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Research Article

Colony-stimulating factor 2 acts from days 5 to 7 of development to modify programming of the bovine conceptus at day 86 of gestation†

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Abstract

Colony-stimulating factor 2 (CSF2) is an embryokine that improves competence of the embryo to establish pregnancy and which may participate in developmental programming. We tested whether culture of bovine embryos with CSF2 alters fetal development and alleviates abnormalities associated with in vitro production (IVP) of embryos. Pregnancies were established by artificial insemination (AI), transfer of an IVP embryo (IVP), or transfer of an IVP embryo treated with 10 ng/ml CSF2 from day 5 to 7 of development (CSF2). Pregnancies were produced using X-sorted semen. Female singleton conceptuses were collected on day 86 of gestation. There were few morphological differences between groups, although IVP and CSF2 fetuses were heavier than AI fetuses. Bicarbonate concentration in allantoic fluid was lower for IVP than for AI or CSF2. Expression of 92 genes in liver, placenta, and muscle was determined. The general pattern for liver and placenta was for IVP to alter expression and for CSF2 to sometimes reverse this effect. For muscle, CSF2 affected gene expression but did not generally reverse effects of IVP. Levels of methylation for each of the three tissues at 12 loci in the promoter of insulin-like growth factor 2 (*IGF2*) and five in the promoter of growth factor receptor bound protein 10 were unaffected by treatment except for CSF2 effects on two CpG for *IGF2* in placenta and muscle. In conclusion, CSF2 can act as a developmental programming agent but alone is not able to abolish the adverse effects of IVP on fetal characteristics.

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Summary Sentence

Production of embryos in vitro (IVP) is associated with alterations in fetal morphology and gene expression at day 86 of gestation; addition of CSF2 to embryo culture altered features of the fetus but did not abolish abnormalities associated with IVP.

Key words: colony-stimulating factor 2, embryokine, programming, fetal development.

Introduction

The developmental program of the embryo displays a plasticity that can be modified by its microenvironment. In species as diverse as amphibians [1], reptiles [2], teleosts [3, 4], and mammals [5–12], resultant changes in trajectory of development can have long-acting effects that extend into postnatal life. Developmental programming can be beneficial when the deviation in adult phenotype prepares the individual for life in the anticipated postnatal environment but harmful when the actual environment is different than expected [7, 13, 14]. In mammals, adult phenotype can be altered by changes in the maternal environment during the preimplantation period. Developmental programming during the periconceptional period has been described for maternal low-protein nutrition [15, 16], exposure to inflammation [17], and feeding a methyl-deficient gestational diet [18, 19].

The mechanisms by which alterations in the maternal environment during the preimplantation period change the developmental program of the embryo is not known. One possibility is that changes in maternal environment alter secretion of embryokines from the maternal reproductive tract that are capable of modifying the developmental program of the embryo. The best evidence for this idea comes from the study of CSF2. This cytokine is produced by the oviduct and endometrium in a variety of species [20–24] and can regulate various aspects of preimplantation development including competence to develop to the blastocyst stage [22, 23, 25–28], gene expression [29–31], and capacity to establish pregnancy after transfer to females [26, 32, 33]. Expression of *CSF2* in the reproductive tract can be modified by level of fatness [34] and exposure to seminal plasma [22, 23, 35]. Moreover, actions of CSF2 on the preimplantation embryo can modify the postnatal phenotype of the offspring. Culture of mouse embryos with Csf2 neutralized alterations in postnatal phenotype caused by derivation from cultured embryos [36]. In cattle, heifer calves derived from embryos cultured with CSF2 from day 5 to 7 of development experienced greater postnatal growth than calves from embryos cultured without CSF2 [37].

The in vitro-produced (IVP) embryo is a good model for studying developmental programming during the preimplantation period both because programming is disturbed and because access to the oocyte and embryo facilitates study of candidate molecules involved in developmental programming. Offspring derived from embryos produced in vitro have increased risk of low birth weight [38], birth defects [39,40], and vascular dysfunction [41] in humans, metabolic disorders [42,43] and vascular dysfunction in mice [44], and epigenetic modifications in humans and cattle [45–48]. In cattle, several studies have described abnormal phenotypes of IVP-derived fetuses and calves, a condition referred to as "large offspring syndrome" [49–52]. Besides disruptions in fetal development [53, 54] and birth weight [55, 56], IVP-derived calves can experience increased incidence of congenital malformations and anomalies [53, 56], neonatal death [57], and altered postnatal growth trajectory and lactational performance [6, 58, 59].

Here, it was hypothesized that exposure of embryos to CSF2 during days 5–7 of development would modify embryonic development to correct abnormal programming caused by IVP. The result would be that fetuses from IVP embryos cultured with CSF2 would exhibit a phenotype more similar to that of embryos produced by artificial insemination (AI) that developed in vivo than that for fetuses from IVP embryos cultured without CSF2. Fetal development was evaluated at the end of the first trimester of gestation because this is a time when morphometric anomalies associated with IVP have been observed in cattle [60–62].

Materials and methods

Procedures described in this experiment were approved by the University of Florida Institutional Animal Care and Use Committee.

Experimental design

The experiment involved production of fetuses using AI with X-sorted semen or by transfer of an IVP embryo produced in either control culture medium or medium containing 10 ng/ml recombinant bovine CSF2 from day 5 to 7 of development. The concentration and timing of CSF2 treatment was chosen because it has been shown to affect embryonic development and survival in cattle [26,28–30,32,37]. Females used to produce fetuses were groups of Holstein heifers and nonlactating cows that were randomly assigned, after blocking by parity (nulliparous vs parous) within replicate (a replicate being a group of cows subject to ovulation synchronization at one time) to one of the three treatments. A total of six replicates were performed. Both cumulus-oocyte complexes (COC) and sexed semen used for IVP were from Holstein animals. A single Holstein sire was used to breed animals in the three experimental groups to minimize effects of sire on fetal morphometry and phenotype. X-sorted semen was used to increase statistical power by eliminating variation due to sex. Only female fetuses were included in the analyses.

In vitro production of embryos

Embryos were produced in vitro following procedures previously described [28]. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Fisher (Pittsburgh, PA, USA) unless otherwise stated. Oocyte washing medium consisted of Tissue Culture Medium-199 with Hanks salts plus 25 mM HEPES. Oocyte maturation medium consisted of Tissue Culture Medium-199 with Earle salts (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 mM sodium pyruvate, 2 mM L-glutamine, 50 ng/ml recombinant human epidermal growth factor (Invitrogen, Waltham, MA, USA), and 5.0 μg/ml of follicle-stimulating hormone (Folltropin; Bioniche Animal Health, Athens, GA, USA). Tyrode albumin lactate pyruvate (TALP) solutions including HEPES-TALP, Sperm-TALP, and IVF-TALP were prepared as described [63] by supplementation with bovine serum albumin (BSA), sodium pyruvate, gentamicin, and heparin (IVF-TALP only) to the base solutions HEPES-TL, SP-TL, and IVF-TL provided as custom preparations from Caisson (Smithfield, UT, USA). Embryos were cultured in a serum-free culture medium termed SOF-BE2 [synthetic oviduct fluid—bovine embryo 2; [64]].

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Straws of X-sorted semen were from a commercially available Holstein sire (DE-SU 527 Spur-ET, NAAB 007HO10723) purchased from Select Sires Inc. (Plain City, OH, USA).

Cumulus-oocyte complexes were retrieved by follicular aspiration of ovaries collected from Holstein females at a commercial abattoir (DeSoto Biosciences, Seymour, TN, USA). Recovered COC were washed three times in oocyte washing and those COC containing at least three layers of compact cumulus cells and a homogeneous cytoplasm were selected for in vitro maturation. Groups of 50 COC were transferred to 2-ml sealed glass sterile vials containing 1 ml of oocyte maturation medium equilibrated with air containing 5% (v/v) CO_2 covered with mineral oil. Tubes with COC were shipped overnight in a portable incubator (Minitube USA Inc., Verona, WI, USA) at 38.5°C to the University of Florida.

At 22 h after shipping, COC were removed from the sealed tubes and used for in vitro fertilization. Groups of 30 COC were washed three times in HEPES-TALP and transferred to fertilization drops covered with mineral oil. Each drop contained 60 μl IVF-TALP and 3.5 μl of a PHE solution [0.05 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine prepared as described [64]]. Drops containing COC were fertilized with 20 μl of X-sorted sperm purified using the Puresperm 40/80 gradient column (Nidacon International AB, Mölndal, Sweden). The sperm purification procedure consisted of centrifugation (2600 x *g* for 5 min) in 2.0 ml microcentifuge tubes of 0.25 ml sperm over two layers of 200 μl of Puresperm (top layer Puresperm 40, bottom layer Puresperm80). The pellet representing the bottom 100 μl was transferred to a new microcentrifuge tube, washed in 1000 μl of IVF-TALP that had been pre-equilibrated at 38.5°C under 5% CO_2, and centrifuged at 600 x *g* for 3 min. The final concentration of sperm in the fertilization drops was approximately 2×10^6 sperm/ml.

