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Physiol Genomics 33: 65–77, 2008.
First published December 18, 2007; doi:10.1152/physiolgenomics.00223.2007.

Aberrant gene expression patterns in placentomes are associated with phenotypically normal and abnormal cattle cloned by somatic cell nuclear transfer

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Submitted 25 September 2007; accepted in final form 11 December 2007

Everts RE, Chavatte-Palmer P, Razzak A, Hue I, Green CA, Oliveira R, Vignon X, Rodriguez-Zas SL, Tian XC, Yang X, Renard JP, Lewin HA. Aberrant gene expression patterns in placentomes are associated with phenotypically normal and abnormal cattle cloned by somatic cell nuclear transfer. *Physiol Genomics* 33: 65–77, 2008. First published December 18, 2007; doi:10.1152/physiolgenomics.00223.2007.—Transcription profiling of placentomes derived from somatic cell nuclear transfer (SCNT, n = 20), in vitro fertilization (IVF, n = 9), and artificial insemination (AI, n = 9) at or near term development was performed to better understand why SCNT and IVF often result in placental defects, hydrops, and large offspring syndrome (LOS). Multivariate analysis of variance was used to distinguish the effects of SCNT, IVF, and AI on gene expression, taking into account the effects of parturition (term or preterm), sex of fetus, breed of dam, breed of fetus, and pathological finding in the offspring (hydrops, normal, or other abnormalities). Differential expression of 20 physiologically important genes was confirmed with quantitative PCR. The largest effect on placentome gene expression was attributable to whether placentas were collected at term or preterm (i.e., whether the collection was because of disease or to obtain stage-matched controls) followed by placentome source (AI, IVF, or SCNT). Gene expression in SCNT placentomes was dramatically different from AI (n = 336 genes; 276 >2-fold) and from IVF (n = 733 genes; 162 >2-fold) placentomes. Functional analysis of differentially expressed genes (DEG) showed that IVF has significant effects on genes associated with cellular metabolism. In contrast, DEG associated with SCNT are involved in multiple pathways, including cell cycle, cell death, and gene expression. Many DEG were shared between the gene lists for IVF and SCNT comparisons, suggesting that common pathways are affected by the embryo culture methods used for IVF and SCNT. However, the many unique gene functions and pathways affected by SCNT suggest that cloned fetuses may be starved and accumulating toxic wastes due to placental insufficiency caused by reprogramming errors. Many of these genes are candidates for hydrops and LOS.

placenta; bovine; reprogramming

ALTHOUGH MAMMALIAN CLONING by somatic cell nuclear transfer (SCNT) has been practiced for the past 10 years (84), the cloning process remains largely inefficient because of losses during gestation (12, 63). For example, the cloning efficiency of transferred cattle embryos that develop through term is generally <10% (27, 44, 90). Approximately 80% of transferred cattle embryos are lost before the beginning of the second trimester (5, 44, 49). The majority of failed pregnancies appear to result from abnormal placental development, such as reduced vascularization, and fewer but enlarged placentomes (16, 20, 40, 49, 50). Recent evidence strongly links errors in reprogramming of the donor nucleus to the placental and fetal developmental anomalies of mammals cloned by SCNT (10, 11, 52, 54, 55, 59). A current hypothesis is that the extraembryonic tissues of the embryo fail to develop normally in SCNT clones, which causes subsequent abnormalities in implantation and development of the placenta (20, 34, 49). Support for this hypothesis derives from the comparison of SCNT- to in vivo-derived bovine embryos, which revealed an altered ratio of inner cell mass (ICM) cells to trophectoderm cells (56). Aberrant gene expression in SCNT-derived extraembryonic tissues and fetuses has been confirmed from the morula stage until day 65 of gestation and seems to become more widespread during later pregnancy (6, 9, 40, 42, 68, 69, 74, 75). However, the genes, timing, and duration of the genetic lesions that cause placental defects in SCNT-cloned cattle have not been determined.

Of those SCNT-derived embryos that develop to term, up to 40% have large offspring syndrome (LOS), which is characterized by enlarged organs, hydrops of the fetus, lethargy, and respiratory distress (16, 18, 34, 59, 92). In all cases of LOS, the placenta is enlarged and edematous. It is noteworthy that LOS is also observed in offspring produced by in vitro fertilization (IVF) and has a human clinical correlate in Beckwith-Wiedemann syndrome, which is associated with imprinting defects (22). Although there are gross placental abnormalities of SCNT-cloned cattle, the general health of surviving offspring appears to be normal. For example, the health status and milk and meat production of cloned cattle surviving past the first year of life was similar to that of artificial insemination (AI)-derived animals (17, 45, 47, 79, 82). In contrast, SCNT-derived mice are obese (78) and have markedly shorter life spans (67). Apparently, for fetal survival the placenta must overcome and compensate for the developmental defects caused by reprogramming errors, and the cloned offspring also must adapt to these changes, which may result in fetal growth retardation (2, 37). The stochastic nature of placental development that occurs as a result of SCNT-induced genomic perturbations would be consistent with such observations.

Comparison of gene expression profiles of placentomes collected from SCNT pregnancies may provide important in-

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sights into the metabolic and cellular pathways that are affected by SCNT, the adaptations that are necessary for fetal survival, and the molecular defects underlying LOS. The design of the present study allowed us to address this issue by examination of gene expression patterns in placentomes collected from SCNT, IVF, and AI pregnancies. The occurrence of hydrops in 30% of the samples permitted an analysis of the genes and gene expression patterns underlying this condition. Results demonstrate gross abnormalities in placentome gene expression associated with SCNT cloning. Surprisingly, IVF also produced a large number of differences in placentome gene expression compared with AI and SCNT samples. Functional annotation of differentially expressed genes (DEG) and pathway analysis revealed multiple affected pathways in SCNT-cloned cattle that may contribute to the development of LOS and hydrops.

MATERIALS AND METHODS

Recipients and calves. Recipient animals for IVF- or SCNT-derived embryos were normal cycling heifers located at the Institut National de la Recherche Agronomique (INRA) experimental farms in Bourges ($n = 4$) and Bressonvillier ($n = 34$), France. The heifers were subjected to estrous synchronization with a progesterone-releasing intravaginal device (Crestar-Intervet, Angers, France). A separate control group of heifers was artificially inseminated with either Holstein or Charolais bull semen at 48 and 60 h after administration of prostaglandin ($PGF_{2\alpha}$).

The SCNT calves were produced by transfer of nuclei from two adult skin fibroblast cell cultures (5538 and 7711) and one fetal fibroblast cell culture (BSF204), as previously described (16, 18, 46, 79a). IVF embryos were cultured in sequential media without fetal bovine serum or oviduct cells, whereas SCNT embryos were cocultured with oviduct cells (16, 18). For both IVF and SCNT, embryos of quality grade 1 were transferred to synchronized recipients after 7 days of culture.

The experiments were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction, and with the European Convention on Animal Experimentation. Research work on cloned animals was approved by the INRA ethics committee (Ethical and Precaution Committee for Agronomical Research Application) in December 1999 as well as by the Comité Scientifique des Utilisateurs (IACUC) of INRA.

Collection of placentomes and RNA isolation. Several of the placentome samples used in the present study were included in the morphometric analysis as described by Constant and coworkers (18), and all samples were collected with the same protocol. The placentome sampling procedure produced relatively consistent sampling of fetal and maternal tissues (~42% fetal, 58% maternal in AI samples and ~47% fetal, 53% maternal in SCNT samples). Briefly, one placentome from the pregnant uterine horn was sampled (1–2 g), using care to harvest from the same area for all placentome samples. The tissue sample was flash frozen in liquid nitrogen and stored at −80°C until RNA extraction. Calves were classified as normal or hydrops, based on the absence or presence of edema in the animal and/or placental tissues. A more detailed description of the diagnosis of hydrops can be found elsewhere (14, 18). Preterm calves were between day 174 and day 260 of gestation; term calves were delivered at 260 days or later. RNA was isolated from the placentome samples with TRIzol reagent (Invitrogen, Carlsbad, CA). Quality and integrity of RNA were assessed by spectrophotometry and denaturing agarose gel electrophoresis. The resulting RNA was resuspended in double-distilled H_2O with 20 mM DTT and 40 U of RNasin (Invitrogen) and stored at −80°C until use.

