

Morphologic and transcriptomic assessment of bovine embryos exposed to dietary long-chain fatty acids

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Abstract

The main objectives of this study were to determine the influence of diets enriched in α -linolenic, linoleic or oleic acid on the development and transcriptomic profile of embryos collected from dairy cattle. Non-lactating Holstein cows received one of the three diets supplemented with 8% rolled oilseeds: flax (FLX, $n=8$), sunflower (SUN, $n=7$) or canola (CAN, $n=8$). After a minimum 35-day diet adaptation, cows were superovulated, artificially inseminated and ova/embryos recovered non-surgically after 7.5 days. Cows fed FLX had less degenerated embryos and more viable embryos than those fed CAN or SUN. In total, 175 genes were differentially expressed in blastocysts from cows fed FLX than in cows fed CAN or SUN. These differentially expressed genes were mainly involved in cellular growth and proliferation, cellular development, and cell survival and viability. In conclusion, dietary n-3 polyunsaturated fatty acids reduced early embryonic degeneration possibly through improving embryonic cell survival and viability.

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Introduction

The establishment of pregnancy in cattle requires ovulation of a competent oocyte, insemination at the optimum time and production of sufficient embryonic interferon τ to inhibit prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release (Hunter 1989, Robinson *et al.* 2006). In this regard, it has been shown that feeding dairy cows diets supplemented with n-3 polyunsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids suppressed $PGF_{2\alpha}$ production, delayed luteal regression and improved conception rate (Burke *et al.* 1997). Although it is widely accepted that dietary fats (Thatcher 2010) and fatty acids (Mattos *et al.* 2000) enhance reproductive performance in dairy cows, the effect of fatty acids on the transcriptomic profile of early embryos remains unknown.

Bovine oocytes contain 63 μ g fat (McEvoy *et al.* 2000) that is used as an energy source during oocyte maturation and the extended period of embryo development before implantation (Paczkowski *et al.* 2013, 2014). Inhibition of fatty acid oxidation in bovine oocytes reduced their capacity to form blastocysts after fertilization (Ferguson & Leese 2006). High concentrations of non-esterified

fatty acids (NEFA) and ketone bodies were found in the follicular fluid of the dominant follicle (Leroy *et al.* 2004) during early postpartum period. *In vitro* culture models indicated that high-NEFA concentrations were detrimental to the oocyte's nuclear maturation, developmental competence, granulosa cell viability and steroidogenic capacity (Leroy *et al.* 2005, Vanholder *et al.* 2005). It has been demonstrated that palmitic and stearic acids predominate the follicular fluid of lactating dairy cows and adding these fatty acids to *in vitro* maturation medium of bovine cumulus–oocyte complexes (COC) inhibited cumulus expansion and reduced progression to metaphase II and blastocyst rate (Leroy *et al.* 2005). However, adding oleic acid to *in vitro* maturation medium reversed the detrimental effects of palmitic and stearic acids on subsequent embryo development (Aardema *et al.* 2011). Canola seed has high amount of oleic acid (61% of total fat) and is a common ingredient in dairy cattle rations in Western Canada; however, its influence on *in vivo* early embryonic development has not been investigated.

Previously, our group (Thangavelu *et al.* 2007) found that feeding lactating dairy cows a diet enriched

in α -linolenic (flaxseed) or linoleic acid (sunflower seed) enhanced embryonic development through a significant increase in blastomere number compared with feeding a diet enriched in saturated fatty acid (high in stearic and palmitic acids). Moreover, cows that were fed a diet supplemented with a partially rumen protected n-3 polyunsaturated fatty acids (PUFA; high in eicosapentaenoic and docosahexaenoic acids) had a lower proportion of degenerated embryos than those fed a diet supplemented with palmitic acid (Childs *et al.* 2008). A combination of *in vivo* and *in vitro* approaches has been used to examine the effect of PUFA on oocyte developmental competence (Moallem *et al.* 2013). In this regard, feeding a diet enriched in α -linolenic acid (flaxseed oil) increased the number of follicles and oocytes collected by transvaginal ultrasonography, enhanced the cleavage rate of *in vitro* fertilized oocytes and tended to improve blastocyst rate compared with feeding a diet enriched in saturated fatty acid (Moallem *et al.* 2013). In another study, adding α -linolenic acid to *in vitro* maturation medium enhanced oocyte maturation and subsequent embryo development, whereas adding linoleic acid significantly inhibited cumulus cell expansion, delayed development of the oocytes to the metaphase II stage and reduced cleavage and blastocyst rates (Marei *et al.* 2009, 2010). These results suggest that α -linolenic acid enhanced embryo development most probably through improved oocyte competence. Although these studies have provided significant information regarding the effect of fatty acids on *in vivo* embryo development or on oocyte competence, very limited information is available about the transcriptomic profile of embryos exposed to different fatty acids during their development. Therefore, the main objectives of this study were to examine the influence of diets enriched in α -linolenic, linoleic or oleic acid on the development and transcriptomic profile of embryos collected from dairy cattle.

Materials and methods

Embryo production

The study was conducted at the Laird W McElroy Environmental & Metabolism Research Centre, University of Alberta, Edmonton, Canada, with all animal experimental procedures approved (Protocol # AUP00000131) by the University of Alberta Animal Care and Use Committee and animals were cared in accordance with the Guidelines of the Canadian Council of Animal Care (1993). Non-lactating Holstein cows (726 ± 11.4 kg) were blocked by parity and body weight and were assigned to one of the three diets supplemented with flax (FLX; high in α -linolenic acid, $n=8$), sunflower (SUN; high in linoleic acid, $n=7$) or canola (CAN; high in oleic acid, $n=8$) oilseed. Cows were individually fed a diet containing hay (8.8 kg/day on a dry matter basis) and concentrate mix (3.8 kg/day on a dry matter basis). The concentrate portion of the diet contained 0.99 kg/day dry matter basis of rolled canola seed, sunflower seed or flaxseed. The energy and protein content of dietary treatments and fatty acid profiles of supplemented oilseeds are presented in Table 1. After a minimum 35-day diet adaptation, ovarian status was synchronized and superstimulated as described previously (Colazo *et al.* 2005) with purified porcine follicle-stimulating hormone (Folltropin-V; Bioniche Animal Health Inc, Belleville, ON, Canada). Cows were then artificially inseminated twice, 12 h apart, with frozen-thawed semen from the same sire and ova/embryos recovered non-surgically 7.5 days post-insemination. A total of 35 embryo collections (uterine flushing) were performed (FLX: 12, SUN: 11 and CAN: 12). Once started on a diet, cows continued to receive the same diet until the end of the experiment. Before embryo collection, ovarian status was determined by transrectal ultrasonography and cows with less than two corpora lutea were not used for embryo collection. Collected embryos were classified according to the *Manual of the International Embryo Transfer Society* (3rd ed, Savoy, IL, USA) as Stage 1 (unfertilized), Stage 2 (2–8 cells), Stage 3 (early morula), Stage 4 (morula), Stage 5 (early blastocyst), Stage 6 (blastocyst) and Stage 7 (expanding or expanded blastocyst). In this study, embryos of Stages 6 and 7 were considered blastocysts. We defined transferable embryos

Table 1 Energy and protein content of dietary treatments and fatty acid profile of supplemented oilseeds.

