CSIRO PUBLISHING

Reproduction, Fertility and Development, 2010, **22**, 564–579 www.publish.csiro.au/journals/rfd

Depletion of BIRC6 leads to retarded bovine early embryonic development and blastocyst formation *in vitro*

Dessie Salilew-Wondim[A], *Micheal Hölker*[A], *Franca Rings*[A], *Chirawath Phatsara*[A], *Abdollah Mohammadi-Sangcheshmeh*[A], *Ernst Tholen*[A], *Karl Schellander*[A] *and Dawit Tesfaye*[A,B]

[A]Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn, Endenicher Allee 15, Bonn, Germany.
[B]Corresponding author. Email: tesfaye@itz.uni-bonn.de

Abstract. Baculoviral inhibitors of apoptosis repeat-containing 6 (BIRC6) is believed to inhibit apoptosis by targeting key cell-death proteins. To understand its involvement during bovine preimplantation embryo development, two consecutive experiments were conducted by targeted knockdown of its mRNA and protein using RNA interference. In Experiment 1, the effect of BIRC6 knockdown during the early stages of preimplantation embryo development was assessed by injecting zygotes with long double-stranded RNA (ldsRNA) and short hairpin RNA (shRNA) against BIRC6 mRNA followed by *in vitro* culturing until 96 h post insemination (hpi). The results showed that in RNA-injected zygote groups, reduced levels of BIRC6 mRNA and protein were accompanied by an increase ($P < 0.05$) in the proportion of 2- and 4-cell and uncleaved embryos and a corresponding decrease ($P < 0.05$) in the number of 8-cell embryos. In Experiment 2, the effect of BIRC6 knockdown on blastocyst formation, blastocyst total cell number and the extent of apoptosis was investigated. Consequently, zygotes injected with ldsRNA and shRNA resulted in lower ($P < 0.05$) blastocyst formation and total blastocyst cell number. Moreover, the apoptotic cell ratio, CASPASE 3 and 7 activity, BAX to BCL-2 ratio and levels of SMAC and CASPASE 9 were higher in blastocysts derived from the ldsRNA and shRNA groups, suggesting increased apoptosis in those blastocysts. The results of this study reveal the importance of BIRC6 expression for embryo survival during bovine preimplantation embryo development. However, whether BIRC6 is essential for implantation and fetal development during bovine pregnancy needs further research.

Additional keywords: apoptosis, RNA interference.

Introduction

Several factors, including the culture conditions, maternal transcripts or transcripts accumulated during embryonic genome activation, are thought to influence the developmental potential of mammalian embryos (Telford *et al*. 1990; Wieschaus 1996; Lonergan *et al*. 2003; Lequarre *et al*. 2004; Thélie *et. al* 2007). However, embryo survival and viability is mainly limited by the degree of fragmentation or the extent of apoptosis (Warner *et al*. 1998; Alikani *et al*. 1999). This, in turn, depends on transcripts that regulate cell survival (anti-apoptotic) and cell death (pro-apoptotic) (Warner *et al*. 1998). Hence, the ratio of pro-apoptotic to anti-apoptotic transcripts determines whether the cell will live or die (Oltval *et al*. 1993; Reed 1997; Bartke *et al*. 2004). Several transcripts in the BCL-2 family rescue cells by inhibiting the release of cytochrome c from mitochondria (Kluck *et al*. 1997; Exley *et al*. 1999). Likewise, the inhibitor of apoptosis (IAP) family of proteins are also thought to be involved in regulating apoptosis against cell death by suppressing active caspases (Deveraux and Reed 1999; Verhagen *et al*. 2001; Salvesen and Duckett 2002; Ren *et al*. 2005).

The baculoviral inhibitor of apoptosis repeat-containing 6 (BIRC6) belongs to the IAP family of proteins and is designated as Apollon, BRUCE, FLJ13726 or KIAA1289. It encodes a protein with a baculoviral inhibition of apoptosis protein repeat (BIR) domain (Ren *et al*. 2005). BIRC6 is believed to be expressed in drug-resistant cancer cells (Chen *et al*. 1999), cancerous tissues (Bianchini *et al*. 2006), mouse embryo (Ren *et al*. 2005), 293T adenovirus-transformed human embryo kidney cells (Gjørret *et al*. 2007), embryonic, extra embryonic and adult mouse tissue (Lotz *et al*. 2004).

Studies have shown that BIRC6 promotes cell survival by blocking second mitochondria-derived activator of caspases (SMAC) and CASPASE 9 (Hao *et al*. 2004). The loss of its function in Drosophila was found to result in degenerated and hyper-condensed spermatid nuclei due to an increased level of CASPASE 9 (Arama *et al*. 2003). Similarly, inhibition of BIRC6 using RNA interference was found to affect the growth of tumour cells in culture (Chu *et al*. 2008). In addition, complete inactivation of the mouse BIRC6 (Lotz *et al*. 2004) was associated with

© CSIRO 2010 10.1071/RD09112 1031-3613/10/030564

Table 1. Sequence of primers used for double-stranded RNA synthesis and mRNA quantification

Gene name	Accession no.	Primer sequence (5′–3′)	Product size (bp)
BIRC6[A]	XM_612946	F: GTAATACGACTCACTATAGGGGTGTTTGTGCGCTGTGACG R: TAATACGACTCACTATAGGGTCTGTGTGCCACTGGGAGTG	418
BIRC6[B]	XM_612946	F: GTGTTTGTGCGCTGTGACG R: TCTGTGTGCCACTGGGAGTG	418
BIRC6[C]	XM_612946	F: TGTCCACCTCACTGCCTCTGT R: GCACACTATGACCACCAGTCGT	248
BAX[C]	NM_173894.1	F: GCGGCTGAAATGTTTTCTGAC R: TCCCAAAGTAGGAGAGGAGGC	255
H2A[C]	NM_174809.2	F: GCCGTATTCATCGACACCTGA R: CTCCACGAATAGCAAGTTGCAA	231
GAPDH[C]	NM_001034034	F: AATGGAGCCATCACCATC R: GTGGTTCACGCCCATCACA	240
BCL-2[C]	NM_001035459.1	F: TCTGCAGGCCTTATGCAAAAC R: GTTAATCTCGGCT CCCAACTG	250
BIRC5[C]	NM_001001855.2	F: AGGAGTTGGAAGGCTGGGA R: TGGCACAGCGGACTTTCTT	219
CASPASE 9[C]	XM_001790068.1	F: CTGCTGAGTCGCGAGCTCTT R: GCTGCTTGTCTGCTGGTCTTC	205
SMAC[C]	NM_001045882.1	F: AGTACCTGAAGCTGGAAACCA R: TCTGTCGGAGTTCCTCTGTCT	209

[A]The primer was coupled with T7 promoter (underlined) for *in vitro* transcription.
[B]Primers used for DNA template amplification.
[C]Primers used for mRNA quantification.

embryonic lethality, suggesting its importance during mouse embryo development.

Despite its anti-apoptotic role and its importance in mouse embryonic development, its significant relevance during bovine preimplantation embryo development is not yet documented. Therefore, two consecutive experiments were conducted to investigate the involvement of BIRC6 during the early and later stages of bovine preimplantation embryo development by targeted knockdown using RNA interference technology.

Materials and methods

Long double-stranded RNA (ldsRNA) in vitro *synthesis*

Long double-stranded RNA (ldsRNA) targeting the BIRC6 gene was synthesised *in vitro* from long double-stranded DNA templates as described in Nganvongpanit *et al*. (2006*a*, 2006*b*) and Tesfaye *et al*. (2007). Briefly, 418-base pair fragments were amplified from the coding sequence of BIRC6 mRNA using sequence-specific primers (Table 1). For this, a thermal cycling protocol of heating at 95°C for 5 min followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min was used. Following the last cycle, a 10 min elongation step at 72°C was performed. The PCR product was cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and further amplified overnight in competent cells (*E. coli*, JM109 strain). Following this, the plasmid DNA was recovered using GenElute Plasmid Miniprep (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's recommendations. The isolated plasmid was then sequenced to evaluate the identity of the BIRC6 product. Following this, the sense and anti-sense double-stranded DNA promoter were separately amplified from plasmid using the primers containing T7 promoter (Table 1). Afterwards, both sense and anti-sense RNA strands were transcribed *in vitro* from sense and anti-sense DNA strands in separate reactions using RiboMAX Large Scale RNA Production T7 Systems (Promega). After DNase treatment, the sense and anti-sense RNA strands were annealed and purified using phenol/chloroform. The double-stranded RNA was then precipitated in 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of 100% ethanol. The RNA pellet was suspended in RNase-free water. The RNA concentration was determined using an Ultraspec 2100 pro spectrophotometer (Amersham Biosciences, Buckinghamshire, UK) and its integrity was determined by agarose gel electrophoresis.

Short hairpin RNA (shRNA) in vitro *synthesis*

In addition to the long double-stranded RNA, short hairpin RNA may be more effective to analyse specific gene functions in cells (Paddison *et al*. 2002). For this reason, we have included *in vitro*-synthesised short hairpin RNA along with ldsRNA throughout the experiment.

Short hairpin RNA (shRNA) targeting BIRC6 mRNA was synthesised *in vitro* from chemically synthesised DNA oligonucleotide templates using the Message-Muter shRNA Production Kit (Epicentre Biotechnologies, Madison, WI, USA). Briefly, 21 nucleotide sequences were selected from the coding region of the BIRC6 mRNA. A loop sequence (CTTGCTTC), inverted repeat of the initial DNA sequence and eight base sequences complementary to the T7 promoter oligo were added to the 3′ end of the 21-nucleotide sequence (Fig. 1). This template (target oligo) was used as a starting material for *in vitro*

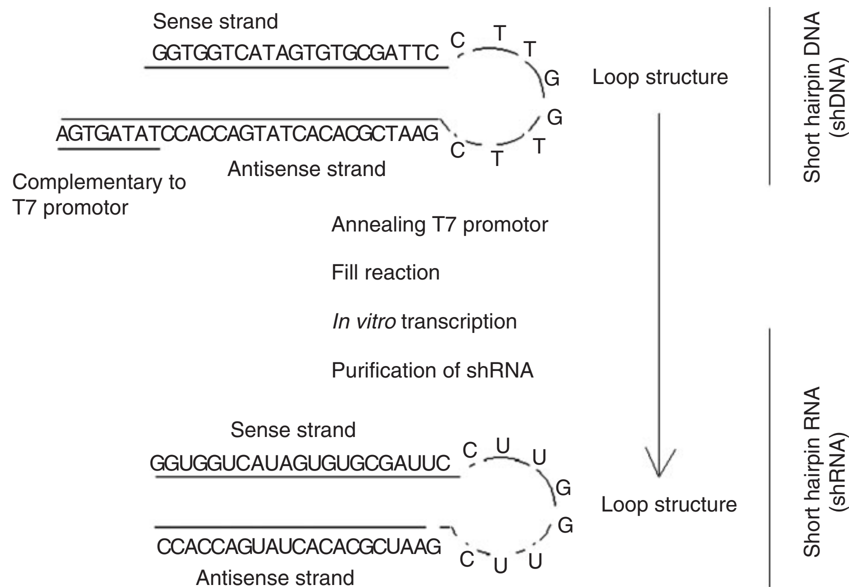

Fig. 1. Systematic representation of shRNA *in vitro* transcription directed against BIRC6. The shRNA produced using the MessageMuter shRNA Production Kit contains a sequence homologous to the target mRNA (sense sequence), a 'loop' region and a sequence complementary to the target sequence (anti-sense sequence).

transcription of the short hairpin. Following this, a mix containing of T7 promoter oligo (75 pmoles), 5× annealing buffer and RNase-free water was added to the target oligo (50 pmoles) and incubated for 2 min at 75°C. After the annealing reaction, the double-stranded DNA (dsDNA) was prepared using a reaction mix containing of 10× Klenow Fill-In buffer, dNTP mix (2.5 mM dNTPs), Exo-minus Klenow DNA Polymerase (20 U mL^{-1}) and RNase-free water. The reaction was incubated for 30 min at 37°C followed by heating for 10 min at 70°C and cooling to room temperature. The shRNA was then transcribed from the dsDNA template using AmpliScribe T7-flash 10× reaction buffer, NTP mix (25 mM each dNTP), 100 mM DTT, AmpliScribe T7-flash enzyme solution and RNase-free water. *In vitro* transcription was performed at 42°C for 90 min· and the shRNA was purified with phenol/chloroform before precipitating with ethanol. The quality and quantity of *in vitro*-synthesised shRNA was determined using the same procedure used for long double-stranded RNA.