Microarrays, RNA labeling, and hybridization. A 13,257-element bovine oligoarray [NCBI Gene Expression Omnibus (GEO): GPL2853] was used for transcript profiling (61). The oligonucleotides were selected from expressed sequence tags (ESTs) obtained from a placenta and spleen cDNA library (30) and cDNA libraries created from a mix of a day 36 fetus, a day 64 fetus, and extraembryonic tissues from days 14–25 (NCBI Libraries 15575, 15980, 15992, 15993, and 17811) and from endometrial tissues (NCBI Library 16609). The annotation for each selected sequence was updated with human UniGene (build 201), mouse UniGene (build 162), and bovine UniGene (build 83) databases. Ten micrograms of sample and reference RNA was used to make aminoallyl-labeled cDNA followed by incorporation of Cy3-ester or Cy5-ester (Amersham, Piscataway, NJ) essentially as described previously (60). The samples were then combined, denatured, and cohybridized on the 13,257 oligoarray at 42°C for 40 h. After stringency washes to remove unbound cDNA, the oligoarrays were scanned with an Axon 4000B scanning densitometer (Molecular Devices, Sunnyvale, CA) and images were processed with GenePix 4.0 software (Molecular Devices). Scanning normalization of the data was accomplished by using the automated global normalization feature of GenePix 6.0. Microarray data are MIAME compliant and deposited in the NCBI GEO database (**www.ncbi.nlm.nih.gov/geo**; accession number GSE8923).

Data analysis. First the local background intensity was subtracted from the foreground (signal) intensity, and only spots with median signal intensity greater than the median intensity of the negative control spots for either one of the dyes (Cy5 or Cy3) were considered "expressed." For each dye, signal intensities for spots with median signal intensity lower than the median background intensity for that spot were set to 1. The resulting output files were loaded into GeneSpring 7.3.1, and the data for hierarchical clustering were normalized with LOESS regression.

A mixed-effects model using SAS software's (73) Proc MIXED (86), employing the aforementioned median negative control spot cutoff strategy, was used to determine gene expression differences. First, $\log_2$-transformed ratios of signal intensity for placentome divided by the reference were normalized for dye and array effects with LOESS regression and array centering. Then a first-stage model fit the normalized $\log_2$-transformed ratio across all genes for the effects of dye and array. The residuals were then analyzed by gene in a second-stage model that included the fixed effects of placentome type (AI, SCNT, or IVF), duration of pregnancy, breed of fetus, breed of dam, phenotype, sex, and dye and the random effect of dye and animal. Cell line (used for SCNT) effects were also tested in preliminary analysis but were removed from the final model because the effects were confounded by sample size. Statistically significant P values for the models were adjusted for multiple comparisons with the false discovery rate (FDR) approach (4). For all analyses, an FDR-adjusted P value <0.3, corresponding to a raw P value of $\sim 10^{-3}$–10^{-4}, and a minimum of 18 degrees of freedom were used as a significance threshold. Unless otherwise noted, ratios used in this paper are the adjusted ratios as derived from the statistical model. To facilitate data mining, MetaCore (GeneGO, **http://trials.genego.com**; version 4.3, build 9787) and Ingenuity Pathway Analysis (IPA; **http://www.ingenuity.com**; release 5.1, 7/8/2007) were used to analyze the DEG and to identify significant Bio Functions and canonical pathways associated with specific lists of DEG. Only IPA Bio Functions having $P < 0.05$ and at least five DEG were used for further analysis. For the analysis of canonical pathways in IPA, only pathways with $P \leq 0.05$ and having three or more DEG were considered as biologically relevant.

Quantitative PCR. Primers were designed to specifically amplify 21 target genes and an endogenous control gene, beta-actin (*ACTB*), with Primer Express Software v2.0 (Applied Biosystems, Foster City, CA). The sequences, product sizes, and accession numbers are listed in Supplemental Table S19.[1] Purification of total RNA and RT-PCR

[1] The online version of this article contains supplemental material.

were carried out as described elsewhere (60). cDNA synthesis was performed in duplicate. Quantitative PCR (qPCR) was done with an ABI Prism 7900HT SDS instrument as described previously (60). Relative quantification of mRNA amounts of the target genes was calculated according to the relative standard curve method (60). Duplicate data were pooled, and the mixed model as described above was run on the qPCR values to assess the differential expression. Genes were considered differentially expressed if the mixed model *P* value was ≤ 0.05.

RESULTS

Overview of analysis. Gene expression profiles of placentomes collected from AI ($n = 9$), IVF ($n = 9$), and SCNT ($n = 20$) pregnancies were obtained by hybridization of placentome cDNAs to oligonucleotide microarrays specific for ~13,000 unique cattle genes. A reference design with dye swap was used for the experiment (76 microarrays total). A total of 12,787 oligonucleotide probes gave signals above the threshold level for expression. Patterns in gene expression among the samples were analyzed by hierarchical clustering of normalized ratios, and multivariate analysis of variance (MANOVA) was used to detect differences in the expression of individual genes according to placentome source (AI, IVF, and SCNT), taking into account the effects of sex of fetus, length of pregnancy, breed of dam, breed of fetus, and fetal pathology. The MANOVA detected 1,747 DEG attributable to all variables in the model. A FDR of 0.30 was used for all contrasts to maximize the sensitivity to detect gene function categories, pathways, and networks affected by each of the variables. qPCR was used to confirm the microarray results and to provide additional support for the functional analysis of DEG. The results of cluster analysis, MANOVA, qPCR, and functional analysis of DEG are summarized below.

Cluster analysis reveals distinct groupings on the basis of placentome source. Hierarchical clustering of the normalized expression ratios of all 12,787 expressed genes relative to the reference sample revealed four major groupings (Fig. 1). The correlation between all sample clusters is 0.62, demonstrating a high degree of similarity in expression profiles among the samples (Fig. 1). Within the four major clusters identified, correlation ranged from 0.70 to 0.86. *Cluster I* contains four of five term AI samples, all of which were phenotypically normal. *Cluster II* contains six of eight preterm samples (2 SCNT, 3 AI, and 1 IVF). This cluster also contains four of six hydrops samples. *Cluster III*, the largest, contains most of the phenotypically normal SCNT (7 of 11) and IVF (6 of 8) samples. *Cluster IV*, the smallest, contains three phenotypically normal SCNT samples and the remaining two AI samples, of which one was preterm. There were two SCNT samples, one phenotypically normal and one preterm with hydrops, that clustered with each other but independently of all other samples. The attributes of the samples within the clusters suggest that placentome source, duration of pregnancy, and fetal pathology account for most of the variation in gene expression. However, the compound groupings indicate that multiple sources of variation contribute to the general patterns in gene expression, thus indicating the importance of MANOVA for discerning the effects of each variable on gene expression among the placentome samples.

Effect of AI, IVF, and SCNT (source) on gene expression in placentomes. To exclude the possibility of biased tissue sampling, we first examined a subset of genes present on the

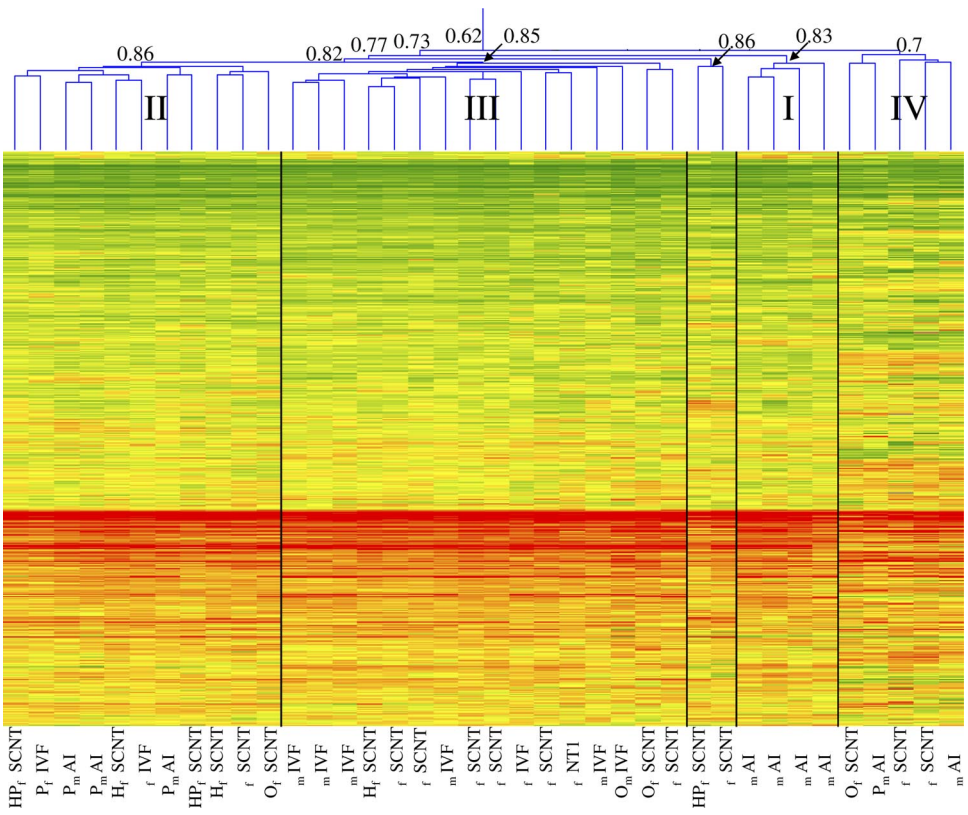

Fig. 1. Cluster diagram of all 38 gene expression profiles based on 12,787 expressed genes. Each column represents the combined data of 2 arrays for an animal, whereas each line represents a specific gene. The clusters are indicated with Roman numerals (as discussed in the text). Pearson correlations (*r*) for major branches are shown. Red indicates high expression in the placentome samples, whereas green indicates low expression in the placentome samples compared with the reference sample. Sample source: artificial insemination (AI), in vitro fertilization (IVF), and somatic cell nuclear transfer (SCNT). Sex: m, male; f, female. All samples are normal term placentomes unless noted otherwise with the following symbols: P, preterm; H, hydrops; O, other phenotype.