After 18 h of coincubation of gametes at 38.5°C and 5% CO_2 in humidified air, oocytes were removed from fertilization drops and denuded of cumulus cells by vortexing for 5 min in 100 μl hyaluronidase solution (10 000 U/ml dissolved in saline) diluted in 600 μl HEPES-TALP. Putative zygotes were then washed twice in HEPES-TALP and a third time in SOF-BE2. Zygotes were cultured in groups of 25–30 in 63-μl microdrops of SOF-BE2 covered with mineral oil at 38.5°C in a humidified atmosphere of 5% CO_2 (v/v), 5% O_2 (v/v), and 90% N_2 (v/v). Cleavage was assessed on day 3 after insemination (72 h postinsemination, hpi). Treatments (control or CSF2) were applied to culture drops on day 5 (120 hpi) by adding 7 μl of vehicle [Dulbecco phosphate buffered saline containing 1% (w/v) BSA] or 7 μl of a 100 ng/ml CSF2 solution. The final concentration of CSF2 in the 70-μl culture drops was 10 ng/ml. The recombinant bovine CSF2 was obtained as a gift from CIBA-GEIGY (Basle, Switzerland); its bioactivity was verified by upregulation of nitric oxide synthase 2 mRNA in bovine monocyte cultures using methods described elsewhere [65].

Presence of blastocysts was determined on day 7 (168 hpi) when embryos were harvested for embryo transfer (ET). Grade 1 expanded blastocysts [66] were transferred to a holding medium that consisted of HEPES-TALP containing 10% (v/v) fetal bovine serum and 50-μM DL-dithiothreitol.

Estrus synchronization, artificial insemination, and embryo transfer

Ovulations were synchronized in groups of Holstein cows and heifers using gonadotropin-releasing hormone (GnRH) and prostaglandin $F_{2\alpha}$ (PGF) treatments following the Ovsynch procedure [GnRH-7 d-PGF-2 d-GnRH; [67]] and were randomly assigned to (1) be inseminated with a single straw of X-sorted semen 16 h after the second GnRH treatment (AI group), (2) receive an IVP embryo cultured in control medium 8 days after the second GnRH treatment (day 7 of the estrous cycle), or (3) receive an IVP embryo cultured in medium containing 10 ng/ml CSF2 at 8 days after GnRH. For AI, 0.25 cc straws of X-sorted semen were thawed at 37°C for 50 s and insemination performed using standard procedures by a single technician. For ET, animals assigned to IVP and CSF2 groups were examined for ovulation 8 days after the second GnRH injection and, when a corpus luteum was present, received a single grade 1 in vitro-produced embryo transferred using standard procedures to the distal third of the uterine horn ipsilateral to the ovary bearing a corpus luteum. Transfers were performed by the same technician used for AI. Pregnancy was determined at day 28–31 of gestation (21–24 days after ET) by visualization of the embryonic vesicle and fetal heartbeat using ultrasonography. After pregnancy was confirmed, weekly blood samples were taken from pregnant females at days (±1) 31, 38, 45, 52, 59, 66, 73, and 80 of gestation. Samples were collected into tubes coated with ethylenediaminetetraacetic acid and, after centrifugation at 600 x *g* for 15 min, plasma was harvested and stored at –20°C until further analysis for pregnancy-associated glycoproteins (PAGs).

Fetal collection and tissue processing

Pregnant cows (n = 29) were sacrificed at day 86 of gestation by stunning and exsanguination. Reproductive tracts were recovered after slaughter and kept on ice until processed within ~2 h for morphometric measurements and collection of tissue samples. Briefly, caruncles of the maternal placenta were detached manually from cotyledons of the fetal placenta until complete separation of the entire conceptus (placenta, extraembryonic fluids, and fetus) from the uterine endometrium was achieved. Cotyledons were counted and the diameters of 10 randomly chosen cotyledons located in regions of the placenta located over the fetus were measured with a tape measure. Samples (approximately 1 × 1 × 1 mm) from the intercotyledonary placenta were collected and snap-frozen in liquid nitrogen for further analysis of gene expression. The entire placenta was weighed and then freeze-dried at –84°C (FreeZone, Labconco, Kansas City, MO, USA) for 72 h to assess dry placenta weight. Fluids were collected separately from the allantois and amniotic sac, aliquoted in 15 ml tubes, centrifuged at 600 x *g* for 15 min, and frozen at –20°C for further chemical analysis. The total volume of the allantoic and amniotic fluids was assessed using a graduated cylinder.

Several morphometric measures of the fetuses were recorded: crown-rump and crown-nose length, heart girth, umbilical cord diameter, fetal body weight before and after evisceration, and weights of heart, liver, kidneys, and brain. Likewise, ~1 × 1 × 1 mm samples collected from the main lobe of the liver and the semitendinosus muscle (from this point on referred to as 'muscle') were snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction. In addition, a blood sample from the fetal heart was collected using a 1-ml syringe and needle, transferred to a microcentrifuge tube, centrifuged at 2000 x *g* for 10 min, and the serum harvested was stored at –20°C for insulin analysis.

Fetuses derived from IVP embryos were assessed for abnormal size using criteria established elsewhere [47] to determine fetal large offspring syndrome (LOS). Body weights of fetuses from the AI group were ranked to determine the weight corresponding to the 97th percentile, which was 161.4 g. Those fetuses in the IVP and CSF2 groups that had body weights above this value were considered to be fetuses experiencing LOS.

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Chemical analysis of allantoic fluid

Frozen samples of allantoic fluid were thawed at room temperature, centrifuged at 2000 x *g* for 15 min, and the supernatant fraction analyzed to determine the concentrations of urea nitrogen, creatinine, glucose, Na, K, Cl^-, HCO_3^-, Ca, P, and Mg. Analyses were performed at the Clinical Pathology Laboratory, College of Veterinary Medicine, University of Georgia using a Roche Diagnostics Cobas c501 analyzer (Roche Diagnostics Corp., Indianapolis, IN, USA). Sample volumes were 2 μl for urea nitrogen, creatinine, glucose, and HCO_3^-, 3 μl for Ca and Mg, 2.5 μl for P, and 9.7 μl for Na, K, and Cl. For most analytes, concentrations were below the limit of analytic linearity for all samples (urea nitrogen, creatinine, glucose, Ca, and Mg) or were below or near the limit of analytic linearity (Na and Cl). Thus, analysis was limited to K, HCO_3^-, and P. In addition, concentrations of total protein in the allantoic fluid were determined using the Quick Start Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the manufacturer's instructions.

For statistical purposes, samples below the limit of detection for any given analyte were given a value equal to the limit of detection.

Insulin assay

Serum insulin concentrations in fetal heart blood were measured using a solid-phase enzyme immunoassay (Bovine Insulin ELISA, Mercodia AB, Uppsala, Sweden) following the manufacturer's instructions. The Research Resource Identifier (RRID) for the insulin antibody was AB_2636872. The limit of detection was 0.025 ng/ml. For statistical purposes, samples below the limit of detection were given a value of 0.025 ng/ml.

Analysis of pregnancy-associated glycoproteins in maternal plasma

Concentrations of bovine PAGs in maternal plasma were determined using weekly blood samples collected from weeks 4 (pregnancy diagnosis, day 28–31) to 11 (day 80) of pregnancy. Analysis of plasma PAGs was performed using a sandwich enzyme immunoassay as described [68] with monoclonal antibodies raised against early secreted PAGs. The RRID for the rabbit anti-PAG polyclonal antibody is AB_2636857 while the RRID for the mouse monocolnal antibody against PAG is AB_2636858. Each PAG assay included a standard curve, a pooled sample from pregnant cows at day 60 of gestation and a pooled sample from nonpregnant cows. The assay sensitivity was 0.28 ng/ml and the intra- and interassay coefficients of variation were <10%.

RNA extraction

RNA from liver, placenta, and muscle was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. The RNA isolation procedure included DNase treatment. Briefly, fragments of each tissue were disrupted and homogenized with a tissue homogenizer (Omni Tissue Master 125, Omni International, Kennesaw, GA, USA) for 30 s in 600 μl of lysis buffer supplemented with 1% (v/v) β-mercaptoethanol. The lysates were centrifuged for 3 min and the supernatant removed and transferred to 1.5-ml microcentrifuge tubes. An additional 600 μl of 70% (v/v) ethanol was added to the lysate and mixed by pipetting. RNA was purified using the RNeasy MinElute spin column following the manufacturer's protocol. Elution of RNA was performed in a volume of 40 μl and eluted RNA was evaluated for integrity (RIN) using the TapeStation 2200 machine (Agilent Technologies, Santa Clara, CA, USA). Extracted RNA was stored at –80°C until further analysis by qPCR.

