



Targeted Suppression of Connexin 43 in Ovine Preimplantation Embryos by RNA Interference Using Long Double-stranded RNA

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ABSTRACT : RNA interference (RNAi) is an acknowledged useful and effective tool to study gene function in various cells. Here, we suppressed the Connexin 43 (Cx 43) gene expression during *in vitro* development of ovine pre-implantation embryos using the RNAi method. The 353 bp Cx 43 double-stranded RNA was microinjected into *in vitro* fertilized ovine zygotes, and the levels of target mRNA and protein were investigated. Control groups included uninjected zygotes or those injected with RNase-free water. The dsRNA injection resulted in the specific reduction of Cx 43 transcripts as analyzed by quantitative real-time RT-PCR and decreased protein levels as shown by Western blot analysis at the blastocyst stage. Microinjection of Cx 43 dsRNA led to 20.3%, 21.7% and 34.5% blastocyst rates and 19.2%, 37.5% and 41.3% hatched blastocyst rates in Cx 43 dsRNA-injected, water-injected and uninjected groups, respectively. Then the RNAi could not significantly affect cell number and cell death rates of blastocysts. Therefore, suppression of Cx 43 dsRNA and proteins did not apparently affect the development potential of ovine pre-implantation embryos but may play a role in embryo quality. RNAi technology is a promising approach to study gene function in early ovine embryogenesis. (**Key Words :** Connexin 43, RNA Interference, Ovine Embryo)

INTRODUCTION

It is a well-documented fact that there are significant differences in quality and developmental competence between embryos produced *in vivo* and *in vitro*. Folliculogenesis in mammals occurs over a long period requiring abundant RNA and protein to be synthesized and accumulated to sustain many processes, including oocyte maturation, fertilization and pre-implantation embryo development (Adona et al., 2008). However, embryos produced *in vitro* often exhibit low quality and developmental ability due to the short duration of maturation and non-stabilizing culture conditions (Rizos et al., 2002b), and these differences are affected by the expression pattern of embryogenesis-related genes (Wrenzycki et al., 1999).

Most recent studies on this subject describe differential gene expression in embryos from varieties of sources in an attempt to understand the underlying regulatory networks

that define embryo quality. However, only a few studies have concentrated on the functionality of the genes described. Some gene knock-out experiments have been performed in mice (Larue et al., 1994; Riethmacher et al., 1995); however, these knock-out technologies are extremely laborious and require a long period of time to achieve the effects, and it is not feasible for larger animals. The RNA interference (RNAi) approach utilizing introduction of sequence specific long double-stranded (ds) RNA into cells was first successfully performed in *Caenorhabditis elegans* and became an effective method to study gene function in this species (Fire et al., 1998). Due to its relative ease of use and effectiveness, the RNAi method has been used to study gene function during pre-implantation embryogenesis in mammalian species, including mouse (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000), pig (Cabot et al., 2002; Cabot and Prather, 2003) and bovine (Nganvongpanit et al., 2006). However, until now, there have been no similar reports utilizing RNAi investigating gene function in ovine pre-implantation embryos.

The mechanism of RNAi is known to be a highly conserved process which interferes or limits the transcript level by either suppression of transcription (transcriptional gene silencing; TGS) or by activating a sequence-specific

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RNA degradation process (post-transcriptional gene silencing; PTGS). In these processes, dsRNA is cleaved into small strands of about 21-23 nucleotides by Dicer which eventually destroys the integrity of target mRNA molecules. However, the application of RNAi in pre-implantation animals was previously limited to studies in mice (Wianny and Zernicka, 2000; MacIntyre et al., 2002; Lykke-Andersen et al., 2008). In recent years, dsRNA was introduced into cattle oocytes and embryos produced *in vitro*. In 2005, CyclinB1 was the first gene to be successfully suppressed by RNAi in bovine embryos produced *in vitro* (Paradis et al., 2005); injection of CyclinB1 dsRNA resulted in significant reductions in the amount of CyclinB1 mRNA and subsequent reduction in its protein synthesis. Gradually, various genes related to embryogenesis have since been studied by dsRNA-mediated RNAi. Some studies showed that sequence-specific dsRNA directed against maternal or embryonic transcripts resulted in a significant reduction of the corresponding mRNA and protein products and affected some aspects of development (Nganvongpanit et al., 2006).