microarray and known to be expressed exclusively in fetal derived ($n = 15$ genes) or maternal derived ($n = 3$ genes) placental tissue (76). Correlations of expression ratios for known maternal and fetal expressed genes both within the three sample sources ($r > 0.88$) and between sample sources ($r > 0.88$) show that the relative proportion of maternal and fetal tissues in the placentome samples was generally consistent (Supplemental Table S1). Thus tissue sampling was unlikely to be a significant source of variation in gene expression.

Among the 1,747 DEG detected by MANOVA, 956 were attributed to sample source (AI, IVF, and SCNT; Table 1, Fig. 2; Supplemental Tables S2–S4). For the three pairwise group comparisons of DEG (AI-SCNT, AI-IVF, and IVF-SCNT), the largest number of differences were detected between SCNT and IVF ($n = 733$; 162 >2-fold), followed by SCNT and AI ($n = 336$; 276 >2-fold). Expression differences for relatively few genes ($n = 66$) were found for the AI-IVF comparison. A Venn diagram of DEG for each of the pairwise group comparisons permitted the identification of DEG unique and overlapping for each comparison (Fig. 2). The comparison with the largest number of unique DEG was SCNT-IVF ($n = 593$), followed by SCNT-AI ($n = 186$), and AI-IVF ($n = 22$). These results demonstrate that the SCNT samples are the outlier in their gene expression profile. Only four genes (*BCKDHB*, *ECT2*, *KIAA1333*, *WDR61*) were differentially expressed in all three pairwise comparisons. These four genes were expressed at the lowest level in SCNT and highest in AI placentomes. Among the 37 genes putatively imprinted in cattle that are represented on the microarray, the expression levels of *CPA4*, *COMMD1*, and *GRB10* were affected by placentome source; all were in comparisons involving IVF samples (Supplemental Tables S3 and S4).

An analysis of the distribution of DEG in IPA Bio Function categories was performed to gain insight into the cellular functions, biochemical processes, and disease-related genes that were affected by placentome source (Supplemental Tables S5–S7). All frequency comparisons were based on the number of genes associated to each IPA term for the genes on the microarray (not the total number of genes associated to each term in the Ingenuity Pathway Knowledge Database). A comparison of the 336 DEG in the SCNT-AI comparison revealed significant associations ($P < 0.05$) for 40 terms having ≥5 genes, including *DNA replication*, *recombination and repair*, *cell cycle*, *cell death*, *RNA posttranslational modification*, *gene expression*, *protein trafficking*, *cell-to-cell signaling and interaction*, *molecular transport*, *cancer*, *cell morphology*, *connective tissue development*, *cellular growth and proliferation*, and *reproductive system disease* (Table 2, Supplemental Table S5). Canonical pathways affected in the SCNT-AI comparison include *oxidative phosphorylation* (7 genes), *cell cycle: G_2/M DNA damage checkpoint regulation* (3 genes), and *purine metabolism* (11 genes) (Supplemental Table S6).

Table 1. *Results of MANOVA*

Variable	Terms	No. Genes (FDR $P < 0.3$)*	No. >2-Fold Different
Placentome source	AI, IVF, SCNT	956	420
AI vs. SCNT		336	276
AI vs. IVF		66	50
IVF vs. SCNT		733	162
Duration of pregnancy	Term, preterm	873	112
Breed of dam	HF, CH, NM, C	76	35
Breed of fetus	HF, CH	1	1
Phenotype	Hydrops, normal	17	7
Sex	Male, female	83	38

MANOVA, multivariate analysis of variance; FDR, false discovery rate; AI, artificial insemination; IVF, in vitro fertilization; SCNT, somatic cell nuclear transfer; HF, Holstein-Friesian; CH, Charolais; NM, Normande; C, crossbred. *No. of differentially expressed genes (DEG) exceeds 1,747 because of overlap between categories.

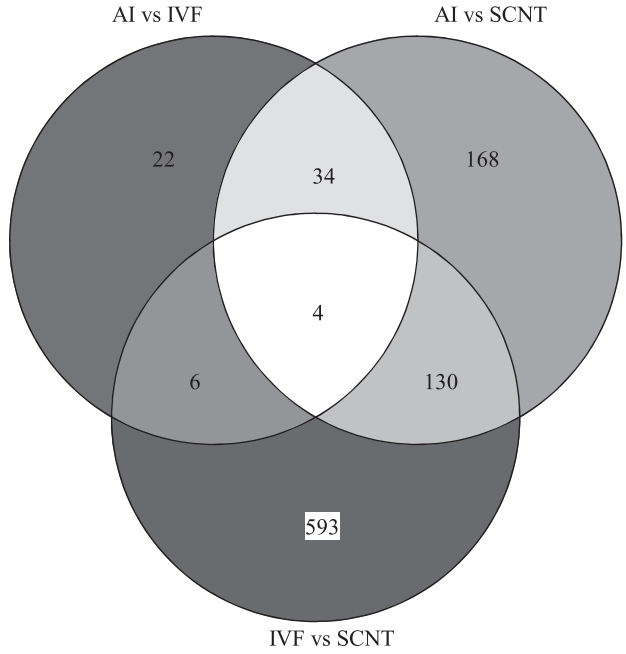

Fig. 2. Overlap of the differentially expressed genes (DEG) for the 3 placentome sources. Each circle represents a pairwise comparison of DEG from among 12,787 expressed genes.

For the SCNT-IVF comparison, 57 IPA Bio Function terms with five or more genes showed significant associations ($P < 0.05$; Supplemental Table S7). There is overlap of 35 of 40 terms with the SCNT-AI list and 22 terms that are unique to the SCNT-IVF comparison. Additional terms include several associated with metabolism (e.g., *lipid metabolism*, *carbohydrate metabolism*, *amino acid metabolism*), with growth, tissue and organ development, and many different diseases. Ten canonical pathways with three or more genes were significantly affected, including *hypoxia signaling in the cardiovascular system* (11 genes), *oxidative phosphorylation* (12 genes), *purine metabolism* (21 genes), *PI3/AKT signaling* (13 genes), and *TGF-β signaling* (11 genes) (Supplemental Table S8).

In contrast to the two pairwise comparisons involving SCNT samples, comparison of AI to IVF samples identified only four significantly affected IPA Bio Function/disease categories: *cancer*, *carbohydrate metabolism*, *small molecule biochemistry*, and *cellular growth and proliferation* (Supplemental Table S9). The only canonical pathway with three or more DEG affected was *oxidative phosphorylation* ($P < 0.05$; Supplemental Table S10). The main effects of IVF on placentome gene expression thus appear to be on cellular metabolism.

Effect of hydrops on gene expression in placentomes. Among the SCNT pregnancies, 30% (6 of 18) of the fetuses showed evidence of hydrops (all hydrallantois). By MANOVA,

Table 2. *Most significant ($P < 0.01$) IPA Bio Function categories and DEG within categories for the comparison of AI and SCNT placentomes*

