the roles of NASP in bovine preimplantation embryonic development. Using *NASP* gene knockdown (KD), we confirmed the reduction of *NASP* mRNA expression during preimplantation development. *NASP* KD did not affect cleavage but significantly decreased development of embryos into the blastocyst stage. Furthermore, blastocyst hatching was significantly decreased in *NASP* KD embryos. Cell numbers in the inner cell mass of *NASP* KD blastocysts were also decreased compared to those of controls. These results suggest that *NASP* mRNA expression is required for preimplantation development into the blastocyst stage in cattle.

Key words: *bovine embryo, NASP, knockdown.*

INTRODUCTION

Following syngamy that is fusion of the male and female pronuclei after in vitro fertilization (IVF), the single-cell embryo, or zygote, undergoes a sequence of mitotic divisions. The process of cellular division without any accompanying increase in the volume of the cytoplasm is called cleavage, and the small cells produced by the series of early cleavage divisions are blastomeres. In mammals including cattle, preimplantation embryos are able to develop in culture media, and some can develop into the blastocyst stage. In cattle, when attempting the transfer of embryos produced by IVF to the uterus of a recipient cow, it is essential to have an in vitro culture (IVC) system for the effective production of blastocysts.

The cell number of a single bovine embryo drastically increases until the blastocyst stage during 8 d of IVC. Therefore, cleavage represents many rounds of mitosis with DNA replication. To assemble newly replicated DNA into chromatin, compensating active histone mRNA/protein synthesis occurs during S phase of the cell cycle, resulting in sufficient histone production. The nucleosome is assembled by wrapping a 146-bp DNA segment around a histone octamer that consists of two each of the core histones, H2A, H2B, H3, and H4. Repeating nucleosome units constitute chromatin. The transport of histones from the cytoplasm to the nucleus and their subsequent assembly into nucleosomes is mediated by a set of proteins that includes histone chaperones. Nuclear autoantigenic sperm protein, NASP, is one of these histone chaperones; it protects the H3–H4 tetramer from degradation in human cells (Cook *et al.* 2011), and transports linker histone H1, which is essential for normal development, into nuclei (Bustin *et al.* 2005; Alekseev *et al.* 2005).

NASP is associated with DNA replication, cell proliferation, and cell cycle progression, specifically binding to histones H1, H3, and H4, and affecting chromatin assembly (Finn *et al.* 2012). NASP was first characterized in rabbit testes, and is conserved in a wide range of species (Welch &

O'Rand 1990; Finn *et al.* 2012). NASP is involved in chromatin remodeling, which maintains the nucleosome spacing that is critical for the regulation of gene expression and for normal development. The essential role of NASP in early murine embryonic development is underscored by the report that *NASP*-/- null mice showed lethality at embryonic day 5.5 with implantation failure (Richardson *et al.* 2006).

In a previously reported study, we addressed the site-predominant expression of the *NASP* mRNA in the inner cell mass (ICM) of in vitro-derived blastocysts (Nagatomo *et al.* 2013). Little is known, however, as to whether NASP plays roles in preimplantation development in cattle. To elucidate the role of NASP during bovine preimplantation development, we used knockdown (KD) of *NASP* gene expression and evaluated the ability of *NASP* KD embryos to develop into the blastocyst stage.

MATERIALS AND METHODS

Preparation of bovine embryos by in vitro fertilization

Bovine oocyte retrieval, in vitro oocyte maturation, fertilization, and subsequent in vitro bovine embryo culture were performed according to the methods described in a previous paper (Nagatomo *et al.* 2013). Briefly, cumulus–oocyte complexes (COCs) collected from slaughterhouse-derived ovaries were matured by culturing in TCM-199 medium (Gibco, Grand Island, NY, USA) at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 20–22 h. In vitro-matured oocytes were transferred to Brackett and Oliphant (BO) medium (Brackett & Oliphant 1975) containing 2.5 mM theophylline (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Subsequently, frozen-thawed semen was centrifuged at 600 × *g* for 8 min in BO medium, and the spermatozoa were added to the COCs at a final concentration of 5×10^6 cells/mL. After 18 h of incubation, presumptive zygotes were denuded and cultured in mSOFaa medium (Aono *et al.* 2013) at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 8 d.