Prior to the compaction, cells contact each other loosely and may communicate each other via cytoplasmic bridges or mid bodies. However, cellular inter-relations changed dramatically during compaction. The cells compact down on each other to minimize the intercellular space and they begin to communicate each other via the intercellular structure, the gap junction. Molecules and information communicate through gap junctions are important for compaction, which ensure the subsequent establishment of the separate identities of outer cells, which will form the trophectoderm, and inner cells, which will produce the embryo proper. So that, blastocyst formation begins at compaction, and the onset of cellular differentiation is mediated by transfer through the intercellular connections between the neighboring blastomeres. The blastomeres subsequently differentiate into the trophectoderm and the inner cell mass. In cattle, compaction occurs at the 16- to 32-cell stage (Betteridge et al., 1989), but the degree of which this happens is lower in *in vitro*-produced embryos compared to those derived *in vivo* (Prather and First, 1993). This is may be attributed to the reduction of intercellular communicative molecules and expression of genes related to compaction and cellular adhesion (Cx43, E-Cadherin, desmocollin II) (Wrenzycki et al., 1996; Wrenzycki et al., 2001; Rizos et al., 2003). Previous studies showed that suppression of gap junctional proteins in mouse compacted embryos could affect the physiological function of junctional apparatus. Injection of Lucifer Yellow, a fluorescent dye injected into single blastomere to detect the effect of RNAi in compacted embryos, could not transfer into all the blastomeres (Bevilacqua et al., 1989). And another experiment showed that single blastomere injected

with anti-sense RNA could be extruded from the compacted embryo (Lee et al., 1987). These results showed that RNAi presumably disrupt gap junction synthesis and the normal pathway of junctional communication, which in turn leads to discompaction (Bevilacqua et al., 1989). Therefore, it appears that functional gap junctions are necessary for the normal progression of embryogenesis.

As an important family member of gap junctional proteins, the abundance of Cx43 is considered to be related to the quality of embryos. Longeran et al. demonstrated that the abundance of Cx43 transcripts could be altered in different culture condition, and this pattern of expression reflects the quality of blastocysts measured in terms of cryotolerance (Rizos et al., 2003). Ghassemifar et al. also showed that the level of junctional transcript was positively correlated to the morphological grade (Ghassemifar et al., 2003). Despite the predicted role of Cx43 in affecting the quality of blastocysts, no information is available on the effect of suppressing this transcript in the developmental potential of embryos in ovine. Therefore, in the present study we targeted the ovine Cx43 gene via microinjection of sequence-specific dsRNA at the zygote stage and observed its effects on mRNA and protein expression and the development potential of ovine embryos.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, all the chemicals were purchased from Wako Pure Chemical Industries, Ltd, Japan. Phosphate-buffered saline (PBS), tissue culture medium 199 (TCM199), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol were purchased from Sigma-Aldrich, St Louis, USA.

Synthesis of DNA template

The method of synthesis of template DNA and the corresponding dsRNA was performed as described by Nganvongpanit et al. (Nganvongpanit et al., 2006). For amplification of Cx43, we designed primers using the Primer 5.0 software according to the cDNA sequence published in Genbank (Table 1). We amplified the corresponding amplicon and the identity of the product was confirmed by sequencing. The Premix Taq Polymerase was used in the first round of PCR with the following conditions: pre-denaturation for 3 min at 94°C; 35 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 40 sec; and a final elongation step at 72°C for 10 min. Subsequently, the pfu polymerase was used in the second PCR with the same conditions as the first PCR. The product of the second PCR was used as template for the third PCR, again with the same conditions, and a T7 promoter was attached to the 5'-end of upstream primer and