Oocyte recovery, in vitro *maturation*

The oocyte recovery, *in vitro* maturation and fertilisation was performed as described in our previous studies (El-Sayed *et al.* 2006; Salilew-Wondim *et al.* 2007; Tesfaye *et al.* 2007). Briefly, bovine ovaries were obtained from the abattoir and transported to the laboratory in a thermo flask containing 0.9% saline. The cumulus oocyte complexes (COCs) were aspirated from follicles with a diameter of 2–6 mm using a sterilised 5-mL syringe fixed to an 18-gauge needle. The COCs were washed with modified Parker medium (MPM) supplemented with 15% oestrus cow serum (OCS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 mg mL^{-1} gentamycin sulfate and 10 µL mL^{-1} FSH (Folltropin; Vetrepharm, London, ON, Canada). A group of 50 COCs were cultured in 400 µL of maturation medium covered with mineral oil (Sigma, Taufenkirchen, Germany) in four-well dishes (Nunc, Roskilde, Denmark). Maturation was performed for 24 h at 39°C in a humidified atmosphere of 5% CO_2.

In vitro *fertilisation and* in vitro *culture*

For *in vitro* fertilisation groups of 50 matured oocytes were transferred into four-well dishes containing fertilisation medium supplemented with 2 mg mL^{-1} heparin (Sigma, St. Louis, MO, USA), 0.2 mM pyruvate (Sigma) and 25 mL mL^{-1} penicillinamine, hypotaurine and epinephrine (PHE). A swim-up procedure was used to obtain motile spermatozoa from frozen–thawed semen. *In vitro* fertilisation was performed using a final concentration of 2×10^6 spermatozoa mL^{-1} in the fertilisation drop containing a group of 50 COCs. Oocytes were co-incubated with spermatozoa for 20 h in a humidified atmosphere of 5% CO_2 at 39°C. At the end of the incubation period, the cumulus cells were removed from presumptive zygotes before microinjection.

Microinjection

In this study, two consecutive experiments were conducted to achieve the objectives. For this, *in vitro* synthesis of ldsRNA and shRNA that would silence the BIRC6 mRNA and protein were diluted to a concentration of 4 µg μL^{-1} using RNase-free water (Promega). Approximately 7 pL or 28 pg of ldsRNA or shRNA was injected into the cytoplasm of each zygote. The microinjection procedure was performed as described in Nganvongpanit *et al.* (2006*a*, 2006*b*).

In vitro *culture and data collection*

In Experiment 1, the effect BIRC6 knockdown on early bovine preimplantation embryo development (until 96 h post

insemination (hpi)) was assessed after introducing exogenous ldsRNA and shRNA against BIRC6 mRNA into zygote stage embryos. For this, *in vitro*-fertilised zygotes were injected with ldsRNA ($n = 886$) or shRNA ($n = 960$). Water-injected ($n = 819$) and uninjected zygotes ($n = 920$) were used as controls. All zygote groups were cultured in Charles Rosenkrans 1 (CR1) culture medium supplemented with 10% OCS, 20 $\mu L\,mL^{-1}$ BME and 10 $\mu L\,mL^{-1}$ MEM at 39°C in a humidified atmosphere of 5% CO_2. At the end of 96 hpi the number of embryos at the 2-, 4- and 8-cell stage and uncleaved embryos were recorded. Furthermore, the transcript abundance of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and survivin (BIRC5) was analysed to evaluate the specificity of the BIRC6 knockdown.

In Experiment 2, the effect of BIRC6 knockdown on later stage bovine preimplantation embryo development (blastocyst formation, blastocyst total cell number) and the extent of apoptosis was investigated. For this, *in vitro*-fertilised zygotes injected with ldsRNA ($n = 302$), shRNA ($n = 352$), water ($n = 399$) and uninjected zygotes ($n = 377$) were cultured *in vitro* until 8 days post insemination (dpi). The number of blastocysts and non-blastocysts derived from each treatment group were recorded at 8 dpi. The non-blastocyst embryos were those embryos that failed to reach blastocyst stage by 8 dpi. The effect of BIRC6 knockdown on genes involved in the apoptotic pathway was evaluated by determining the abundance of the pro-apoptotic BAX mRNA, CASPASE 9, SMAC and anti-apoptotic BCL-2. In addition, the transcript abundance of GAPDH and survivin (BIRC5) were analysed on blastocyst and non-blastocyst embryos to evaluate the sequence specificity of BIRC6 knockdown.

RNA isolation and cDNA synthesis

Messenger RNA was isolated from uncleaved, 2-, 4- and 8-cell, blastocyst and non-blastocyst embryos derived from ldsRNA, shRNA, water-injected and uninjected zygote groups using Dynabead Oligo $(dT)_{25}$ (Dynal Biotech, Oslo, Norway). Briefly, five independent pools of embryos each consisting of twenty 2-, 4-, 8-cell or uncleaved embryos collected at 96 hpi and three independent biological replicates each consisting of 10 blastocyst or 30 non-blastocyst embryos collected at 8 dpi were used for RNA isolation. The embryos were lysed with binding buffer (20 mM TRIS-HCl, pH 7.5, 1 M LiCl, 2 mM EDTA, pH 8.0) at 70°C for 5 min. The cell lysate was incubated with Dynabead Oligo $(dT)_{25}$ suspension at room temperature for 30 min. The beads hybridised with mRNA were washed three times with washing buffer (10 mM Tris HCL, pH 7.5; 0.15 mM LiCl, 1 mM EDTA, pH 8.0) and mRNA was eluted in RNase-free water.

First-strand cDNA was synthesised in a 20-μL reaction volume. For this, the mRNA was incubated with 1 μL anchored oligo $(dT)_{23}$ primer (20 μM) (Sigma, Germany) for 3 min at 70°C, to which 8 μL reverse transcription mix (4 μL of 5× first-strand synthesis buffer (50 mM TRIS-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$), 2 μL 0.1 mM dithiothreitol (DTT), 1 μL 0.3 M deoxynucleotide-triphosphate (dNTP), 10 U of RNase inhibitor (Promega) and 200 U superscript II (Invitrogen, Karlsruhe, Germany)) was added. This reaction was incubated at 42°C for 90 min followed by reaction termination at 70°C for 15 min.

Semiquantitative PCR

Semiquantitative RT-PCR was performed to determine the temporal expression and presence of BIRC6 mRNA in the bovine preimplantation embryos. For this, total RNA was isolated from two pools each consisting of germinal vesicle (GV) oocytes ($n = 50$), matured oocytes ($n = 50$), zygotes ($n = 50$), 2-cell ($n = 50$), 3-cell ($n = 50$), 4-cell ($n = 50$), 8-cell ($n = 50$), morula ($n = 30$), blastocysts ($n = 10$) and expanded blastocysts ($n = 10$). The reverse transcription was performed as described above. Following cDNA synthesis, the PCR amplification was performed for BIRC6 and GAPDH in a 20-μL reaction volume using sequence-specific primers (Table 1) at a final concentration of 4 picomolar in a standard thermo cycler program with a first denaturation step at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and 1 min elongation at 72°C. The PCR reaction was terminated after final elongation at 72°C for 10 min. The products were loaded onto ethidium bromide-stained 2% agarose gels and subjected to electrophoresis running in 1× TAE buffer. The image of the PCR product from the agarose gel was acquired using a UV trans-illuminator.

Quantitative real-time PCR (qPCR)

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009) were employed during the qPCR. For each primer used (Table 1), a serial dilution of 10^1–10^9 copy numbers of molecules was prepared from the plasmid DNA. Each plasmid DNA was sequenced to confirm the identity of our gene of interest. Prior to qPCR, the concentration of the cDNA samples from each treatment group were determined using the Nanodrop 8000 spectrophotometer (Biotechnology GMBH, Erlangen, Germany) and the same amount of cDNA was used to compare samples from different treatment groups. The qPCR was performed in a 20-μL reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad laboratories, Munich, Germany), the cDNA sample and the specific forward and reverse primer in an ABI PRISM 7000 sequence detection system instrument (Applied Biosystems, Foster city, CA, USA). The primer concentrations used during the qPCR were optimised for each gene and found to be in the range of 100 to 400 nM. The qPCR thermal cycling parameters were set at 95°C for 3 min, 40 cycles of 15 s at 95°C and 45 s at 60°C. At the end of the qPCR reaction, the specificity of amplification for each gene was evaluated by monitoring the dissociation (melting) curve. Transcript abundance of the samples was determined using the relative standard curve method using histone (H2A) for internal normalisation as indicated in Paddison *et al.* (2002), Lonergan *et al.* (2003), Tesfaye *et al.* (2004), Hitz *et al.* (2005) and Salilew-Wondim *et al.* (2007). The data generated were considered for further analysis provided that the slope was between −3.2 and −3.6 and the regression line (R^2) of the standard curve was ≥0.99.

Caspase activity assay

CASPASE 3 and 7 activity was monitored by means of fluorochrome-labelled inhibitor of caspase (FLICA) using the

CaspaTag Pan-Caspase *in situ* Assay kit, Fluorescein (Millipore Corporation, Billerica, MA, USA). Briefly, the FLICA staining solution was diluted 150 times and 2 μL of this stock solution was mixed with 400 μL of culture medium (CR1). Blastocysts from ldsRNA ($n = 9$), shRNA ($n = 12$), water injected ($n = 10$) and uninjected ($n = 10$) groups were used for the assay. The blastocysts were incubated in the culture medium–FLICA complex (CR1/FLICA) for 1 h at 39°C in a humidified atmosphere with 5% CO_2. At the end of the staining period, the embryos were washed 5 times in drops of 1× washing buffer supplied with the kit. Caspase-positive control blastocysts were produced by inducing apoptosis using 50 U mL^{-1} DNase (Roche Diagnostics, Indianapolis, IN, USA) followed by incubation in CR1/FLICA for 1 h at 37°C. Caspase-negative controls were produced by incubating DNase-treated blastocysts in culture medium (CR1) in the absence of FLICA.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) and determining blastocyst total cell number

The blastocysts obtained from ldsRNA, shRNA, water injected and uninjected zygote groups were fixed in 4% paraformaldehyde (Sigma, Germany) and washed in phosphate buffered saline (PBS) followed by permeabilisation in PBS containing 0.5% Triton X-100 (Sigma, Germany) and 0.1% sodium citrate for 30 min. After three times washing in PBS, blastocysts were incubated for 1 h at 37°C in 50 μL TUNEL reaction mixture (Roche Diagnostics) containing 5 μL of 10× TUNEL in storage buffer and 45 μL 1× label solution (nucleotide mixture in reaction buffer). TUNEL-positive controls were produced by inducing apoptosis in blastocysts using DNase (50 IU mL^{-1}) by incubating for 1 h at 37°C as indicated previously (Makarevich and Markkula 2002; Roccheri *et al.* 2002; Antunes *et al.* 2008). TUNEL-negative controls were produced by incubating apoptosis-induced blastocysts with label solution in the absence of the enzyme terminal deoxynucleotidyle transferase. Counter-staining was performed by incubating blastocysts in the PBS solution containing 6.5 μg mL^{-1} bisbenzemide (Hoechst H33528; Sigma) for 15 min followed by three times washing in PBS.

