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Paternal breed effects on expression of *IGF-II*, *BAK1* and *BCL2-L1* in bovine preimplantation embryos

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Summary

The effects of the paternal breed on early embryo and later pre- and postnatal development are well documented. Several recent studies have suggested that such paternal effects may be mediated by the paternally induced epigenetic modifications during early embryogenesis. The objective of this study was to investigate the effects of the paternal breed on the early embryonic development and relative expression of the maternally imprinted gene, *IGF-II*, and the apoptosis-related genes *BAK1* and *BCL2-L1* in *in vitro* produced (IVP) bovine embryos derived from two unrelated paternal breeds (Holstein and Brown Swiss). The degree of correlation of *IGF-II* expression pattern with embryo developmental competence and apoptosis-related genes was also investigated. The relative abundance of *IGF-II*, *BCL2-L1* and *BAK1* transcripts in day 8 embryos was measured by quantitative reverse-transcription polymerase chain reaction using the comparative C_p method. Our data revealed that the paternal breed did not influence cleavage rate, blastocyst rate and relative abundance of *IGF-II*, *BAK1* and *BCL2-L1* in day 8 blastocysts ($P > 0.05$). Nevertheless, *IGF-II* expression levels were highly correlated with embryonic developmental competence ($r = 0.66$, $P < 0.1$), relative expression of *BCL2-L1* ($r = 0.72$, $P < 0.05$) and ratio of *BCL2-L1*/*BAK1* ($r = 0.78$, $P < 0.05$). In conclusion, our data show that *IGF-II*, *BCL2-L1* and *BAK1* expression is not related to the chosen combination of paternal breed, but that *IGF-II* expression is correlated with embryonic viability and apoptosis-related gene expression.

Keywords: *BAK1*, *BCL2-L1*, Bovine blastocyst, *IGF-II*, Paternal breed

Introduction

Reproductive efficiency, the ability of cows to become pregnant and produce a healthy live calf, is one the primary factors affecting productivity in the beef or dairy industry. Reproductive efficiency has been shown to be determined by a number of factors, including maternal environment and nutrition, embryo quality, heat stress (Bilodeau-Goeseels & Kastelic, 2003; Hansen, 2007) and maternal and/or paternal breeds (Fryer *et al.*, 1958; Fischer *et al.*, 2000; Casas *et al.*, 2011).

Paternal effects on reproductive efficiency have been shown to operate through various mechanism, including influence on fertilization and embryonic survival rates (Giritharan *et al.*, 2007), blastocyst apoptotic index (Jeong *et al.*, 2005; Giritharan *et al.*, 2007) and placental development and characteristics (Constancia *et al.*, 2002); however, the exact mechanisms are still unclear. There is a growing body of evidence which shows that new sets of epigenetic marks, established during both gametogenesis and in early embryonic development

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followed by genome-wide reprogramming, could significantly influence gene expression pattern in the embryo and beyond (for review, see Chason *et al.*, 2011). In addition, some of the epigenetics marks that are established during the preimplantation period are stable during replication, and may affect gene expression at later stages of development (Dean *et al.*, 1998; Khosla *et al.*, 2001; Shao *et al.*, 2008).

Previous studies have provided strong evidence for the importance of expression of imprinted genes in embryonic (Rappolee *et al.*, 1992; Perecin *et al.*, 2009) and fetal development (Wu *et al.*, 2004; Kwong *et al.*, 2006). The maternally imprinted insulin-like growth factor II (*IGF-II*) gene on bovine chromosome 29 is one of the best characterized epigenetically regulated loci (Goodall & Schmutz, 2003; Gebert *et al.*, 2009). Insulin-like growth factors have been shown to play important roles in early embryonic (Rappolee *et al.*, 1992; Wang *et al.*, 2009), placental, and fetal development (Sibley *et al.*, 2004; Su *et al.*, 2011), probably through combined activation of multiple cell signaling pathways critical to survival, growth, and differentiation (Kim *et al.*, 2008). In addition, the *IGF-II* gene provides an excellent model for studying RNA involvement in maintenance of nucleosome-retained regions of the sperm chromatin in spite of nearly complete replacement of DNA-bound histones with protamines (Jenkins & Carrell, 2011). It is suggested that retained histones may contain modifications that play a critical role in epigenetic regulation (Jenkins & Carrell, 2012). Accordingly, given that epigenetic patterns govern gene expression (for review see Niemann *et al.*, 2008), it can be hypothesized that paternal breed effect on reproductive efficiency is mediated through, at least in part, influence on expression levels of *IGF-II* gene.

The aim this study was to assess the effects of paternal breed on the early embryo development and relative expression of *IGF-II* in day 8 bovine blastocysts. As a second objective, we examined whether the paternal breed influences blastocyst quality, which is considered the most reliable factor influencing embryo viability, by analysis of blastocyst transcript abundance of selected apoptosis-associated genes such as *BCL2-L1* (anti-apoptotic) and *BAK1* (pro-apoptotic) (Jeong *et al.*, 2005).