Table 1. Primers used for dsRNA preparation and quantitative RT-PCR

Gene	GenBank accession number	Primer sequences	Annealing temperature (°C)	Product size (bp)
Connexin 43 ^A	AY074716	5'-ATACGACTCACTATAGGG TCCGAATCCTGCTACTGG-3' 5'-GATTTAGGTGACACTATAGA CCCTCGCATCTTCACCTT-3'	55	353
Connexin43 ^B	AY074716	5'- TCCGAATCCTGCTACTGG-3' 5'- CCCTCGCATCTTCACCTT-3'	55	353
Connexin 43 ^C	AY074716	5'-TCTTCCTTTCGCATCACAT-3' 5'-GACAAATCCTTCCCAATCT-3'	60	113
Connexin 45	NM001080383.1	5'-CAGCAAGCCCTACGCAATG-3' 5'-AAACCCGCCTCAAACACG-3'	60	234
β-actin	NM_173979.3	5'-TCTTCCAGCCGTCCTTCT-3' 5'-TGCCAGGGTACATGGTGGT-3'	60	144
β-catenin	BT020888	5'-ATTCAGCAGAAGGTCCGAGTGC-3' 5'-GTTGAAGCTACTGCCTCCGGTC-3'	60	208
E-Cadherin	AY508164	5'-GACACTGGAGGTATCAGCGCAC-3' 5'-TGATCTGGACCAGCGACTT-3'	60	194

^A Primers coupled with T7 or SP6 promoter used for dsDNA template amplification.

^B Primers used for dsRNA preparation. ^C Primers used for Real-time quantitative PCR.

a SP6 promoter was used by the downstream primer. The modified primers were used to generate two different templates for *in vitro* transcription to produce sense and antisense RNA strands; all the reagents including T7 and SP6 polymerase used for this procedure were obtained from Takara Biotechnology Co., Ltd.).

Synthesis of dsRNA

The templates for producing dsRNA were digested by RNA-free DNase I at 37°C for 10 min. Subsequently, equal volumes of sense and antisense RNA strands were mixed for 4 min at 68°C to denature and then maintained at room temperature at least 4 h. Following a phenol/chloroform extraction, a 0.1 volume of 3 M Sodium acetate and 2.5 volume of 100% ethanol were used to precipitate the dsRNA and pelleted by centrifugation at 12,000 rcf at 4°C. Finally, the dsRNA pellet was washed by 70% ethanol and resuspended by a suitable volume of DEPC treated water. The concentration of dsRNA was evaluated by a spectrophotometer, and the solution was diluted to a final concentration of 200 ng/μl as a final concentration with DEPC treated water to be stored at -80°C until use. The size and the purity of the dsRNA was evaluated by loading 2 μl per well onto a 2% agarose gel and visualizing under UV light (Figure 1).

Oocytes collection and *in vitro* maturation

Ovine ovaries were obtained from a local slaughterhouse and transported to the laboratory within 2-3 hours in a thermo flask containing 0.9% saline at 37°C. Cumulus-oocyte complexes (COCs) were recovered by

cutting the follicles on the ovary with a blade in PBS solution. Those COCs with multiple layers of cumulus cells were selected for *in vitro* maturation. The COCs were washed three times in maturation medium (TCM199+2×10⁻⁴ mol/L pyruvate+10 IU/ml LH+10%FSH+10⁻² mol/Hepes+10% ovine estrus serum (OES, prepared by our lab)) before culturing. The COCs were cultured in groups of 80 in 800 μl maturation medium for 24 h at 38.5°C in an incubator with a humidified atmosphere of 5% CO₂.

In vitro fertilization (IVF) of oocytes

After maturation, COCs were washed three times in fertilization medium (SOF+20% ovine estrus serum+amino acids) before transfer into fertilization droplets covered by mineral oil (20 COCs/90 μl). For IVF, frozen thawed semen was prepared by a 45%-90% percoll gradient centrifugation technique (Wang et al., 1998), and separated spermatozoa were added to the fertilization droplet at a final concentration of 2×10⁶ sperm/ml. The mixture was cultured for 20 h under the same conditions as that for maturation.