	Dietary treatments		
	Flaxseed	Sunflower seed	Canola seed
NEL (Mcal/kg)	1.43	1.48	1.55
Crude protein (%)	16.90	17.20	16.60
Fatty acid profile of oilseeds (g/100g of total fatty acids)			
Palmitic acid (SFA)	5.41	6.80	4.08
Stearic acid (SFA)	3.72	5.29	1.80
Oleic acid (MUFA)	18.04	12.47	61.18
Linoleic acid (PUFA)	15.37	73.13	18.84
α -linolenic acid (PUFA)	56.52	0.71	9.62
$n-6:n-3$ ratio	0.27	10.3	1.95
Total SFA	9.57	13.55	7.175
Total MUFA	18.11	12.63	63.79
Total PUFA	71.91	73.84	28.50
PUFA:SFA ratio	7.51	5.45	3.97

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

and the proportion of viable embryos as total number of grade 1 and 2 blastocysts and the proportion of grade 1 and 2 blastocysts over total collected embryos respectively. Grade 1 and 2 blastocysts from each cow were pooled to create two or more biological replicates per cow with each pool consisting of one to five blastocysts.

Total RNA isolation for microarray analysis

We extracted total RNA from four biological replicates per dietary treatment. Total RNA was extracted using Arcturus PicoPure RNA Isolation Kit (Applied Biosystems) in both the experiments (microarray and real-time q-PCR). Total RNA quality and integrity of each sample were evaluated by Bioanalyzer RNA 6000 Pico LabChip (Agilent Technologies), and all samples showed RNA integrity number greater than 7.5 in both the experiments.

RNA amplification and labelling for microarray analysis

The extracted RNA was amplified using RiboAmp HSPlus kit (Applied Biosystems) as per the manufacturer's instructions and generated amplified RNA (aRNA) targets for microarray reactions. Thereafter, the quantity and quality of the aRNA products from RNA amplification reactions were evaluated by spectrophotometry (ND-2000, Nanodrop Technologies, Wilmington, DE, USA) and microelectrophoresis (2200 TapeStation, Agilent Technologies) respectively. aRNA weighing 2 µg was used in each labelling reaction. All labelling reactions were performed using the ULS Fluorescent Labelling Kit (Kreatech Diagnostics, Amsterdam, The Netherlands) as per the manufacturer's instructions. The labelling of aRNA targets was processed under an ozone-free environment. The labelling efficiency of each labelled sample was evaluated using spectrophotometry (ND-2000, Nanodrop Technologies, Wilmington, DE, USA).

Microarray experimental procedure

The amplified RNA (825 ng per replicate) was hybridized on Agilent-manufactured EmbryoGENE slides in a two-colour dye swap design (Robert *et al.* 2011). After 17 h at 65°C, the microarray slides were washed in Gene Expression Wash Buffer 1 (room temperature; 1 min), in Gene Expression Wash Buffer 2 (42°C, 3 min), in 100% acetonitrile (room temperature, 10 s), and in Stabilization and Drying Solution (30 s; Agilent Technologies) and scanned with Axon 4200AL scanner (Molecular Device, Sunnyvale, CA, USA). Intensity files were analyzed with FlexArray (Blazejczyk *et al.* 2007).

Intensity raw data were corrected by background subtraction and normalized within (green or red) and between each array (Loess and quartile respectively) (Tsoi *et al.* 2012). The dataset of the microarray results has been deposited in NCBI's Gene Expression Omnibus and is accessible through Gene Expression Omnibus series accession number GSE67686.

To identify differentially expressed genes, the normalized microarray data were analyzed using the 'limma' package (Smyth 2005) of Bio-conductor through FlexArray (Robert *et al.* 2011).

For any particular analysis, a 2 × 2 comparison was performed (FLX vs CAN; SUN vs CAN; and FLX vs SUN), and only genes with *P* value ≤ 0.05 and a fold-change (FC) ≥ 2.0 were considered significantly upregulated or downregulated.

Functional analysis of differential gene expression profile

The expression data obtained from the comparative transcriptomic analysis were analyzed using the Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com).

Biological function analysis

The biological functions were performed under BH-FDR multiple testing correction conditions. Only the biological functions with a BH-FDR-corrected *P* value (B-H *P* value) < 0.05 and with a -log (B-H *P* value) > 2.0 were considered significant.

Network generation

Differentially expressed genes (*P* value ≤ 0.05 and a fold-change (FC) ≥ 2.0) called network-eligible molecules, were overlaid onto a global molecular network developed from the information contained in Ingenuity's Knowledge Base.

IPA upstream regulator analysis

The upstream regulator analysis predicted the activation status of the upstream regulators by calculating a regulation Z-score and an overlap *P* value, which were based on the number of known regulation target genes from the dataset of interest, expression changes of these target genes, and their agreement with the literature findings. Upstream regulators with an overlap *P* value ≤ 0.05 and an IPA activation Z-score ≥ 2.0 (or ≤ -2.0) were considered significantly activated (or inhibited).

IPA regulation Z-score and overlap *P* value were calculated as described in IPA white papers 'A Novel Approach to Predicting Upstream Regulators' and a full description is available on the IPA website (<http://www.ingenuity.com>) under 'Upstream Regulator Analysis', 'Biological Functions Analysis' and 'Ingenuity Canonical Pathways Analysis'.

Validation of microarray results

For validation, total RNA was extracted from another three biological replicates per dietary treatment (each with a pool of three to five grade 1 and 2 blastocysts). The extracted RNA was used to confirm the expression of six genes (*PTGS2*, *LGALS3*, *ANXA1*, *IL1RN*, *KRT19* and *SRXN1*) based on the microarray data analysis by real-time quantitative PCR (q-PCR).

