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Aberrant placenta gene expression pattern in bovine pregnancies established after transfer of cloned or in vitro produced embryos

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Salilew-Wondim D, Tesfaye D, Hossain M, Held E, Rings F, Tholen E, Looft C, Cinar U, Schellander K, Hoelker M. Aberrant placenta gene expression pattern in bovine pregnancies established after transfer of cloned or in vitro produced embryos. *Physiol Genomics* 45: 28–46, 2013. First published October 23, 2012; doi:10.1152/physiolgenomics.00076.2012.—In the present study, we used the global transcriptome profile approach to identify dysregulated genes, molecular pathways, and molecular functional alterations in bovine placentas derived from somatic cell nuclear transfer (SCNT) and in vitro embryo production (IVP) pregnancies compared with their artificial insemination (AI) counterparts at *day 50* of gestation. For this, *day 7* blastocysts derived from AI, IVP, or SCNT were transferred to oestrus-synchronized cows. The pregnant animals were slaughtered at *day 50* of gestation, and the placentas were then recovered and used for transcriptome analysis using Affymetrix GeneChip bovine genome array. Results showed the SCNT placenta to be different from its AI counterpart in the expression of 1,196 transcripts. These genes were found to be associated with alterations in key biological processes and molecular pathways in SCNT placenta, and the dysregulation of 9% ($n = 110$) of these genes was due to transcriptional reprogramming error. IVP placenta also displayed alterations in the expression of 72 genes, of which 58 were common to SCNT placenta. Gene enrichment analysis revealed that the expression of genes involved in organ development, blood vessel development, extracellular matrix organization, and the immune system was affected in both SCNT and IVP placentas. However, 96% of the affected genes in SCNT were not significantly altered in IVP groups. Thus, the higher transcriptome dysregulation in SCNT placenta followed by IVP would reflect the degree of placental abnormality in SCNT and IVP pregnancies at *day 50* of the gestation, which may have a profound effect on subsequent fetal development and health of the offspring.

bovine placenta; transcriptome reprogramming; gene expression

THE DISCOVERY OF SPERM CAPACITATION (5, 6, 14) and advancements in reproductive biotechnology have revolutionized in vitro embryo production (IVP) to be a routine activity in human and animal fertility centers. In addition, the creation of Dolly, the first cloned sheep, by somatic cell nuclear transfer (SCNT) by Wilmut and coworkers (112) unraveled the possibilities of adult animal cloning in goats (121), cattle (49), mice (104), pigs (75), horses (30), and rabbits (18). Thus, SCNT, along with IVP, has emerged as a useful tool for in vitro embryo production in farm animals.

Despite somatic cloning's speculated importance in the field of precision animal agriculture and its potential applicability to preserve animal species that are on the verge of extinction (110), obtaining live offspring from somatic cloning has been inefficient (21, 56), and pregnancy success rates remain between 2 and 10% (16, 111). Several factors related to cloning procedures and culture environment contribute to pregnancy failure of clones at different stages of embryonic and fetal development (21). To address this issue in the bovine, investigators have used a variety of donor cells including cumulus (50), fibroblasts (38, 50, 51, 53, 117), ovarian/granulosa (109, 110), mammary epithelial cells (51, 117), oviduct (49, 50), and uterine cells (50), along with several technical modifications (23, 99, 101). Despite all these efforts the incidence of pre- and postimplantation pregnancy failure and postnatal deaths in SCNT-derived pregnancy remains higher (80). For instance, ~80–90% of the pregnancy losses occur between *days 30* and *180* (37, 57, 109), and pregnancy failures also occur in the third trimester, between *days 200* and *265* of the gestation period (21).

Regarding IVP embryos, which are exposed to the same culture environment as SCNT embryos, pregnancy success after embryo transfer is considerably higher compared with SCNT-derived embryos, but substantially lower compared with in vivo blastocysts. Moreover, the majority of pregnancy failures in IVP embryos occur between *days 30* and *90* of gestation (26). For instance, neonatal and prenatal abortion rates up to 19% after transfer of IVP embryos have been reported (60). A study by Agca et al. (2) indicates ~11–33% pregnancy losses after *day 40* of the gestation period, while other reports show 13% pregnancy losses after *day 60* of gestation (35, 36). In another study (12), 16% pregnancy loss between *days 56* and *81* and 9.7% loss between *day 81* of gestation and calving have been reported after transfer of IVP embryos.