Materials and methods

Chemicals and reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise stated.

In vitro production (IVP) of embryos

Oocyte collection and in vitro *maturation (IVM)*

Bovine ovaries, originating mainly from culled Danish dairy cows and heifers, were obtained from a local abattoir, (Herlufmagle Slaughtery, Denmark) and transported to the laboratory within 3 h of retrieval in 0.9% physiological saline solution at 29–32°C in a thermocontainer. The temperature from ovary collection throughout all manipulations outside the incubator was kept within this temperature range. Immature cumulus–oocyte complexes (COCs) were retrieved from antral follicles (2–20 mm in diameter) by use of a vacuum pump (KNF-Neuberger N86 KN.18, VWR International, Copenhagen, Denmark) connected with an 18-gauge needle (18-G × 1.5, 1.2 × 40, Terumo Neolus Luer) and collected into a 50-ml tube containing 140 μl heparin (5000 i.u./ml, Leo Pharma, Ballerup, Denmark). The COCs were retrieved and washed three times in HEPES-buffered TCM-199 wash medium (M 2520, Sigma) supplemented with, 50 μg/ml gentamycin (G 3632, Sigma) and 0.5 mg/ml bovine serum albumin (BS; A4919, Sigma). The COCs were washed once after retrieval in *in vitro* maturation (IVM) medium before maturation. Groups of 35–40 COCs were placed in 500 μl maturation medium in four-well dishes (Nunc, Roskilde, Denmark) and cultured without oil overlay for 24 h at 38.8 ± 0.2°C in 5% CO_2 in ambient humidified air. The IVM medium consisted of Dulbecco's Eagle's Modified Medium (DMEM; D6046, Sigma) supplemented with 5% fetal calf serum (FCS; produced by the Danish Veterinary Institute, Copenhagen, Denmark), 15 IE/ml equine serum gonadotrophin (PMSG)-HCG (Suigonan, VetPharm, Løgstør, Denmark), 0.2 mM pyruvate (P3662, Sigma), 50 ng/ml epidermal growth factor (EGF; E4127, Sigma) and 50 μg/ml gentamicin (G1264, Sigma).

Sperm preparation and in vitro *fertilization (IVF)*

The COCs were evaluated for cumulus expansion and viscoelasticity and scored from 0–3 on an arbitrary scale and transferred in groups of 40 into four-well dishes containing 400 μl fertilization TALP medium. All experiments were carried out using frozen–thawed semen from proven unrelated high fertile Brown Swiss and Holstein bulls, (both from Viking Genetics, Randers, Denmark). Straws containing frozen semen from the test bulls were thawed at 37°C and washed twice by centrifugation at 328 *g* for 5 min in non-capacitation medium (NCM: 0.5 mM $NaHCO_3$ (S 4019, Sigma); 113 mM NaCl (S 5886, Sigma); 4.78 mM KCl (P 5405, Sigma); 1.19 mM $MgSO_4$ (M 2643, Sigma); 1.2 mM KH_2PO_4 (P 5655, Sigma); 21.6 mM sodium lactate (L4263, Sigma); 50 μg/ml gentamicin (G1264, Sigma); pH 7.4). The spermatozoa were counted in

a Makler chamber and diluted in Sperm-TALP to give a concentration of 50 × 10^6 sperm cells/ml. An appropriate volume of this suspension was added to fertilization wells to obtain a final concentration of 2 × 10^6 spermatozoa/ml. The spermatozoa were co-incubated with matured COCs in TALP medium for 22–24 h without oil overlay at 38.8 ± 0.2°C in 5% CO_2 in ambient humidified air. The TALP medium contained 25 mM sodium bicarbonate (S4019, Sigma), 10 mM lactate (L4263, Sigma), 6 mg/ml BSA (A4919, Sigma), 0.25 mM pyruvate (P3662, Sigma), 30 μg/ml heparin (H3149,Sigma), 50 μg/ml gentamicin (G1264, Sigma), 20 μM penicillamine (P4875, Sigma), 10 μM hypotaurine (H1384, Sigma), and 1 μM epinephrine (E4250, Sigma).

Embryo culture after fertilization

After 22–24h of *in vitro* fertilization (IVF), the inseminated oocytes were denuded of surrounding cumulus cells and excess spermatozoa were removed by vortex agitation. After three washes in HEPES-buffered tissue culture medium (TCM-199, M 2520, Sigma), the zygotes were washed once in culture medium (BA1216 medium, Origio, Måløv, Denmark). The inseminated oocytes were cultured in groups of 35 in 500 μl culture medium (BA1216 medium) with an overlay of oil in low oxygen, 5% O_2 and 5% CO_2, at 38.8 ± 0.2°C for 168 h. On day 8, embryos from all groups were scored for the rate of blastocyst development (day 0 = IVF). The resulting blastocyst rates were expressed per inseminated oocyte placed into *in vitro* culture (IVC). Day 8 blastocysts were used for mRNA transcript abundance analysis using reverse-transcription–qualitative polymerase chain reaction (RT-qPCR).