Real-time q-PCR

Primers were designed (Supplementary Table 1, see section on supplementary data given at the end of this article) using Integrated DNA Technologies (<https://www.idtdna.com/scitools/Applications/RealTimePCR>) and analyzed by BLAST

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to verify primer specificity. A total of 1 ng of total RNA extracted from each pool of embryos was reverse transcribed into cDNA using a high-capacity reverse transcriptase (SuperScript VILO cDNA Synthesis Kit, Invitrogen) in a 20 µL reverse transcription (RT) reaction volume, as per the manufacturer's instructions. The cDNA products were then diluted once, and 1 µL of the diluted cDNA was used as the template with the StepOnePlus Real-Time PCR System (Applied Biosystems) and Fast SYBR Green Master Mix (Applied Biosystems). Melting curve analyses were performed at the end of each run to ensure the specificity of the amplification. Each assay included negative controls with no template, and each sample was analyzed in duplicate. Primer efficiency was determined using the standard curve method (with at least five serial dilutions) in a pool of all samples.

The mRNA abundance of target genes was normalized to the expression level of a reference gene (ribosomal protein L19 (*RPL19*)), according to the comparative Ct method ($\Delta\Delta C_t$) with correction for amplification efficiency of each primer pair (Pfaffl 2001, Abedini *et al.* 2015).

Serum metabolites and fatty acid composition

To evaluate the effect of dietary treatments on serum fatty acid profiles and metabolites (NEFA, BHBA and glucose), blood samples were collected in non-heparinized, silicone-coated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) at oestrus and kept 4 h at room temperature for clotting. Blood samples were then centrifuged (3000 g, 20 min, 4°C) and serum harvested. Serum NEFA (NEFA-C kit; Wako), β -hydroxybutyric acid (BHBA; Roche Diagnostics) and glucose (P7119; Sigma-Aldrich) were determined using commercially available kits in triplicate. The intra- and inter-assay coefficients of variation for NEFA, BHBA and glucose were 1.49 and 3.32, 2.51 and 6.71, and 1.38 and 2.42% respectively. The minimum sensitivity of the kits used for NEFA, BHBA and glucose were 62.5 mEq/dL,

2.5 mg/dL and 6.25 mg/dL respectively. Fatty acids in serum samples were extracted (Folch *et al.* 1957) and fatty acid composition was assessed by gas chromatography (Cruz-Hernandez *et al.* 2007).

Statistical analysis

The abundance of mRNA, serum metabolites and fatty acid profile as well as the total number of corpora lutea, anovulated follicles, embryos, blastocysts, unfertilized ova and degenerated embryos were analyzed using the MIXED procedure of SAS (version 9.3, 2011; SAS Institute, Cary, NC, USA). The statistical model included dietary treatment as the main effects. Moreover, fertilization rate and proportion of viable embryos were analyzed using the GENMOD procedure of SAS. The statistical model included dietary treatment as main effect. Model specifications included a binomial distribution and logit link function. The results are expressed as proportion or as mean \pm S.E.M. For all results, $P \leq 0.05$ were considered significant, whereas $P > 0.05$ but < 0.10 was considered trends.

Results and discussion

The evaluation of serum fatty acid profiles indicated that the main differences were found between serum fatty acid profiles of cows fed FLX (high in α -linolenic acid) and SUN (high in linoleic acid) (Table 2). Moreover, the concentration of serum metabolites (NEFA, BHBA and glucose) did not differ between dietary groups (Table 2), indicating that cows were in similar metabolic states. Superovulatory response did not differ among dietary treatments based on the number of CL, anovulated follicles and total ova/embryos collected (Table 3). Cows fed SUN tended ($P = 0.06$) to produce more transferable embryos (total number of grade 1 and 2 embryos) than

Table 2 The concentrations of metabolites and fatty acid profile of serum collected from cows fed diets supplemented with flax (FLX), sunflower (SUN) or canola (CAN) oilseed.

	Treatments		
	FLX*	SUN†	CAN‡
NEFA (mEq/dL)	125.62 \pm 14.82	91.81 \pm 14.82	134.02 \pm 14.82
BHBA (mg/dL)	11.00 \pm 1.19	9.23 \pm 1.19	11.17 \pm 1.19
Glucose (mg/dL)	61.27 \pm 1.96	63.02 \pm 1.96	58.89 \pm 1.96
Fatty acid profile of serum (g/100 g of total fatty acids)			
Palmitic acid (SFA)	11.86 \pm 0.71	11.76 \pm 0.71	12.76 \pm 0.71
Stearic acid (SFA)	22.88 \pm 1.04	22.19 \pm 1.04	21.89 \pm 1.04
Oleic acid (MUFA)	9.85 \pm 1.20	9.79 \pm 1.07	10.33 \pm 1.20
Linoleic acid (PUFA)	32.13 \pm 3.06 ^{a,c}	41.63 \pm 2.74 ^b	41.05 \pm 3.06 ^{a,b,d}
α -linolenic acid (PUFA)	17.69 \pm 2.09 ^a	6.08 \pm 1.87 ^b	10.55 \pm 2.09 ^b
Total n-6 fatty acids	33.17 \pm 2.97 ^a	44.14 \pm 2.66 ^b	42.67 \pm 2.97 ^b
Total n-3 fatty acids	20.37 \pm 2.24 ^a	10.89 \pm 2.00 ^b	11.79 \pm 2.24 ^b
n-6:n-3 ratio	1.65 \pm 1.19 ^a	5.35 \pm 1.06 ^b	4.02 \pm 1.19 ^{a,b}
Total SFA	34.87 \pm 1.34	34.69 \pm 1.20	34.90 \pm 1.34
Total MUFA	11.46 \pm 2.06	9.79 \pm 1.84	13.62 \pm 2.06
Total PUFA	53.55 \pm 2.65	55.03 \pm 2.37	54.76 \pm 2.65
PUFA:SFA ratio	0.86 \pm 0.22	0.85 \pm 0.19	0.81 \pm 0.22

Within a row, values with no common superscripts differ ^{a,b} P value ≤ 0.05 ; ^{c,d} P value < 0.10 .

*Serum collected from cows fed a diet supplemented with flaxseed; †Serum collected from cows fed a diet supplemented with sunflower seed;

‡Serum collected from cows fed a diet supplemented with canola seed.

BHBA, β -hydroxybutyric acid; MUFA, monounsaturated fatty acids; NEFA, non-esterified fatty acid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Table 3 Superovulatory response and embryo production* in cows fed diets supplemented with flax (FLX), sunflower (SUN) or canola (CAN) seed.