IPA Function	No. Genes Up	No. Genes Down	Genes Upregulated (log_2 ratio)	Genes Downregulated (log_2 ratio)
Cell Cycle	46	5	*DLG7*(3.2); *NCAPG*(4.0); *RAN*(2.2); *SMC4*(1.9); *CCNB2*(1.4); *HES1*(0.8); *SET*(2.0); *SMC1A*(1.6); *METAP2*(2.0); *GSPT1*(1.9); *BMI1*(1.7); *CENPE*(2.5); *CSNK2A1*(0.8); *SUGT1*(0.8); *DNAJA2*(1.4); *ECT2*(2.9); *SMC3*(1.1); *TOPBP1*(1.6); *CKAP2*(2.0); *NASP*(3.4); *GNL3*(1.9); *EBNA1BP2*(1.2); *NPM1*(2.0); *TMPO*(1.5); *SOCS5*(1.9); *TRIM33*(2.2); *KPNA2*(1.5); *PFDN1*(1.4); *RAD51*(2.6); *CCNA2*(2.2); *NEK2*(2.1); *CREB1*(1.3); *TFDP2*(2.0); *RAD21*(1.4); *CCNB1*(2.2); *KHDRBS1*(1.3); *RACGAP1*(2.1); *TUBG1*(1.1); *MAPK9*(1.5); *RPL23*(2.3); *CDC2*(2.7); *BUB1*(2.4); *MAD2L1*(2.3); *PES1*(0.9); *RPL5*(2.4); *CENPH*(1.9)	*EME1*(−1.2); *NEK6*(−1.2); *CABLES1*(−1.2); *FAS*(−1.0); *RASSF2*(−1.9)
Cellular Assembly and Organization	25	3	*DLG7*(3.2); *NCAPG*(4.0); *SMC4*(1.9); *RAN*(2.2); *CCNB2*(1.4); *SET*(2.0); *METAP2*(2.0); *SMC1A*(1.6); *RAD51*(2.6); *CASP6*(1.8); *CCNA2*(2.2); *NEK2*(2.1); *CENPE*(2.5); *VAMP3*(0.8); *ECT2*(2.9); *CCNB1*(2.2); *TUBG1*(1.1); *CDC2*(2.7); *BUB1*(2.4); *MAD2L1*(2.3); *PES1*(0.9); *HELLS*(3.2); *EBNA1BP2*(1.2); *TMPO*(1.5); *NPM1*(2.0);	*LIPE*(−1.2); *FAS*(−1.0); *NEK6*(−1.2)
DNA Replication, Recombination, and Repair	28	3	*DLG7*(3.2); *KPNA2*(1.5); *NCAPG*(4.0); *SMC4*(1.9); *RAN*(2.2); *CCNB2*(1.4); *SET*(2.0); *DFFA*(1.6); *METAP2*(2.0); *SMC1A*(1.6); *RAD51*(2.6); *CASP6*(1.8); *CCNA2*(2.2); *NEK2*(2.1); *CENPE*(2.5); *ECT2*(2.9); *RAD21*(1.4); *DUT*(2.4); *CCNB1*(2.2); *ENTPD1*(2.9); *ASB2*(1.1); *TOPBP1*(1.6); *MAPK9*(1.5); *BUB1*(2.4); *MAD2L1*(2.3); *NASP*(3.4); *EBNA1BP2*(1.2); *TMPO*(1.5)	*EME1*(−1.2); *FAS*(−1.0); *NEK6*(−1.2)
Cancer	32	6	*TRIM33*(2.2); *DLG7*(3.2); *KPNA2*(1.5); *HES1*(0.8); *ODC1*(1.9); *CCNA2*(2.2); *BMI1*(1.7); *CENPE*(2.5); *CREB1*(1.3); *CSNK2A1*(0.8); *TFDP2*(2.0); *SEC63*(1.0); *ECT2*(2.9); *TMEM97*(1.6); *RAD21*(1.4); *CCNB1*(2.2); *ASB2*(1.1); *RACGAP1*(2.1); *TUBG1*(1.1); *MAPK9*(1.5); *CKAP2*(2.0); *RPL23A*(1.0); *RPL23*(2.3); *USP1*(1.9); *CDC2*(2.7); *GNAI3*(1.0); *MAD2L1*(2.3); *NASP*(3.4); *RPL5*(2.4); *NPM1*(2.0); *CENPH*(1.9); *RP6-213H19.1*(2.4)	*IL12A*(−1.3); *LMO2*(−0.8); *FAS*(−1.0); *CABLES1*(−1.2); *NME3*(−0.6); *PTPN21*(−1.1)
Reproductive System Disease	15	3	*CYP11A1*(2.4); *DLG7*(3.2); *KPNA2*(1.5); *RPL23A*(1.0); *CDC2*(2.7); *MAD2L1*(2.3); *CCNA2*(2.2); *NASP*(3.4); *CENPE*(2.5); *CSNK2A1*(0.8); *NPM1*(2.0); *ECT2*(2.9); *SERBP1*(1.6); *RAD21*(1.4); *RP6-213H19.1*(2.4)	*IL12A*(−1.3); *CABLES1*(−1.2); *FAS*(−1.0)
RNA Posttranscriptional Modification	7		*CCNB1*(2.2); *KHDRBS1*(1.3); *TCERG1*(1.1); *EBNA1BP2*(1.2); *NPM1*(2.0); *DIS3*(1.4); *CDC2*(2.7)	
Cellular Compromise	10	2	*BUB1*(2.4); *MAD2L1*(2.3); *CASP6*(1.8); *PLK4*(1.6); *CCNB1*(2.2); *NEK2*(2.1); *CENPE*(2.5); *ECT2*(2.9); *DFFA*(1.6); *CDC2*(2.7);	*EME1*(−1.2); *FAS*(−1.0)
Gene Expression	7	2	*CYP11A1*(2.4); *GTF2B*(2.3); *CCNB1*(2.2); *KHDRBS1*(1.3); *NCAPG*(4.0); *CREB1*(1.3); *CDC2*(2.7)	*ZHX2*(−1.2); *MEF2D*(−1.1)
Gastrointestinal Disease	11	1	*GNAI3*(1.0); *MAD2L1*(2.3); *CREB1*(1.3); *CKAP2*(2.0); *HES1*(0.8); *USP1*(1.9); *NPM1*(2.0); *TFDP2*(2.0); *SEC63*(1.0); *CENPH*(1.9); *TMEM97*(1.6)	*PTPN21*(−1.1)
Cell Morphology	8	2	*MAD2L1*(2.3); *CASP6*(1.8); *NEK2*(2.1); *RACGAP1*(2.1); *CDC42SE2*(1.5); *HES1*(0.8); *TMPO*(1.5); *ECT2*(2.9)	*MCAM*(−1.6); *FAS*(−1.0)
Connective Tissue Development and Function	11	5	*SMC3*(1.1); *KHDRBS1*(1.3); *ANP32A*(0.9); *ODC1*(1.9); *PLAG1*(1.6); *METAP2*(2.0); *MAD2L1*(2.3); *BMI1*(1.7); *CDC42SE2*(1.5); *NPM1*(2.0); *C1GALT1*(2.2)	*CES2*(−1.1); *ADRB3*(−1.2); *FAS*(−1.0); *MCAM*(−1.6); *RASGRP2*(−0.6)
Molecular Transport	10	6	*SLC35A1*(1.4); *KHDRBS1*(1.3); *KPNA2*(1.5); *RAN*(2.2); *ANP32A*(0.9); *ODC1*(1.9); *RAD51*(2.6); *PARK7*(1.0); *NPM1*(2.0); *RNUXA*(1.9)	*CHGA*(−1.6); *PPP1R3C*(−1.5); *G6PD*(−0.7); *SLC22A6*(−0.9); *FAS*(−1.0); *GBA*(−1.0)
Protein Trafficking	4	2	*PPIH*(1.8); *KPNA2*(1.5); *RAN*(2.2); *SEC63*(1.0)	*PPP1R3C*(−1.5); *FOLR2*(−1.1)
Cellular Growth and Proliferation	11	3	*DLG7*(3.2); *SMC3*(1.1); *KHDRBS1*(1.3); *RPL23A*(1.0); *HES1*(0.8); *ANP32A*(0.9); *ODC1(1.9); PLAG1*(1.6); *PLK4*(1.6); *NPM1*(2.0); *RP6-213H19.1*(2.4)	*CABLES1*(−1.2); *CES2*(−1.1); *RASGRP2*(−0.6)