Apart from lower pregnancy rates, offspring obtained from IVP or SCNT embryos are reported to be marred with placental physiological and anatomical complications (26, 114). In addition, the occurrence of large offspring syndrome is also evidenced in both SCNT- and IVP-derived pregnancies (8, 26, 57, 114, 116). Indeed, the low efficiency of SCNT and IVP is triggered by the confounding effects of multidimensional factors. However, aberrant expression of developmentally related genes induced by culture condition (69, 114) and/or deficiencies in genome reprogramming (11, 44, 69, 85) could account for the larger share of the factors contributing to the lower efficiency of SCNT and IVP embryo-derived pregnancies in the bovine. In this regard, cumulative evidence (20, 33, 64, 96, 118) indicates altered gene expression in SCNT- and IVP-derived embryos during preimplantation development. Thus, epigenetic modifications, abnormal DNA methylation, and altered expression of developmentally relevant genes during

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embryonic development may result in abnormal placental development (4, 20, 37, 41, 85, 114), which in turn could affect the viability of SCNT and IVP pregnancies (19, 40, 48). Apparently, malfunctioning of the placenta is more often associated with abnormal fetal development and pregnancy failure (13, 37, 62). Hence, identification of genes, key molecular functions, and pathways associated with placental physiology and development could help us plan an inclusive road map toward improving the rate of successful pregnancies after transfer of SCNT- or IVP-derived embryos. Therefore, this study aimed to investigate the placental transcriptome profile alteration in SCNT- and IVP embryo-derived pregnancies at *day 50* of the gestation period using the Affymetrix bovine genome array. *Day 50* of gestation is believed to be critical for placenta formation and placental angiogenesis. Moreover, in bovines, *day 50* of gestation is the time when the placenta attaches fully to the endometrium. Apart from this, the probability to obtain pregnancy from cloned embryos after *day 50* is critical. Thus due to the period's critical importance, this study was conducted on *day 50* gestation as a baseline stage from which to study the transcriptome profile of placenta in SCNT and IVP embryo derived pregnancies.

MATERIALS AND METHODS

Animal handling and management. Animals used for artificial insemination (AI) and embryo transfer were selected based on general clinical examination. They were all managed under the same farm and housed in a free-stall barn with slotted floors and cubicles, lined with rubber mats, and they were fed a total mixed ration. All experimental procedures involving animals were licensed by the Animal Protection Office of the University of Bonn based on the rules and regulations of the German Animal Protection Law (1972).

IVP. In vitro fertilization and in vitro culture was performed as described previously (84). In brief, bovine ovaries were obtained from the local abattoir and transported to the laboratory in a thermo flask containing 0.9% saline solution. Cumulus-oocyte complexes (COCs) were aspirated from follicle size of 2- to 8 mm-diameter follicles. A group of 50 COCs, with evenly granulated oocyte cytoplasm surrounded by more than three compact layers of cumulus cells, was washed and transferred to four-well dishes (Nunc, Roskilde, Denmark) containing 400 ml of maturation medium that consisted of TCM-199 with Earle salts buffered with 4.4 mM HEPES and 33.9 mM sodium bicarbonate (Sigma-Aldrich Chemie, Steinheim, Germany) supplemented with 10% estrous cow serum (OCS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 mg/ml gentamycin sulphate, and 10 μl/ml FSH (Folltropin, Vetrepharm). The oocytes were then set to mature in incubator at 38.7°C and 5% CO_2 in humidified air. After maturation (22 h), all COCs were coincubated with frozen semen (1×10^6 spermatozoa/ml) in a fertilization medium consisting of Fert-TALP medium supplemented with 10 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml BSA, 1 μg/ml heparin, 10 μM hypotaurine, 20 μM penicillamine, and 2 μM epinephrine at 38.7°C in 5% CO_2 in humidified air. About 18 h after fertilization, the cumulus cells were removed, and cumulus-free presumptive zygotes were washed three times in CR1aa supplemented with 10% heat-inactivated OCS. The zygotes were then cultured in 400 μl of culture medium in four-well dishes (Nunc) and covered with mineral oil at 38.7°C in 5% CO_2 in humidified air, and the developmental rates were recorded until *day 7*.