	Dietary treatment		
	FLX	SUN	CAN
Corpora lutea (n)	13.1 ± 1.5	14.5 ± 1.2	12.8 ± 2.6
Anovulated follicles (n)	2.6 ± 0.9	2.0 ± 1.1	3.0 ± 1.4
Total ova/embryos (n)	7.3 ± 1.2	8.6 ± 1.7	7.5 ± 2.0
Transferable embryo (n)	4.8 ± 1.3 ^{c,d}	6.1 ± 1.7 ^c	4.3 ± 1.5 ^d
Unfertilized (n)	1.8 ± 0.9 ^b	0.6 ± 0.5 ^a	1.3 ± 0.6 ^b
Degenerated (n)	0.7 ± 0.4 ^a	1.9 ± 0.5 ^b	1.9 ± 0.6 ^b
Fertilization rate (%)	75.3 ^b	93.0 ^a	82.7 ^b
Proportion of viable embryos (%)	87.3 ^{a,c}	76.3 ^{a,b,d}	69.4 ^b

Within a row, values with no common superscripts differ ^{a,b}*P* value ≤ 0.05; ^{c,d}*P* value < 0.10.

*Embryos were harvested from a total of 35 embryo collections (FLX, *n* = 12; SUN, *n* = 11; and CAN, *n* = 12).

those fed CAN, but it did not differ from those fed FLX. Cows fed SUN had fewer unfertilized ova and, hence, greater fertilization rate than those fed either CAN or FLX. Previous findings indicated that a diet enriched in linoleic acid had either a positive (Cerri *et al.* 2009) or no (Thangavelu *et al.* 2007) effect on the fertilization rate in lactating dairy cows. Conversely, using an *in vitro* model, it has been demonstrated that linoleic acid negatively influences oocyte maturation and subsequent blastocyst development (Marei *et al.* 2010), a response attenuated by adding antioxidants during the *in vitro* maturation (Khalil *et al.* 2013). Although these contradictory results could be associated with the status of animals (lactating vs non-lactating) or the model used (*in vitro* vs *in vivo*), the effect of linoleic acid on bovine oocyte competence and fertilization rate remains controversial.

Cows fed FLX had fewer degenerated embryos than those fed either CAN or SUN (Table 3). Feeding FLX also increased (*P* < 0.05) or tended to increase (*P* < 0.10) the proportion of viable embryos (the proportion of grade 1 and 2 embryos over total collected embryos) compared with feeding CAN or SUN respectively. Similarly, cows fed a diet supplemented with a partially rumen protected n-3 PUFA (high in eicosapentaenoic and docosahexaenoic acids) had a lower proportion of

degenerated embryos compared with those fed a diet supplemented with palmitic acid (Childs *et al.* 2008). It has also been reported that feeding cows a diet enriched in α-linolenic acid (flaxseed oil) increased the presence of α-linolenic acid in collected oocytes and subsequently those oocytes had greater cleavage rate and tended to have higher blastocyst rate compared with those collected from cows fed a diet enriched in saturated fatty acid (Moallem *et al.* 2013). Therefore, it is plausible that the reduced number of degenerated embryos in cows fed FLX resulted from enhanced embryo development.

Evaluation of the transcriptomic profile revealed that 175 genes were differentially expressed in blastocysts from cows fed FLX compared with those from cows fed SUN (Fig. 1; FC > 2.0 and *P* value < 0.05; full list of genes provided in Supplementary Table 2). However, when FLX vs CAN and SUN vs CAN were compared, only four and 14 differentially expressed genes, respectively, were evident (Fig. 1). Given that the serum fatty acid profile of CAN and SUN groups were mostly similar, the absence of a greater difference in gene expression between embryos of these two diets is not surprising. However, the lack of difference in gene expression between CAN and FLX groups is somewhat intriguing, although it may

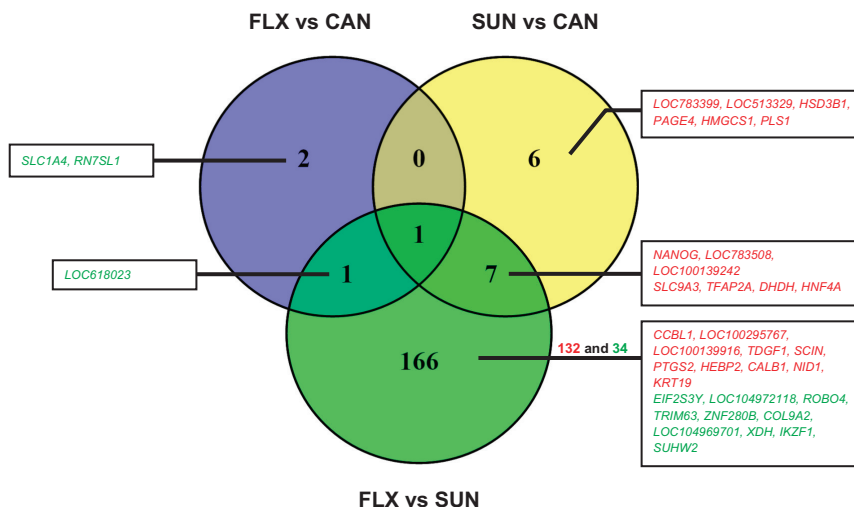


Figure 1 Number of differentially expressed genes (≥2.0-fold changes with a *P* value ≤ 0.05) in embryos (Day 7.5) collected from cows fed a diet supplemented with flax (FLX), sunflower (SUN) or canola (CAN) oilseed. The 175 differentially expressed genes were used for further functional analysis with Ingenuity Pathway Analysis. Gene names appearing in red and green colour indicate up- and down-regulated genes respectively.

be partially explained by the differences in n-6:n-3 ratio among FLX, SUN and CAN groups. Although the n-6:n-3 ratio was significantly lower in FLX compared with SUN, it neither differed between FLX and CAN or between SUN and CAN, leading to the speculation that n-6:n-3 ratio in maternal diets could influence embryonic gene expression.

As most of the differences were between FLX and SUN (175 differentially expressed genes), further functional analysis of IPA was performed on only this comparison. The list of biological functions influenced (BH-FDR-corrected *P* value (B-H *P* value) <0.05 with a -log (B-H *P* value) >2.0) by FLX vs SUN, which includes cellular growth and proliferation, cellular development, lipid metabolism and molecular transport, is presented in Table 4. Moreover, cell viability and survival functions were activated functions having Z-score >2.0 (Fig. 2). IPA analysis also identified 13 activated pathways based on differentially expressed genes (Supplementary Table 3).

Blastocysts collected from cows fed FLX had greater mRNA expression of claudin 4 (*CLDN4*, 2.4-fold) and solute carrier family 9, subfamily A, member 3 (*SLC9A3*, 7.0-fold) compared with those collected from cows fed

SUN. It has been reported that *Cldn4* and *Cldn6* are essential for tight junctions and blastocyst development (Moriwaki *et al.* 2007). In this regard, when embryos were cultured with an inhibitor of *Cldn4* and *Cldn6*, the development of normal blastocysts was remarkably reduced (Moriwaki *et al.* 2007). The solute carrier family 9 (SLC9A) (previously known Na⁺/H⁺ exchangers (NHEs)) consists of five members (SLC9A1, 2, 3, 4 and 5), which are located in the plasma membrane and involved in intracellular pH maintenance, cell volume regulation and transduction of signals that promote cell proliferation (Yun *et al.* 1995). It was found that the addition of specific SLC9A3 inhibitor to embryo cultures did not affect the embryonic development from two-cell stage to morula stage but inhibited the formation of blastocyst in a concentration-dependent manner (Kawagishi *et al.* 2004).