Continued

Table 2.—*Continued*

IPA Function	No. Genes Up	No. Genes Down	Genes Upregulated (log_2 ratio)	Genes Downregulated (log_2 ratio)
Amino Acid Metabolism	13	5	*CCNB1*(2.2); *HAT1*(2.7); *MAPK9*(1.5); *COL4A3BP*(2.6); *CDC2*(2.7); *DYRK3*(1.6); *SRP72*(1.2); *VRK1*(1.9); *PLK4*(1.6); *ASRGL1*(1.3); *CSNK2A1*(0.8); *SGK3*(2.7); *RP6-213H19.1*(2.4)	*LIPE*(−1.2); *GBA*(−1.0); *NEK6*(−1.2); *PTPN21*(−1.1); *PDK4*(−3.7)
Posttranslational Modification	17	5	*HUWE1*(2.7); *CCNB1*(2.2); *HAT1*(2.7); *UBE2D2*(1.2); *MAPK9*(1.5); *COL4A3BP*(2.6); *SET*(2.0); *CDC2*(2.7); *DYRK3*(1.6); *SRP72*(1.2); *VRK1*(1.9); *CASP6*(1.8); *PLK4*(1.6); *BMI1*(1.7); *CSNK2A1*(0.8); *SGK3*(2.7); *RP6-213H19.1*(2.4)	*LIPE*(−1.2); *NEK6*(−1.2); *IMMP2L*(−1.1); *PTPN21*(−1.1); *PDK4*(−3.7)
Small Molecule Biochemistry	22	14	*SQLE*(2.4); *SLC35A1*(1.4); *ODC1*(1.9); *DYRK3*(1.6); *VRK1*(1.9); *SRP72*(1.2); *PLK4*(1.6); *PARK7*(1.0); *CSNK2A1*(0.8); *OXCT1*(1.7); *CYP11A1*(2.4); *CCNB1*(2.2); *ENTPD1*(2.9); *HAT1*(2.7); *MAPK9*(1.5); *COL4A3BP*(2.6); *CDC2*(2.7); *PPA1*(1.6); *GNAI3*(1.0); *APLN*(2.3); *SGK3*(2.7); *RP6-213H19.1*(2.4)	*PPP1R3C*(−1.5); *LIPE*(−1.2); *CES2*(−1.1); *ADRB3*(−1.2); *FAS*(−1.0); *GBA*(−1.0); *NEK6*(−1.2); *PDK4*(−3.7); *CHGA*(−1.6); *G6PD*(−0.7); *CMAH*(−1.2); *EMR2*(−1.1); *CHIA*(−1.5); *PTPN21*(−1.1)
Cell-to-Cell Signaling and Interaction	6	2	*SMC3*(1.1); *HES1*(0.8); *C1GALT1*(2.2); *ANP32A*(0.9); *METAP2*(2.0); *PLAG1*(1.6)	*EMR2*(−1.1); *FAS*(−1.0)
Cellular Function and Maintenance	13	3	*SMC3*(1.1); *TRIM33*(2.2); *ASB2*(1.1); *RACGAP1*(2.1); *ANP32A*(0.9); *CDC2*(2.7); *PLAG1*(1.6); *METAP2*(2.0); *GNAI3*(1.0); *NEK2*(2.1); *CSNK2A1*(0.8); *VAMP3*(0.8); *NPM1*(2.0)	*LIPE*(−1.2); *FAS*(−1.0); *NME3*(−0.6);
Skeletal and Muscular Disorders	7		*CCNA2*(2.2); *RPL5*(2.4); *RPL23*(2.3); *NPM1*(2.0); *PAPSS2*(2.2); *CDC2*(2.7); *LBR*(1.9);	
Lipid Metabolism	4	4	*CYP11A1*(2.4); *GNAI3*(1.0); *BDH2*(1.2); *OXCT1*(1.7)	*GBA*(−1.0); *LIPE*(−1.2); *ADRB3*(−1.2); *FAS*(−1.0)

Ratios are given as log_2 fold changes of AI compared with SCNT samples. IPA, Ingenuity Pathway Analysis.

17 DEG associated with hydrops were detected (Table 3). Although the small number of DEG precluded a robust analysis of functions affected specifically by hydrops, functional annotations for 14 of 17 DEG were available. These include the downregulated genes (>3-fold in hydrops) *GREB1*, an estrogen-responsive gene involved in control of cell proliferation, and *TCRG*, which encodes the gamma chain of the T-cell receptor for antigen. Genes that were overexpressed (>2-fold) in hydrops relative to normal placentomes included *GRSF1*, which encodes a G-rich RNA sequence binding factor-associated morphogenesis of embryonic epithelium, *KLF11*, which encodes a zinc-finger protein involved in negative regulation of cell growth and proliferation, and *TXNDC12* (confirmed by qPCR, Table 4), a thioredoxin domain-containing protein involved in defense against oxidative stress. Notably, the transcript encoding *PRKAR2A,* a regulatory α-subunit of a cAMP-dependent protein kinase, was also upregulated. *PRKAR2A* is known to undergo dramatic changes in expression in human myometrium during gestation and parturition (62). Among the 17 hydrops-associated DEG, 16 (94%) overlapped with DEG identified in the AI-SCNT comparison, thus indicating relatedness in the placentome gene expression patterns attributable to both SCNT and hydrops.

Table 3. *Genes differentially expressed in hydrops and normal SCNT placentomes (FDR $P < 0.30$)*

Gene Symbol	Ratio	NCBI Gene ID
ABLIM2	0.6	84448
ANKRD29	1.5	147463
COL4A3BP	2.5	10087
GREB1	0.3	9687
GRSF1	2.5	2926
JOSD1	2.0	9929
KLF11	2.1	8462
LOC614899	0.7	614899
PRKAR2A	1.7	19087
TCRG	0.3	6965
TMPIT	2.5	83862
TXNDC12	2.5	51060
USP6NL	1.9	9712
OLIGO_00241*	0.7	Bt.33247
OLIGO_01814*	1.4	Hs.661988
OLIGO_04290*	0.7	None
OLIGO_09660*	0.6	Bt.45678

Ratios are given as hydrops over normal samples. *Oligo IDs are given for DEG without any gene annotation.

Effect of pregnancy duration (term, preterm) on placentome gene expression. Among the variables in the MANOVA, "duration of pregnancy" had the largest effect on gene expression other than placentome source, with 873 genes differentially expressed, of which 112 were more than twofold different (Supplemental Table S11). The majority of genes that were differentially expressed were upregulated in term compared with preterm samples. Among the genes with the largest differences in expression were *PTGS2* (20-fold increase), *IGFBP1* (10-fold increase), *CYP19A1* (4-fold increase), *SOD2* (4-fold

Table 4. *Results of confirmatory qPCR on selected genes*

Gene	GenBank ID	Comparison	Microarray Fold Change*	TaqMan Fold Change†
APRIN	BF043498	AI vs. SCNT	2.6	3.3
C6ORF204	BM365636	Preterm vs. term	0.4	0.2
CASP6	BF040727	AI vs. SCNT	3.5	4.0
CDC2	L26547	AI vs. SCNT	6.0	13.7
CDC2	L26547	IVF vs. SCNT	2.2	5.6
CDC2	L26547	Hydrops vs. normal	1.8	2.6
CSH1	AW465564	AI vs. SCNT	9.9	2.3‡
CSH1	AW465564	Preterm vs. term	0.3	0.6
CSPG6	BF040906	AI vs. SCNT	2.2	4.2
CYP19A1	BF041489	Preterm vs. term	0.2	0.2
EBAG9	BF042900	AI vs. SCNT	2.4	5.7
FAS	U34794	AI vs. SCNT	0.5	2.0§
GAPVD1	AW465643	AI vs. SCNT	3.8	6.0
GAPVD1	AW465643	AI vs. IVF	2.7	2.4
GLUL	AW465870	Preterm vs. term	0.5	0.5
HAT1	AW464112	AI vs. SCNT	6.5	12.4
HES1	AW465398	AI vs. SCNT	1.7	3.5
LBR	CN440990	AI vs. SCNT	3.8	6.3
NCAPG	CN435656	AI vs. SCNT	16.4	104.1
NCAPG	CN435656	IVF vs. SCNT	3.1	10.8
PDPN	AW461783	Preterm vs. term	0.4	0.4
PTGS2	AW462092	Preterm vs. term	0.1	0.1
RAD51	CR452998	AI vs. SCNT	6.3	18.1
RAD51	CR452998	IVF vs. SCNT	2.0	4.7
SLC30A4	BF040441	Preterm vs. term	0.3	0.5
TXNDC12	BF046280	Hydrops vs. normal	2.5	1.6
XLKD1	BF041814	Preterm vs. term	0.5	0.4

qPCR, quantitative PCR. *Gene significant at FDR $P < 0.3$ on microarray; †gene significant at $P < 0.05$ by qPCR unless otherwise noted; ‡gene significant at $P < 0.10$ by qPCR. The statistical model was the same for qPCR and microarray analyses, §not significant.

increase), *CSH1* (*PL* in cattle; 3-fold increase), *TCRG* (6-fold decrease), *CYP4F2* (3-fold decrease), *CD19* (2-fold decrease), *HGF* (2-fold decrease), and *SLC1A6* (2-fold decrease). A very large number of IPA Bio Function terms ($n = 63$; Supplemental Table S12) were significantly associated to genes affected by duration of pregnancy, including (in order of highest significance, all $P < 0.001$) *cell death*, *cell cycle*, *connective tissue development*, *cell signaling*, and *embryonic development*. Canonical pathways affected included *cell cycle: G2/M DNA damage checkpoint regulation* and several related to signaling (*neutrophin/Trk signaling*, *SAPK/JNK signaling*, *FGF signaling*, and *integrin signaling*) and metabolism (*sulfur*, *nitrogen*, and *inositol phosphate metabolism*) (Supplemental Table S13). These results indicate major effects on functions related to cellular stress responses. The levels of expression of five imprinted genes, *CPA4*, *GNAS*, *KCNQ1*, *MEG3*, and *NAP1L4*, were also affected by duration of pregnancy. *CPA4* and *GNAS* were expressed at a higher level, whereas *KCNQ1*, *MEG3*, and *NAP1L4* were expressed at a lower level in term placentomes.