Microinjection of *NASP* short hairpin RNA (shRNA) expression vectors

An RNA interference hairpin, with antisense/sense regions, a 12-bp stem loop (GCTTCCTGTCAC), and a 6-bp terminator element (TTTTTT), was designed to target nucleotides 878-898 of the NASP mRNA (GenBank accession number NM_001038088.2). The sense- and antisense-strand oligonucleotides (Sigma-Aldrich Japan, Tokyo, Japan) were denatured at 95°C for 5 min, and then gradually cooled to 25°C for annealing. The double-strand DNA was ligated downstream of the U6 promoter in the pBAsi/mU6 Neo vector (Stratagene, La Jolla, CA, USA). The following sequence was used: 5′- GATCCGGAACTACAGGAGAAAGTTAAGCTTCCTGTCACTTAACTTTCTCCTG TAGTTCCTTTTTTA -3′ (underlined and double-underlined portions indicate the stem-loop region

and terminator element, respectively). The shRNA expression vector (pBAsi/mU6/NASP-I) targeting the *NASP* mRNA (*NASP* shRNA) was isolated using the EndoFree Plasmid ezFlow Mini Kit (Biomiga, Inc., San Diego, CA, USA). Twelve h after the completion of insemination, the synthesized *NASP* shRNA (10 nG/μL) was then injected into cytoplasm of each bovine zygote by using a FemtoJet® injection device (Eppendorf, Hamburg, Germany). After *NASP* KD, *NASP* mRNA expression was confirmed by quantitative reverse transcription-PCR using the same procedure as reported previously (Nagatomo *et al.* 2013). The zygotes injected with *NASP* shRNA were cultured, to examine the effect of *NASP* KD on subsequent embryonic development into the blastocyst stage. Zygotes injected with the pBAsi/mU6 Neo vector without the *NASP* shRNA insert were used as a control in these experiments.

Assessment of development to the blastocyst stage and the competence of hatching from the zona pellucida

Rates of cleavage and blastocyst formation were assessed on day 2 (D2) and day 8 (D8) of IVC, respectively. Embryos that developed into the blastocyst stage on D8 were allotted to experiments of differential staining or cultured for a further 24 h to assess the number of blastocysts that showed hatching from the zona pellucida.

Differential staining of blastocysts

The differential staining of the ICM and trophectoderm (TE) within a blastocyst was performed according to the method of Thouas et al. (Thouas *et al.* 2001). Briefly, the blastocysts were stained with propidium iodide (Life Technologies, Carlsbad, CA, USA) as a 0.1 mg/mL solution in phosphate-buffered saline containing 0.2% (v/v) Triton X-100 at room temperature for approximately 2 min, followed by staining with 25 μg/mL bisbenzimide (Sigma-Aldrich) in 99.5%

ethanol at 4°C for 3 h. After washing with glycerol, the blastocysts were mounted on a glass slide, and observed with a fluorescence microscope (EVOS® Cell Imaging System, Advanced Microscopy Group, Mill Creek, WA, USA). The ICM and TE nuclei were differentially stained blue and pink, respectively.

Statistical analysis

Statistical analyses of all data were carried out by one-way analysis of variance and Fisher's post-hoc least significant difference test using the Statview statistical-analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). A value of $P < 0.05$ was considered significant.

RESULTS

Knockdown of *NASP* mRNA expression in bovine preimplantation embryos

To examine the role of NASP in early bovine embryogenesis, we first performed an RNA interference experiment by microinjecting *NASP* shRNA expression vectors into in vitro-derived zygotes. The relative expression level of the *NASP* mRNA was significantly decreased in *NASP* KD embryos during preimplantation development (at the 2–4-cell and blastocyst stages; $P < 0.05$) (Fig. 1). Although there were no significant differences at the 8–16-cell and morula stages, the *NASP* mRNA tended to be decreased. During these stages, the *NASP* mRNA expression might be low in bovine embryos. The *NASP KD* embryos showed a lower rate of blastocyst formation compared to controls (43.2 ± 5.8% vs 28.4 ± 7.2%; $P < 0.05$) (Table 1). However, KD of *NASP* did not affect the cleavage rate. Taken together, these results clearly show the importance of *NASP* mRNA expression for embryonic survival during preimplantation bovine embryonic development.

Effects of *NASP* knockdown on ICM cell number and subsequent hatching of blastocysts

Because we previously observed ICM-predominant expression of the *NASP* mRNA (Nagatomo *et al.* 2013), we further investigated the ratio of ICM cell number to total cell number in *NASP* KD blastocysts by the detergent procedure for differential staining of ICM and TE cells (Thouas *et al.* 2001). The proportion of ICM cells in blastocysts was significantly decreased in the *NASP* KD compared to the control (Fig. 2A and B). Next, we investigated the number of blastocysts showing hatching from the zona pellucida in *NASP* KD blastocysts. At 24 h after IVC for 8 d, the ratio of blastocysts showing hatching to total blastocysts was significantly decreased in the *NASP* KD compared to the control (Table 1). These findings clearly demonstrate the requirement of *NASP* gene expression for the normal formation of blastocysts in cattle.