Gene expression analysis

Isolation of total RNA and cDNA synthesis

In vitro-produced day 8 blastocysts were first transferred from the culture medium into TCM-199 and groups of 10 blastocysts were rinsed three times in phosphate-buffered saline (PBS), and finally transferred into 500-μl centrifuge tubes with a minimal amount of PBS. Samples were immediately snap-frozen in liquid nitrogen and stored at –80°C until RNA isolation. Total RNA was isolated from a pool of eight *in vitro* produced (IVP) blastocysts using a NucleoSpin® RNA XS Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. To eliminate any contaminating DNA, a DNase digestion step was performed. The quantity and purity (260 nm/280 nm ratio) of total RNA was estimated using a NanoDrop ND-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE, USA). The total RNA of each pool was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo-Scientific) with random hexamer primers according to the manufacturer's instructions. The reverse-transcription reaction was carried out in thermo-cycler using the following temperature conditions: 25°C for 10 min, 42°C for 60 min, 95°C for 5 min, and cooled down to 4°C.

Primer design

PCR primers except *H2A* and *BCL2-L1* were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA) based on public transcript and genomic sequence data from cattle, mice and humans (see Table 1). Primer sequences for *H2A* and *BCL2-L1* genes were obtained from previously published data (Robert *et al.*, 2002, Hwang *et al.*, 2008).

For each gene analyzed, a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) also was conducted to ensure the specificity of the designed primers and to assure that they were not designed from any homologous regions, coding for other genes. The primer sequences, expected fragment sizes and the sequence references are shown in Table 1.

Reverse-transcription quantitative real-time PCR (RT-qPCR)

Relative expression levels were quantified in real time on a LightCycler® 480 Instrument (Roche Diagnostics, Indianapolis, IN, USA) with LightCycler® 480 SYBR Green I Master mix (Roche Applied Science, Germany) as detection chemistry. The PCR mixture consisted of 2 μl diluted cDNA (10 ng), 0.5 μl forward and reverse primers (10 mM), and 5 μl SYBR Green I master mix, and 2 μl water to a total volume of 10 μl. PCR reactions were performed according to the manufacturer's instructions. First, cDNA was denatured and pre-incubated by heating for 10 s at 95°C. Template was then amplified by 45–55 cycles consisting of denaturation for 10 s at 95°C, annealing for 10 s at 58–60°C, and extension for 20 s at 72°C. At least three replicates were used for each reaction, and a reaction without template served as negative control. To verify RT-PCR product identity, melting point curves were analyzed following amplification.

The relative quantification of gene expression was analyzed by the comparative C_p method (Pfaffl *et al.*, 2002), using LightCycler 480 software (release 1.5.0 SP4). Quantification was normalized to the endogenous control, *H2A* (Robert *et al.*, 2002). The mathematical model used is based on the PCR efficiencies and the crossing point deviation between targets and reference gene of each sample. The PCR product sizes were confirmed by gel electrophoresis on a standard 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

Table 1 Primer sequences and cycling conditions used in quantitative real-time PCR and for sequencing

Gene	GenBank accession no.	Primer sequence	No. of cycles/ annealing temp. (°C)	Amplicon size (bp)
IGF-II	X53553	F: 5′-GGCAAGTTCTTCCAATATGA-3′ R: 5′-TGGCAGAATTACGACACT-3′	55/58	239
BAK1	NM_001077918.1	F: 5′-AGAACCTAGCAGCACCAT-3′ R: 5′-CGATCTTGGTGAAGTACTC-3′	45/60	150
BCL2-L1	NM_001077486.2	F: 5′-GTTGACTTTCTCTCCTACAAGC-3′ R: 5′-GGTACCTCAGTTCAAACTCATC-3′	45/60	277
H2A	U62674	F: 5′-GTCGTGGCAAGCAAGGAG-3′ R: 5′-GATCTCGGCCGTTAGGTACTC-3′	45/58	182
IGF-II[a]	X53553	F: 5′-GGCAAGTTCTTCCAATATGAC-3′ R: 5′-AAAGCCAATTCGTTTCAAGG-3′	37/55	553

[a]This primer pair was designed for amplification of the bovine IGF-II DMR located at exon10 for DNA sequencing.