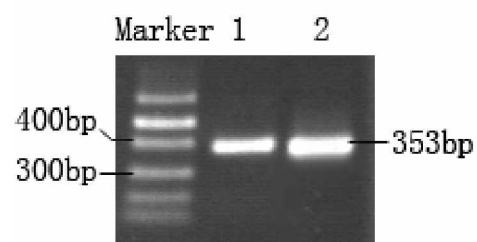


Figure 1. Agarose gel electrophoresis of Cx43 (353 bp) dsRNA compared with DNA template used for *in vitro* transcription. 1- Cx43 DNA, 2- Cx43 dsRNA.

***In vitro* culture of embryos**

After fertilization for 18-20 h, the presumptive zygotes were denuded of cumulus cells and attached sperms by repeated puffing with a pipette. Zygotes were divided into 3 groups: injected with Cx43 dsRNA (43 group), injected with DEPC treated water (water group) and uninjected controls (control group). After manipulation, zygotes were washed 3-4 times by fresh culture medium and cultured in groups of 25-30 zygotes per 30 μ l culture medium until day 7 after fertilization. The culture medium was SOF medium supplemented with 10% OES, 3% essential amino acids and 1% nonessential amino acids. Cleavage rate was detected 48 h after insemination, and the morula, blastocyst and hatching blastocyst rates were assessed at day 5, day 7-8 and day 8-10 after fertilization, respectively. 7-10 blastocysts from each group were treated by PI and Hoechst 33342 at day 7 to assess the cell numbers and cell dead rate of blastocysts. *In vitro* culture was performed at 38.5°C in an incubator with a humidified atmosphere of 5% CO₂.

Microinjection of dsRNA

Three groups of 40 presumptive zygotes were placed into a 100 μ l injection droplet (TCM-199+20 mM HEPES+7.5 μ g/ml cytochalasin B) covered by mineral oil. The injection was performed under an inverted microscope at magnification 200 \times . The injection volume was estimated from the displacement of the meniscus of mineral oil in the capillary. After manipulation, zygotes were washed three times with the culture medium and placed back in culture. The dead zygotes were removed 4 h later.

RNA extraction and reverse transcription

Total RNA was extracted from 10 frozen embryos in different groups using an RNA isolation mini kit (Wastonbiot, Shanghai, China) following the manufacturer's instructions. The reverse transcription reaction was performed with Reverse Transcriptase XL (AMV) (Takara biotechnology, Dalian, China).

Quantitative real-time PCR

Quantitative real-time PCR was performed to assess the expression of Cx 43 and β -actin mRNA in the embryos of each treatment group. In addition, three transcripts related to embryo cell-to-cell adhesion, Connexin 45 (Cx 45), β -catenin and E-Cadherin, were also quantified in all three groups to detect the specificity of mRNA suppression by the dsRNA. The quantitative analysis using SYBR[®] Premix Ex TaqTM (Takara Biotechnology (Dalian) Co., Ltd.) was performed by the ABI Prism[®] 7300 system (Applied Biosystems, Foster City, CA). Quantitative analysis of embryo Cx 43, Cx 45, E-cadherin and β -catenin were run in

separate wells and compared with β -actin as an endogenous control. Real-time PCR was carried out using 2 μ l of each sample cDNA along with specific primers. The primer sequences were designed for PCR amplification according to the sequences published on NCBI (Table 1) using Primer Express[®] Software v3.0 (Applied Biosystems). The PCR was performed with triplicate 20 μ l reactions for each cDNA sample to ensure reproducibility of the results. Universal thermal cycling parameters (first denaturation step at 95°C for 30 sec, 40 cycles of denaturation at 95°C for 5 sec, and 60°C for 31 sec) were used to detect the expression of target genes. After the last cycle, a dissociation curve was generated by first collecting fluorescent signals at 60°C and taking measurements at 7 sec intervals with increasing temperature until it reached 95°C. The quantity of dsRNA was calculated using the $\Delta\Delta$ Ct method (Winer et al., 1999; Schmittgen et al., 2000; Livak and Schmittgen, 2001). The $\Delta\Delta$ Ct method uses a single sample, termed the calibrator sample, to compare the unknown sample gene expression level. The formula is:

$$\text{Fold induction} = 2^{-\Delta\Delta\text{Ct}}$$

where $\Delta\Delta$ Ct = (CtGI (unknown sample)-Ct β -actin (unknown sample))-(Ct GI (calibrator sample)-Ct β -actin (calibrator sample)). GI represent the gene of interest. The uninjected control group was chosen to be the calibrator sample which represented 1 \times expression of the gene of interest. β -Actin as a universal housekeeping gene was used as the endogenous control. For the $\Delta\Delta$ Ct calculation to be valid, the amplification efficiencies of the target and reference were optimized to be approximately equal. Finally, the results are reported as the relative expression level compared to the calibrator cDNA after normalization of the transcript levels to the endogenous control.

Western blot analysis

Groups of 50 blastocysts from treated groups were used for Western blot analysis. Embryos were transferred into homogenization buffer containing 1 mM EDTA, 10 mM Tris (pH 7.6), 100 mM NaCl, 1 μ g/ml Aprotinin and 1 mM PMSF. Then, 2 \times loading buffer containing 100 mM Tris (pH 6.8), 1% β -mercaptoethanol, 0.2% bromophenol blue, 20% glycerol and 2% SDS was added before boiling at 95°C for 4 min. Lysates of embryos at different stages were loaded on 5% stacking gel and 12% separating polyacrylamide gel. After electrophoresis, the proteins were transferred onto a PVDF membrane (Amresco Inc, Ohio, USA). The membrane was incubated with primary antibody (rabbit polyclonal anti-cnx43, Santa Cruz, 1:200 dilution) for 3 h after blocking overnight, followed by incubation with the anti-rabbit secondary IRD700 antibody (1:3,000

Table 2. Effect of injection on embryos viability

Groups	Total zygotes	Viable zygotes	Dead zygotes	Rate of viability (%)
Cx43 group	864	679	185	78.6 ^A
Water group	655	502	153	76.6 ^A
Control group	308	302	6	98.1 ^B

The different letter superscripts indicate significant difference in the same column ($p < 0.01$).

dilution, Rockland Inc, Pennsylvania, USA). The immunoreactive bands were detected by Odyssey Infrared Imaging System (LI-COR Bioscience, Nebraska, USA).

Statistical analysis

The relative expression data were analyzed using the SPSS 11.0 software. The frequencies of dead cells in blastocysts and the development rates were analyzed by χ^2 -test. Differences in mean values between experimental groups or developmental stages were tested using one way ANOVA with a multiple pair wise comparisons. P values of less than 0.05 were considered statistically significant.

RESULTS

In vitro development of zygotes after microinjection

Assessment of the mechanical damage to the zygotes was performed 4 h after microinjection. The percentages of zygotes from the Cx43 group, water group and control group died from the microinjection procedure were 21.4%, 23.4% and 1.9%, respectively (Table 2). To rule out the effect by mechanical damage, only zygotes surviving microinjection were chosen to calculate subsequent developmental statistics, and all dead zygotes were removed. Zygotes with grey cell plasma and damaged zona pellucida were considered to be dead. The cleavage rate of embryos was estimated 48 hours after microinjection, and no significant differences were observed between groups from

different treatment ($p > 0.05$). The type and proportion of embryos present at this development stage between the three groups was comparable, because of the presence of heterogeneous stages of embryos. We observed that 64-68% embryos were at the 8-cell stage, 10-14% were at the 4-cell stage and 4-5% were at the 2-cell stage. The remaining 15-18% were noncleaved and abnormal zygotes (Table 3).

In vitro development of 4- and 8-cell stages embryos

The *in vitro* development competence of 4- and 8-cell stages embryos was assessed at day 8 after fertilization, and the hatching rate was assessed at day 8-10. As shown in Table 4, blastocyst and hatch blastocyst rates of 4- and 8-cell stages embryos were 20.3%, 21.7% and 34.5% and 19.2%, 37.5% and 41.3% from the Cx43 group, water group and control group, respectively. Also in Table 4, cell numbers and means of cell dead rates of blastocyst were 74, 76 and 83 and 24.6%, 15.4% and 18.2%, respectively. The different letter superscripts indicate significant differences in the same column ($p < 0.05$).