In vivo embryo production. To be used as controls, in vivo embryos were generated from Simmental cows after estrous synchronization and followed by AI. Estrous synchronization was performed as previously indicated (83). In brief, six Simmental heifers aged from 2 to 2.5 yr were presynchronized by intramuscular administration of 500 mg of the prostaglandin $F_{2\alpha}$ (PGF2α) analog cloprostenol (Estrumate, Munich, Germany) twice within 11 days. Cows were then received 0.02 mg of GnRH analog buserelin (Receptal; Intervet, Boxmeer, The Netherlands), and cows were artificially inseminated with semen collected from the same bull whose semen was used for in vitro fertilization and whose ear biopsy was used for derivation of donor fibroblast for SCNT embryo generation.

Derivation and preparation of donor cells. Bovine fibroblast cells were used as donor cells for production of SCNT blastocysts. Primary fibroblast cell lines were established from biopsies taken from the ear of the Holstein Frisian bull whose semen was used for in vivo and in vitro embryo production. The age of the bull was 9 yr at the time of biopsy sampling. The biopsies were minced, washed several times, and dispersed in a T25 cell culture flask. The cells were then cultured in DMEM supplemented with 2 mM glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich Chemie), and 10% fetal calf serum (Gibco, Karlsruhe, Germany) in 5% CO_2 at 37°C in humidified air. At 90% confluence, the cells were trypsinized, subpassaged, and stored frozen as aliquots in cell culture medium supplemented with 10% DMSO until use. Cells of *passage 3* were cultured and induced to enter a period of quiescence (presumptive G0) for 7 serum starvation before being used for nuclear transfer.

Generation of SCNT embryos. To generate SCNT blastocysts, oocytes were matured for 19 h. We enucleated metaphase II oocytes by removing polar bodies and the metaphase plate via a 25-μm beveled glass pipette. The absence of the metaphase plate was confirmed by minimum exposure of oocytes to ultraviolet fluorescence. A single donor cell was placed into the perivitelline space of the oocyte in close contact with the oocyte membrane via a 30-μm beveled glass pipette. We induced fusion between oocyte and donor cell 2 h after complex formation by applying a single electrical pulse of 25 V for 45 μs between two electrodes placed 150 μm apart in Kruess electro fusion CFA 400 (Hamburg, Germany). The oocyte and donor cell complex were then activated in 5 μM ionomycin for 4 min and for 3.5 h in 2 mM 6-dimethylaminopurine in CR1aa medium. Subsequently, the SCNT-derived embryos were in vitro cultured in CR1aa medium covered with mineral oil at 38.7°C in 5% CO_2 in humidified air until the blastocyst stage.

Chromosome spread of the donor cells. The chromosomal spread of the donor fibroblast cells was performed in the fibroblast cells that were obtained from ear of the bull. For this, the fibroblast cells were cultured as indicated above. After 50–70% confluence, the cells were treated with 10 μg/ml colchicin (Sigma-Aldrich) and cultured for 14 h. The cells were trypsinized with 0.25% Trypsin-EDTA. The fibroblast cells were then resuspended in 0.56% KCl or 1.2% sodium citrate and incubated at 37°C for 30 min. At the end of incubation, the cells were pelleted and washed two times in cold fixative (methanol-glacial acetic acid, 3:1). The cell suspension in fixative was then applied onto precleaned slide at the height of ~8 inches followed by brief exposure to water steam at 70–80°C. The slides were then air-dried and stained in 4% Giemsa stain (Promega, Mannheim, Germany) for 2 h. The chromosomal spreads were examined with ×1,000 magnification under oil immersion.

Embryo transfer and placenta sample collection. Prior to embryo transfer, 20 Simmental heifers aged 2–2.5 yr and weighing 350–400 kg were estrus-synchronized to be used as recipients by a similar protocol described for generating AI pregnancies, except recipient animals did not receive FSH and AI. Following this, morphologically good-quality, fresh blastocysts produced in vitro (IVP) were transferred to 10 Simmental heifers, and 10 blastocysts produced by SCNT were transferred to other 10 Simmental heifers at *day 7* of the estrous cycle. In each case, each recipient received a single embryo. Pregnancy diagnosis was performed on *day 28* and *day 42* of gestation by transrectal ultrasonography and rectal palpation, respectively. Following this, recipients of IVP embryos, SCNT embryos, and AI were slaughtered on *day 50* of the gestation. The placenta and the fetus

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were collected, washed twice in PBS, and stored at −80°C for gene expression analysis.