DNA extraction from sperm and amplification

Genomic DNA was extracted from frozen/thawed sperm using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After extraction, the quality and quantity of total DNA was determined using a NanoDrop spectrophotometer. Sperm DNA was amplified in the differentially methylated region (DMR) and shore of exon 10 of the bovine *IGF-II* gene using primers (Table 1). This region included all 27 putative CpG sites between nucleotides 257 and 676 bp of the coding region (Gebert *et al.*, 2006). Amplification was performed in a 25-μl reaction volume, using *Taq* PCR master mix (Qiagen), which included 1 μl of sperm DNA, 10 mM each primer, 12.5 μl of *Taq* PCR master mix and 10.5 μl RNase-free water. The temperature cycles were as follows: 95°C for 5 min, followed by 37 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. Amplified gene-specific PCR products were analyzed on a 2% agarose in 1× TBE buffer gel containing 0.2 mg/ml ethidium bromide (EtBr) which were run for 40 min at 90 V. A 100-bp DNA ladder (Fermentas, Germany) was used as a size marker to determine the size of PCR products.

Sequencing of bovine DMR2 DNA Fragments

PCR products were separated by gel electrophoresis and gel bands were cut out and purified using the Wizard® SV Gel and PCR Clean-up kit (Promega, Fitchburg, WI, USA) according to the manufacturer's protocol to remove primers and dNTPs, and then sequenced to detect possible differences across samples using commercial sequencing service (DNA Technology, Risskov, Denmark).

Statistical analysis

The IVP experiments were repeated four times. Relative quantification of target gene expression levels as fold-differences was based on at least three sets of biological samples and three technical samples. Embryos development data were analyzed with the chi-squared test using logistic regression model. The percentage of cleaved embryos and blastocysts formed are presented as the mean ± standard error of the mean (SEM).

Linear mixed model (Chen *et al.*, 2004) followed by multiple pair-wise comparisons using the Student–Newman–Keuls multiple comparisons test was used for analysis of variance components of gene expression data obtained by RT-qPCR. The following mixed model was used was for analysis gene expression data: $y_{ijk} = \mu + S_i + O_j + K_{ij} + e_{ijk}$, where: y_{ijk} is the gene expression in blastocysts, μ is the overall mean, S_i is the fixed effect of the sperm genotype (1, 2), O_j is the random effect of the batch of ova from ovary collected in each replicate, K_{ij} is the interaction effect of sperm genotype and batch of oocyte, and e_{ijk} is the random residual error. Pearson's pair-wise correlation coefficient was used to determine the degree of correlation between *in vitro* embryo development and gene expression in blastocyst stage embryos. Data were statistically analyzed using the computer program JMP 7.0 (SAS Institute Inc., Cary, NC, USA).

Results

In the present study, the embryonic developmental competence and mRNA expression profiles of *IGF-II*, *BAK1* and *BCL2-L1* were compared in bovine preimplantation embryos derived from two different paternal genetic backgrounds (breeds).

In four replicates, in total 897 oocytes were utilized for IVP of bovine embryos using sperm from two different bulls' breeds: Holstein and Swiss Brown. The cleavage and blastocyst rates are presented in Table 2.

Table 2 Cleavage and blastocyst rates following IVP using semen from either Holstein or Brown Swiss bulls[a]

Sperm donor	Oocyte no.	Cleaved embryo no. (%; mean ± SEM)	Blastocysts no. (%; mean ± SEM)
Holstein	462	419[a] (86 ± 1.07)	158 (34.2 ± 2.4)[a]
Brown Swiss	435	392[a] (88 ± 1.23)	17 (39.2 ± 2.5)[a]

[a]Four replicated trials were carried out, and blastocysts were analyzed on day 8.
The blastocyst rate was calculated from the number of inseminated oocytes.
Values with same letters are not statistically different ($P > 0.05$).
IVP, *in vitro* production; SEM, standard error of the mean.

Table 3 Analysis of variance for *IGF-II*, *BAK1* and *BCL2-L1* gene expression in blastocysts derived from two unrelated sperm origins

		P-value		
Source of variation	DF	*IGF-II*	*BCL2-L1*	*BAK1*
Sperm genotype[a]	1	0.5318	0.5151	0.9124
Batch of oocyte	3	0.3158	0.9066	0.1328
Batch of oocyte × sperm genotype	3	0.0001[b]	0.0001[b]	0.0001[b]

[a]Sperm genotype was considered a fixed effect while all other effects were considered random.
[b]Interaction between sperm genotype and batch of oocyte significantly influence on gene expression ($P < 0.05$).
DF = degree of freedom.

No statistical differences were found in both cleavage and blastocyst rates between the two sperm breeds derived embryos ($P > 0.05$) (Table 2).