Microscopy

The caspase activity, TUNEL-positive cells and total cell count were determined using a fluorescence microscope (DMIRB; Leica, Bensheim, Germany). A setting of 490-nm excitation and 520-nm emission was employed to observe the green fluorescence signal of fluorescein, which is a direct measure of the amount of active CASPASE 3 and 7 present in the blastocyst. A setting of 365-nm excitation and 480-nm emission was used to determine the total cell number. A 512-nm emission wavelength was used to detect TUNEL-stained nuclei.

Western blot analysis

Pools of 8-cell ($n = 40$), uncleaved embryos ($n = 31$), Day 8 blastocysts ($n = 40$) or non-blastocyst embryos ($n = 50$) from different treatment groups were used for protein analysis. The embryos were boiled at 95°C for 5 min in the presence of loading buffer (26% 1 M Tris pH 6.8, 12% SDS, 20% 2-mercaptoethanol and 40% glycerol) and analysed by SDS polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane, pore size 0.45 μm (Millipore, Schwalbach, Germany) using transfer buffer containing 10 mM N-cyclohexyl-3-amino propane sulfonic acid (CAPS) and 10% methanol. The membrane was then blocked for 1 h in 1% polyvinylpyrolidone (PVP) (Sigma, Germany) in Tris-buffered saline Tween-20 (TBST). The membrane was incubated overnight at 4°C with goat anti-BIRC6/KIAA1289 antibody diluted at 1 : 500 in TBST containing 0.1% PVP. At the end of incubation period, the membrane was washed twice in TBST and incubated with the rabbit anti-goat secondary antibody diluted at 1 : 10 000 in TBST containing 0.1% PVP. The peroxidase activity was detected using the ECL Plus western blotting detection system (Amersham Bioscience) following the manufacturer's instructions and visualised using Kodak BioMax XAR film (Sigma-Aldrich).

Statistical analysis

The embryonic developmental data, blastocyst cell number and the apoptotic cell ratio were analysed using one-way analysis of variance (ANOVA) using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test or least significant difference tests were used for analysis of mRNA abundance. Differences with $P < 0.05$ were considered as significant. Moreover, the uninjected control was considered to be 100% each time during interpretation of mRNA knockdown in reference to the control (Amanai *et al.* 2006; Nganvongpanit *et al.* 2006*a*, 2006*b*; Sato *et al.* 2006; Corteling *et al.* 2007; Tesfaye *et al.* 2007).

Results

Experiment 1

BIRC6 is expressed in bovine oocytes and preimplantation embryos

Semiquantitative RT-PCR was performed to determine the temporal expression of BIRC6 mRNA in the bovine preimplantation embryos. The results indicated that the transcript was detected in bovine oocytes and in all stages of preimplantation embryos (Fig. 2). Its expression in bovine preimplantation embryos implicates the possible involvement of this gene during bovine embryo development and suggests the need to understand its functional contribution using RNA interference.

Depletion of BIRC6 and developmental phenotype at 96 hpi

The effect of BIRC6 knockdown on early bovine preimplantation embryo development (until 96 h post insemination (hpi)) was assessed after introducing exogenous ldsRNA and shRNA against BIRC6 mRNA into zygotic stage embryos. The numbers of uncleaved, 2-, 4- and 8-cell embryos were evaluated at 96 hpi and the level of BIRC6 mRNA and protein was determined at each stage for each treatment group of embryos. The results showed that the level of BIRC6 mRNA was reduced in all embryo groups derived from ldsRNA- and shRNA-injected zygotes. In 2-cell-stage embryos, the BIRC6 mRNA was reduced ($P < 0.05$)

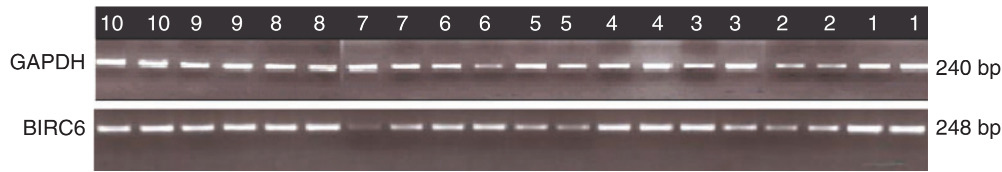

Fig. 2. Semiquantitative RT-PCR showing the expression of BIRC6 in bovine preimplantation embryo. Expression of BIRC6 and GAPDH in GV stage oocyte (Lane 1), matured oocyte (Lane 2), zygote (Lane 3), 2-cell embryo (Lane 4), 3-cell embryo (Lane 5), 4-cell embryo (Lane 6), 8-cell embryo (Lane 7), morula (Lane 8), blastocyst (Lane 9) and expanded blastocyst (Lane 10).

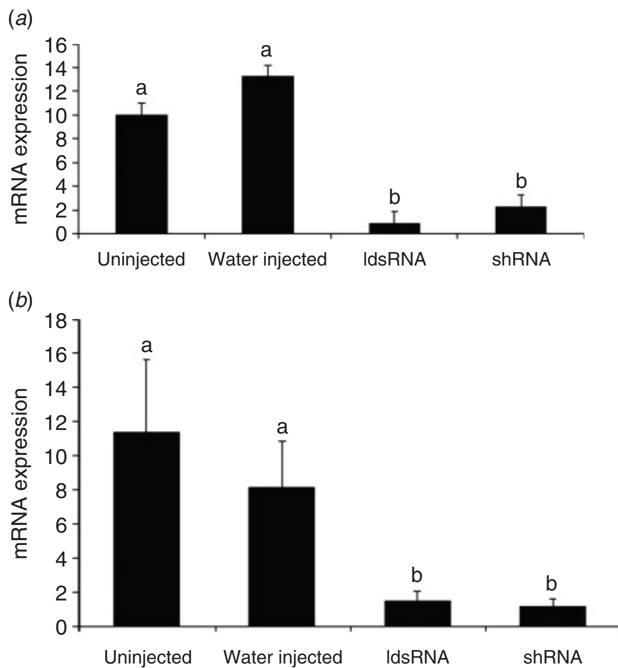

Fig. 3. The relative mRNA level of BIRC6 at 96 hpi in (*a*) 2-cell embryo and (*b*) 4-cell embryo. The BIRC6 mRNA was reduced both in ldsRNA- and shRNA-injected groups. Five replicates each consisting of a pool of 20 2- or 4-cell embryos were used for the analysis. Bars show mean $\pm$ s.e.m. [a,b]Values with different letters indicate statistical significance ($P < 0.05$).

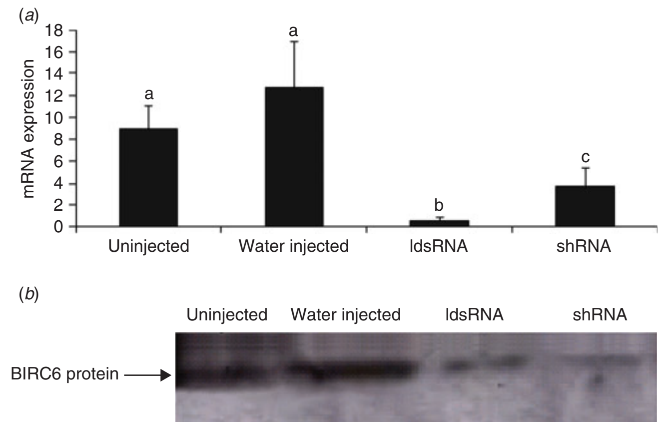

Fig. 4. (*a*) The relative mRNA level of BIRC6 at 96 hpi in uncleaved embryos. Five replicates each consisting of a pool of 30 uncleaved embryos were used for the analysis. Bars show mean $\pm$ s.e.m. [a,b,c]Values with different letters indicate statistical significance ($P < 0.05$). (*b*) Western blot analysis conducted to detect the BIRC6 protein level at 96 hpi in uncleaved embryos.

by 92% in ldsRNA- and 78% in shRNA-injected embryos compared with those 2-cell embryos derived from uninjected or water-injected control groups (Fig. 3*a*). There were no significant differences in BIRC6 mRNA abundance between uninjected and water-injected controls. Similarly, in 4-cell stage embryos, the BIRC6 mRNA was reduced by 82 and 87% in ldsRNA- and shRNA-injected groups, respectively, compared with those 4-cell embryos derived from uninjected control zygote groups (Fig. 3*b*). Furthermore, in uncleaved embryos the BIRC6 mRNA was decreased ($P < 0.05$) by 80 and 70% in ldsRNA and shRNA groups, respectively, compared with uninjected groups (Fig. 4*a*). The corresponding protein reduction associated with downregulation of BIRC6 mRNA in uncleaved embryos is shown in Fig. 4*b*. Similarly, in 8-cell embryos the BIRC6 mRNA was reduced by 90% in both the ldsRNA- and shRNA-injected groups (Fig. 5*a*). The BIRC6 protein level in 8-cell embryos derived from different treatment groups of zygotes is shown in (Fig. 5*b*). The abundance of GAPDH mRNA in 8-, 4-cell and uncleaved embryos was not significantly different between the treatment groups (Fig. 6*a*, *b*, *c*). Similarly, the expression of survivin (BIRC5) was not significantly different in any of the embryo groups derived from different zygote groups (Fig. 7*a*, *b*, *c*).

Corresponding to the BIRC6 mRNA and protein reduction, at 96 hpi zygotes injected with ldsRNA and shRNA resulted in lower numbers of ($P < 0.05$) 8-cell embryos and higher numbers of 2- and 4-cell and uncleaved embryos compared with water-injected and uninjected control treatment groups (Table 2).

Experiment 2

Depletion of BIRC6 resulted in reduced bovine blastocyst formation

To investigate the effect of knockdown of BIRC6 mRNA on blastocyst formation, bovine zygotes injected with ldRNA and shRNA against BIRC6, water and uninjected groups were cultured until 8 dpi and blastocyst formation and corresponding mRNA knockdown was evaluated. Results revealed that a lower ($P < 0.05$) total blastocyst rate was obtained from shRNA- and ldsRNA-injected zygote groups compared with water-injected and uninjected control groups (Table 3). Furthermore, the number of hatched and expanded blastocysts obtained at 8 dpi was

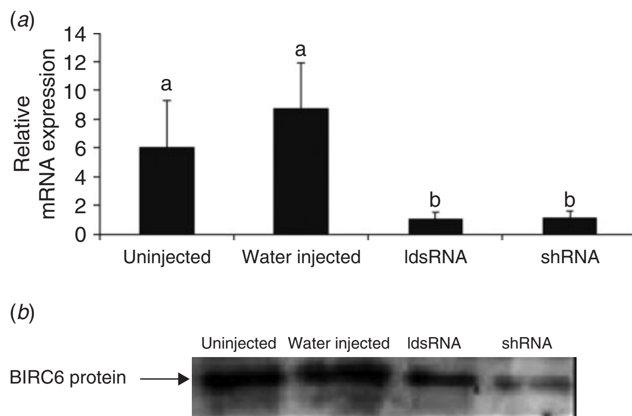

Fig. 5. (*a*) The relative mRNA expression of BIRC6 in 8-cell embryos at 96 hpi. Five replicates each consisting of 20 8-cell embryos were used for the analysis. Bars show mean ± s.e.m. [a,b]Values with different letters indicate statistical significance ($P < 0.05$). (*b*) Western blot analysis conducted to detect the expression of BIRC6 protein in 8-cell embryos at 96 hpi.

lower ($P < 0.05$) in ldsRNA- and shRNA-injected compared with uninjected and water-injected zygote groups (Table 3). On the other hand, the proportion of non-blastocyst embryos was higher ($P < 0.05$) in ldsRNA (73.9 ± 3.2) and shRNA (70.6 ± 3.0) groups compared with water-injected (61.9 ± 4.6) and uninjected controls (60.9 ± 2.9).