PCR primers

A set of 96 PCR primers corresponding to 92 genes of interest plus four housekeeping genes actin beta (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) was designed by Fluidigm for the Delta Gene assay system (Fluidigm Co., San Francisco, CA, USA). Among the collection of genes of interest, there were 55 genes previously demonstrated to be disrupted in the fetus by culture [47, 62, 69–71] or maternal undernutrition [72], 14 genes important for embryonic development, 16 imprinted genes [73], and seven genes involved in fetal growth. Gene names, gene symbols, sequences of each primer and the reason for inclusion of each gene in the assay are listed in Supplemental File S1.

A qualification run was performed to validate the primers for all 96 genes using three control samples of RNA (one from each tissue). The qualifications were performed with eight-point, two-fold dilution series (replicated three times), and relationships between log of the RNA amount and amplification cycles (40-Ct) values were analyzed as described previously [74]. Briefly, an initial 3.5 μl of each sample was preamplified in a 10-μl reaction for 18 cycles followed by exonuclease I treatment. Then, each sample was diluted in two-fold serial dilutions for a total of eight dilutions and three replicates per sample. A water sample was included as a no template control. The quality control criteria for passing a primer were an $R^2 \geq 0.97$, efficiency of 0.8–1.3, and a slope of –3.92 to –2.76.

Reverse transcription and cDNA synthesis

RNA from fetal tissues was subjected to reverse transcription and cDNA synthesis using the Cells Direct Kit (Life Technologies, Carlsbad, CA, USA), per manufacturer's instructions. A total of 18 cDNA synthesis cycles were performed on 500-pg RNA, followed by exonuclease I treatment and loading into the microfluidic chip (1:4 dilution). Each sample was run once, except for eight samples from muscle and liver that were analyzed in technical duplicates so that an intra-assay coefficient of variation (CV) could be assessed. The average intra-assay CV for all genes examined was 0.58%. A no template control sample was included on the microfluidic chip as well.

Analysis of gene expression

Gene expression was analyzed by the Fluidigm qPCR procedure, using the microfluidic device Biomark HD system. Primer-probe sets and samples were transferred to an integrated fluidic circuits (IFC) plate and loaded into an automated controller that prepares the nanoliter reactions. The IFC plate was ran into the Biomark machine, which uses a thermal cycler for real-time quantitative PCR. The software Fluidigm Real-Time PCR Analysis was used to establish standard curves and calculate cycle threshold (Ct) values. Forty cycles of PCR were performed, using the 96.96 dynamic array IFC plate developed by the manufacturer. Nondetectable expression was considered to be a Ct of 27. The gene *YWHAZ* was rejected as a housekeeping gene because of its high CV among samples for a given tissue. Thus, for gene expression analysis, three housekeeping genes were used (*ACTB, GAPDH,* and *SDHA*). The ΔCt values were calculated relative to the geometric mean of the three housekeeping genes in the 95-gene set. Fold changes were calculated as $2^{-\Delta Ct}$ (i.e., amount of the gene relative to abundance of the housekeeping genes).

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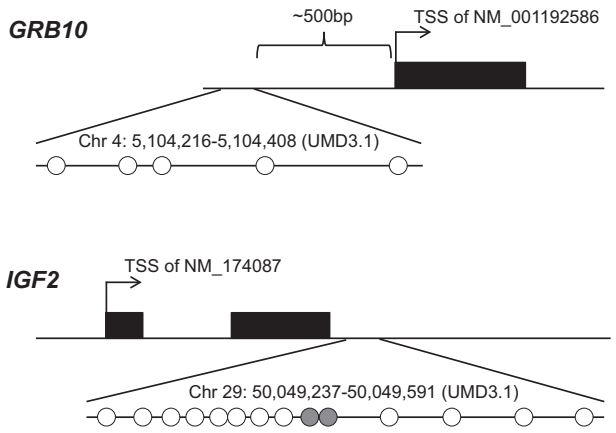

Figure 1. Schematic of the regions analyzed for DNA methylation of *GRB10* and *IGF2* loci. Bent arrows and black boxes represent transcription start sites (TSS) and exons, respectively. Open circles illustrate CpGs within the PCR amplicons for each gene (drawn to scale). Gray circles represent CpGs that were not successfully analyzed by the EpiTYPER array. Note that the fifth and sixth CpG units of *IGF2* were of the same mass and could not be distinquished from each other.

Determination of DNA methylation levels at loci for imprinted genes insulin-like growth factor 2 and growth factor receptor bound protein 10

Genomic DNA was isolated from liver, muscle, and placenta of the fetuses using the phenol–chloroform method, and the integrity of the DNA was verified by agarose gel electrophoresis. The DNA methylation levels of insulin-like growth factor 2 (*IGF2*) and growth factor receptor bound protein 10 (*GRB10*) loci were determined using a MALDI-TOF mass spectrometry-based method (EpiTYPER, Agena Bioscience Inc., San Diego, CA, USA) at the Weill Cornell Medical College Epigenomics Core (http://epicore.med.cornell.edu). In brief, genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research Corp., Irvine, CA, USA) following the manufacturer's instructions. In this reaction, the unmethylated cytosines are converted to uracil, while the methylated cytosines remain cytosines. Further, the regions of interest were amplified by PCR with the reverse primer containing a T7 promoter tag and the PCR amplicons were subject to in vitro RNA transcription followed by base-specific RNA cleavage. Lastly, cleaved products were analyzed using MassARRAY analyzer, and the proportion of cytosines that were methylated cytosines at each CpG site was determined using the EpiTYPER software.

The PCR primers for bisulfite-converted DNA were designed using the Methyl Primer Express Software (version 1.0, Applied Biosystems, Foster City, CA, USA). For EpiTYPER analyses, a 10-bp tag and a T7 promoter tag (31 bp) were added to the 5'end of the forward and reverse primers, respectively. The *IGF2* amplicon is located within the second intron of the transcript NM_174087 and is adjacent to the exon 2 (UMD 3.1; Chr 29: 50,049,237-50,049,591; 14 CpGs). Of the 14 CpGs within the *IGF2* amplicon, two sites could not be analyzed by the EpiTYPER array and two other sites produced CpG units of the same mass. The *GRB10* amplicon is located at the promoter region and is ~500 bp upstream of the transcript NM_001192586 (UMD 3.1; Chr 4: 5,104,216-5,104,408; 5 CpGs). Location of CpGs within each gene's amplicon is illustrated in Figure 1. Primer information was as follows (lower cases are the sequences of the tags):

*IGF2*_Forward aggaagagagTAGGGGATTTTTGGAGAATGAG
*IGF2*_Reverse cagtaatacgactcactatagggagaaggctCCCCCAACACAA AACTTAACTAATA
Product: 355 bp + 10 + 31 = 396 bp
*GRB10*_Forward aggaagagagGGATTGTTTTTTTGGTTTTAAAA AGGT
*GRB10*_Reverse cagtaatacgactcactatagggagaaggctAAACTACCCC-TAAAAATCCCTAATCC
Product: 193 bp + 10 + 31 = 234 bp

Statistical analysis

This was carried out using SAS software (version 9.4: SAS Institute Inc., Cary, NC, USA). Unless otherwise stated, data are presented as least-squares means ± SEM.

Morphometric endpoints measured in each fetus were analyzed by analysis of variance using the General Linear Models procedure (PROC GLM) for effects of treatment (AI, IVP, CSF2). When the main effect of treatment was at $P \leq 0.10$, contrasts were used to determine pairwise comparisons between treatments using the following comparisons: AI vs IVP, AI vs CSF2, and IVP vs CSF2. Concentrations of PAGs in maternal plasma from week 4 to 11 of gestation were analyzed by analysis of variance using the PROC MIXED procedure with a repeated statement to account for autocorrelations over time (weeks of gestation). The analysis was run separately to specify three types of covariance structures: compound symmetry, autoregressive, and variance components. Autoregressive was the best structure based on the fit statistics parameters of the model, so results were based on the autoregressive component. Chemical constituents of allantoic fluid were analyzed by analysis of variance using the GLM procedure of SAS (K and total protein) or, when residuals were not normally distributed, by the Kruskal–Wallis nonparametric test using the NPAR1WAY procedure of SAS. Pairwise comparisons were performed as described above using the contrast statement of GLM or by performing pairwise Kruskal–Wallis nonparametric tests. Fetal insulin concentrations were also analyzed using PROC GLM.