The effect of Cx43 dsRNA on target mRNA expression at the blastocyst stage after culture *in vitro*

Embryos microinjected with Cx 43 dsRNA at the zygote stage could trigger the suppression in the amount of target mRNA at the blastocyst stage. Compare to the uninjected control group, the relative expression level of Cx 43 mRNA were 0.26 \times and 1.2 \times in Cx 43 dsRNA injected group and

Table 3. *In vitro* development of ovine zygotes during the 48 h period after microinjection with dsRNA or water

Groups	Zygote alive	Cleavage rate (%)	No.(%) of embryos 48 h after treatment			
			2-cell	4-cell	8-cell	Non-cleaved or abnormal
Cx43 group	679	83.7	31 (4.6)	99 (14.6)	438 (64.5)	111 (16.3)
Water group	277*	84.5	13 (4.7)	30 (10.8)	191 (68.9)	43 (15.50)
control group	302	81.5	14 (4.6)	31 (10.3)	201 (66.6)	56 (18.5)

* Not all 502 live zygotes were used in this experiment; only 277 presumptive embryos were used.

Table 4. *In vitro* development of 4- and 8-cell stage embryos from each group until day 8 or day 10

Groups	No.of embryos	No.of blastocysts	No.of hatching blastocysts	Means of cell numbers of blastocysts	Means of dead cells and dead rates (%)	Blastocyst rate (%)	Hatching blastocyst rate (%)
Cx43 group	537	109	21	76	18.7(24.6)	20.3 \pm 1.83 ^A	19.2 \pm 1.18 ^A
Water group	221	48	18	74	11.7(15.4)	21.7 \pm 1.16 ^A	37.5 \pm 1.06 ^B
Control group	232	80	33	83	15.1(18.2)	34.5 \pm 2.18 ^B	41.3 \pm 3.38 ^B

The different letter superscripts indicate significant difference in the same column ($p < 0.05$).

Table 5. (a) The $\Delta\Delta\text{Ct}$ method of interpreting gene expression data

Treatment	Cx43 Ct	Mean \pm SEM	β -actin Ct	Mean \pm SEM	E-cadherin Ct	Mean \pm SEM	Cx45 Ct	Mean \pm SEM
Cx43 dsRNA injected group	22.17	21.97 \pm 0.13	15.86	16.00 \pm 0.15	27.37	27.30 \pm 0.06	29.02	29.05 \pm 0.06
	21.71		15.79		27.37		29.10	
	22.03		16.34		27.17		29.03	
Water group	21.68	21.62 \pm 0.12	17.46	17.33 \pm 0.05	29.15	29.10 \pm 0.20	29.47	29.10 \pm 0.16
	21.36		17.29		29.47		28.83	
	21.81		17.25		28.68		28.99	
Control group	20.58	20.37 \pm 0.13	16.47	16.34 \pm 0.07	28.22	28.05 \pm 0.08	28.55	29.18 \pm 0.15
	20.29		16.36		28.01		29.16	
	20.24		16.21		27.92		28.91	

The Ct values for Cx43, β -actin, E-cadherin and Cx45 mRNA extracted from different groups were showed. All the groups were repeated triplicate.

Table 5. (b) Comparison of fold change in RNA transcript levels over control calculated by the $\Delta\Delta\text{Ct}$ method

Treatment	Cx43	Cx45	β -catenin	E-Cadherin
Uninjected control	1	1	1	1
Water injected	1.2	1.13	1.16	1.23
Cx43 dsRNA injected	0.26	1.03	0.9	0.92

All relative amounts of mRNA in water injected groups and Cx 43 dsRNA injected groups were compared to corresponding uninjected group which was considered as 1 fold.

water injected group, respectively. The result showed that there was a sharp decrease in the amount of target mRNA. In order to confirm the specificity of dsRNA in suppression of target transcripts, three functionally related genes (Cx 45, β -catenin and E-Cadherin) and one housekeeping gene were used for the analysis of mRNA relative abundance. Compare to the uninjected control group, the results showed that the expression of Cx 45, β -catenin and E- Cadherin transcripts were 1.03 \times , 0.9 \times , 0.92 \times in Cx 43 dsRNA injected groups and 1.13 \times , 1.16 \times , 1.23 \times in water injected groups, respectively. And there were nearly no change of mRNA amounts in these control genes. The statistical results of Ct value and concrete fold changes are shown in Table 5(a) and Table 5(b), respectively.