Effect of fetal sex on placentome gene expression. Sex of the fetus had a significant effect on placentome gene expression (Supplemental Table S14). Among the 83 sex-related DEG, 9 were expressed at higher levels in male-bearing pregnancies, the remainder being expressed at higher levels in female-bearing pregnancies. The IPA terms significantly associated with the DEG included *cell cycle*, *cellular assembly and organization*, and *cell morphology* (Supplemental Table S15). No canonical pathways with three or more DEG were significantly affected by sex of the offspring.

qPCR analysis of selected genes. Twenty-one genes were picked to confirm the differences in gene expression detected with microarrays. The genes were picked on the basis of differences in placentome gene expression that were attributed to placentome source, hydrops, or duration of pregnancy. A total of 26 comparisons were made for the 21 genes; only one gene (*FAS*; AI-SCNT comparison) was not confirmed by qPCR (Table 4).

DISCUSSION

The gene expression profiles of AI, IVF, and SCNT placental biopsies were compared to elucidate the genes, pathways, and networks that produce placental defects, hydrops, and LOS. Furthermore, we aimed to gain insights into the genes and pathways that are affected by early reprogramming errors that occur during the SCNT process. Adjusting for all effects, the model detected 1,747 DEG (Table 1), of which most were attributed to placentome source and duration of pregnancy (term or preterm). By analyzing the expression of several genes reported to be expressed primarily in fetal or maternal tissue (76), we excluded the possibility that gene expression differences between AI, IVF, and SCNT placentomes were due to biased tissue sampling. qPCR was used to confirm key genes in specific pathways in order to substantiate conclusions drawn from the analysis. Expression differences were confirmed for 20 of 21 selected genes in 25 of 26 comparisons tested (Table 4).

Genes and pathways affected by in vitro maturation, fertilization, and culture: relation to LOS. The IVF procedure in cattle involves in vitro oocyte maturation and fertilization, embryo culture to the blastocyst stage (typically 7 days), and transfer to a synchronized recipient in diestrus. Similarly, SCNT involves all of the above except for IVF. Recently a number of abnormalities similar to LOS have been associated with mouse and human IVF, such as increased anaerobic glycolysis (35), loss of imprinting (22, 25), increased incidence of retinoblastoma (64), Beckwith-Wiedemann syndrome (22), and altered behaviors and spatial memory (26, 33). It has been hypothesized that the embryo culture procedures, particularly the use of serum that contains hormones and growth factors, cause the dysregulation of gene expression and imprinting (81, 91). Furthermore, cattle IVF (unlike that in humans) employs oocyte maturation in vitro, which also appears to have a dramatic effect on gene expression patterns in the developing embryo (74). In the human diseases Beckwith-Wiedemann and Angelman syndromes, both with a phenotype similar to LOS, loss of imprinting appears to play a major role in disease development (23, 38). Using microarray analysis, we (74) and others (36) have shown large numbers of DEG in cultured IVF blastocysts compared with those produced by AI, providing further evidence for a "culture effect" on gene expression in embryos. Thus a central question is to what extent differences in gene expression in cultured embryos are compensatory for the culture environment and result in normal offspring, or whether such compensatory changes may lead to developmental abnormalities.

To address this question, we compared gene expression in IVF and AI placentomes and found 66 DEG, of which 50 (76%) were more than twofold different. Caution should be used in interpreting results on the basis of the smaller number of DEG (as compared with the IVF-SCNT comparison) be-

cause of the reduced statistical power of the comparison resulting from a smaller number of AI ($n = 9$) and IVF ($n = 9$) samples relative to the number of SCNT samples ($n = 20$). Still, cluster analysis showed that IVF placentomes have distinctly different expression profiles relative to AI (Fig. 1). These results suggest a significant and long-lasting effect of the cattle IVF procedure on placentome gene expression patterns. Many DEG are likely due to in vitro embryo culture, while others may be due to oocyte maturation and fertilization in vitro (see further discussion below). In total, 34 DEG common to the AI-IVF and AI-SCNT comparisons are specific for the long-term culture effect associated with IVF and SCNT (Fig. 2; Supplemental Table S16). These 34 DEG have significant functional associations with *cell death* (6 genes), *posttranslational modifications* (5 genes), and *amino acid metabolism* (3 genes). Our data are thus consistent with the hypothesis that LOS observed in IVF and SCNT pregnancies has, at least in part, a common molecular basis.

Analysis of the functional annotations of DEG in common among the IVF-AI and IVF-SCNT comparisons supports and extends previous studies of the effects of embryo culture on metabolism (8, 31). For the AI to IVF comparison, transcripts of genes involved in the *pentose phosphate pathway* (*BCKDHB* and *ALDOC*) and *oxidative phosphorylation* (*PPA1*, *COX6A1*, and *NDUFA4*) were the only canonical pathways affected. *COX6A1*, a nuclear gene that encodes a subunit of cytochrome-*c* oxidase, was expressed at fivefold greater levels in IVF compared with AI placentomes. These results suggest an increase in overall metabolic activity of IVF placentomes, consistent with the data of others (7, 8). The fact that *COX6A1* was among the six DEG in common between the AI-IVF and IVF-SCNT comparisons suggests that differential expression of this gene may be due to the effects of fertilization in vitro. The branched-chain keto acid dehydrogenase E1 beta polypeptide encoded by *BCKDH* was downregulated twofold in IVF compared with AI placentomes. Mutations in *BCKDH* are causal for *Maple Syrup Urine Disease* type 1B (branched-chain α-keto dehydrogenase deficiency) in humans and cattle (24). Interestingly, *BCKDHB* was one of four genes differentially expressed in all comparisons (Fig. 2), which suggests that embryo culture, fertilization in vitro, and SCNT all affect its expression. Finally, the nearly threefold reduced expression of *CPA4*, an imprinted gene that may be involved in histone hyperacetylation, supports the hypothesis that embryo culture affects epigenetic modifications. Thus embryo culture clearly has long-range effects on gene expression and imprinting that are manifested in the core metabolism of placental tissues. These surprising results may have important implications for assisted reproduction in humans and in animals. Moreover, our results supply additional candidate genes and pathways to explain the relatively high frequency of LOS associated with IVF.

Genes, pathways, and networks affected by SCNT. Accumulating evidence points to a failure of complete reprogramming of the donor nucleus as a major reason for the placental defects observed after SCNT (53, 89). In cattle, demethylation begins after nuclear transfer and continues through the four- to eight-cell stage but does not reach the level observed in embryos produced by AI (21, 89). Active remethylation begins at the eight-cell stage and reaches "normal" levels by the blastocyst stage (21). However, in cloned cattle and mouse embryos, methylation patterns are abnormal in the trophectoderm, leading to the hypothesis that imprinting errors contribute to the placental defects observed from the preimplantation period through parturition (55, 89). As additional evidence in support of this hypothesis, morphometric studies clearly point to abnormalities in the formation of the trophectoderm and placenta in SCNT clones (18). These abnormalities may be extreme, especially in cases of hydrops. By comparing transcript profiles of AI, hydrops, and phenotypically normal SCNT placentomes at or near term, our study captured the molecular and biochemical features of the SCNT hydrops placenta and the possible molecular defects leading to its development.

A large fraction of DEG in the AI-SCNT comparison differed by more than twofold in their transcript levels ($n = 275/335$; 82%), thus demonstrating extraordinary long-term effects of SCNT on placentome gene expression. We cannot exclude the possibility that some of these differences are due to differences in embryo culture conditions (e.g., the use of oviduct cells). Although the comparison of IVF to SCNT placentomes revealed the largest number of DEG for any pairwise comparison ($n = 733$, 593 unique to the IVF-SCNT comparison), the fraction more than twofold different was much lower (22%) for the AI-IVF comparison than for the AI-SCNT comparison (82%). Furthermore, 130 DEG in the IVF-SCNT comparison overlap with DEG in the AI-SCNT comparison (SCNT-specific set), and 35 of 40 affected IPA functional categories unique to the AI-SCNT comparison were also found for the IVF-SCNT comparison. The many shared DEG (as discussed above) and overlapping functions among the IVF and SCNT gene lists support the hypothesis that common pathways are affected by the embryo culture methods used for IVF and SCNT. However, SCNT also affects the expression of at least 202 additional genes (Fig. 2) that likely contribute to development of placental abnormalities and pathologies not observed in IVF.