The BIRC6 mRNA and protein was determined from those blastocysts and non-blastocyst embryos to verify whether differences in embryonic development were associated with the BIRC6 gene knockdown. Results showed that blastocysts derived from ldsRNA- and shRNA-injected zygotes exhibited a 40% reduction ($P < 0.05$) in BIRC6 mRNA expression (Fig. 8*a*). The corresponding BIRC6 protein reduction due to downregulation of its mRNA in those blastocysts is indicated in Fig. 8*c*. Similarly, BIRC6 mRNA level was reduced by 45% and 83% in non-blastocyst embryos derived from zygotes injected with ldsRNA and shRNA, respectively (Fig. 8*b*). The corresponding BIRC6 protein knockdown due to reduction of its mRNA in non-blastocyst embryos is indicated in (Fig. 8*d*). We also determined the expression GAPDH and survivin (BIRC5) in blastocysts and non-blastocyst embryos to assess whether knockdown affects the expression of other non-targeted genes; the expression of GAPDH mRNA (Fig. 8*e*, *f*) and survivin (BIRC5) (Fig. 9*a*, *b*) were not different ($P > 0.05$) between the blastocysts or non-blastocyst embryos of different origin indicating the specificity of the knockdown.

Knockdown of BIRC6 resulted in lower blastocyst cell numbers at 8 dpi

Blastocysts obtained from zygotes of different treatment groups were stained using Hoechst 33342. The results indicated that blastocysts derived from zygotes injected with ldsRNA and shRNA exhibited lower ($P < 0.05$) total cell numbers compared with blastocysts derived from water-injected or uninjected zygote groups (Fig. 10).

Caspase activity and TUNEL assays

The green fluorescent signal, which is a direct measure of the amount of active CASPASE 3 and 7, was higher in the blastocysts derived from ldsRNA- and shRNA-injected groups than water-injected and uninjected control groups at 8 dpi (Fig. 11). Moreover, the apoptotic cell ratio (TUNEL-positive nuclei to the total cell number) on Day 8 blastocysts tended to be higher in blastocysts derived from ldsRNA- and shRNA-injected zygote groups compared with water-injected and uninjected controls (Table 4).

Depletion of BIRC6 leads to an increase in BAX, BAX to BCL-2 ratio, SMAC and CASPASE 9 in blastocyts and non-blastocyst embryos at 8 dpi

BAX, SMAC and CASPASE 9 (pro-apoptotic genes) and BCL-2 (anti-apoptotic gene) were analysed to understand the shift of apoptotic regulatory genes due to suppression of BIRC6 mRNA. The results showed that BAX mRNA was increased by 50% and 100% in blastocysts derived from zygotes injected with ldsRNA and shRNA, respectively, compared with the control groups (Fig. 12*a*). However, BCL-2 expression did not differ between these treatment groups. Consequently, the BAX to BCL-2 ratio was higher ($P < 0.05$) in blastocysts derived from ldsRNA- and shRNA-injected embryos compared with water-injected and uninjected zygote groups (Fig. 12*b*). Furthermore, the expression of SMAC and CASPASE 9 was found to be higher in blasotcysts derived from the ldsRNA- and shRNA-injected groups compared with water-injected and uninjected controls (Fig. 13*a*, *b*) suggesting that there is an increase in apoptosis in the RNA-injected groups. Therefore, the increase in the BAX/BCL2 ratio, and in levels of SMAC and CASPASE 9 may indicate an increase in apoptosis in blastocysts derived from ldsRNA- and shRNA-injected zygote groups.

Discussion

Introducing exogenous double-stranded RNA into oocytes, embryos or organisms has been widely used to investigate the function of a gene in mosquito (Kennerdell and Carthew 2000), *C. elegans* (Fire *et al*. 1998), pig (Cabot and Prather 2003), mouse (Wianny and Zernicka-Goetz 2000; Soares *et al*. 2005; Amanai *et al*. 2006), cow (Paradis *et al*. 2005; Golding *et al*. 2006; Nganvongpanit *et al*. 2006*a*, 2006*b*; Park *et al*. 2007; Tesfaye *et al*. 2007) and chicken (Sato *et al*. 2006). Similarly, in the present study, *in vitro*-synthesised long double-stranded RNA (ldsRNA) and short hairpin RNA (shRNA) were introduced to the zygotic stage of bovine embryos to investigate whether BIRC6 is required during bovine preimplantation embryo development. For this, the pattern of BIRC6 mRNA expression was assessed across the different developmental stages of bovine preimplantation embryos as preliminary data before RNA interference experiments. Consequently, BIRC6 was found to be expressed in all stages of bovine preimplantation embryos including GV stage and mature oocytes (Fig. 2). Although information is lacking in cows, studies in other species showed that BIRC6 is expressed in Day 7.5 to Day 11.5 mouse embryo (Ren *et al*. 2005), 293T adenovirus-transformed human

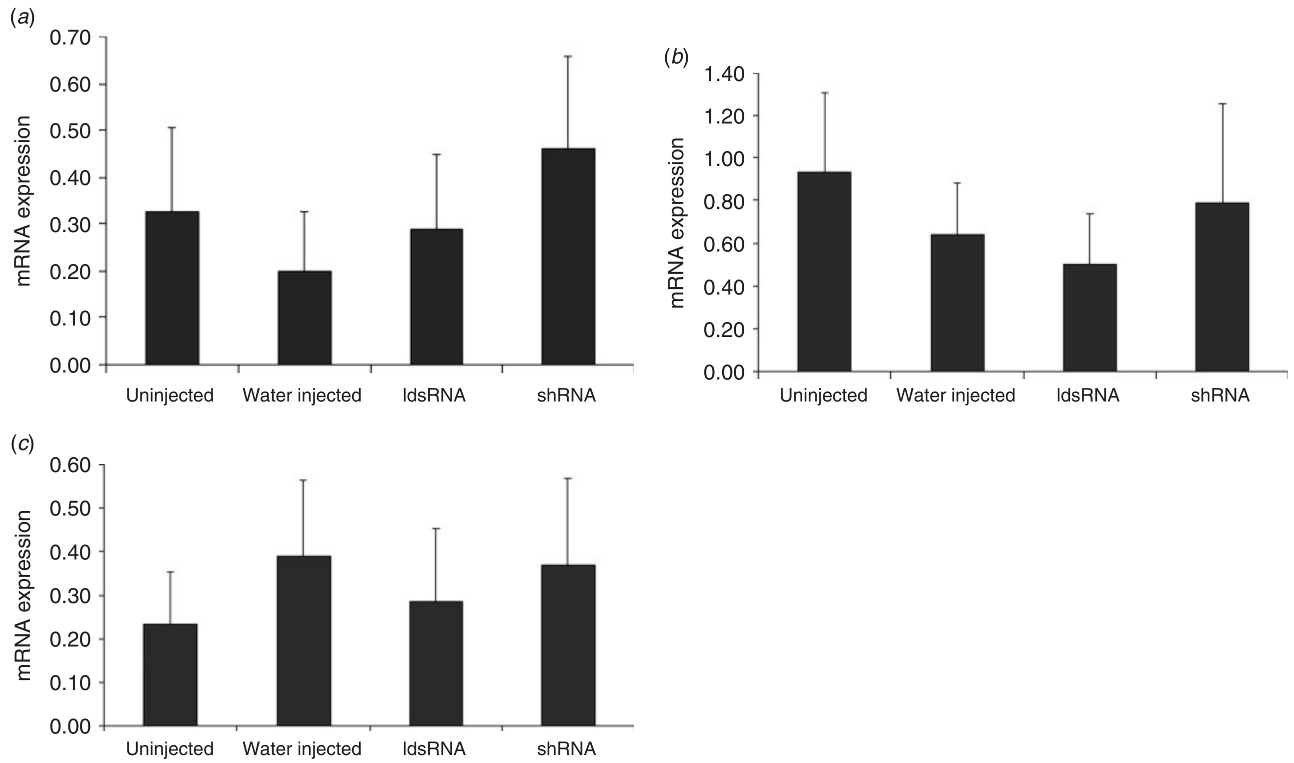

Fig. 6. The mRNA level of GAPDH at 96 hpi in (*a*) 8-cell embryo, (*b*) 4-cell embryo and (*c*) uncleaved embryo.

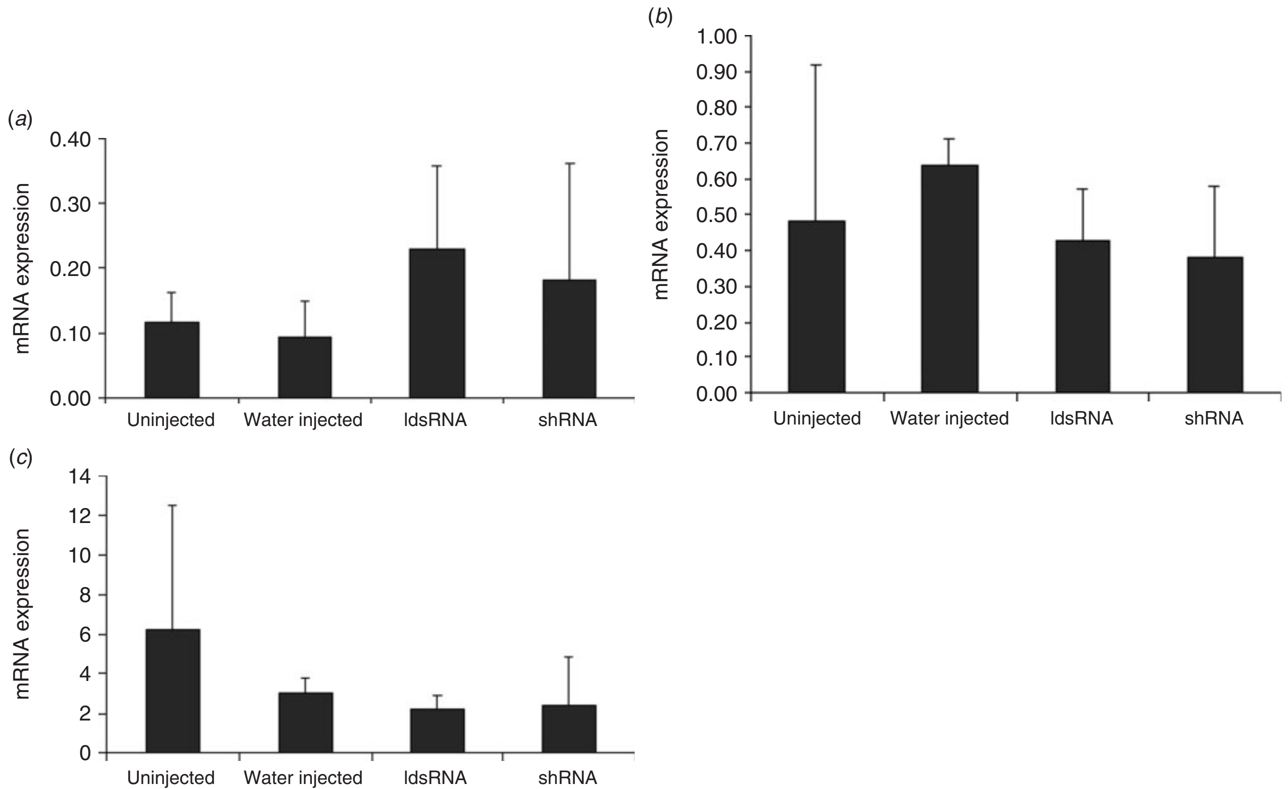

Fig. 7. The mRNA level of Survivin at 96 hpi in (*a*) 8-cell embryo, (*b*) 4-cell embryo and (*c*) uncleaved embryo.