Gene expression data were analyzed as follows. Data on ΔCt values for each gene were analyzed for the effect of treatment by analysis of variance using PROC GLM as described above for morphometric data except that RNA integrity number of each sample was used as a covariate. Data are presented as least-squares means ± SEM of fold change relative to the geometric mean of the housekeeping genes. Data on DNA methylation at specific CpG were also analyzed by analysis of variance using PROC GLM as described for morphometric analysis. Data were analyzed two ways. First, data for each tissue were analyzed separately with main effects of treatment. Secondly, effects of tissue on DNA methylation were determined by combining data across tissues and performing analysis of variance with effects of tissue, treatment and the tissue by treatment interaction and with the error term being fetus nested within treatment.

Results

Morphometric features of the fetus and placenta

The overall pregnancy rate was not affected by treatment and was 23% (11 of 48 cows) for AI, 52% (16 of 31 cows) for IVP, and 34% (12 of 35 cows) for CSF2. A total of 29 pregnancies with single female fetuses were collected (9 AI, 12 IVP, and 8 CSF2). Distributions of body weight, eviscerated body weight, umbilical

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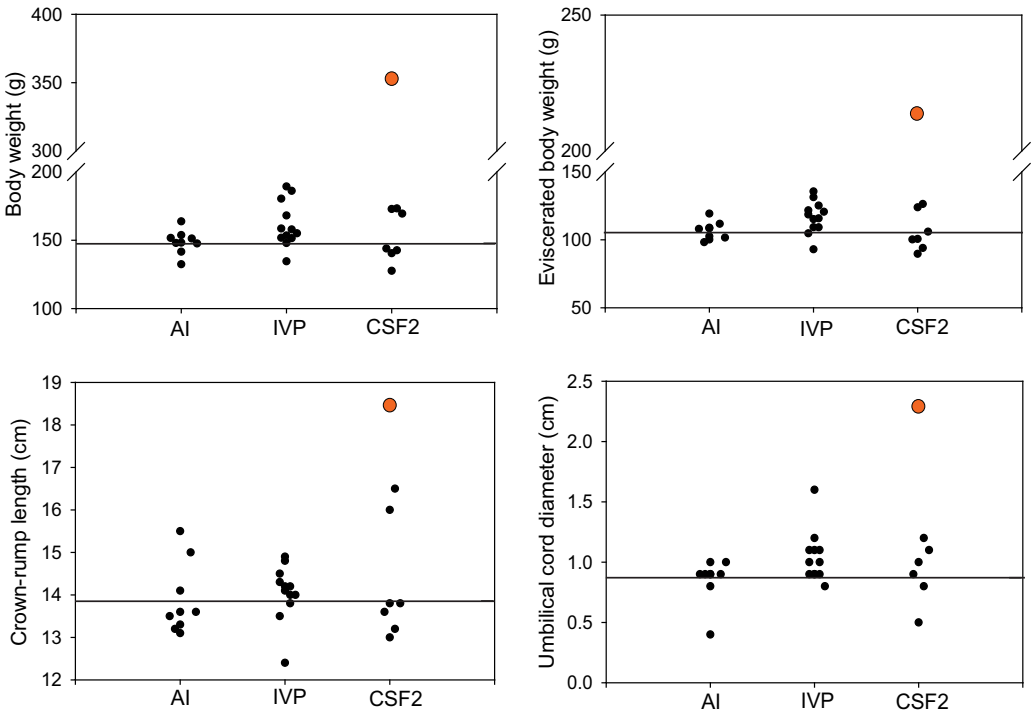

Figure 2. Individual distribution of morphometric features that were affected by treatment. The average value of the AI group is indicated by a horizontal line. Abbreviations: AI, artificial insemination and in vivo development; IVP, in vitro-produced embryos; CSF2, in vitro-produced embryos exposed to CSF2 from day 5 to 7. The orange circle represents the one fetus that was grossly abnormal.

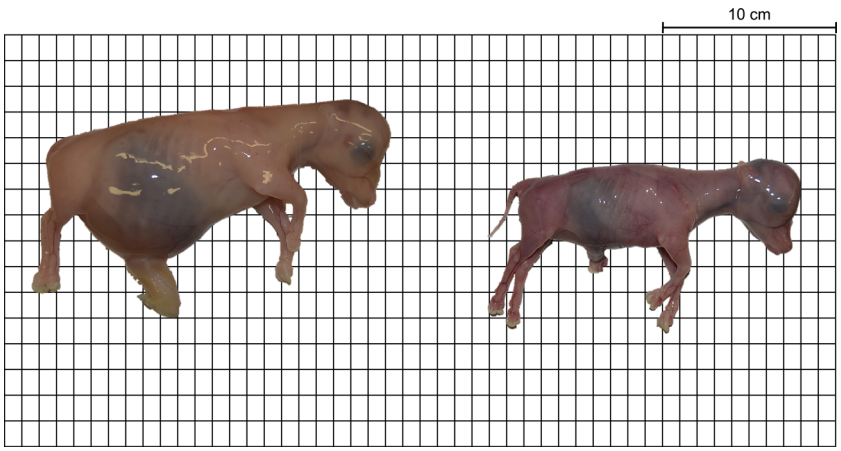

Figure 3. Morphological distinct phenotypes observed in day 86 fetuses. Left: fetus from the CSF2 group presenting a phenotype consistent with the abnormal offspring syndrome in cattle. Right: fetus from the AI group with a typical phenotype and a body weight close to the average of the AI group.

cord diameter, and crown-rump length is illustrated in Figure 2. The number of fetuses characterized as large offspring represented 11% (1 of 9) of the fetuses in the AI group, 30% (4 of 12) of the IVP group, and 50% (4 of 8) of the CSF2 group. Note that one fetus (marked in orange in Figure 2) was grossly abnormal as compared to the other fetuses and had characteristics of LOS. As compared to the average of other fetuses, conceptus weight was three times greater, body weight two-fold greater, placental weight seven-fold greater, and allantoic fluid volume was three-fold greater (see Supplemental Table S1 for details). In addition, most organs were enlarged. Severe ascites in the fetus (Figure 3) and hemorrhagic cotyledons were also observed. This fetus, a clear outlier, was excluded from all subsequent analyses.

Characteristics of each of 23 morphometric measurements on the remaining 28 conceptuses are shown in Table 1. Differences between treatments were found for four measurements—body weight, eviscerated weight, crown-rump length, and umbilical cord diameter. The IVP fetuses differed from AI fetuses for body weight ($P = 0.0460$), eviscerated weight ($P = 0.0378$), and umbilical cord diameter ($P = 0.0519$). A similar difference was found for CSF2 vs AI ($P = 0.0381$) and, despite lack of statistical significance, eviscerated body weight. For umbilical cord diameter, in contrast, CSF2 was more similar to AI than IVP. The fourth morphological measurement affected by treatment was crown-rump length. For this endpoint, there was no difference between AI and IVP, but crown-rump length was

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Table 1. Morphometric measurements of bovine conceptuses at day 86 of gestation generated by three different techniques: artificial insemination (AI; in vivo control), in vitro embryo production (IVP), and in vitro embryo production with treatment with CSF2 from days 5 to 7 of culture (CSF2).[a]

Endpoints	AI (n = 9)	IVP (n = 12)	CSF2 (n = 7)
Extraembryonic			
Conceptus length (cm)	101.0 ± 5.5	89.9 ± 5.1	87.4 ± 7.0
Conceptus weight (g)	1212 ± 115.4	1186 ± 108.2	1301 ± 148.2
Number of cotyledons	76 ± 8.8	74 ± 8.2	75 ± 11.3
Mean cotyledon diameter (cm)	3.3 ± 0.2	3.5 ± 0.2	3.4 ± 0.3
Allantoic fluid (ml)	349.6 ± 137.1	329.6 ± 125.9	414.1 ± 164.1
Amniotic fluid (ml)	493.7 ± 30.9	505.9 ± 29.4	531.4 ± 39.8
Wet placenta weight (g)	210.8 ± 20.3	211.9 ± 19.0	188.4 ± 26.0
Dry placenta weight (g)	9.8 ± 0.8	9.8 ± 0.7	9.7 ± 1.0
Fetal			
Fetal body weight (g)[b]	142.9 ± 4.8	157.1 ± 4.5	162.7 ± 6.1
Fetal eviscerated weight (g)[c]	102.9 ± 3.4	113.6 ± 3.2	112.2 ± 4.4
Crown-rump length (cm)[d]	13.7 ± 0.2	14.0 ± 0.2	14.7 ± 0.3
Crown-nose length (cm)	4.4 ± 0.1	4.2 ± 0.1	4.2 ± 0.2
Heart girth (cm)	11.1 ± 0.2	11.4 ± 0.1	11.3 ± 0.2
Liver weight (g)	6.2 ± 0.6	6.8 ± 0.5	7.3 ± 0.7
Paired ovary weight (g)	0.06 ± 0.01	0.05 ± 0.01	0.08 ± 0.01
Paired kidney weight (g)	1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.2
Whole brain weight (g)	5.2 ± 0.1	5.2 ± 0.1	5.2 ± 0.2
Heart weight (g)	1.3 ± 0.08	1.5 ± 0.07	1.5 ± 0.1
Aortic root diameter (cm)	0.39 ± 0.03	0.40 ± 0.03	0.35 ± 0.04
Umbilical cord diameter (cm)[e]	0.9 ± 0.08	1.1 ± 0.07	0.9 ± 0.11
Ratios			
Fetal:placental weight (wet)	0.74 ± 0.60	0.78 ± 0.65	0.87 ± 0.69
Fetal:placental weight (dry)	15.0 ± 1.0	16.4 ± 1.0	17.4 ± 1.3

[a]Group abbreviations: AI, artificial insemination and in vivo development; IVP, in vitro-produced embryos; CSF2, in vitro-produced embryos exposed to CSF2 from day 5 to 7. Data are least-squares means ± SEM.