Functional analysis of the large number of DEG in the SCNT-specific set (Fig. 2) is important for understanding the molecular basis for the development of placental abnormalities and hydrops. The most prominent functional effects of SCNT on placentome gene expression involved *cell cycle*, *cell signaling*, *molecular transport*, and *DNA replication, recombination, and repair* (Supplemental Table S17). All of the significantly affected molecular functions and pathways appear to be downregulated in SCNT placentomes. Many of the individual genes whose expression patterns were dramatically altered are known to have important biological effects, i.e.., in *cancer*, *cell morphology*, *connective tissue development*, *cell growth and proliferation*, and *reproductive diseases*. For example, *LBR* (lamin B receptor) is associated with *Hydrops-ectopic calcification-moth-eaten*, an autosomal recessive disease in humans that is characterized by fetal hydrops and short limbs. *LBR* transcript levels were found to be nearly fourfold greater in AI than SCNT placentomes (confirmed by qPCR; Table 4). Several genes associated with *DNA replication* and *cell cycle* were expressed at lower levels in SCNT placentomes, including *NASP* (nuclear autoantigenic sperm protein), a histone binding protein that has a somatic form that is expressed in all mitotic cells. *NASP* was downregulated >10-fold in SCNT placentomes. *RAD51* (RAD51 homolog) was expressed at sixfold greater levels in AI than SCNT placentomes (confirmed by qPCR at 18-fold differential expression; Table 4). In yeast, Rad51 is known to be involved in homologous recombination

and DNA repair. Similarly, *HELLS*, thought to encode a lymphoid-specific helicase, showed >10-fold increase in expression in AI compared with SCNT placentomes. *HCAP-G* (orthologous to human *NCAPG*) was expressed 16-fold greater in AI compared with SCNT placentomes. *NCAPG* is involved in DNA modifications, including direct interaction with DNA methyltransferase, a key enzyme involved in epigenetic modifications. These results suggest that mitotic activity is reduced or there is a loss of mitotic subpopulations of cells in SCNT placentomes. It is noteworthy that in our present comparison of AI and SCNT term placentas we did not find major histocompatibility complex (MHC) class I genes among the DEG. This is consistent with a recent report by Chavatte-Palmer et al. (15), who did not find differential expression of MHC class I genes, as opposed to earlier reports (19, 51). However, the latter studies used samples collected at day 30 to day 60 of pregnancy, whereas Chavatte-Palmer et al. (15) compared day 30 to day 60 and term samples, and our study used only samples collected at term. Thus SCNT effects on MHC class I gene expression remain to be verified.

The paradox between the occurrence of placentomegaly in SCNT and placentome gene expression associated with reduced mitotic activity and increase in apoptosis can be explained because the major period of placental growth occurs before term rather than at term, the time when our samples were collected. This is supported by the observations of Redmer et al. (71), who showed that the major period of placental growth in ruminants is during the first and second trimesters. Furthermore, it was shown that normal growth and functioning of the placenta is dependent on placental vascularization. The gross differences in gene expression patterns we observed in SCNT placentomes, including genes involved in *cell signaling*, *cell assembly and organization*, *cell morphology*, *cell function and maintenance*, and *tissue-specific transcription factors* (Supplemental Table S5), are consistent with the placental abnormalities (i.e., maternal epithelium thinning and mesenchyme overgrowth) observed by morphometric analysis (18). The occurrence of these abnormalities in SCNT placentas is consistent with results demonstrating that normal placental and fetal development is dependent on adequate exchange of gases and nutrients across the placenta during mid- and late gestation (71, 72). Effectively, a large fraction of cloned fetuses may be starved and/or accumulating toxic wastes due to placental insufficiency caused by reprogramming errors, depending on the time of onset of the placental abnormalities. This in turn may lead to embryonic and fetal morbidity and mortality. This hypothesis is supported by the decreased fetal-to-placental weight ratio in SCNT pregnancies, suggesting that placental function is affected (18). Furthermore, work on early fetal growth also showed that SCNT fetuses are growth retarded compared with AI controls in the first 2 mo of pregnancy (14).

If it is true that placental insufficiency results in hydrops and LOS in clones (5), then there should also be a metabolic footprint in SCNT placentomes. In humans, respiratory chain enzyme deficiencies are associated with clinical features similar to those observed in LOS (48, 80). In addition, the biochemical composition of fetal fluids between day 50 and day 180 of IVF, SCNT, and AI pregnancies shows remarkable differences in the levels of glucose, fructose, and lactate in the allantoic fluid. For example, in IVF placentas glucose and fructose levels are higher than in AI placentas, whereas in SCNT placentas lactate and fructose levels are higher than in AI placentas (8, 58, 70). Recently, it was also shown that neonatal SCNT calves have high fructose levels and low glucose and lactate levels in their blood (3). Glucose, fructose, and lactate serve as energy sources for the placenta and fetus (77), and artificial elevation of lactate levels in the fetal plasma in near-term pregnancies results in polyhydramnios and/or hydrops fetalis (70). Under normal conditions maternal glucose enters the fetal circulation through the placenta and under aerobic conditions is reduced to pyruvate. Subsequently, pyruvate enters the TCA cycle and is reduced to CO_2, H_2O, and ATP by oxidative phosphorylation. Data mining revealed that seven genes involved in oxidative phosphorylation (*SDHA*, *NDUFB5*, *COX6C*, *PPA1, UQCRC2*, *ATP5B*, *NDUFA4*) are downregulated in SCNT placentomes. The downregulated genes are distributed over four of the five protein complexes of the oxidative phosphorylation pathway. Therefore, because of the constraints in supplying oxygen and nutrients, the anaerobic pathway may be used more in SCNT placentomes, which would result in pyruvate being reduced to lactate and less ATP production (13). In sheep, asphyxiation of the placenta for 30 min during midterm pregnancy produced phenotypes with similarities to hydrops in cattle (65). On the basis of the DEG found in the SCNT and hydrops comparisons, our data are consistent with the hypothesis that abnormal placental development in clones leads to reduced functioning of the placenta, resulting in less oxygen to the fetus. The compensatory response leading to enlarged placentomes appears to be insufficient to meet the energy demands of the growing fetus and placenta, and may lead to LOS and/or hydrops.

Construction of gene networks from the lists of DEG associated with SCNT (Fig. 3) clearly demonstrated that many DEG with known interactions associated with *cell growth and proliferation*, *gene expression*, and *cell signaling* are downregulated in SCNT placentomes. Hydrops thus appears to be an extreme form of the typical placental abnormalities that are observed in SCNT clones. We propose that hydrops is a manifestation of the extensive dysregulation of gene expression caused by reprogramming errors that occur during the SCNT procedure. This may involve many genes, although the same pathways appear to be consistently affected. In clones that survive to late term, the functional abnormalities may be severe enough to cause a complete breakdown in tissue homeostasis, leading to the hydrops phenotype. What tips the balance toward hydrops is unknown, but it could be related to cumulative errors in tissue function or metabolism, or perhaps other external factors.

The period prior to parturition is associated with induction of a wide array of gene expression differences in placenta (1, 29, 41, 43, 85). This had to be taken into account in our analysis because several of the hydrops cases were preterm deliveries. Between term and preterm placentomes, 873 DEG were found, of which 61 (7%) overlap with the AI-SCNT comparison. Functional analysis of DEG showed that *cell signaling* (182 genes) was a major affected category. The canonical pathway analysis complemented this view, showing mostly signaling-related pathways, such as *IL-6*, *GPCR*, and *integrin signaling*, to be affected. In our study we found differential expression of 12 genes in the IL-6 canonical pathway, which compares favorably to other studies that showed *IL-6* and *NFKBIA* to be overexpressed in human placental tissues from normal term