Table 2. Preimplantation embryonic development (mean ± s.e.m.) of zygotes injected with ldsRNA, shRNA and water and uninjected control at 96 hpi

n = number of zygotes cultured after microinjection. [a,b]Values with different letters indicate significant difference ($P < 0.05$). The embryonic developmental data was analysed from 14 independent experimental replicates

Treatment	*n*	Developmental stages at 96 hpi				
		8-cell	4-cell	2-cell	Sum of 2- and 4-cell	Uncleaved
Uninjected control	920	59.8 ± 2.9[a]	12.8 ± 1.1[a]	5.6 ± 0.7[a]	17.4 ± 1.2[a]	21.4 ± 2.4[a]
Water-injected	819	58.2 ± 2.4[a]	12.4 ± 0.9[a]	7.6 ± 0.8[a]	20.1 ± 1.4[a]	21.7 ± 2.2[a]
ldsRNA-injected	866	45.5 ± 3.3[b]	16.5 ± 1.3[b]	11.9 ± 1.7[b]	28.7 ± 2.1[b]	30.4 ± 2.9[b]
shRNA-injected	960	47.4 ± 3.8[b]	16.1 ± 1.5[b]	9.8 ± 1.55[b]	25.9 ± 2.2[b]	27.5 ± 2.4[b]

Table 3. Blastocyst formation (mean ± s.e.m.) of zygotes injected with ldsRNA, shRNA and water and uninjected control

n = number of zygotes cultured after microinjection. [a,b]Values with different letters indicate significant difference ($P < 0.05$). The embryonic developmental data was analysed from seven independent experimental replicates

Treatment	*n*	Total blastocyst rate at 7 dpi	Total blastocyst rate at 8 dpi	Hatched and exp. blastocyst rate at 8 dpi
Uninjected control	377	25.4 ± 3.7[a]	39.1 ± 2.9[a]	29.2 ± 3.4[a]
Water-injected	399	24.6 ± 4.7[a]	38.1 ± 4.6[a]	29.2 ± 5.3[a]
ldsRNA-injected	302	14.8 ± 2.4[b]	26.1 ± 3.2[b]	18.4 ± 2.2[b]
shRNA-injected	352	20.3 ± 2.6[a]	29.4 ± 2.9[b]	23.2 ± 3.0[b]

embryo kidney (Gjørret *et al.* 2007), embryonic, extra embryonic and adult mouse tissue (Lotz *et al.* 2004).

BIRC6 is involved in bovine preimplantation embryo development

In the present study, knockdown of BIRC6 impaired the developmental kinetics and the developmental potential of bovine embryos. For example, the embryonic developmental data at 96 hpi showed that the number of bovine zygotes reaching 8-cell stage was significantly reduced in ldsRNA- and shRNA-injected zygote groups (Table 2). On the contrary, a higher percentage of zygotes injected with ldsRNA and shRNA remained at the uncleaved, 2- or 4-cell embryo stage until 96 hpi. Considering normal bovine embryo developmental kinetics (Gjørret *et al.* 2007), those embryos remaining uncleaved or arrested at the 2- and 4-cell stage at 96 hpi will not develop to the blastocyst stage. Similarly, Vandaele *et al.* (2006) indicated that even those embryos that cleaved at 48 hpi had a lower chance of reaching blastocyst stage, signifying that those embryos that deviated from normal developmental kinetics have a very low chance of successful maturation. To validate if the higher percentage of uncleaved, 2- and 4-cell embryos or the reduced number of 8-cell embryos is attributed to the knockdown of BIRC6 gene expression, we have analysed the level of BIRC6 in those embryos. Consequently, transcript analysis in those 2-, 4-cell and uncleaved embryos revealed a substantial reduction in BIRC6 mRNA. Furthermore, at 96 hpi the BIRC6 mRNA and its protein level were reduced in the 8-cell embryos derived from ldsRNA- and shRNA-injected groups that ultimately resulted in significantly lower blastocyst rates at 8 dpi (Table 3). Therefore, the lower proportion of 8-cell embryos, the higher proportion of 2-, 4-cell and uncleaved embryos from zygotes injected with ldsRNA and shRNA accompanied by the reduction in BIRC6 expression at 96 hpi suggest the involvement of BIRC6 in the early stages of bovine preimplantation embryo development.

Since bovine preimplantation embryonic development covers all developmental stages until blastocyst formation, we extended our experiment to investigate the involvement of BIRC6 in blastocyst formation and blastocyst quality and apoptosis. We found that the total blastocyst rate at 8 dpi was significantly ($P < 0.05$) reduced in ldsRNA- and shRNA-injected zygote groups (Table 3). Moreover, the proportion of hatched and expanded blastocysts obtained at 8 dpi was reduced ($P < 0.05$) in the ldsRNA- and shRNA-injected zygote groups compared with water-injected and uninjected groups, suggesting a developmental delay in the RNA-injected groups. The proportion of non-blastocyst embryos (embryos that did not reach the blastocyst stage) was increased in ldsRNA- and shRNA-injected zygote groups, indicating an increase in developmental arrest of the RNA-injected zygotes. The expression level of BIRC6 was determined in those non-blastocyst and blastocysts at 8 dpi, indicating that blastocyst-stage embryos derived from ldsRNA- and shRNA-injected zygotes exhibited a 40% reduction in BIRC6 mRNA and protein compared with the uninjected controls (Fig. 8*a*, *c*). Of course, the 40% knockdown obtained at the blastocyst stage seems to be modest. However, this can be explained by the fact that the potency and efficiency of RNA interference

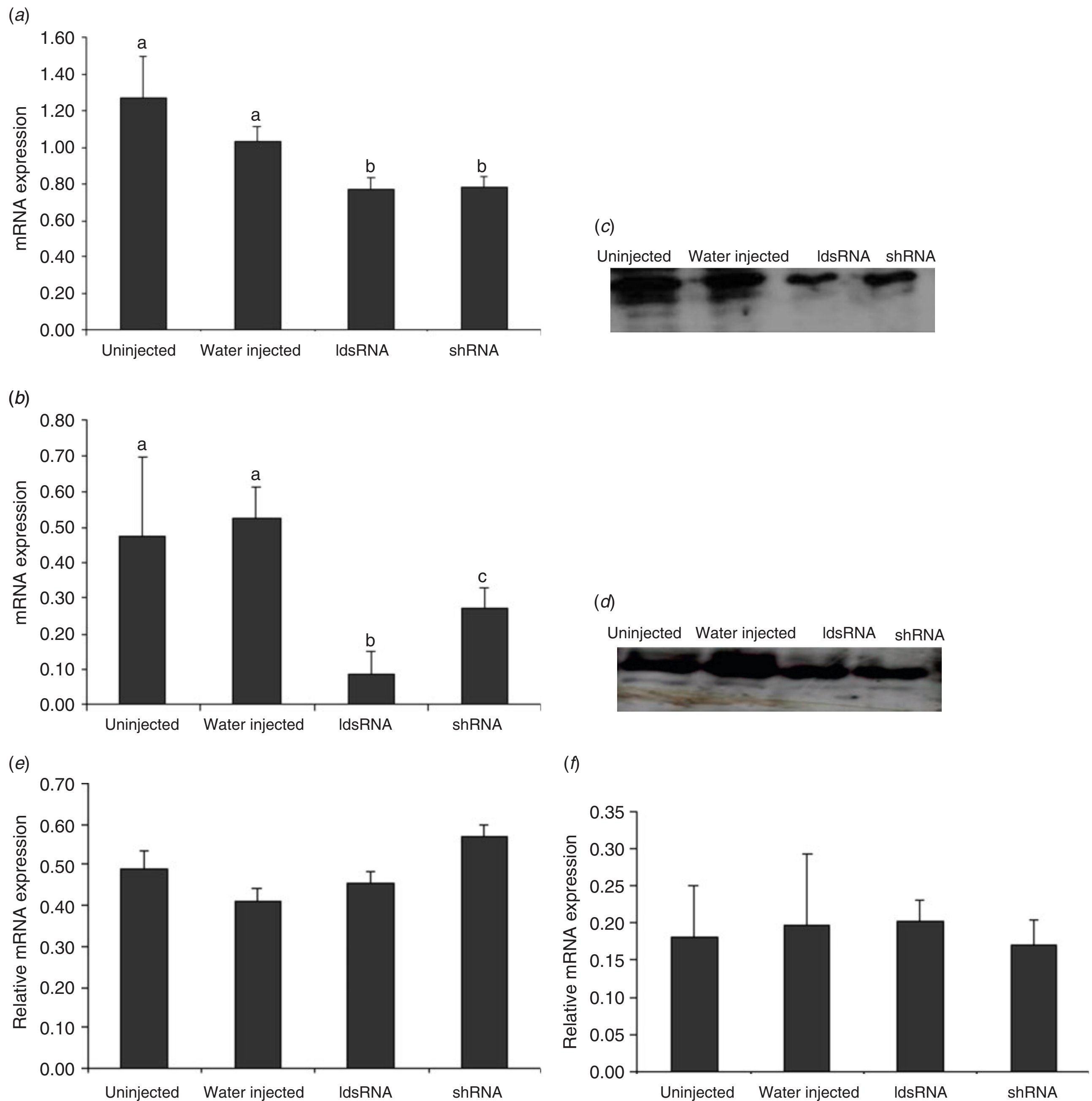

Fig. 8. The mRNA expression of BIRC6 in (*a*) blastocysts and (*b*) non-blastocyst embryos at 8 dpi. Three independent replicates each consisting of 15 blastocysts or 30 non-blastocyst embryos were used for the analysis. Bars show mean ± s.e.m. [a,b,c]Values with different letters indicate statistical significance ($P < 0.05$). Western blot analysis conducted to detect the BIRC6 protein level in (*c*) blastocysts and (*d*) non-blastocyst embryos at 8 dpi. The relative mRNA expression of GAPDH in (*e*) blastocysts and (*f*) non-blastocyst embryos at 8 dpi.

(RNAi) 7 days after transfection can be reduced due to the fact that those embryos reaching the blastocyst stage do have more blastomeres and cytoplasm size in which the accumulation of the transcript can mask degradation induced by RNAi. Secondly, the number of RNAi molecules that can be found at 8 dpi in each blastomere of the blastocyst may be reduced in a temporal fashion. Therefore, as the embryos grow to the blastocyst stage the RNAi molecules introduced at the zygotic stage may not persist to achieve the strong knockdown seen in the 8-cell embryo. However, in non-blastocyst embryos, which are developmentally arrested and unable to divide, the RNAi molecules are not dividing per cytoplasm, hence it was possible to achieve a 45 and 85% reduction in BIRC6 in ldsRNA- and shRNA-injected zygotes, respectively, even at 8 dpi. Hence, reduction of blastocyst

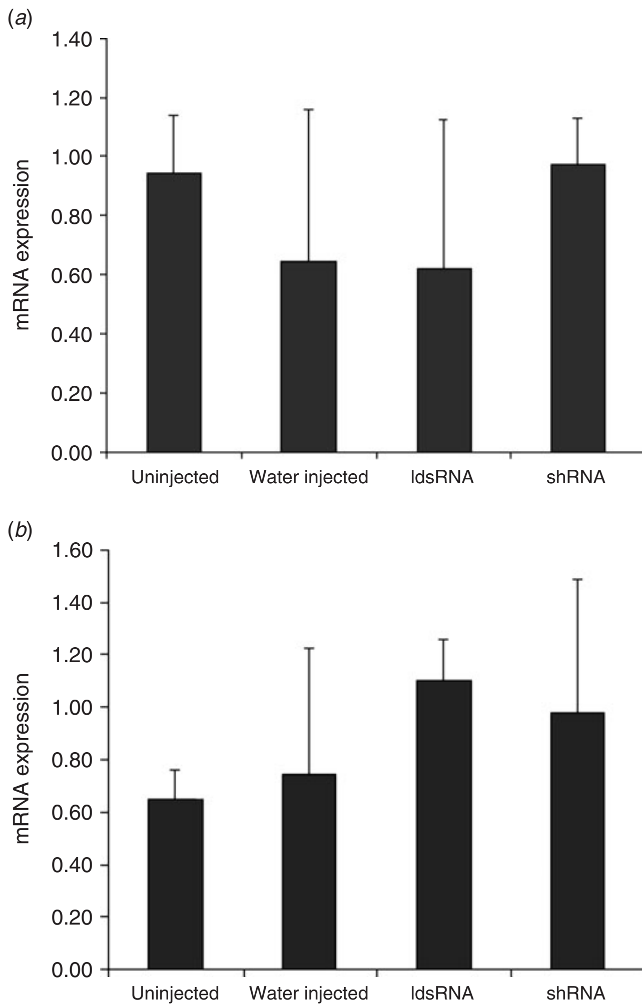

Fig. 9. The mRNA expression of Survivin (BIRC5) in (*a*) blastocysts and (*b*) non-blastocyst embryos at 8 dpi.