[b]AI vs IVP, $P = 0.0460$; AI vs CSF2, $P = 0.0381$

[c]AI vs IVP, $P = 0.0378$

[d]AI vs CSF2, $P = 0.0212$; IVP vs CSF2, $P = 0.0631$

[e]AI vs IVP, $P = 0.0519$

increased for CSF2 compared with AI ($P = 0.0212$) and IVP ($P = 0.0631$).

Characteristics of allantoic fluid

In general, the composition of allantoic fluid was not affected by treatment. The exception was for HCO_3^- concentration, which was lower ($P = 0.0174$) for IVP (7.0 ± 0.9 mmol/l; mean ± SEM) than for CSF2 (10.0 ± 1.2 mmol/l). Concentrations for AI were similar to CSF2 (10.0 ± 1.4 mmol/l), although not statistically different from IVP.

Concentrations of insulin in fetal heart

Insulin concentrations were low and, for six samples, nondetectable. There were no differences in insulin levels among groups. Values were 0.07 ± 0.01, 0.08 ± 0.01, and 0.07 ± 0.01 ng/ml for AI, IVP, and CSF2, respectively.

Circulating maternal pregnancy-associated glycoproteins from 4 to 11 weeks of gestation

Concentrations of PAGs varied over time ($P < 0.0001$) in a quadratic pattern, with higher concentrations at week 4, a nadir at weeks 7–8, and a second increase in concentrations thereafter (Figure 4). Treatment did not affect concentrations of PAGs in maternal blood ($P = 0.5196$), and there was no interaction between treatment and week ($P = 0.6100$).

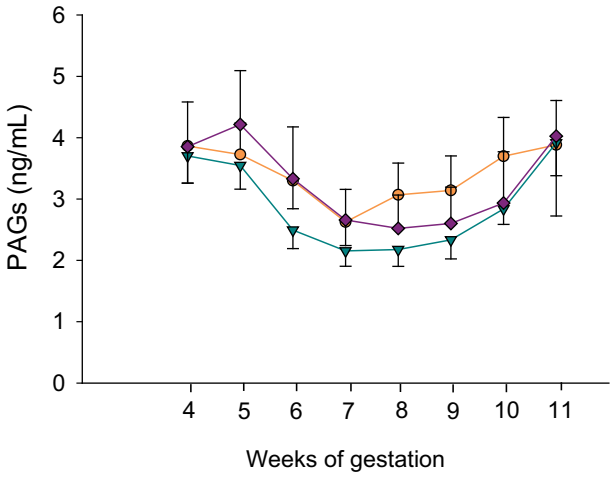

Figure 4. Pregnancy-associated glycoprotein (PAGs) in maternal circulation from weeks 4 to 11 of gestation in dams gestating a conceptus conceived by AI (artificial insemination and in vivo development; orange circles), IVP (in vitro-produced embryos; dark green triangles), or CSF2 (in vitro-produced embryos exposed to CSF2 from day 5 to 7; purple diamonds). Concentrations were affected by week ($P < 0.0001$) but not treatment ($P = 0.5196$) or the interaction between treatment and week ($P = 0.6100$).

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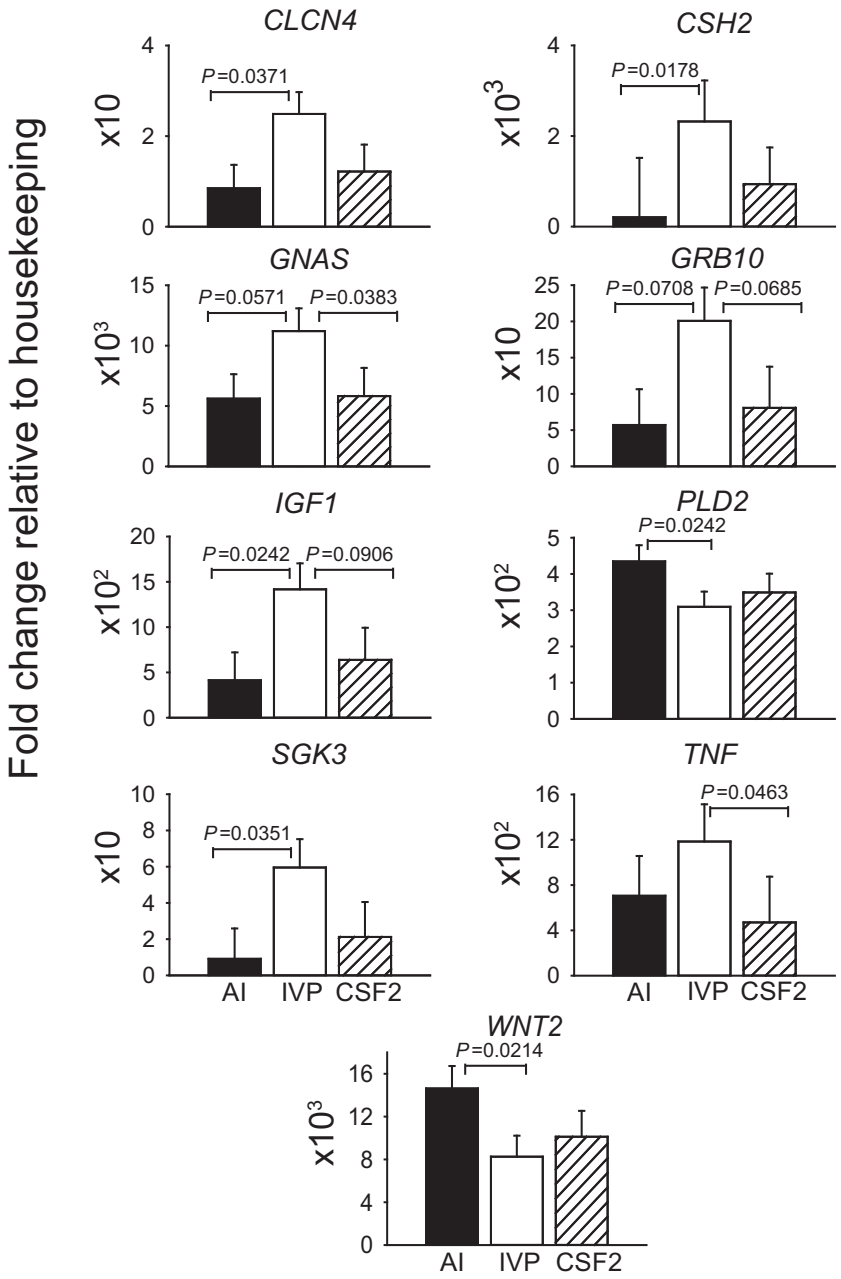

Figure 5. Genes affected by treatment in fetal liver. Data are least-squares means ± SEM. Horizontal lines between bars indicate differences between groups with $P < 0.10$. The actual P value of the pairwise comparison is indicated above the line. Abbreviations: AI, artificial insemination and in vivo development; IVP, in vitro-produced embryos; CSF2, in vitro-produced embryos exposed to CSF2 from day 5 to 7.

Gene expression in liver, muscle, and placenta

There were eight genes whose expression in liver was different between AI and IVP, with IVP having higher expression for chloride voltage-gated channel 4 (*CLCN4*), chorionic somatomammotropin hormone 2 (*CSH2*), GNAS complex locus (*GNAS*), *GRB10*, insulin-like growth factor 1 (*IGF1*), and serum/glucocorticoid regulated kinase family member 3 (*SGK3*) and lower expression for phospholipase D2 (*PLD2*), and Wnt family member 2 (*WNT2*) (Figure 5). The difference between AI and IVP was abrogated or reduced by CSF2 for each of these genes as indicated by a lack of difference between AI and CSF2 and, in some cases, by differences between IVP and CSF2 (*GNAS*, *GRB10*, *IGF1*, and tumor necrosis factor).