Effect of Cx 43 dsRNA on protein levels in blastocysts derived *in vitro*

In order to investigate the effect of Cx 43 dsRNA on the expression of corresponding protein products, Western blot analysis was performed using protein extracted from blastocysts of the three treatment groups: Cx 43 dsRNA-injected, water-injected and uninjected groups after *in vitro* culture. A significant reduction Cx 43 protein was observed in the Cx 43 dsRNA-injection group compared to the water-injected and uninjected groups (Figure 2). This result was consistent with the observations from the measurements of mRNA transcript abundance.

DISCUSSION

In the present study, we confirmed that microinjection

of Cx 43 dsRNA to *in vitro*-derived ovine zygotes could not significantly alter the proportion of blastocyst yield but Cx 43 may reflect in blastocyst quality. In addition, we investigated for the first time the effect of suppressing developmentally relevant transcripts in ovine embryos. We demonstrated that microinjection of dsRNA into ovine embryos before the first cleavage could trigger sequence-specific suppression of target mRNA and lead to post-transcriptional silencing of gene expression.

RNAi can down-regulate gene expression by either inducing degradation of the target RNA or by inhibiting its translation. In mammals, dsRNA for RNAi must be transferred into embryo cells by microinjection. Small interfering RNA (siRNA), which are short synthetic ~ 21-22 nt dsRNA, has been used for RNAi in mouse early embryos; however, in cattle, blastocyst formation *in vitro* normally requires 8-9 days and RNAi mediated by siRNA cannot to be maximally effective throughout this development time frame. Since siRNA would not be feasible for use in the ovine model cattle, we considered

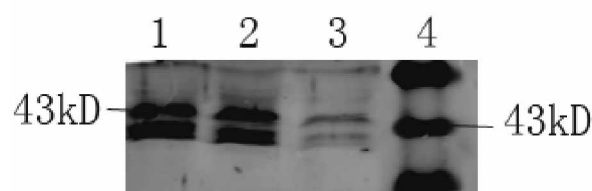


Figure 2. Western blot analysis for detection of Cx43 protein derived from the three treatment groups. 1- uninjected group, 2- water-injected group, 3-Cx43 dsRNA-injected group, 4-protein marker. The molecular weight of Cx43 is 43kD.

using dsRNA because it is a more potent silencing agent than antisense siRNA (Svoboda et al., 2000). Furthermore, long chain dsRNA is regarded as a useful tool to study gene function in early mammalian embryos because it would not trigger an interferon-mediated antiviral response that would lead to general inhibition of protein synthesis and non-specific degradation of mRNAs (Stein et al., 2005). Studies in mice and cattle have demonstrated that the effect of dsRNA-mediated suppression of gene expression could be maintained at least for six rounds of cell division under *in vitro* culture conditions (Wianny and Zernicka-Goetz, 2000; Nganvongpanit et al., 2006). It was shown that microinjection of sequence-specific dsRNA into bovine zygote could trigger a significant decrease of target transcripts until at least day 8 (Tsfaye et al., 2007).

In the present study, target mRNA and protein decreased significantly after injection with Cx 43 dsRNA at the blastocyst stage compared to untreated and water groups. A housekeeping gene and homolog gene were detected at the same time, and no obvious change was observed. Therefore, RNAi in ovine pre-implantation embryos by Cx 43 long chain dsRNA was efficient and specific.