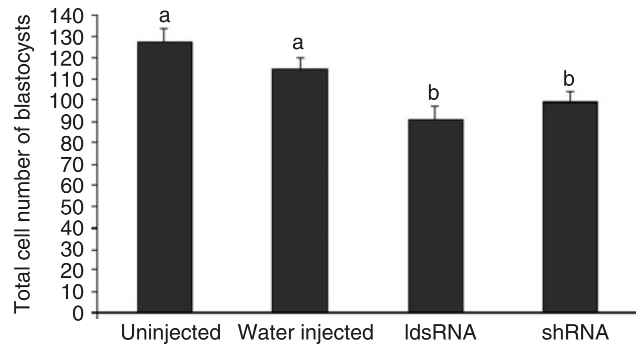

Fig. 10. The total cell number of blastocysts obtained from the different treatment groups namely: blastocysts derived from uninjected controls ($n = 54$), water-injected ($n = 62$), ldsRNA-injected ($n = 37$) and shRNA-injected ($n = 55$). Bars show mean ± s.e.m. The data was analysed from three independent biological replicates. [a,b]Values with different letters indicate statistical significance ($P < 0.05$).

formation in RNA-injected zygote groups can be attributed, in part, to the reduction in 8-cell embryos due to the reduced level of BIRC6 mRNA at 96 hpi. Furthermore, at 8 dpi those blastocyst embryos derived from ldsRNA- and shRNA-injected groups exhibited lower cell numbers, suggesting their inferior quality compared with control counterparts. Therefore, a significantly lower blastocyst rate and reduced blastocyst total cell number in addition to a reduction in BIRC6 mRNA and protein in ldsRNA- and shRNA-injected zygote groups indicate the involvement of the BIRC6 gene in the later stages of bovine preimplantation embryo development. Similar to this study, the functional contribution of BIRC6 has been documented in the later stage of mouse embryonic development by various studies. For instance, a BIRC6 mutant mouse has been reported to die due to progressive loss of the placental spongiotrophoblast layer between Day 11.5 and 14.5 of embryonic development (Hitz *et al*. 2005). In addition, inactivation of BIRC6 has resulted in mouse embryonic lethality between Day 11.5 and 16.5 due to increased apoptosis (Ren *et al*. 2005). BIRC6 may also be involved in normal trophoblast differentiation and embryo survival (Lotz *et al*. 2004).

Knockdown of BIRC6 was associated with increased level of apoptotic cell ratio, CASPASE 3 and 7 activity and lower total cell number

Although suppression of BIRC6 resulted in reduced developmental competence of zygotes in early and later stages of bovine preimplantation embryo development, the mechanism by which BIRC6 is involved is not clear. However, in a cell culture model it is found that BIRC6 knockdown was associated with a time-dependant decline of cell growth and an increased rate of apoptosis (Lopergolo *et al*. 2009). Furthermore, downregulation of the BIRC6 gene by RNA interference has been shown to promote cell death and sensitise cells to apoptotic stimuli (Qiu *et al*. 2004). In the present study, the apoptotic cell ratio, CASPASE 3 and 7 activity and expression profile of apoptosis-related transcripts were investigated in blastocysts derived from different treatment groups to verify whether BIRC6 is playing a role in bovine embryonic development as an apoptotic modulator. To understand the presence of active caspases, CASPASE 3 and 7 activity was monitored using fluorochrome inhibitors of caspases (FLICA), which binds covalently to the active caspase and results in a green fluorescent signal. The image from fluorescence microscopy (Fig. 11) indicated that blastocysts from zygotes injected with ldsRNA and shRNA exhibited a strong green fluorescent signal compared with blastocysts obtained from water-injected and uninjected controls, suggesting an increased level of level of CASPASE 3 and 7 activity in RNA-injected groups. Similarly, in mouse it has been documented that a deletion of the C-terminal half of BRUCE (the mouse BIRC6) and its ubiquitin-conjugating domain resulted in an increase in activation of CASPASE 3 and 7 in the placenta and yolk sack, leading to apoptosis (Ren *et al*. 2005).

Besides to caspase activity assay, we have also evaluated the incidence of apoptosis as DNA fragmentation using the TUNEL assay in blastocysts from different treatment groups. Consequently, it has been shown that the apoptotic cell ratio tended to be higher in RNA-injected groups (4.1 ± 0.7–4.2 ± 0.6)

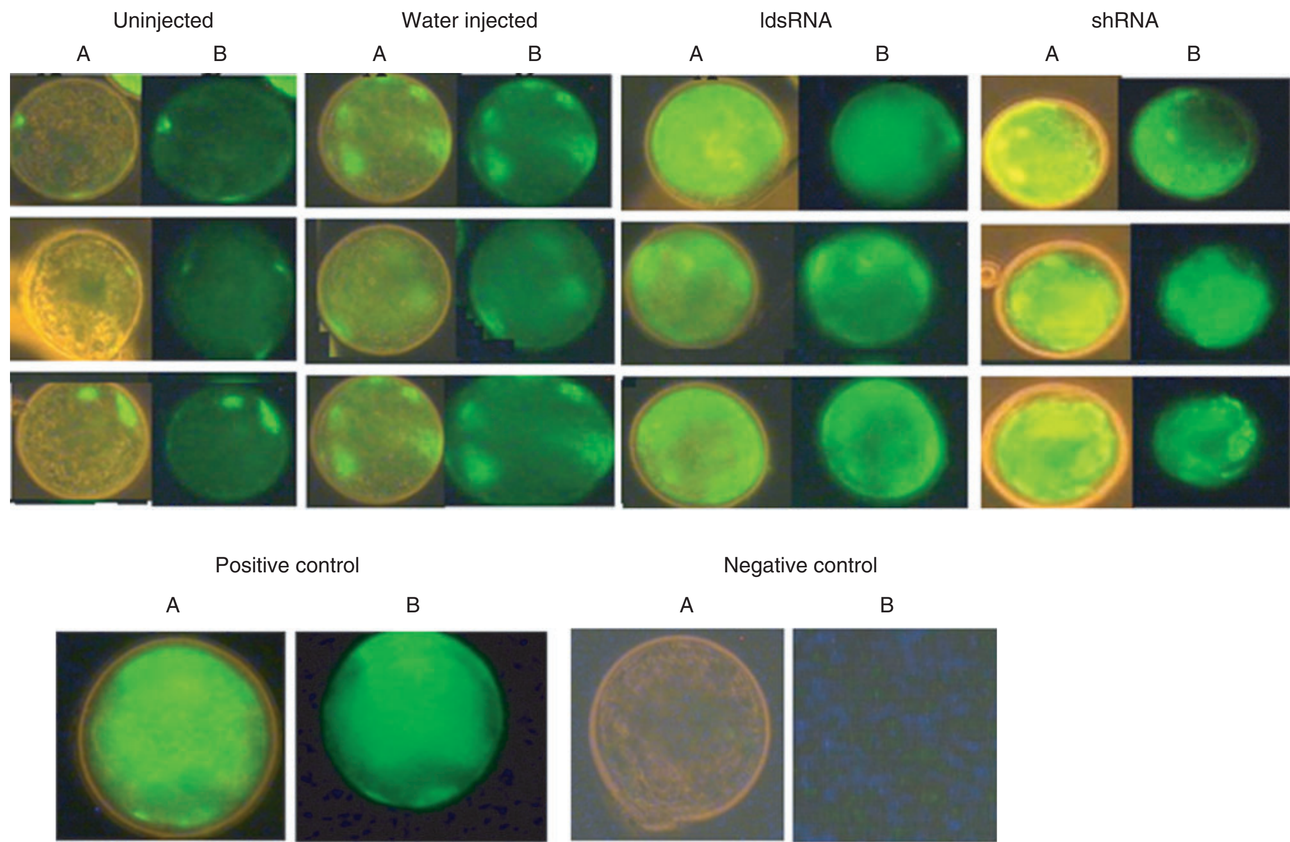

Fig. 11. Representative pictures of caspase-positive blastocysts in Day 8 blastocysts derived from different treatment groups. Lane A represents the caspase activity at 490 nm excitation and 520 nm emission in combination with visible light and Lane B represents the caspase activity at 490 nm excitation and 520 nm emission.

Table 4. Apoptotic cell ratio (mean ± s.e.m.) of blastocysts derived from zygotes injected with ldsRNA, shRNA and water and uninjected control

[a,b]Values with different letters indicate significant difference ($P < 0.05$). The apoptotic cell ratio was analysed from seven independent experimental replicates

Treatment	No. of blastocysts	Apoptotic cell ratio
Uninjected control	54	2.9 ± 0.4^{ab}
Water-injected	62	2.6 ± 0.3^{a}
ldsRNA-injected	36	4.1 ± 0.7^{b}
shRNA-injected	55	4.2 ± 0.6^{b}

compared with the controls (2.6 ± 0.3–2.9 ± 0.4) (Table 4). The lower total cell number in blastocysts derived from RNA-injected zygote groups may also be attributed to increased apoptosis in those embryos (Fig. 10).

Knockdown of BIRC6 was associated with increased BAX to BCL-2 ratio, SMAC and CASPASE 9 expression in blastocysts

In addition to TUNEL, CASPASE activity assay and total cell count, transcript analysis of apoptosis-related genes is thought to be a useful method for examining the quality of embryos subjected to environmental changes (Gutiérrez-Adán *et al*. 2004; Park *et al*. 2006). Hence, in the present study we have analysed pro-apoptotic BAX, CASPASE 9 and SMAC and anti-apoptotic BCL-2 to understand the shift of apoptotic regulatory genes due to inhibition of BIRC6 gene transcriptional abundance. We have found that although BCL-2 was not altered between blastocysts derived from treatment groups, BAX expression was increased significantly in blastocysts derived from zygotes injected with ldsRNA and shRNA. This is consistent with the report of Park *et al*. (2007), in which the anti-apoptotic Bcl-XL was not altered in blastocysts derived from double-stranded injected zygotes against bovine survivin compared with sham-injected zygotes, and the results of Yang and Rajamahendran (2002) who reported higher BAX expression in fragmented compared with good quality bovine embryos.

The mechanism for upregulation of BAX due to modest downregulation of BIRC6 in bovine embryos is not clear. To assess the relationship between BIRC6 and BAX or other apoptosis-related genes, gene symbols were uploaded in to the Ingenuity pathway analysis (Ingenuity Systems, www.ingenuity.com). As shown in (Fig. 14), BAX has indirect molecular interaction with BIRC6, suggesting an intermediate molecule is required to activate or deactivate BAX depending

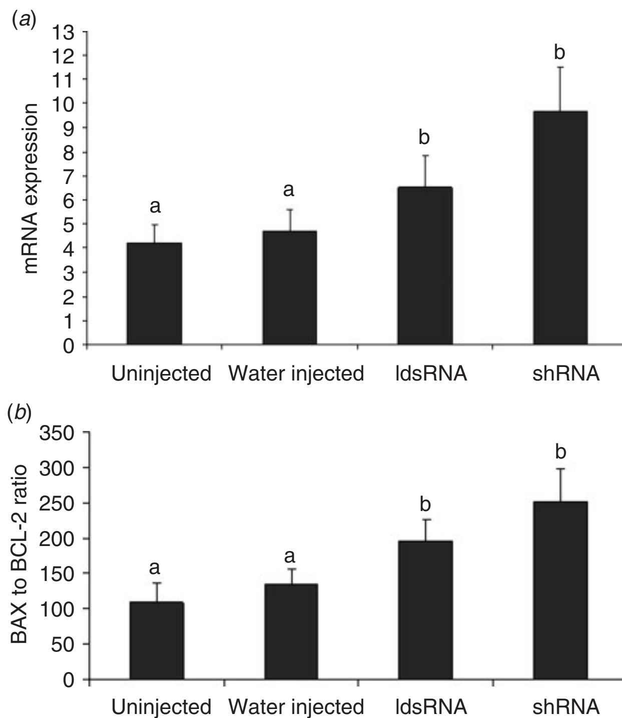

Fig. 12. (*a*) The mRNA expression of BAX in blastocysts. (*b*) BAX to BCL-2 transcript ratio in blastocysts. BAX to BCL-2 ratio was analysed from three independent replicates each consisting of 15 blastocysts. The mRNA level of BAX and BCL-2 was normalised using H2A as internal standard. The ratio was calculated by dividing the normalised value of BAX by the normalised value of BCL-2. Bars show mean ± s.e.m. [a,b]Values with different letters indicate statistical significance ($P < 0.05$).