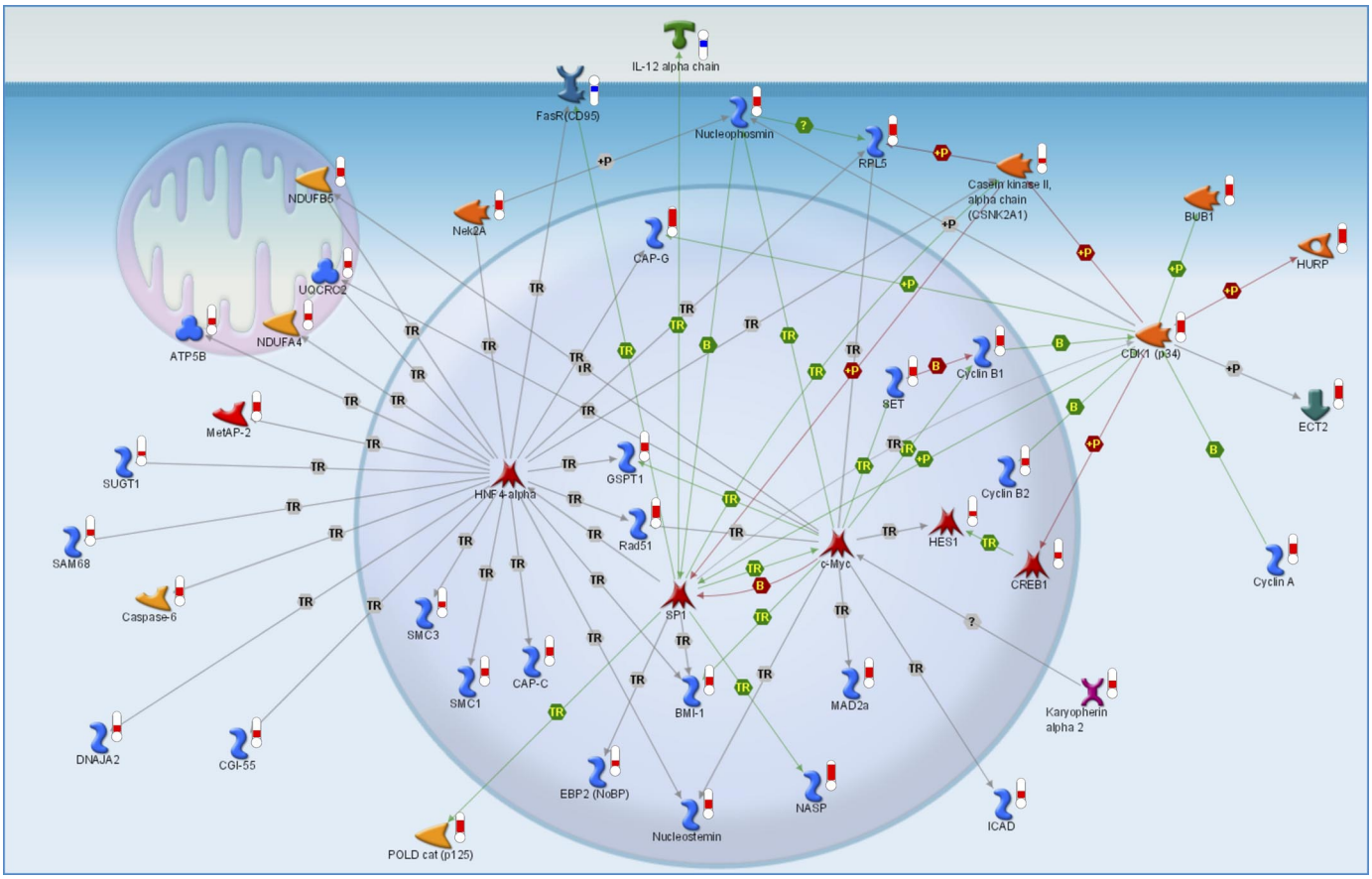

Fig. 3. Interaction network for a subset of DEG identified in the AI-SCNT comparison. Selected DEG are from the Ingenuity Pathway Assist (IPA) Bio Function categories *cell cycle* and *cell death* and the IPA canonical pathway *oxidative phosphorylation*. Subnetworks were identified, merged, and drawn with MetaCore by GeneGO. The "autoexpand" algorithm was employed, stopping the expansion when the subnetworks intersected. The expanded network was uploaded into GeneGO MapEditor, which allows representation of protein localization (nucleus, cytoplasm, mitochondrion, transmembrane, and extracellular). Each transcript in the DEG list is identified by a thermometer symbol. Additional proteins in the network but not represented in the DEG list have only a symbol. Red within thermometers indicates increased gene expression in AI samples, with the height of the bar representing the fold change in expression. Blue within thermometers denotes increased gene expression in SCNT samples, with the height of the bar representing the fold change in expression. Green lines denote positive effect on gene expression, whereas a red line indicates a negative effect. Gray lines denote unknown effects. A complete description of the symbols used can be obtained at **http://ftp.genego.com/files/MC_legend.pdf**.

labor compared with term or preterm and not in labor (28, 39, 87). The gene *CYP19A1*, part of the IL-6 pathway, was increased 4-fold, and expression of *PTGS2* was 20-fold greater in term placentomes compared with preterm placentomes, which is consistent with other studies and with their function in prostaglandin synthesis (41, 83). After 230 days of gestation, prostaglandins are essential for the promotion of myometrial contractions (83).

Another factor accounted for was sex of the calves (23 female and 15 male) because this might contribute to variation in gene expression patterns (88). The majority of the genes were upregulated in females (89%), and most annotated genes were associated with the terms *cell cycle* or *cellular assembly*. One of the genes, *BCL6B*, was approximately twofold upregulated in placentomes from male fetuses. This gene is involved in spermatogonial stem cell renewal in vivo (66). The effects detected for sex were rather large, because a placentome is composed of both maternal and fetal tissue, with less than half of the tissue being of fetal origin. For this reason, the number of genes whose expression is affected by sex is probably underestimated in our study.

Overlap with genes differentially expressed in day 7 embryos. We (74) and others (69) have shown that blastocysts derived by IVF and SCNT exhibit differences in gene expression patterns. It is unknown whether these differences reflect variation that can be tolerated by the developing embryo or primary reprogramming errors that later manifest as developmental anomalies due to failure of redifferentiation (89). A comparison of 142 overlapping DEG with those found by Smith and coworkers (74) for day 7 cattle embryos revealed only seven DEG to be common for the comparison of IVF to SCNT placentomes. Two genes were in common for the comparison of AI to SCNT, but this may be due in part to the different microarray platform (7,872 cDNAs), the different donor cell lines used for SCNT, and/or the manner in which Smith et al. (74) analyzed their data. Reanalysis of the day 7 blastocyst data with the present study's statistical approach (and FDR $P < 0.05$) uncovered 11 and 79 genes common to the IVF-AI and IVF-SCNT comparisons, respectively (data not shown). These results suggest that not all variation in SCNT embryo gene expression is lethal, and that many differences in gene expression observed in SCNT and IVF are maintained from early

embryonic development to term despite the vastly different tissue complexity between embryos and placentomes. Those non-stage-specific genes that are differentially expressed during the zygote to blastocyst stage in SCNT embryos but not at later stages of development may be prime candidates for primary reprogramming errors that lead to early mortality and reduced cloning efficiency.

Summary. Our results provide a detailed snapshot of the underlying genes, pathways, and networks that are responsible for and associated with the placental abnormalities caused by SCNT. An extensive body of experimental evidence indicates that SCNT results in abnormal development of the placenta, which in turn leads to a high frequency of pregnancy failure at different gestational stages (16, 20, 32, 46, 49). In late gestation and term SCNT cattle pregnancies, placentomegaly is highly frequent (~50%) and is characterized by hydrallantois and fetal mortality (18, 57). Live SCNT offspring have increased birth weight, which is correlated with placental abnormalities (18). The results of morphometric analysis were suggested to indicate that the placental abnormalities observed in SCNT result from adaptation(s) to placental dysfunction, and that dysregulation of cellular metabolism and cell signaling are responsible for these underlying defects (18). Our findings support these earlier results, but until the present study it was unclear as to whether all SCNT placentas are abnormal or only those that show gross pathological changes. We clearly demonstrated that all SCNT term and preterm placentas, whether appearing normal or not, have radically altered gene expression profiles, thus suggesting that compensatory mechanisms play a key role in overcoming fundamental defects in placental function that occur during the peri-implantation period. Predictably, clones with placentas that cannot compensate because of the cumulative effects of reprogramming errors will die, whereas those that can compensate may be abnormal yet support pregnancy to term. It is also likely that a large proportion of the clone pathologies, such as increased birth weight and hydrops, result from placental dysfunctions and not from genetic or epigenetic defects of the developing fetus. It is thus important to determine when and in what part of the developing SCNT embryo critical genes for normal development of the placenta are differentially expressed so that strategies can be devised to either avoid or correct reprogramming errors. Finally, although adult clones have been reported to be healthy, it is important to obtain long-term follow-up studies in these animals because placental abnormalities may lead to an increased incidence of metabolic pathologies later in adulthood.

ACKNOWLEDGMENTS

R. E. Everts, X. C. Tian, X. Yang, J.-P. Renard, and H. A. Lewin designed research; R. E. Everts, P. Chavatte-Palmer, I. Hue, A. Razzak, C. A. Green, R. Oliveira, and X. Vignon performed research; R. E. Everts, and H. A. Lewin contributed new reagents/analytic tools; R. E. Everts, H. A. Lewin, and S. L. Rodriguez-Zas analyzed data; and R. E. Everts and H. A. Lewin wrote the paper. All authors contributed to the editing of the paper.

GRANTS

This work was supported by US Department of Agriculture (USDA)-Agricultural Research Service Contract AG 58-1265-2-020 (to H. A. Lewin), USDA-Agricultural Research Service Contract AG 58-1265-2-018 (to X. Yang, X. C. Tian), and awards from INRA No. 20 058 (to J.-P. Renard) and from the French Ministry of Research No. 20085 (to I. Hue, J.-P. Renard). The support of the International Relationship Direction of INRA is warmly acknowledged.

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