Rates of death were calculated about 4 h after injection when we discarded dead embryos to exclude those with mechanical damage from the injection process. Embryos with loose and grey cell plasma, damage in zona pellucida and disintegration in cell membrane were considered to be dead. Of course the handling time, depth of injection and temperature might also affect the death rate.

The cleaved and development rates were calculated at 48 h, in the blastocyst and hatched blastocyst stage, respectively, and there was no significant differences between the Cx 43 treated and control groups in 2-cell, 4-cell and 8-cell stages ($p > 0.05$). This result suggests that microinjection of Cx 43 dsRNA prior to the first cleavage did not affect the first cleavage rate and further development of the zygotes during the 48 h post-injection, consistent with a previous report in the bovine model (Tsfaye et al., 2007). Non-cleaved and 2-cell stage embryos were discarded 48 h after injection because, in principle, they would have lost the ability to develop further. The remaining embryos were cultured until day 8-10 to count the number of blastocysts and hatched blastocysts. The rate of blastocyst development in the dsRNA and water groups was significantly lower than the control group ($p < 0.01$), but the effect was similar between dsRNA-injection and water injection group ($p > 0.05$). Although the reason for the low blastocyst rate in the water-injected group was not clear, it may reflect the damage by injection to the embryos. However, the rates of hatched blastocysts in dsRNA-injection group were significantly lower than control and water-injection groups ($p < 0.01$). It appeared that suppression of Cx 43 may not affect the cell number of

blastocysts, but it increase the dead rates of cells involved in blastocysts, even if not significantly. Cell apoptosis, which plays an important role in early embryo development, is a widely known biophysical process during blastocyst formation in many mammalian species. Previous studies found that the degree of cell death seems to correlate with embryo quality and cell number in mouse blastocyst (Hardy, 1997). Then it is hypothesized that down-regulate Cx 43 may decrease the communication of molecules that relates to cell viability, resulting in decreasing embryonic cell survival. The hatching ability and cell number of a blastocyst is directly correlated to the quality of the embryo, and Cx 43 may contribute to the blastocyst quality. Our result was similar to some studies in bovine showing that the Cx 43 was related to blastocyst quality but not developmental competence as measured by blastocyst yield (Lonergan et al., 2003; Rizos et al., 2003). Cx 43 is thought to play putative roles in blastocyst quality and development in terms of cryotolerance (Enright et al., 2000; Rizos et al., 2002b). The Cx 43 protein is expressed in nearly all the mammalian tissues. Some knock-out and transgenic experiments demonstrated that mice lacking or overexpressing the Cx 43 gene died from heart defects shortly after birth (Reaume et al., 1995; Ewart et al., 1997). Some evidence exists that show the efficiency of RNAi in terms of RNA degradation could be affected by the volume of target transcripts existing in the embryos (Hu et al., 2004), and the level of transcripts could be changed by the culture conditions. Cx 43 expression was significantly higher in bovine blastocysts produced *in vivo* than those produced *in vitro* (Rizos et al., 2002a), and RNAi induced by dsRNA was efficient in bovine embryos produced *in vivo* but had no effect *in vitro* (Tsfaye et al., 2007). Wrenzycki et al. reported that bovine Cx43 is expressed from the zygotes to morula stages but not in blastocysts and hatched blastocysts *in vitro* (Wrenzycki et al., 1996); however, the same authors subsequently observed that Cx43 mRNA disappeared at the 8- to 16- cell stage and reappeared at the hatched bovine blastocyst stage in the presence of serum (Wrenzycki et al., 1999). Rizos et al. showed that the expression pattern of Cx43 could be altered by the addition of serum (Rizos et al., 2003). In our previous unpublished studies, the Cx 43 transcripts and proteins could be detected throughout all the developmental stages in ovine, and efficient reduction in target RNA and lead to post-translation silencing. While there was previously no report on RNAi usage in the ovine model, the present study has demonstrated the effective suppression of Cx 43 at the mRNA and protein levels during ovine embryogenesis under *in vitro* culture conditions; this suppression had no effect on the development of embryos culture *in vitro*, but may play a role in the process of blastocyst and contribute to blastocyst quality in ovine.

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