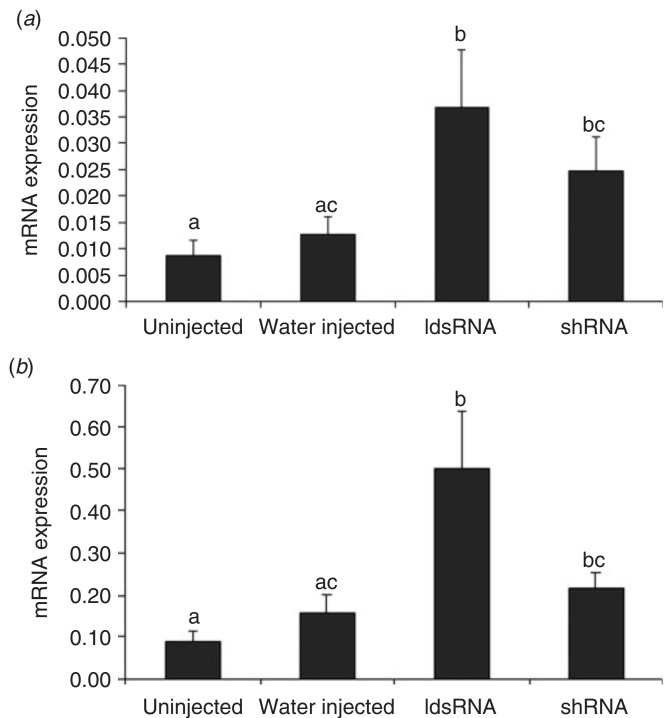

Fig. 13. mRNA expression of (*a*) SMAC and (*b*) CASPASE 9 in blastocysts at 8 dpi. [a,b,c]Values with different letters indicate statistical significance ($P < 0.05$).

on the level of BIRC6. Similarly, Ren *et al*. (2005) indicated an increase in BAX expression in a BIRC6 mutant mouse. According to those authors the increase of BAX in the BIRC6 mutant mouse was due to increased p53 activity, which in turn increases BAX and other pro-apoptotic genes, leading to mitochondrial apoptosis, suggesting p53 to be a direct target of BRUCE (mouse BIRC6). Therefore, the increase of BAX expression in blastocysts derived from zygotes injected with ldsRNA and shRNA against BIRC6 may be due to an increase in p53.

The ratio of BCL-2 to BAX is believed to be the main determinant of the fate of the cell (Chao and Korsmeyer 1998) and a higher BAX/BCL-2 ratio suggests cells to be vulnerable to apoptosis (Jarskog *et al*. 2004). Here we determined the BAX to BCL-2 ratio to obtain an insight into the incidence of apoptosis in blastocysts derived from the different treatment groups. The BAX to BCL-2 ratio was significantly higher in blastocysts derived from the RNA-injected groups compared with water-injected and uninjected zygote groups, showing elevated apoptosis (Fig. 12). Consistent with this, a study by Groc *et al.* (2001) demonstrated a significant increase in the BAX to BCL-2 ratio during the apoptotic death of nigral dopamine neurons in development, suggesting that a higher BAX to BCL-2 ratio is more often associated with cell death.

BIRC6 is believed to have cytoprotective activity by degradation of SMAC and by inhibiting caspase activity (Hao *et al.* 2004; Qui and Goldberg 2005). Furthermore, the Ingenuity pathway analysis (Fig. 14) shows that SMAC and CASPASE 9 have a direct relationship to BIRC6. Here we determined the expression of SMAC and CASPASE 9 to see whether knockdown of BIRC6 leads to activation of SMAC and CASPASE 9 in the bovine embryo and the results indicated that SMAC mRNA was increased 4.2- and 2.8-fold in ldsRNA and shRNA groups, respectively, compared with uninjected controls and 2.9- and 1.9-fold in ldsRNA and shRNA groups, respectively, compared with the water-injected group (Fig. 13*a*). Furthermore, CASPASE 9 mRNA was increased 5.6- and 2.4-fold in ldsRNA and shRNA groups, respectively, compared with uninjected controls and 3.2- and 1.4-fold in ldsRNA and shRNA groups, respectively, compared with the water-injected group (Fig. 13*b*). The increase of SMAC and CASPASE 9 suggests an increase in apoptosis in the blastocysts derived from the RNA-injected groups, and that these genes may be targets of BIRC6 during bovine *in vitro* embryo development.

In conclusion, our data reveals that suppression of BIRC6 using ldsRNA and shRNA resulted in a higher proportion of 2- and 4-cell and uncleaved embryos but a lower proportion of 8-cell embryos at 96 hpi, indicating higher embryonic arrest during bovine early preimplantation embryo development. In addition, targeted knockdown of the BIRC6 gene resulted in lower blastocyst formation and total blastocyst cell number and

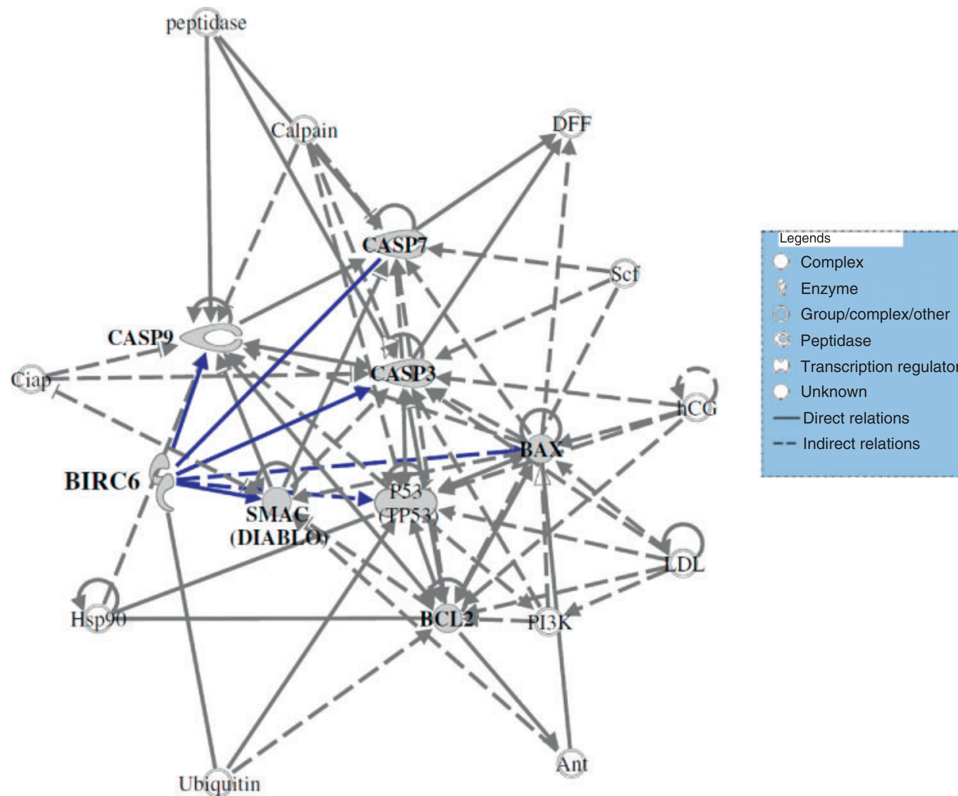

Fig. 14. The molecular network interaction between pro- and anti-apoptotic genes.

increased apoptosis levels as indicated by elevated caspase activity and BAX to BCL-2 ratio, SMAC and CASPASE 9 mRNA during the later stages of bovine preimplantation embryo development. Consequently, the results of this study revealed the importance of BIRC6 expression for embryo survival during bovine preimplantation embryo development. However, whether BIRC6 is essential for implantation and fetal development during pregnancy in cows needs further research.

References

Alikani, M., Cohen, J., Tomkin, G., Garrisi, G. J., Mack, C., and Scott, R. T. (1999). Human embryo fragmentation *in vitro* and its implications for pregnancy and implantation. *Fertil. Steril.* **71**, 836–842. doi:10.1016/S0015-0282(99)00092-8

Amanai, M., Shoji, S., Yoshida, N., Brahmajosyula, M., and Perry, A. (2006). Injection of mammalian metaphase II oocytes with short interfering RNAs to dissect meiotic and early mitotic events. *Biol. Reprod.* **75**, 891–898. doi:10.1095/BIOLREPROD.106.054213

Antunes, G., Chaveiro, A., Santos, P., Marques, A., Jin, H. S., and Moreira da Silva, F. (2008). Influence of apoptosis in bovine embryo's development. *Reprod. Domest. Anim.*, in press. doi:10.1111/J.1439-0531.2008.01131.X

Arama, E., Agapite, J., and Steller, H. (2003). Caspase activity and a specific cytochrome C are required for sperm differentiation in Drosophila. *Dev. Cell* **4**, 687–697. doi:10.1016/S1534-5807(03)00120-5

Bartke, T., Pohl, C., Pyrowolakis, G., and Jentsch, S. (2004). Dual role of BRUCE as an antiapoptotic IAP and a chimeric E2/E3 ubiquitin ligase. *Mol. Cell* **14**, 801–811. doi:10.1016/J.MOLCEL.2004.05.018

Bianchini, M., Levy, E., Zucchini, C., Pinski, V., Macagno, C., De Sanctis, P., Valvassori, L., Carinci, P., and Mordoh, J. (2006). Comparative study of gene expression by cDNA microarray in human colorectal cancer tissues and normal mucosa. *Int. J. Oncol.* **29**, 83–94.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., *et al.* (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–622. doi:10.1373/CLINCHEM.2008.112797

Cabot, R. A., and Prather, R. (2003). Cleavage stage porcine embryos may have differing developmental requirements for karyopherins alpha2 and alpha3. *Mol. Reprod. Dev.* **64**, 292–301. doi:10.1002/MRD.10238

Chao, D. T., and Korsmeyer, S. J. (1998). BCL-2 family: regulators of cell death. *Annu. Rev. Immunol.* **16**, 395–419. doi:10.1146/ANNUREV.IMMUNOL.16.1.395

Chen, Z., Naito, M., Hori, S., Mashima, T., Yamori, T., and Tsuruo, T. (1999). A human IAP-family gene, apollon, expressed in human brain cancer cells. *Biochem. Biophys. Res. Commun.* **264**, 847–854. doi:10.1006/BBRC.1999.1585

Chu, L., Gu, J., Sun, L., Qian, Q., Qian, C., and Liu, X. (2008). Oncolytic adenovirus-mediated shRNA against Apollon inhibits tumor cell growth and enhances antitumor effect of 5-fluorouracil. *Gene Ther.* **15**, 484–494. doi:10.1038/GT.2008.6

Corteling, R. L., Brett, S. E., Yin, H., Zheng, X. L., Walsh, M. P., and Welsh, D. J. (2007). The functional consequence of RhoA knockdown by RNA interference in rat cerebral arteries. *Am. J. Physiol. Heart Circ. Physiol.* **293**, H440–H447. doi:10.1152/AJPHEART.01374.2006

Deveraux, Q. L., and Reed, J. C. (1999). IAP family proteins suppressors of apoptosis. *Genes Dev.* **13**, 239–252. doi:10.1101/GAD.13.3.239

El-Sayed, A., Hoelker, M., Rings, F., Salilew, D., Jennen, D., Tholen, E., Sirard, M. A., Schellander, K., and Tesfaye, D. (2006). Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiol. Genomics* **28**, 84–96. doi:10.1152/PHYSIOLGENOMICS.00111.2006

Exley, G. E., Tang, C., McElhinny, A. S., and Warner, C. (1999). Expression of caspase and BCL-2 apoptotic family members in mouse preimplantation embryos. *Biol. Reprod.* **61**, 231–239. doi:10.1095/BIOLREPROD 61.1.231

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.