Expression of a total of 12 genes in placenta differed between groups (Figure 6). In 10 of these cases, expression was highest for IVP as indicated by effects of AI vs IVP or IVP vs CSF2. This was the case for cytochrome c oxidase subunit 6A1 (*COX6A1*), DNA methyltransferase 1 (*DNMT1*), *IGF2*, insulin-like growth factor binding protein 2 (*IGFBP2*), methionyl aminopeptidase 2 (*METAP2*), nucleosome assembly protein 1 like 5 (*NAP1L5*), nuclear receptor subfamily group C member 1 (*NR3C1*), Salvador family WW domain containing protein 1 (*SAV1*), *WNT2*, and X inactive specific transcript (*XIST*). CSF2 reduced the effect of in vitro production of embryos on expression of *COX6A1*, *IGF2*, *IGFBP2*, *METAP2*, *SAV1*, and *WNT2* but not on expression of *DNMT1*, *METAP2*, *NAP1L5*,

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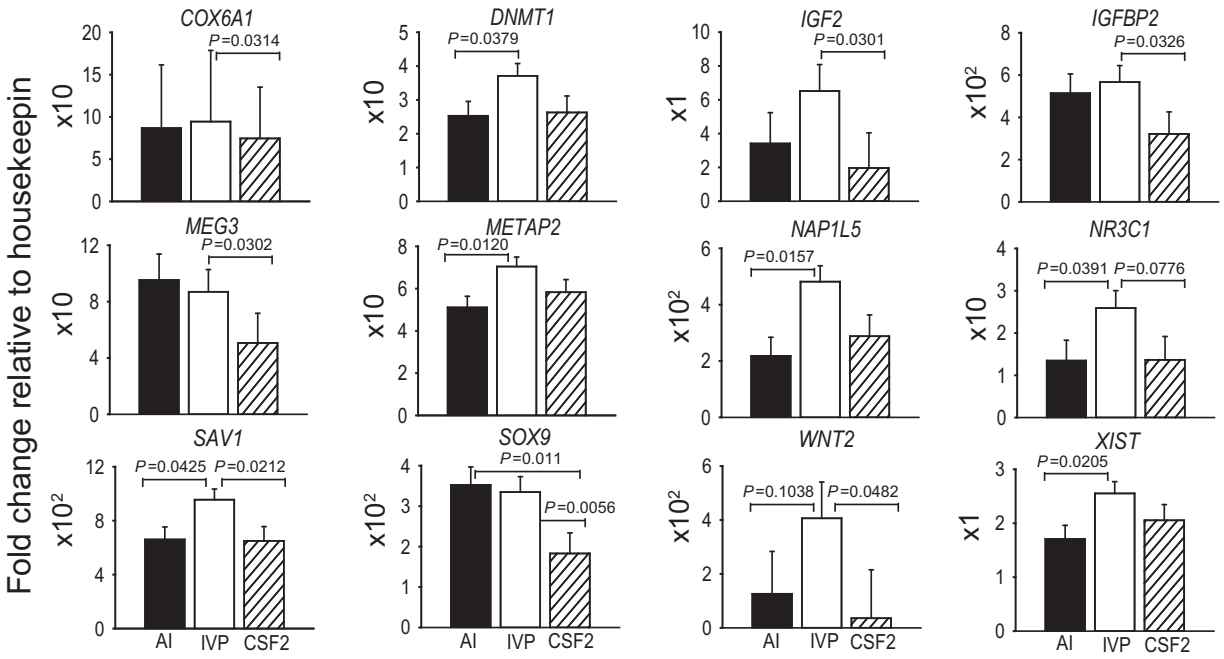

Figure 6. Genes affected by treatment in fetal placenta. Data are least-squares means ± SEM. Horizontal lines between bars indicate differences between groups with $P < 0.10$. The actual P value of the pairwise comparison is indicated above the line. Abbreviations: AI, artificial insemination and in vivo development; IVP, in vitro-produced embryos; CSF2, in vitro-produced embryos exposed to CSF2 from day 5 to 7.

NR3C1, and *XIST*. CSF2 also decreased expression of *COX6A1*, *IGF2*, *IGFBP2*, maternally expressed 3 (*MEG3*), *SAV1*, and *WNT2*. For SRY-box 9 (*SOX9*), CSF2-treated fetuses had the lowest expression, which differed significantly from both AI and IVP fetuses.

In muscle, the pattern of disruption of gene expression by IVP was different than for the other two organs (Figure 7). The vast majority of the 27 affected genes were downregulated in IVP compared to AI, except for *COX6A1*, which was upregulated in IVP and, to a greater extent, for CSF2 compared with AI. Moreover, CSF2 was not able to correct aberrant gene expression caused by in vitro production of embryos because transcript abundance for most genes was similar between IVP and CSF2. There were six genes in which expression was lower for IVP than AI and where CSF2 was either intermediate or similar to AI. These were DIS3 homolog, exosome endoribonuclease and 3'-5'exoribonuclease (*DIS3*), general transcription factor IIB (*GTF2B*), heart and neural crest derivatives expressed 1 (*HAND1*), solute carrier family 22 member 2 (*SLC22A2;* previously known as *LOC521027*), *METAP2* and ZPR1 zinc finger (*ZPR1*). The same tendency occurred for *DNMT1*.

Although not included in the statistical analysis, gene expression for the abnormal fetus was generally within the range of expression levels for other fetuses. Exceptions were for four genes in placenta: *IGFBP2* (fold change relative to housekeeping was 0.13 compared with 0.05 in the other fetuses), *MEG3* (fold change 1.89 vs 0.8), *NAP1L5* (1.08 vs 0.03), and *SOX9* (0.12 vs 0.03).

DNA methylation of insulin-like growth factor 2 and growth factor receptor bound protein 10 loci

Treatment did not affect the degree of DNA methylation at five CpG sites evaluated for *GRB10* in any of the three tissues examined (Figure 8). For *IGF2*, treatment affected two of the 12 CpGs examined for placenta and two for muscle but there were no treatment effects for liver (Figure 9). In each case and for each tissue, lowest methylation was for the CSF2 group.

Discussion

It is well established that the environment of the preimplantation embryo can cause changes in development that have consequences for fetal development and postnatal life [15, 18, 75, 19, 76]. Changes in the developmental program could be due to actions of maternally derived regulatory molecules called embryokines that alter development. Here, we used the in vitro-produced embryo to test the hypothesis that CSF2 is one embryokine responsible for developmental programming. Fetuses derived from in vitro production procedures can sometimes experience aberrant development that has consequences that extend into postnatal life [6, 41, 58, 70, 77]. In the present study, as well, IVP was associated with some alterations in morphological characteristics and gene expression in muscle and, less extensively, in placenta and liver. We hypothesized that lack of maternal regulatory signals is a major factor involved in the detrimental effects of embryo production in vitro on development and that CSF2 is one of the embryokines involved. This hypothesis, which would mean that exposure of the preimplantation embryo to CSF2 would correct adverse effects of in vitro production and lead to less severe abnormalities in fetal development during the first trimester of gestation, was partially supported. Treatment of embryos with CSF2 modified the developmental program of the embryo as indicated by alterations in morphology, gene expression, and DNA methylation in conceptuses at day 86 of gestation. Only in some cases, however, did CSF2 reverse the aberrant phenotype associated with IVP. In other cases, CSF2 resulted in alterations in phenotype compared to AI that were not seen for IVP. Thus, CSF2 can act as a developmental programming agent with specific roles in embryonic and fetal development. Nonetheless, this embryokine is not able to abolish the adverse effects of in vitro production on fetal characteristics.