Our results also indicated that blastocysts collected from cows fed FLX had higher mRNA expression of keratin 19 (*KRT19*, 8.8-fold), lectin galactoside-binding soluble 3 (*LGALS3*, 2.1-fold) and annexin A1 (*ANXA1*, 2.5-fold) compared with those collected from cows fed SUN. A previous study has shown that *Krt19* is essential for the integrity of trophoblast cells and embryo survival

Table 4 List of biological functions based on differentially expressed genes in Day 7.5 embryos collected from cows fed diets supplemented with flaxseed (FLX) vs sunflower seed (SUN).

Category	B-H <i>P</i> value	Genes
Cellular growth and proliferation	2.33E-03-3.65E-02	<i>B2M</i> , <i>ID2</i> , <i>LGALS3</i> , <i>GADD45B</i> , <i>EMILIN2</i> , <i>XDH</i> , <i>HMG1</i> , <i>NR3C1</i> , <i>XRCC2</i> , <i>CLDN4</i> , <i>TIMP1</i> , <i>ANXA1</i> , <i>CXCL14</i> , <i>IFI30</i> , <i>MGLL</i> , <i>IKZF1</i> , <i>ABCG2</i> , <i>ULBP1</i> , <i>TDGF1</i> , <i>PLCL2</i> , <i>EFNA1</i> , <i>FOS</i> , <i>IL1RN</i> , <i>KRT19</i> , <i>BTG2</i> , <i>ZAP70</i> , <i>EDN3</i> , <i>PTGS2</i> , <i>GLDC</i> , <i>SFN</i>
Cellular development	2.33E-03-3.65E-02	<i>B2M</i> , <i>ID2</i> , <i>GADD45B</i> , <i>LGALS3</i> , <i>XDH</i> , <i>HMG1</i> , <i>CD8B</i> , <i>NR3C1</i> , <i>CLDN4</i> , <i>TIMP1</i> , <i>ANXA1</i> , <i>CXCL14</i> , <i>GIMAP4</i> , <i>PLAC8</i> , <i>TBX3</i> , <i>IKZF1</i> , <i>ABCG2</i> , <i>ULBP1</i> , <i>TDGF1</i> , <i>SCIN</i> , <i>PLCL2</i> , <i>EFNA1</i> , <i>UBD</i> , <i>FOS</i> , <i>IL1RN</i> , <i>ZAP70</i> , <i>BTG2</i> , <i>EDN3</i> , <i>PTGS2</i> , <i>SFN</i> , <i>GLDC</i>
Lipid metabolism	3.45E-03-3.65E-02	<i>SCIMP</i> , <i>LIPH</i> , <i>LTA4H</i> , <i>LGALS3</i> , <i>XDH</i> , <i>ABCG2</i> , <i>HMG1</i> , <i>PLA2R1</i> , <i>SLC34A2</i> , <i>NR3C1</i> , <i>FOS</i> , <i>MGAT4A</i> , <i>MGST2</i> , <i>IL1RN</i> , <i>TIMP1</i> , <i>ANXA1</i> , <i>CXCL14</i> , <i>MGLL</i> , <i>EDN3</i> , <i>PTGS2</i>
Molecular transport	3.45E-03-3.65E-02	<i>SCIMP</i> , <i>LTA4H</i> , <i>LGALS3</i> , <i>XDH</i> , <i>ABCG2</i> , <i>HMG1</i> , <i>PLA2R1</i> , <i>SLC34A2</i> , <i>NR3C1</i> , <i>GSTO1</i> , <i>FOS</i> , <i>SLC23A1</i> , <i>MGAT4A</i> , <i>IL1RN</i> , <i>TIMP1</i> , <i>ANXA1</i> , <i>CXCL14</i> , <i>MGLL</i> , <i>EDN3</i> , <i>PTGS2</i>
Small molecule biochemistry	3.45E-03-3.65E-02	<i>SCIMP</i> , <i>LTA4H</i> , <i>DPYS</i> , <i>LGALS3</i> , <i>XDH</i> , <i>PLA2R1</i> , <i>HMG1</i> , <i>HSD17B11</i> , <i>PFKF</i> , <i>NR3C1</i> , <i>GFPT2</i> , <i>SLC23A1</i> , <i>TIMP1</i> , <i>ANXA1</i> , <i>CXCL14</i> , <i>MGLL</i> , <i>LIPH</i> , <i>ABCG2</i> , <i>SLC34A2</i> , <i>GSTO1</i> , <i>FOS</i> , <i>MGAT4A</i> , <i>MGST2</i> , <i>IL1RN</i> , <i>GCSH</i> , <i>PTGS2</i> , <i>EDN3</i> , <i>GLDC</i>
Amino acid metabolism	3.88E-03-3.02E-02	<i>GCSH</i> , <i>GLDC</i>
Post-translational modification	3.88E-03-3.16E-02	<i>GCSH</i> , <i>BTG2</i> , <i>AS3MT</i> , <i>GLDC</i>
Cell death and survival	4.82E-03-3.65E-02	<i>B2M</i> , <i>ID2</i> , <i>EMILIN2</i> , <i>GADD45B</i> , <i>LGALS3</i> , <i>XDH</i> , <i>HMG1</i> , <i>AS3MT</i> , <i>PTPN5</i> , <i>CAMK2N2</i> , <i>NR3C1</i> , <i>SCARB2</i> , <i>XRCC2</i> , <i>CLDN4</i> , <i>TIMP1</i> , <i>PRSS8</i> , <i>ANXA1</i> , <i>ZNF280B</i> , <i>MAP1LC3A</i> , <i>CALB1</i> , <i>GIMAP4</i> , <i>HEBP2</i> , <i>PLAC8</i> , <i>CCBL1</i> , <i>TBX3</i> , <i>IKZF1</i> , <i>ABCG2</i> , <i>ULBP1</i> , <i>TDGF1</i> , <i>ANXA4</i> , <i>SCIN</i> , <i>SRXN1</i> , <i>EFNA1</i> , <i>UBD</i> , <i>FOS</i> , <i>IL1RN</i> , <i>KRT19</i> , <i>ZAP70</i> , <i>BTG2</i> , <i>PTGS2</i> , <i>SFN</i>
Cell cycle	5.76E-03-3.65E-02	<i>ID2</i> , <i>GADD45B</i> , <i>LGALS3</i> , <i>TBX3</i> , <i>ABCG2</i> , <i>IKZF1</i> , <i>HMG1</i> , <i>ORC6</i> , <i>CAMK2N2</i> , <i>NR3C1</i> , <i>UBD</i> , <i>XRCC2</i> , <i>FOS</i> , <i>TACC2</i> , <i>TIMP1</i> , <i>KRT19</i> , <i>ZAP70</i> , <i>BTG2</i> , <i>EDN3</i> , <i>PTGS2</i> , <i>SFN</i> , <i>PLAC8</i>
Nucleic acid metabolism	6.03E-03-3.65E-02	<i>DPYS</i> , <i>XDH</i> , <i>ABCG2</i> , <i>GLDC</i>
Cellular assembly and organization	9E-03-3.65E-02	<i>UBD</i> , <i>LGALS3</i> , <i>CLDN4</i> , <i>IKZF1</i> , <i>KRT19</i> , <i>XDH</i> , <i>HMG1</i> , <i>SFN</i> , <i>NR3C1</i>
Cellular function and maintenance	9E-03-3.65E-02	<i>B2M</i> , <i>ID2</i> , <i>LGALS3</i> , <i>GADD45B</i> , <i>XDH</i> , <i>HMG1</i> , <i>CD8B</i> , <i>NR3C1</i> , <i>CLDN4</i> , <i>ANXA1</i> , <i>IFI30</i> , <i>MAP1LC3A</i> , <i>CALB1</i> , <i>DSC2</i> , <i>GIMAP4</i> , <i>PLAC8</i> , <i>IKZF1</i> , <i>ABCG2</i> , <i>SLC34A2</i> , <i>PLCL2</i> , <i>SRXN1</i> , <i>EFNA1</i> , <i>UBD</i> , <i>FOS</i> , <i>IL1RN</i> , <i>ZAP70</i> , <i>EDN3</i> , <i>PTGS2</i>
DNA replication, recombination and repair	9E-03-3.65E-02	<i>UBD</i> , <i>XRCC2</i> , <i>IKZF1</i> , <i>SFN</i> , <i>GLDC</i> , <i>NR3C1</i>