DISCUSSION

The distinctiveness of bovine embryos is reflected in the lack of a precedent for the successful establishment of embryonic stem (ES) cells, trophoblast stem cells, and even induced pluripotent stem (iPS) cells, which might be due to insufficient genetic information on reliable marker genes in the ICM and TE cell lineages in cattle. We established a technique to determine which genes are expressed in a lineage-specific manner in the ICM and TE of bovine blastocysts, and reported 12 genes with novel site-predominant expression in bovine blastocysts. However, the biological functions of each gene have remained unknown (Nagatomo *et al.* 2013).

NASP was one of the genes showing ICM-predominant expression in bovine blastocysts, and encodes a histone chaperone protein that plays essential roles in mitosis. The mammalian *NASP* gene has a high degree of sequence similarity to a *NASP* homologue in *Xenopus laevis*, called N1/N2 (O'Rand *et al.* 1992). A significant relationship between NASP and carcinogenesis has been indicated in a variety of cancers, such as renal cell carcinoma, ovarian cancer, and prostate cancer (Alekseev *et al.* 2011; Chatterjee *et al.* 2006; Fang *et al.* 2015). In the cancer model, it is assumed that an alteration of NASP levels changes the amount of histone H3/H4 within a cell, resulting in impaired genome integrity, chromatin stability, and replication error (Cook *et al.* 2011); eventually, cells that show an abnormal level of NASP would be transformed into tumor cells.

Interestingly, pluripotent cells such as ES cells and iPS cells share a number of characteristics with cancer cells, including self-renewal, proliferation, and expression of stem-cell markers (Grskovic & Ramalho-Santos 2008). In the present study, the stable KD of *NASP* mRNA expression during bovine preimplantation development caused not only a decrease in blastocyst formation but also a reduction of ICM cell proportion. These results are consistent with the previous observation in murine research that blastocysts from the *NASP-/-* null mouse completely failed to

proliferate after outgrowth on a culture dish (Richardson *et al.* 2006). We previously found that the *NASP* mRNA was predominantly expressed in the ICM of bovine blastocysts (Nagatomo *et al.* 2013), suggesting that NASP plays an essential role in the normal proliferation of the ICM in bovine blastocysts. Furthermore, the present study revealed that blastocyst hatching from the zona pellucida was inhibited in *NASP* KD embryos. This might be due to the depletion of histones H3 and H4 being required for acute DNA replication during embryo cleavage in *NASP* KD embryos. In fact, *NASP* expression is high in ES cells and low in differentiated cells (Sun *et al.* 2008; Yocum *et al.* 2008). Further studies are required to elucidate the function of NASP, and possibly other histone chaperones, in lineage specification, maintenance, proliferation, and differentiation in bovine embryos during preimplantation development.

Conclusions

Here, we have demonstrated that KD of the *NASP* gene disrupts development into the blastocyst stage and subsequent hatching from the zona pellucida in bovine embryos. These findings show that *NASP* mRNA expression is essential for normal bovine embryonic development. To the best of our knowledge, this is the first report of *NASP* function in early embryos of cattle.

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FIGURE LEGENDS

Figure 1 Effect of *NASP* gene knockdown from the 2–4-cell stage to the blastocyst stage in bovine embryos. The *NASP* mRNA level was assessed by quantitative revere transcription PCR. The results are shown as the mean ± SEM (error bars) of three replicate experiments, * $P < 0.05$. KD (knockdown): black. Control: white.

Figure 2 Effect of *NASP* gene knockdown on development of the inner cell mass. (A) The ratio of inner cell mass (ICM) cell number to total cell number in control and *NASP* knockdown (KD) blastocysts. The results are shown as the mean ± SEM (error bars), * $P < 0.05$. The number of embryos analyzed was 12 for the control and 10 for the *NASP* KD. (B) Representative blastocysts differentially stained by both bisbenzimide and propidium iodide in control and *NASP* KD embryos. Arrowheads indicate the ICM. Blue: nuclei in the ICM; pink: nuclei in the TE.

Table 1. Effect of *NASP* gene knockdown on the in vitro development of bovine embryos

	No. of embryos examined	No. of embryos cleaved (% ± SEM)	No. of blastocyst (% ± SEM)	No. of hatched blastocyst (% ± SEM) (/blastocyst)
control	74	59 (67.6 ± 4.0)	32 (43.2 ± 5.8)	7 (21.8 ± 2.9)
KD	95	67(70.5 ± 9.2)	27 (28.4 ± 7.2)*	2 (7.4 ± 3.1)*