The mRNA expressions of *IGF-II*, *BAK1*, and *BCL2-L1* genes were determined by RT-qPCR. The genes selected for the analysis have been shown to play decisive roles in the regulation and execution of apoptosis. There was no significant effect of sperm breeds and/or batch of oocytes on relative expression of mRNA of *IGF-II* (Fig. 1*A*), *BCL2-L1* (Fig. 1*B*), *BAK1* (Fig. 1C) and the ratio of *BCL2-L1/BAK1* (Fig. 1*D*), however relative expression of *IGF-II*, *BCL2-L1* and *BAK1* were significantly influenced by the interaction between sperm genotype and four separate batches of oocytes ($P < 0.05$) (Table 3).

Pearson's pair-wise correlation coefficient shows that the blastocyst production rate was positively correlated ($r = 0.66$, $P < 0.1$) with *IGF-II* gene expression levels of the blastocyst. Furthermore, level of *IGF-II* expression highly correlated with *BCL2-L1* ($r = 0.72$, $P < 0.05$) and ratios of *BCL2-L1/BAK1* ($r = 0.78$, $P < 0.05$), however level of *IGF-II* expression had no correlation with *BAK1* expression.

In order to explore breed-specific genetic markers and determination of their potential role in explanations of interaction between sperm breeds and batches of oocytes and/or environment (stochastic extrinsic factors in replicate) on the *IGF-II* expression pattern, we compared sequences of DMR within the last exon of the bovine *IGF-II* gene between Holstein and Brown Swiss sperm (Fig. 2*A*). The resulting DNA sequences were compared with GenBank database entries using a BLAST search. DNA sequence alignment analysis indicated that no sequence differences exist between the analyzed sperm DNA (Fig. 2*B*).

Discussion

The preimplantation period of mammalian embryogenesis is notable for many critical events that affect the genome both before and after implantation. During this time, reshuffling of the parental genomes, as well as genome-wide epigenetic reprogramming provide a new set of instructions that guide both early embryonic and life-long growth and development (Chason *et al.*, 2011).

In the present study, we found that sperm species neither influenced the rate of fertilization as assessed by the cleavage rates ($P > 0.0.5$), nor blastocyst formation rate ($P > 0.0.5$) (Table 2). These results are in agreement with those reported in other breeds of cattle in which Holstein oocytes have been fertilized with semen from Nelore or Angus bulls (Barros *et al.*, 2006; Eberhardt *et al.*, 2009). In contrast, several studies reported differences in cleavage and/or blastocyst formation rate between sire breeds; i.e. Nelore oocytes fertilized with semen from Nelore or Angus bulls (Barros *et al.*, 2006; Eberhardt *et al.*, 2009) or Holstein oocytes, fertilized with semen from Holstein or brown Swiss bulls (Lazzari *et al.*, 2011). The cause of this discrepancy is not clear, but it could be attributed to the inbreeding depression (Lazzari *et al.*, 2011) or species-specific sperm–egg interactions (Fischer *et al.*, 2000; Zi *et al.*, 2009). Using norm-finder software, we found that the mRNA levels of bovine histone *H2A* are more constant across the replicate than bovine B-actin (*ACTB*) and ubiquitin B (*UBB*) (data not shown). Our data are in agreement with reports showing that mRNA levels of bovine histone *H2A* are more constant across the preimplantation period than other endogenous housekeeping genes (Robert