in mice (Hesse *et al.* 2000). Several studies have also reported that LGALS3 is involved in cell proliferation, growth, adhesion and polarization (Liu *et al.* 2002, Friedrichs *et al.* 2007, Nangia-Makker *et al.* 2007) and, more importantly, in cell cycle progression and cell survival (Liu *et al.* 2002). Furthermore, it was 2.8 times more abundant at blastocyst stage compared with morula stage in bovine (Demant 2012), suggesting that LGALS3 is important for blastocyst development. It has been reported that all annexin family members participate in various signalling pathways that lead to cell differentiation, migration, proliferation and maintenance of cellular calcium homeostasis (Gerke *et al.* 2005, Mussunoor & Murray 2008, Grewal & Enrich 2009,

Grewal *et al.* 2010). Additionally, ANXA1 had higher expression in bovine *in vivo*-produced 16 cell embryos compared with *in vitro*-produced 16-cell embryos (Gad *et al.* 2012). Collectively, greater expression of genes involved in blastocyst development (CLDN4 and SLC9A3), trophectoderm integrity (KRT19), and cell proliferation, growth and anti-apoptosis (LGALS3 and ANXA1) in embryos collected from FLX-fed cows compared with those fed SUN likely enhanced and supported the development of embryos that otherwise may have undergone degeneration.

Compared with those from cows fed with SUN, embryos from FLX-fed cows exhibited higher expression of the T-box 3 (TBX3, 2.4-fold) and Nanog homeobox