For the most part, CSF2 did not modify alterations in fetal morphology associated with IVP. Fetuses from embryos produced in vitro were heavier than fetuses produced by AI regardless of whether CSF2 was added to culture medium. In addition, CSF2 exacerbated

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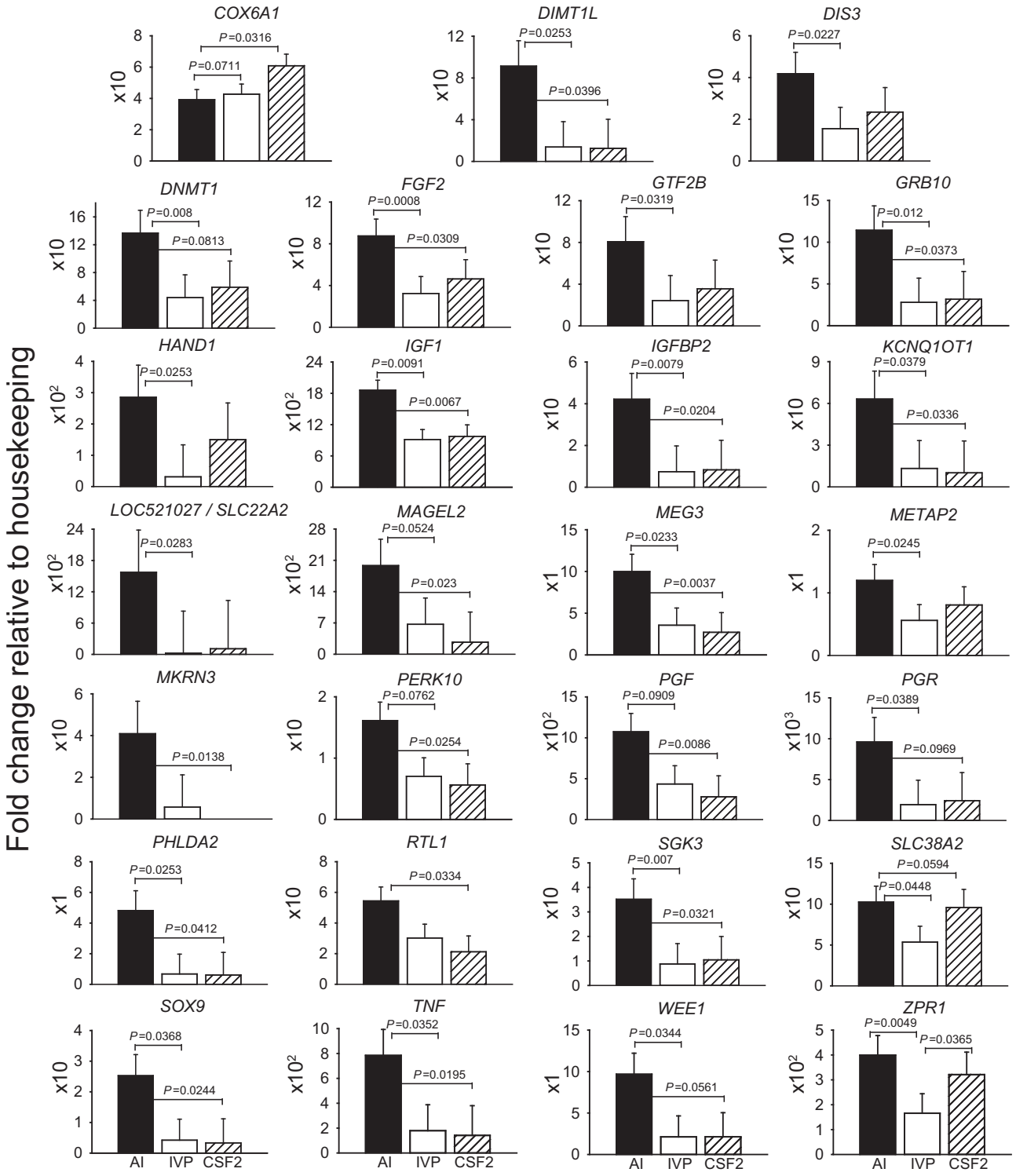

Figure 7. Genes affected by treatment in fetal muscle. Data are least-squares means ± SEM. Horizontal lines between bars indicate differences between groups with $P < 0.10$. The actual P value of the pairwise comparison is indicated above the line. Abbreviations: AI, artificial insemination and in vivo development; IVP, in vitro-produced embryos; CSF2, in vitro-produced embryos exposed to CSF2 from day 5 to 7.

consequences of culture on crown-rump length. Thus, much of the aberrant development associated with IVP is caused by mechanisms independent of actions of CSF2. This is not surprising both because there are many potential causes of abnormal developmental programming associated with IVP and because CSF2 is unlikely to be the only maternal regulatory molecule affecting development. Treatment with CSF2 would not reverse errors in developmental programming caused by inadequate oocyte development or maturation, semen preparation, fertilization, or development before day 5 when CSF2 was added to culture. The oviductal environment plays

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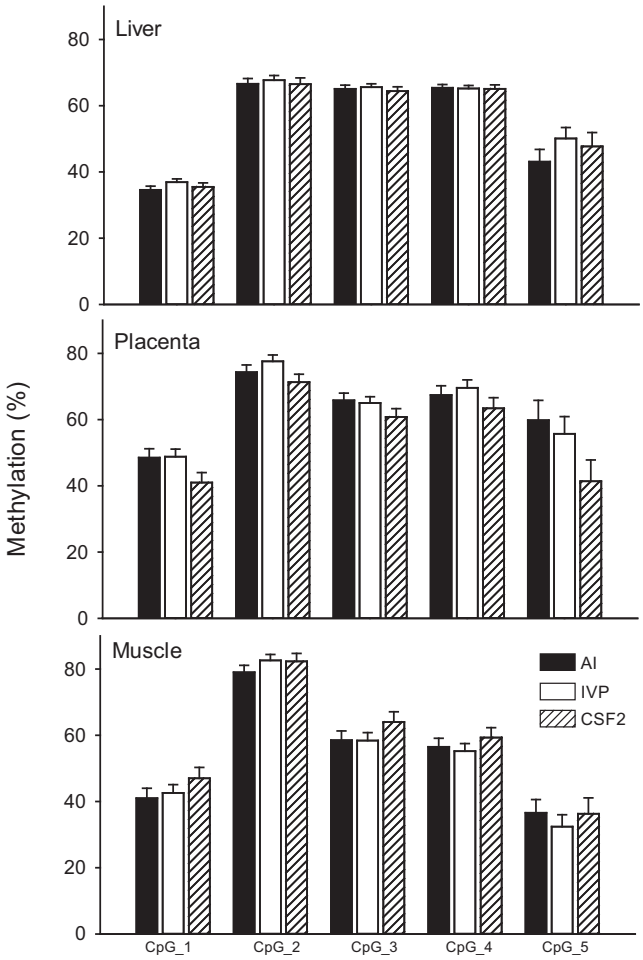

Figure 8. DNA methylation at *GRB10* loci in liver, placenta, and muscle. Data are least-squares means ± SEM for AI (filled), IVP (open), and CSF2 (hatched) conceptuses. Abbreviations: AI, artificial insemination and in vivo development; IVP, in vitro-produced embryos; CSF2, in vitro-produced embryos exposed to CSF2 from day 5 to 7. The amount of methylation differed between tissues for all loci ($P < 0.05$), but there were no significant effects of treatment for any CpGs ($P > 0.10$).

a crucial role for embryonic gene expression during the two-cell to four-cell stages [78]. Abnormal development may be programmed as a result of characteristics of the oocyte or sperm [79, 80] and CSF2 would be unlikely to reverse these effects. In addition, however, there are other maternally derived molecules that can affect embryonic development [in cattle, for example, IGF1 [81, 82] and DKK1 [32]], and lack of these molecules during culture may result in atypical fetal development.

Nonetheless, CSF2 did reverse some of the consequences of IVP on fetal characteristics as reflected by prevention of abnormalities in umbilical cord diameter, HCO_3^- concentration in allantoic fluid, and gene expression, particularly for placenta and liver. Treatment with CSF2 reduced or eliminated effects of IVP on expression of all eight affected genes in liver, 6 of 12 genes affected by IVP in placenta and 6 of 27 genes affected by IVP in muscle. The fact that CSF2 affected programming in some tissues to a greater extent than in others (liver>placenta>muscle) means that developmental programming occurs in a tissue-specific manner and may depend upon the embryonic origin of each tissue. This was the case even in the absence of CSF2 because IVP caused more extensive disruption in gene expression in muscle than in liver and placenta. Moreover, the nature of aberrant gene expression varied between tissues; the predominant effect of IVP was for increased gene expression in liver and placenta and decreased expression in muscle. Chemical analysis of the allantoic fluid revealed no major differences in fetal excretion of electrolytes and other products of protein and carbohydrate metabolism, except for an effect on bicarbonate concentrations. The reduced bicarbonate concentration in the allantois of IVP, which was reversed by CSF2 to concentrations similar to AI conceptuses, indicates that IVP fetuses may have experienced a certain degree of metabolic acidosis. Finally, there was no effect of fetal origin (AI, IVP, or CSF2) on maternal plasma PAGs from weeks 4 to 11 of gestation.