Gjørret, J. O., Fabian, D., Avery, B., and Maddox-Hyttel, P. (2007). Active caspase-3 and ultrastructural evidence of apoptosis in spontaneous and induced cell death in bovine *in vitro*-produced pre-implantation embryos. *Mol. Reprod. Dev.* **74**, 961–971. doi:10.1002/MRD.20714

Golding, M. C., Long, C. R., Carmell, M. A., Hannon, G. J., and Westhusin, M. (2006). Suppression of prion protein in livestock by RNA interference. *Proc. Natl. Acad. Sci. USA* **103**, 5285–5290. doi:10.1073/PNAS.0600813103

Groc, L., Bezin, L., Jiang, H., Jackson, T. S., and Levine, R. A. (2001). Bax, Bcl-2, and cyclin expression and apoptosis in rat substantia nigra during development. *Neurosci. Lett.* **306**, 198–202. doi:10.1016/S0304-3940(01)01897-3

Gutiérrez-Adán, A., Rizos, D., Fair, T., Moreira, P. N., Pintado, B., de la Fuente, J., Boland, M. P., and Lonergan, P. (2004). Effect of speed of development on mRNA expression pattern in early bovine embryos cultured *in vivo* or *in vitro. Mol. Reprod. Dev.* **68**, 441–448. doi:10.1002/MRD.20113

Hao, Y., Sekine, K., Kawabata, A., Nakamura, H., Ishioka, T., *et al*. (2004). Apollon ubiquitinates SMAC and caspase-9 and has an essential cytoprotection function. *Nat. Cell Biol.* **6**, 849–860. doi:10.1038/NCB1159

Hitz, C., Vogt-Weisenhorn, D., Ruiz, P., Wurst, W., and Floss, T. (2005). Progressive loss of the spongiotrophoblast layer of BIRC6/Bruce mutants results in embryonic lethality. *Genesis* **42**, 91–103. doi:10.1002/GENE.20128

Jarskog, L. F., Selinger, E. S., Lieberman, J. A., and Gilmore, J. H. (2004). Apoptotic proteins in the temporal cortex in schizophrenia: high Bax/Bcl-2 ratio without caspase-3 activation. *Am. J. Psychiatry* **161**, 109–115. doi:10.1176/APPI.AJP.161.1.109

Kennerdell, J. R., and Carthew, R. W. (2000). Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nat. Biotechnol.* **18**, 896–898. doi:10.1038/78531

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**, 1132–1136. doi:10.1126/SCIENCE.275.5303.1132

Lequarre, A. S., Traverso, J. M., Marchandise, J., and Donnay, I. (2004). Poly(A) RNA is reduced by half during bovine oocyte maturation but increases when meiotic arrest is maintained with CDK inhibitors. *Biol. Reprod.* **71**, 425–431. doi:10.1095/BIOLREPROD.103.026724

Lonergan, P., Rizos, D., Gutierrez-Adán, A., Moreira, P. M., Pintado, B., de la Fuente, J., and Boland, M. P. (2003). Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage *in vitro* or *in vivo*. *Biol. Reprod.* **69**, 1424–1431. doi:10.1095/BIOLREPROD.103.018168

Lopergolo, A., Pennati, M., Gandellini, P., Orlotti, N. I., Poma, P., Daidone, M. G., Folini, M., and Zaffaroni, N. (2009). Apollon gene silencing induces apoptosis in breast cancer cells through p53 stabilisation and caspase-3 activation. *Br. J. Cancer* **100**, 739–746. doi:10.1038/SJ.BJC.6604927

Lotz, K., Pyrowolakis, G., and Jentsch, S. (2004). BRUCE, a giant E2/E3 ubiquitin ligase and inhibitor of apoptosis protein of the trans-Golgi network, is required for normal placenta development and mouse survival. *Mol. Cell. Biol.* **24**, 9339–9350. doi:10.1128/MCB.24.21.9339-9350.2004

Makarevich, A. V., and Markkula, M. (2002). Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor I during *in vitro* maturation and culture. *Biol. Reprod.* **66**, 386–392. doi:10.1095/BIOLREPROD66.2.386

Nganvongpanit, K., Müller, H., Rings, F., Gilles, M., Jennen, D., Hölker, M., Tholen, E., Schellander, K., and Tesfaye, D. (2006*a*). Targeted suppression of E-cadherin gene expression in bovine preimplantation embryo by RNA interference technology using double-stranded RNA. *Mol. Reprod. Dev.* **73**, 153–163. doi:10.1002/MRD.20406

Nganvongpanit, K., Müller, H., Rings, F., Hoelker, M., Jennen, D., Tholen, E., Havlicek, V., Besenfelder, U., Schellander, K., and Tesfaye, D. (2006*b*). Selective degradation of maternal and embryonic transcripts in *in vitro* produced bovine oocytes and embryos using sequence-specific double-stranded RNA. *Reproduction* **131**, 861–874. doi:10.1530/REP.1.01040

Oltval, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609–619. doi:10.1016/0092-8674(93)90509-O

Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* **16**, 948–958. doi:10.1101/GAD.981002

Paradis, F., Vigneault, C., Robert, C., and Sirard, M. (2005). RNA interference as a tool to study gene function in bovine oocytes. *Mol. Reprod. Dev.* **70**, 111–121. doi:10.1002/MRD.20193

Park, S. Y., Kim, E. Y., Cui, X. S., Tae, J. C., Lee, W. D., Kim, N. H., Park, S. P., and Lim, J. H. (2006). Increase in DNA fragmentation and apoptosis-related gene expression in frozen–thawed bovine blastocysts. *Zygote* **14**, 125–131. doi:10.1017/S0967199406003649

Park, S. Y., Kim, E. Y., Jeon, K., Cui, X. S., Lee, W. D., Kim, N. H., Park, S. P., and Lim, J. (2007). Survivin acts as anti-apoptotic factor during the development of bovine pre-implantation embryos. *Mol. Reprod. Dev.* **74**, 582–590. doi:10.1002/MRD.20641

Qiu, X. B., and Goldberg, A. (2005). The membrane-associated inhibitor of apoptosis protein, BRUCE/Apollon, antagonizes both the precursor and mature forms of Smac and caspase-9. *J. Biol. Chem.* **280**, 174–182.

Qiu, X. B., Markant, S. L., Yuan, J., and Goldberg, A. (2004). Nrdp1-mediated degradation of the gigantic IAP, BRUCE, is a novel pathway for triggering apoptosis. *EMBO J.* **23**, 800–810. doi:10.1038/SJ.EMBOJ.7600075

Reed, J. C. (1997). Double identity for proteins of the Bcl-2 family. *Nature* **387**, 773–776.

Ren, J., Shi, M., Liu, R., Yang, Q. H., Johnson, T., Skarnes, W. C., and Du, C. (2005). The Birc6 (Bruce) gene regulates p53 and the mitochondrial pathway of apoptosis and is essential for mouse embryonic development. *Proc. Natl. Acad. Sci. USA* **102**, 565–570. doi:10.1073/PNAS.0408744102

Roccheri, M. C., Tipa, C., Bonaventura, R., and Matranga, V. (2002). Physiological and induced apoptosis in sea urchin larvae undergoing metamorphosis. *Int. J. Dev. Biol.* **46**, 801–806.

Salilew-Wondim, D., Rings, F., Hölker, M., Gilles, M., Jennen, D., *et al*. (2007). Dielectrophoretic behaviour of *in vitro*-derived bovine metaphase II oocytes and zygotes and its relation to *in vitro* embryonic developmental competence and mRNA expression pattern. *Reproduction* **133**, 931–946. doi:10.1530/REP-06-0277

Salvesen, G., and Duckett, C. S. (2002). IAP proteins: blocking the road to death's door. *Nat. Rev. Mol. Cell Biol.* **3**, 401–410. doi:10.1038/NRM830

Sato, F., Kurokawa, M., Yamauchi, N., and Hattori, M. A. (2006). Gene silencing of myostatin in differentiation of chicken embryonic myoblasts

by small interfering RNA. *Am. J. Physiol. Cell Physiol.* **291**, C538–C545. doi:10.1152/AJPCELL.00543.2005

Soares, M. L., Haraguchi, S., Torres-Padilla, M. E., Kalmar, T., Carpenter, L., *et al*. (2005). Functional studies of signalling pathways in peri-implantation development of the mouse embryo by RNAi. *BMC Dev. Biol.* **5**, 28. doi:10.1186/1471-213X-5-28

Telford, N. A., Watson, A. J., and Schultz, G. A. (1990). Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol. Reprod. Dev.* **26**, 90–100. doi:10.1002/MRD.1080260113

Tesfaye, D., Wimmers, K., Gilles, M., and Schellander, K. (2004). A comparative expression analysis of gene transcripts in post-fertilization developmental stages of bovine embryos produced *in vitro* or *in vivo*. *Reprod. Domest. Anim.* **39**, 396–404. doi:10.1111/J.1439-0531.2004.00531.X

Tesfaye, D., Lonergan, P., Hoelker, M., Rings, F., Nganvongpanit, K., Havlicek, V., Besenfelder, U., Jennen, D., Tholen, E., and Schellander, K. (2007). Suppression of connexin 43 and E-cadherin transcripts in *in vitro*-derived bovine embryos following culture *in vitro* or *in vivo* in the homologous bovine oviduct. *Mol. Reprod. Dev.* **74**, 978–988. doi:10.1002/MRD.20678

Thélie, A., Pennetier, S., Perreau, C., Traverso, J. M., Uzbekova, S., Mermillod, P., Joly, C., Humblot, P., and Dalbiès-Tran, R. (2007). Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation *in vitro* and *in vivo*. *BMC Dev. Biol.* **7**, 125. doi:10.1186/1471-213X-7-125

Vandaele, L., Maes, D., de Kruif, A., and Van Soom, A. (2006). Is apoptosis in bovine *in vitro*-produced embryos related to early developmental kinetics and *in vivo* bull fertility? *Theriogenology* **65**, 1691–1703. doi:10.1016/J.THERIOGENOLOGY.2005.09.014

Verhagen, A. M., Coulson, E. J., and Vaux, D. L. (2001). Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol.* **2**, reviews3009.1–reviews3009.10. doi:10.1186/GB-2001-2-7-REVIEWS3009

Warner, C. M., McElhinny, A. S., and Tang, C. (1998). Genetic regulation of preimplantation mouse embryo survival. *J. Exp. Zool.* **282**, 272–279. doi:10.1002/(SICI)1097-010X(199809/10)282:1/2<272::AID-JEZ29>3.0.CO;2-U

Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell Biol.* **2**, 70–75. doi:10.1038/35000016

Wieschaus, E. (1996). Embryonic transcription and the control of developmental pathways. *Genetics* **142**, 5–10.

Yang, M. Y., and Rajamahendran, R. (2002). Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced *in vitro*. *Anim. Reprod. Sci.* **70**, 159–169. doi:10.1016/S0378-4320(01)00186-5

Manuscript received 1 May 2009, accepted 16 September 2009