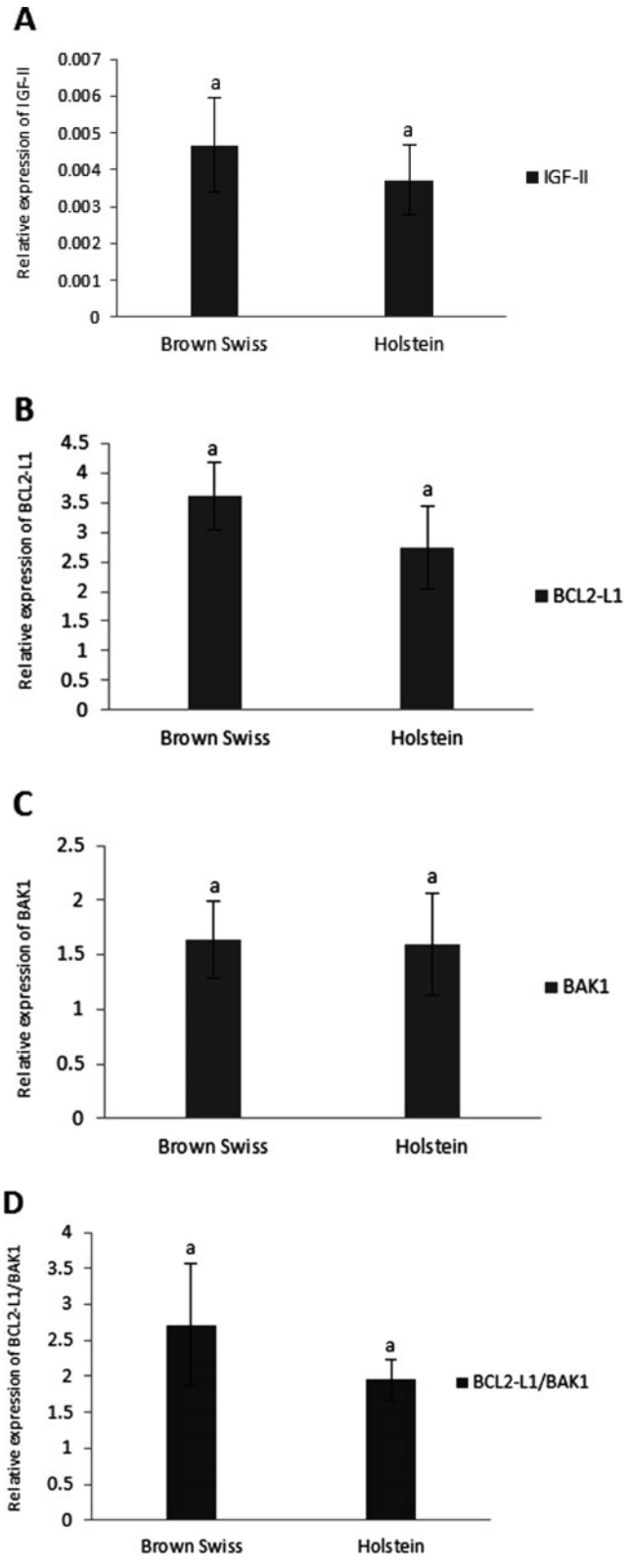


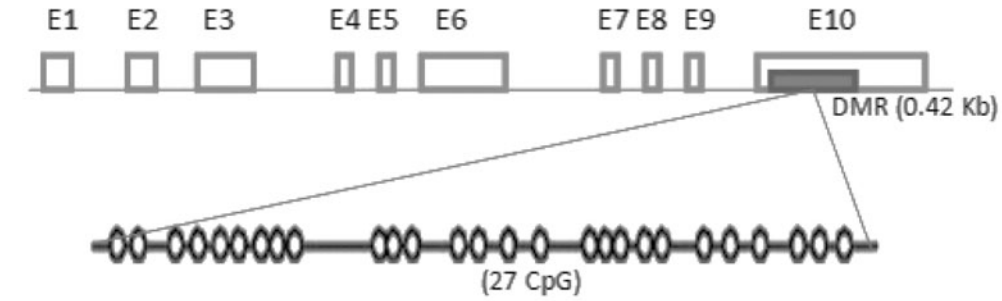
Figure 1 Levels of *IGF-II (A)*, *BCL2-L1 (B)*, *BAK1 (C)* and ratio of *BCL2-L1/BAK1 (D)* expression in day 8 bovine blastocysts following *in vitro* production (IVP) using semen from either Holstein or Brown Swiss bulls (mean ± standard error of the mean (SEM)). Bars with same letters are not statistically different (P > 0.05).

et al., 2002; Lonergan *et al.*, 2003a; Vigneault *et al.*, 2007).

The experimental results and statistical analysis show that neither the sperm breed, nor the oocyte population (batch of ova) affected *IGF-II*, *BAK1* and *BCL2-L1* expression (P > 0.05). In contrast, their interaction significantly influence on the level of *IGF-II*, *BAK1* and *BCL2-L1* expression in blastocysts (*P* < 0.05) (Table 3). Overall, the lack of significant effects of sperm breed and the presence of significant interactions tend to imply that the *IGF-II*, *BCL2-L1*and *BAK1* expression can be modulated by heritable and non-heritable factors. Precise interpretation of gene expression results is difficult given the probable differences in oocyte populations (breed, age, quality) and/or stochastic extrinsic factors connected with the experimental procedure (environment factors). However, numerous studies have demonstrated that variable culture conditions [due to maturation (Warzych *et al.*, 2007) and/or culture media composition (Natale *et al.*, 2001), incubation temperatures (Jin *et al.*, 2007; Yadav *et al.*, 2013), oxygen concentration (Harvey, 2007) and media osmolality (Hwang *et al.*, 2008)] can lead to alteration in expression levels of developmentally important genes in *in vitro* developed embryos. Along with these reports, Feinberg & Irizarry have recently developed an evolutionary model in which DNA mutations that do not change the mean phenotype could modify the variability of phenotype; and this could be mediated epigenetically through tissue-specific differentially methylated regions (T-DMRs) (Feinberg & Irizarry, 2010). In consistence with their evolutionary model, a set of orthogonal contrasts within replicates among the transcript level of the endogenous *IGF-II* in blastocysts derived from cattle oocytes fertilized either with Brown Swiss or Holstein spermatozoa, shows significant differences in levels of *IGF-II* expression (p < 0.05); Thus for investigation of this likelihood we explored the sequence variation at a recently identified intragenic DMR within the last exon (exon10) of the bovine *IGF-II* gene (Gebert *et al.*, 2006, 2009). The sequencing result revealed that no differences existed in this DMR of *IGF-II* between the analyzed sperm samples, which may imply that these regions are conserved in evolution (Gebert *et al.*, 2006; Han *et al.*, 2008a). In addition, the effects of certain combinations of parental alleles (dominance, over dominance and epistatic components) on propensity to blastomere cytofragmentation and/or embryo development which are well documented in the cow and mouse, may partially explain these interactions (Han *et al.*, 2008b; Zi *et al.*, 2009).