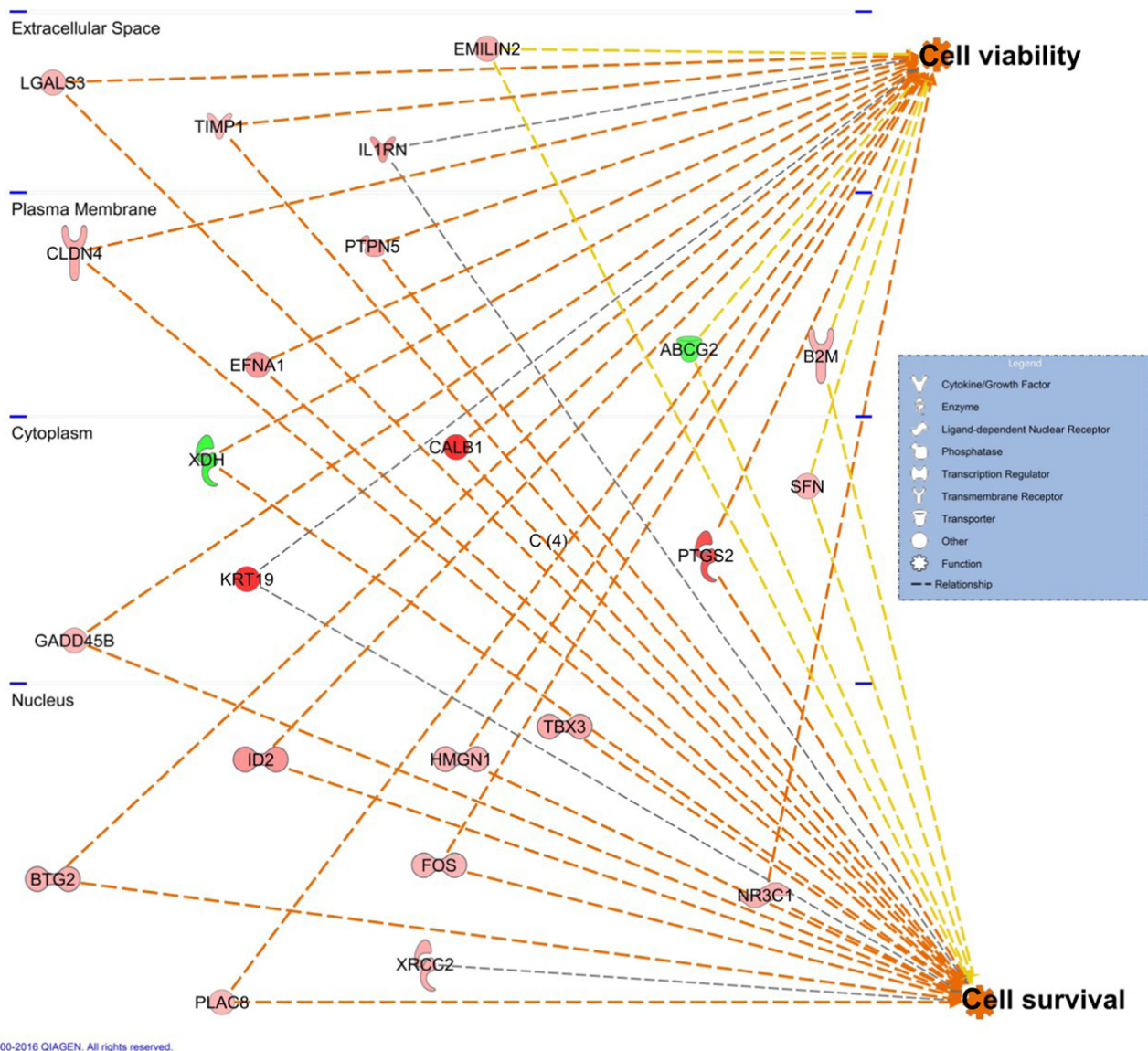


Figure 2 Biological function analysis using Ingenuity Pathway Analysis software. Genes involved in cell viability and survival (Z-score >2.0) functions that are differentially expressed in embryos (day 7.5) collected from cows fed a diet supplemented with flaxseed (FLX) compared with those collected from cows fed sunflower seed (SUN). Up- or downregulated genes involved in these functions are indicated with red and green color symbols respectively. The 175 differentially expressed genes were used for further functional analysis with ingenuity pathway analysis.

(*NANOG*, 12.4-fold) genes, which are responsible for early embryonic cell differentiation and lineage segregation. The lack of proper cell differentiation during blastocyst development would result in embryonic death. Several transcription factors have been found being responsible for proper embryonic cell differentiation; for instance, *CDX2* is considered as trophectoderm-specific marker at the blastocyst stage (Dietrich & Hiiragi 2007), whereas the pluripotency marker *NANOG* is specifically expressed in inner cell mass in bovine embryos (Kuijk *et al.* 2008). *Nanog*-null mouse embryos fail to establish epiblast identity because of inner cell mass degeneration (Mitsui *et al.* 2003, Silva *et al.* 2009). Additionally, *Tbx3*,

a pluripotency-related transcription factor of the T-box gene family, is necessary for both self-renewal of mouse embryonic stem cells and for their differentiation into embryonic endoderm (Chapman *et al.* 1996). Among T-box family members, *Tbx3* is the earliest expressed gene in mouse inner cell mass cells (Chapman *et al.* 1996), and its deletion results in embryonic lethality or deficiencies in the mammary gland, limbs and yolk sac (Davenport *et al.* 2003). Therefore, it is suggested that the reduction in the proportion of degenerated embryos reported in cows fed a diet enriched in FLX is the result of a higher embryonic expression of *TBX3* and *NANOG* genes.

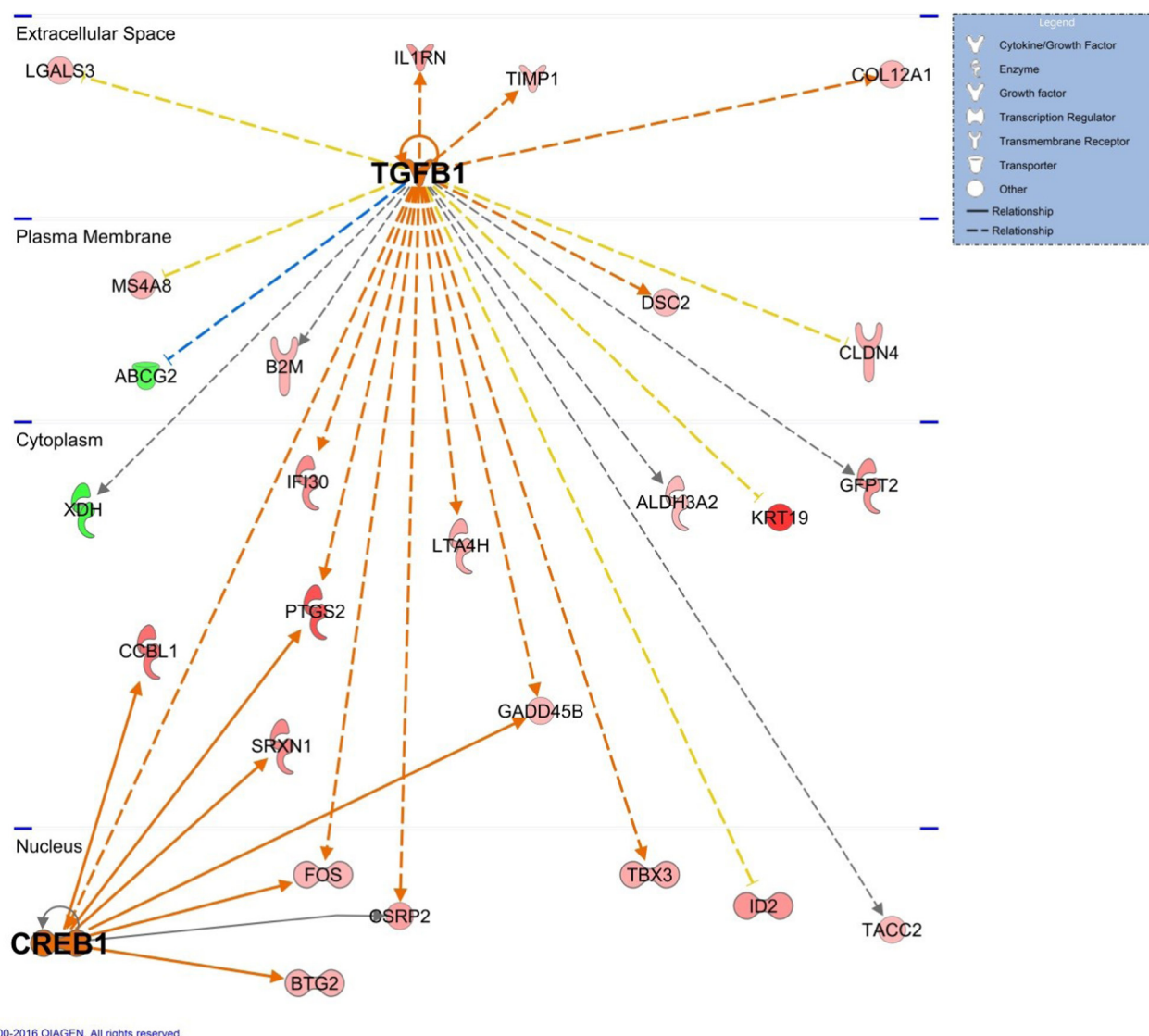


Figure 3 Upstream regulator of differentially expressed genes in embryos (Day 7.5) collected from cows fed a diet supplemented with flaxseed (FLX) compared with those collected from cows fed sunflower seed (SUN). cAMP-responsive element binding protein 1 (CREB1; transcription factor) and transforming growth factor beta 1 (TGF-β1; growth factors) are the upstream regulators of differentially expressed genes in embryos (Day 7.5) collected from cows fed a diet supplemented with flaxseed (FLX) compared with those collected from cows fed a diet supplemented with sunflower seed (SUN). Up- or downregulated genes are indicated with red and green colour symbols respectively. Direct or indirect relationships between molecules are indicated by solid or dashed connecting lines respectively. The analysis of upstream regulator of differentially expressed genes was done with Ingenuity Pathway Analysis software.