* $P < 0.05$

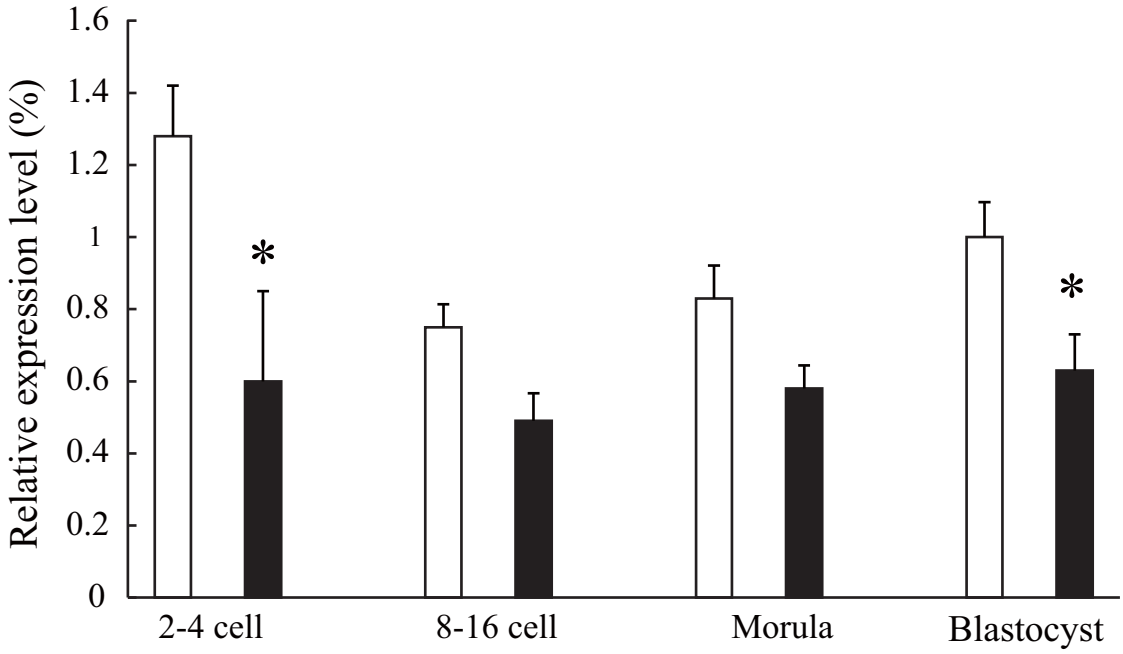
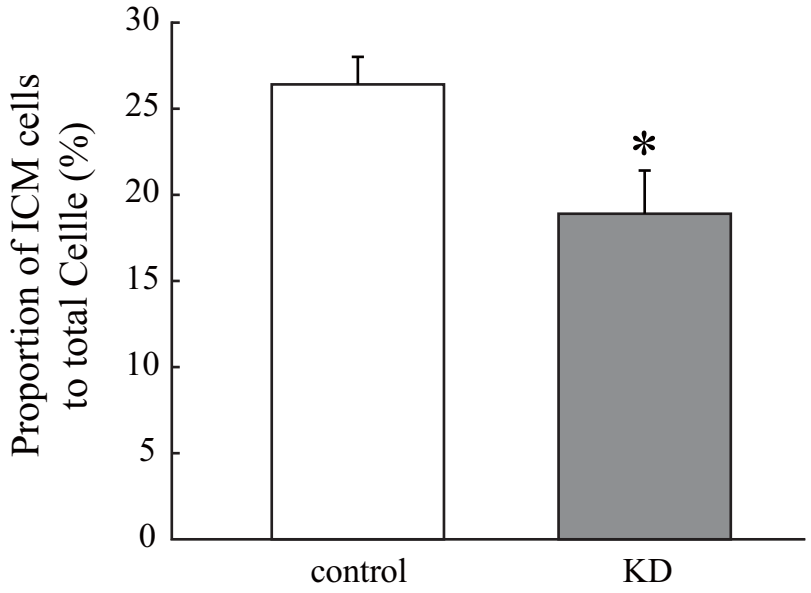

Relative expression level (%)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
2-4 cell
8-16 cell
Morula
Blastocyst

Figure 1

A


Proportion of ICM cells to total Cellle (%)
30
25
20
15
10
5
0
*
control
KD

B

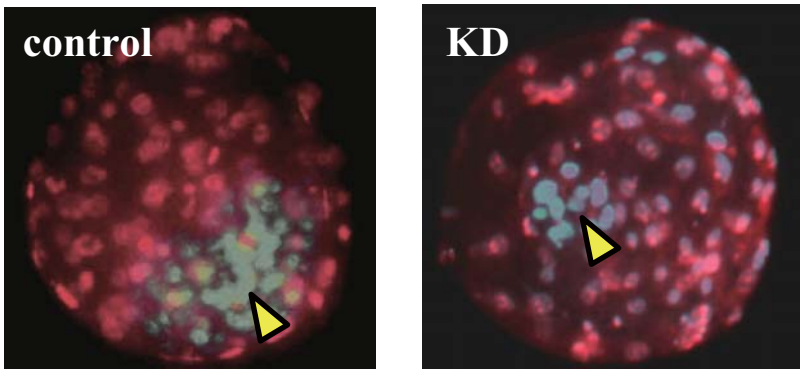

control
KD

Figure 2