Tissue-dependent programming could reflect two phenomena. One is that culture itself or actions of CSF2 could affect one cell lineage differently than another. It is known, for example, that CSF2 can affect gene expression in the trophectoderm differently than in the inner cell mass [30]. A second mechanism is that culture and/or CSF2 causes epigenetic changes in the developing embryo that has different consequences for certain cell lineages than others. Alterations in the epigenome are a major cause of abnormalities resulting from embryo culture [83–86]. In the present experiment, there is evidence that IVP and CSF2 caused epigenetic changes in the resultant fetuses. First, several genes affected by IVP [*GNAS* and *GRB10* in liver; *IGF2*, *MEG3*, *NAP1L5*, and *XIST* in placenta; *GRB10*, KCNQ1 opposite strand/antisense transcript 1 (*KCNQ1OT1*), MAGE family member L2 (*MAGEL2*), *MEG3*, makorin ring finger protein 3 (*MKRN3*), proline-rich receptor-like protein kinase PERK10 (*PERK10*, previously known as *MEG8*), pleckstrin homology like domain family A member 2 (*PHLDA2*), and retrotransposon-like 1 (*RTL1*) in muscle] are imprinted genes and alterations in gene expression could conceivably reflect loss of imprinting. Of the imprinted genes whose expression was affected by IVP, CSF2-modified expression of *GNAS* and *GRB10* in liver and *IGF2*, *MEG3*, *NAP1L5*, and XIST in placenta, whereas CSF2 did not correct the expression pattern of any disrupted by IVP in muscle. In the current experiment, *DNMT1* (involved in maintenance of DNA methylation) was upregulated by IVP in placenta and downregulated in fetal muscle compared with AI. For both tissues, CSF2 fetuses were intermediate in expression of this gene. Expression of *DNMT1* has been reported to be overexpressed in blastocysts produced in vitro as compared to blastocysts produced in vivo [87]. Finally, there were some effects of CSF2 to reduce DNA methylation at specific CpG loci for *IGF2* in placenta and muscle. A more extensive analysis of genome-wide DNA methylation as well as other epigenetic mechanisms (e.g., post-translational modifications of histones and actions of noncoding RNA) will allow better elucidation of the molecular mechanism of action of CSF2 to modify gene expression patterns through epigenetic modifications.

Effects of culture on embryonic and fetal development have been well described [49, 54, 55, 88–90]. Common anomalies include abnormal placentation, fetal growth, and metabolism, which are most likely a result of errors in embryonic reprogramming [51, 91] due to suboptimal conditions provided by culture media used for IVP [92]. In fact, compared with their in vivo counterparts, in vitro-derived embryos have distinct metabolism [93], transcriptome [94–96], and epigenome [8, 86]. While the association of IVP with aberrant fetal growth (either over- or undergrowth) has been well described in ruminants [49, 97] and humans [38, 98], this condition is often ascribed to the use of serum in IVP media [49, 51, 99, 100]. Interestingly, we performed IVP in the absence of serum, using an optimized

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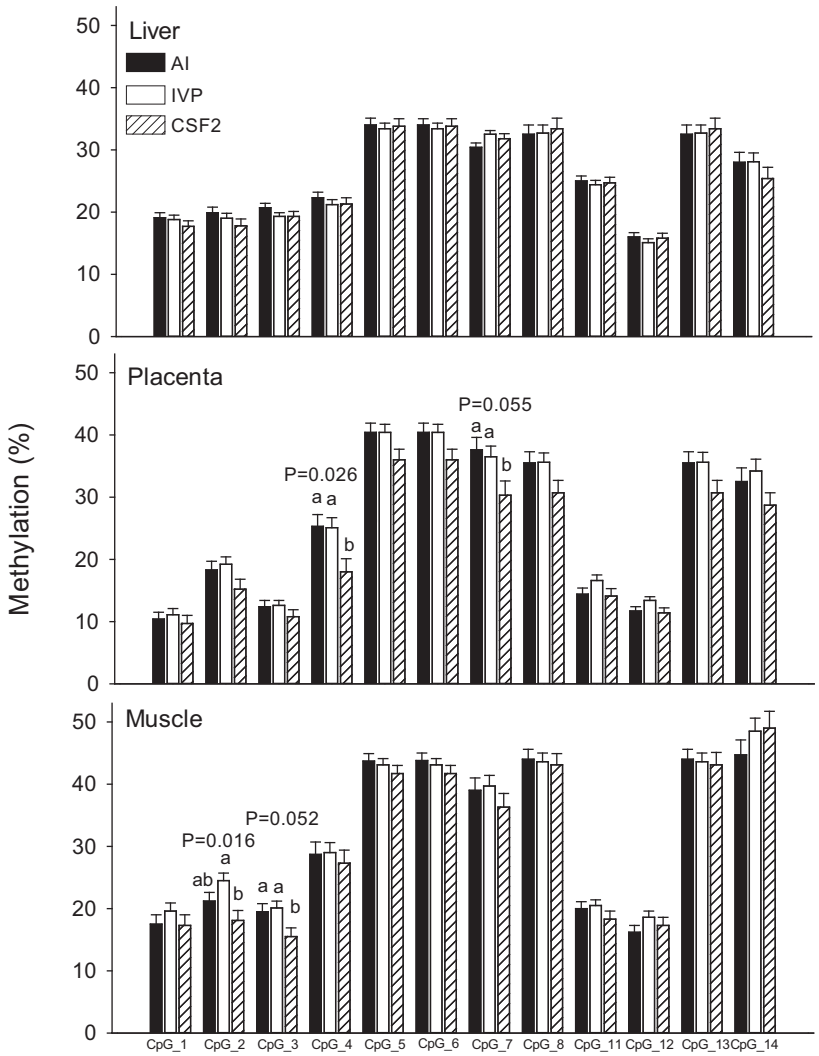

Figure 9. DNA methylation at *IGF2* loci in liver, placenta, and muscle. Data are least-squares means ± SEM of AI (filled), IVP (open), and CSF2 (hatched) fetuses. Abbreviations: AI, artificial insemination and in vivo development; IVP, in vitro-produced embryos; CSF2, in vitro-produced embryos exposed to CSF2 from day 5 to 7. The *P* value for differences between treatments, when $P < 0.10$, is indicated above each group of bars. Means with different superscripts differ ($P < 0.05$). Note that the CpG_5- and CpG_6-produced units of the same mass that could not be distinguished from each other and gave identical signals.

system with a defined medium in low oxygen (designed to increase development; [101]) and still observed alterations in embryonic programming that led to altered morphology and gene expression of fetuses at the end of the first trimester of gestation. This indicates that abnormalities associated with IVP are not only due to the presence of serum, but may reflect loss of other key regulatory molecules during development. In addition, characteristics of the oocyte or sperm can affect the developmental program [79, 80], and it is possible that properties of the sperm and oocyte used for IVP contribute to alterations in developmental programming.

At the molecular level, overgrowth of IVP fetuses observed in this study might be due, in part, to upregulation of *CSH2*, *IGF1*, and *GRB10* in liver since these genes promote growth as well as upregulation of expression of *IGF2* in placenta. Placental IGFs, specifically IGF2, are known as main regulators of fetal growth [102]. Increased *IGF1* in the liver of IVP-derived fetuses contrasts with a previous report about IVP fetuses produced using serum-containing culture medium [62]. Perhaps, the culture medium exerts distinct effects depending upon its composition. In contrast to liver, a different pattern was seen in muscle, in which *IGF1* and *IGFBP2* were downregulated in IVP and CSF2 fetuses compared with AI. Decreased *IGFBP2* expression in muscle of IVP and CSF2 fetuses could conceivably promote growth by removal of inhibition of IGF1 caused by binding to IGFBP2. The fact that expression of the same gene was affected by IVP and CSF2 differently in different tissues leads to the inference that aberrant development of IVP is regulated by distinct molecular pathways in different tissues.

The degree of fetal abnormalities associated with IVP varied among fetuses. In the most extreme case, we observed a phenotype consistent with the large offspring syndrome in cattle. Other fetuses, however, were subtly affected and some fetuses derived by in vitro production (IVP and CSF2 groups) appeared normal regardless of whether treated with CSF2 or not. Therefore, we conclude that culture affects each individual differently and the degree of abnormalities may depend on variations in the oocyte from which embryos were produced and the severity of changes in epigenetic reprogramming occurring during culture. Some fetuses may even have engaged compensatory mechanisms that prevented major

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alterations to be observed at the time of gestation examined in this study.

In summary, here we provide evidence that actions of CSF2 on the preimplantation embryo alter the developmental program to affect fetal phenotype at day 86 of gestation. For some aspects of development, CSF2 alleviated fetal abnormalities associated with IVP but, in other cases, it caused an altered phenotype distinct from AI and IVP. We conclude that CSF2 can act as a developmental programming agent, but this embryokine alone is not able to eliminate undesirable effects of culture on fetal development.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental File S1. Genes and primers used for quantitative PCR using the Biomark microfluidic PCR system. Tab 1 lists the genes subjected to analysis, primers used for amplification, and other details of the genes. Tab 2 is a description of the reason genes were included in the analysis with a list of supporting references.

Supplemental Table S1. Morphometric measurements of one large conceptus at day 86 of gestation with a phenotype consistent with large offspring syndrome.

Acknowledgments

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Conflict 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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