Feeding a diet enriched in FLX increased the blastocyst expression of transmembrane receptor beta-2-microglobulin (*B2M*, 2.3-fold) and placenta-specific 8 (*PLAC8*, 2.0-fold) involved in cell survival. Recent studies have shown that *B2M* expression increased in embryos at blastocyst stage (Tanaka *et al.* 2005) and in endometrial tissue around the time of maternal recognition of pregnancy (Forde *et al.* 2011). The increased expression of *B2m* gene is positively correlated with the expression of blastocyst MHC class Ia, which protects embryonic trophoblast cells from maternal NK cells (Tanaka *et al.* 2005), thus avoiding maternal rejection of the embryo (Lanier 1998). Interestingly, it has been found that *B2m* expression was downregulated in *in vivo*-derived bovine embryos that resulted in no pregnancy or greater pregnancy loss relative to those embryos that resulted in pregnancies carried to term (Ghanem *et al.* 2011). Similarly, the expression of *PLAC8* gene was upregulated (26-fold) in blastocyst biopsies obtained from embryos that resulted in full-term calves compared with those associated with pregnancy loss (El-Sayed *et al.* 2006), as well as in the endometrium of pregnant cows compared with non-pregnant ones (Galvez *et al.* 2003, Klein *et al.* 2006), suggesting a possible role of *PLAC8* in placenta development and fetus–maternal interface. Hence, the increased expression of genes involved in maternal immunity modulation (*B2M*) and placenta development (*PLAC8*) in blastocysts collected from cows fed FLX compared with those obtained from cows fed SUN supports previous findings regarding reduced pregnancy loss in lactating cows fed a diet supplemented with flaxseed (Ambrose *et al.* 2006, Petit & Twagiramungu 2006).

The IPA upstream regulator analysis predicts that cAMP-responsive element binding protein 1 (CREB1) and transforming growth factor beta 1 (TGF- β 1) are the upstream regulators of activated (or inhibited) genes by FLX vs SUN (Fig. 3). The CREB family consists of a family of transcription factors that support somatic cell survival, and this has been most fully investigated in neuronal signalling (Walton & Dragunow 2000). FBJ murine osteosarcoma viral oncogene homolog (*FOS*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) are a part of CREB1 and TGF- β 1 target genes respectively. In this study, *FOS* and *PTGS2* mRNA expression was greater by 2.2- and 5.0-fold in blastocysts collected from cows

fed FLX compared with SUN. *FOS* mainly regulates cell proliferation, differentiation and survival (Hess *et al.* 2004) and is present in pre-implantation embryos of several species such as the cow, sheep, pig and mouse (Muller *et al.* 1982, Whyte & Stewart 1989, Pal *et al.* 1993, Xavier *et al.* 1997, Tetens *et al.* 2000). In knock-out experiments, *Fos*-deficient mice died just before birth, indicating the importance of *FOS* in foetal survival (Johnson *et al.* 1992). It has been suggested that in sheep and cows, conceptus-secreted prostaglandins modulate endometrial gene expression, which subsequently leads to enhanced embryo elongation and maternal recognition of pregnancy (Dorniak *et al.* 2012, Spencer *et al.* 2013). Moreover, greater expression of *PTGS2* was detected in biopsies derived from blastocysts resulting in successful pregnancy and calf delivery (El-Sayed *et al.* 2006). *Ptgs2*-null mice have multiple reproductive impairments characterized by poor ovulation, reduced fertilization rates and failure of implantation and decidualization, which are responsive to prostaglandin replacement (Dinchuk *et al.* 1995, Lim *et al.* 1997).

Our microarray results indicate that sulfiredoxin 1 (*SRXN1*, 3.4 fold) and interleukin 1 receptor antagonist (*IL1RN*, 3.0 fold) are more expressed in blastocysts collected from cows fed FLX than those collected from cows fed SUN. In addition, the IPA upstream regulator analysis predicted that *SRXN1* and *IL1RN* are CREB1 and TGF- β 1 target genes respectively. *Srxn1* eliminates reactive oxygen species (e.g., H_2O_2 and NO) and protects cells from apoptosis (Baek *et al.* 2012). Three members of the interleukin-1 (IL-1) family, IL-1 β , IL1RN and IL-1 receptor type I, are present in oocytes and in all stages of human embryo (De los Santos *et al.* 1996). Exposure of bovine oocytes to a mixture of stearic, palmitic and oleic acid during *in vitro* maturation reduced the expression of *IL1RN* in formed blastocysts (Van Hoeck *et al.* 2015). Interestingly, in our study, *IL1RN* expression increased in embryos collected from cows fed a diet enriched in FLX. Collectively, these findings indicate that *IL1RN* expression in bovine embryos is affected by different types of fatty acids. However, further investigations are required to elucidate the exact function of *IL1RN* genes during the development of bovine embryo.

Based on the microarray analysis, six genes were validated by q-PCR in three additional biological replicates (each containing a pool of three to five grade 1 and 2 blastocysts) per dietary treatment (Fig. 4). They

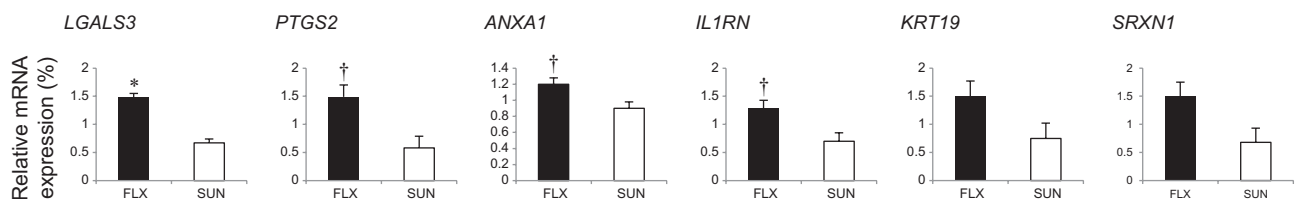


Figure 4 q-PCR confirmation of microarray results in embryos (Day 7.5) collected from cows fed a diet supplemented with flax (FLX) or sunflower (SUN) seed. * $P < 0.05$; † $P < 0.10$.

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