The present study has shown for the first time a strong correlation between *BCL2-L1/BAK1* ratios and endogenous *IGF2* expression at the bovine blastocyst stage. The ratio of *BCL2-L1* to *BAK1* has been shown

A



B

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                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                              10        20        30        40        50        60        70        80

Brown Swiss         GGCAAGTTCT TCCAATATGA CATCTGGAAG CAGTCCACCC AGCGCCTGCG CAGGGGCCTG CCCGCCTTCC TGCGAGCACG
Holstein            GGCAAGTTCT TCCAATATGA CATCTGGAAG CAGTCCACCC AGCGCCTGCG CAGGGGCCTG CCCGCCTTCC TGCGAGCACG
Consensus           GGCAAGTTCT TCCAATATGA CATCTGGAAG CAGTCCACCC AGCGCCTGCG CAGGGGCCTG CCCGCCTTCC TGCGAGCACG

                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                              90        100       110       120       130       140       150       160

Brown Swiss         CCGGGGTCGC ACGCTCGCCA AGGAGCTGGA GGCGCTCAGA GAGGCCAAGA GTCACCGTCC GCTGATCGCC CTGCCCACCC
Holstein            CCGGGGTCGC ACGCTCGCCA AGGAGCTGGA GGCGCTCAGA GAGGCCAAGA GTCACCGTCC GCTGATCGCC CTGCCCACCC
Consensus           CCGGGGTCGC ACGCTCGCCA AGGAGCTGGA GGCGCTCAGA GAGGCCAAGA GTCACCGTCC GCTGATCGCC CTGCCCACCC

                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                              170       180       190       200       210       220       230       240

Brown Swiss         AGGACCCTGC CACCCACGGG GGCGCCTCTT CCAAGGCATC CAGCGATTAG AAGTGAGCCA AAGTGTCGTA ATTCTGCCAA
Holstein            AGGACCCTGC CACCCACGGG GGCGCCTCTT CCAAGGCATC CAGCGATTAG AAGTGAGCCA AAGTGTCGTA ATTCTGCCAA
Consensus           AGGACCCTGC CACCCACGGG GGCGCCTCTT CCAAGGCATC CAGCGATTAG AAGTGAGCCA AAGTGTCGTA ATTCTGCCAA

                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                              250       260       270       280       290       300       310       320

Brown Swiss         GTGGCACCAT CTACCTCGCG CCGACCTCCT GACCGGGACC GCCCCACTAG GTCTCTCTCT GAAATCCCTG TACCGTCCTG
Holstein            GTGGCACCAT CTACCTCGCG CCGACCTCCT GACCGGGACC GCCCCACTAG GTCTCTCTCT GAAATCCCTG TACCGTCCTG
Consensus           GTGGCACCAT CTACCTCGCG CCGACCTCCT GACCGGGACC GCCCCACTAG GTCTCTCTCT GAAATCCCTG TACCGTCCTG

                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                              330       340       350       360       370       380       390       400

Brown Swiss         TCTGCGGGCT CCCCTGCCCC GGCCTCTGTG CCCCAACCTC CCCACGTCAG GCGAATCCCC CTCGGCCCCC TCCATCTGGC
Holstein            TCTGCGGGCT CCCCTGCCCC GGCCTCTGTG CCCCAACCTC CCCACGTCAG GCGAATCCCC CTCGGCCCCC TCCATCTGGC
Consensus           TCTGCGGGCT CCCCTGCCCC GGCCTCTGTG CCCCAACCTC CCCACGTCAG GCGAATCCCC CTCGGCCCCC TCCATCTGGC

                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                              410       420       430       440       450       460       470       480

Brown Swiss         TGAGGGGATC AGAACAACAT CTCTAAAAAT GTACAAAACC AATTGGCTTT AAATATCCCC CCAAATTATC ACCCCCCAAA
Holstein            TGAGGGGATC AGAACAACAT CTCTAAAAAT GTACAAAACC AATTGGCTTT AAATATCCCC CCAAATTATC ACCCCCCAAA
Consensus           TGAGGGGATC AGAACAACAT CTCTAAAAAT GTACAAAACC AATTGGCTTT AAATATCCCC CCAAATTATC ACCCCCCAAA

                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
                              490       500       510       520       530       540       550

Brown Swiss         TTACCCCCAA ATTACACAAC CAAAATTGCA ATCATGAACC CCTCAATCAG CCCCCTTGAA ACGAATTGGC TTT
Holstein            TTACCCCCAA ATTACACAAC CAAAATTGCA ATCATGAACC CCTCAATCAG CCCCCTTGAA ACGAATTGGC TTT
Consensus           TTACCCCCAA ATTACACAAC CAAAATTGCA ATCATGAACC CCTCAATCAG CCCCCTTGAA ACGAATTGGC TTT
```

Figure 2 (*A*) Molecular structure of the bovine *IGF2* gene. Differentially methylated regions (DMRs) in the last exon of bovine *IGF-II* containing 27 CpG islands is indicated in the blue box. (*B*) Alignment with the previously published sequence of bovine *IGF-II* gene (GenBank accession no. X53553) revealed no sequence differences between the analyzed sperm DNA.

to be a critical determinant of cell fate (Willis *et al.*, 2005) such that elevated *BCL2-L1* favors extended survival of cells and increasing levels of *BAK1* expression accelerate cell death (Jeong *et al.*, 2005; Hwang *et al.*, 2008). Typically, *BCL2-L1* transcripts are more abundantly expressed in *in vivo* rather than *in vitro* produced embryos and/or morphologically grade 1 than grade 2 blastocyst (which are generally accepted to differ in their developmental potential) (Jeong *et al.*, 2005; Gyu-Jin Rho *et al.*, 2007; Lloyd

et al., 2009). These correlations tend to imply that the level of endogenous *IGF-II* may act as an anti-apoptotic factor by enhanced expression of the anti-apoptotic gene *BCL2-L1* and influence the ratio of anti-apoptotic to pro-apoptotic related gene expression. This observation supported previous findings that during preimplantation development *in vitro*, *IGF-II* reduce apoptosis and increase embryo viability in bovine (Byrne *et al.*, 2002) and mouse embryos (Kurzawa *et al.*, 2004; Glabowski *et al.*, 2005).

The mechanism whereby *IGF-II* interferes with the pro-apoptotic signaling cascade triggered by intrinsic or extrinsic factors is unclear. However, anti-apoptotic effects of *IGF-II* are considered mainly mediated through the *IGF-IR* (Nussbaum *et al.*, 2008), which is expressed in bovine embryos from zygote to blastocyst stages (Yaseen *et al.*, 2001; Lonergan *et al.*, 2003a). It has been demonstrated that activation of phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB), upon binding of *IGF-II* to insulin-like growth factor receptor (*IGF-1R*), could in turn lead to activation of transcription of the anti-apoptotic *BCL2-L1* gene, which can prevent the apoptosis through binding to pro-apoptotic *BAK1* (Willis *et al.*, 2005). Furthermore, activation IRS-mediated pathway upon binding of *IGF-II* to insulin-like growth factor receptor (*IGF-1R*), could lead to the phosphorylation of BCL2-associated agonist of cell death (*BAD*) (Peruzzi *et al.*, 1999; Vincent & Feldman, 2002), which has been shown to neutralize *BCL2-L1*'s anti-apoptotic function through heterodimerization. However, in its phosphorylated form, *BAD* cannot bind to *BCL2-L1* protein (Peruzzi *et al.*, 1999; Fu *et al.*, 2000). Hence, either one or all of these mechanisms may support our correlation analysis results.

In the present study, the levels of *IGF-II* expression were positively correlated ($r = 0.66$, $P < 0.1$) with *in vitro* embryo development measured by blastocyst production rates, indicating that the viability of embryos is represented by *IGF-II* expression. This observation supports previous finding by Rappolee *et al.* (1992), which reported that the amount of endogenous *IGF-II* transcripts is directly correlated with the development competence of cleaving zygotes. This result is consistent with those found in previous reports showing that the developmentally more competent embryos had higher *IGF-II* mRNA concentrations than their lower developmentally competent counterpart at the blastocyst stage (Yaseen *et al.*, 2001; Lonergan *et al.*, 2003a, 2003b).

In conclusion, our data show that *IGF-II*, *BCL2-L1* and *BAK1* expression is not related to the chosen combination of paternal breed, but that *IGF-II* expression is correlated with embryonic viability and apoptosis-related gene expression. Furthermore, our results indicate that a mechanism contributing to the anti-apoptotic effects of *IGF-II* might be connected with the change in ratio between the mRNA levels of the pro-apoptotic *BAK1* and the anti-apoptotic *BCL2-L1* genes in bovine blastocysts.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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