Total RNA extraction. Total RNA was isolated from 15 mg samples of three biological replicates of IVP, SCNT, or AI placentas or fetuses and 4×10^6 donor cells using miRNeasy mini kit (QIAGEN, Hilden, Germany). Each placenta from each animal group was considered as one biological replicate. In all cases the part of the placenta that includes placentomes was used for study. The fetuses from each corresponding placenta group were also crashed and homogenized prior to RNA isolation. Total RNA from placenta was used for placental global gene expression analysis, and total RNA from fetuses was used to quantify some of the candidate genes that were differentially expressed between placentas of the AI, IVP, and SCNT pregnancy groups. The concentration of the RNA was analyzed with the Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific). RNA integrity and quality were evaluated with Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip Kit (Agilent Technologies). The ribosomal RNA ratio (28S/18S ratio) of the RNA samples was between 1.8 and 2.1, and the RNA integrity was between 7 and 9.

RNA amplification. The total RNA isolated from placenta from three biological replicates of AI, IVP, and SCNT pregnancy groups was subjected to global gene expression with the GeneChip bovine Genome array (Affymetrix). Thus, RNA amplification, cDNA synthesis, and labeling were performed with the GeneChip3' IVT Express Kit following instructions described in the GeneChip3' IVT Express Kit user manual (P/N 702646 Rev. 8, Affymetrix). In brief, first-strand cDNA containing a T7 promoter sequence was generated from 250 ng of total RNA (to which 2 μl of diluted Poly-A RNA controls was added) using T7 oligo(dT) primer. A eukaryotic poly-A RNA control kit was used as a SPIKE-IN control to monitor the entire target labeling process. The controls were then amplified and labeled together with the samples as each eukaryotic GeneChip probe array contains probe sets for several *Bacillus subtilis* genes (lys, phe, thr, and dap) that are absent in eukaryotic samples. Following this, the first-strand cDNA was converted to double stranded with DNA polymerase and RNase H. The resulting double-stranded cDNA was in vitro transcribed and biotin-labeled using IVT master mix at 37°C for 16 h. The amplified RNA was then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate using magnetic beads. The biotin-labeled cRNA was fragmented and analyzed using a Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip Kit (Agilent Technologies).

Affymetrix array hybridization, washing, staining, and scanning. The GeneChip Bovine Genome Array (Affymetrix) was used for hybridization. For this, a hybridization cocktail consisting of fragmented and labeled cRNA, control oligonucleotide B2 (3 nM), 20× eukaryotic hybridization controls (bioB, bioC, bioD, cre) (Affymetrix), 2× hybridization mix, DMSO, and RNase-free water were mixed to a final volume of 200 μl. The mix was then heated at 99°C for 5 min followed by 5 min incubation at 45°C. The samples were then hybridized to GeneChip Bovine Genome Arrays. Hybridization was performed for 16 h, and each sample was hybridized three times. The arrays were then washed and stained with the Fluidics Station 450/250 (Affymetrix) and scanned using the GeneChip3000 laser confocal slide scanner (Affymetrix) integrated with GeneChip Operating System as recommended in the GeneChip expression wash, stain, and scan user manual (P/N 702232 Rev. 3).

Affymetrix data analysis and visualization. After scanning, we evaluated the quality of the arrays by assessing the absent and present calls of the control probe sets using Affymetrix expression console tool (http://www.affymetrix.com). The microarray data normalization and background correction were performed using GC robust multiarray average analysis (32) implemented in the Bioconductor package (http://bioconductor.org). The CEL files were first converted into an expression set and normalized using the R environment (http://www.r-project.org). The CELL files and normalized data can be accessed from the Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/ series entry GSE38512, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38512. We used linear models for microarray data (LIMMA) to obtain differentially expressed genes (95). *P* values were adjusted by the Benjamini-Hochberg procedure that controls the false discovery rate (FDR) (10). The genes were further classified according to their gene ontology (GO) of molecular functions and biological processes using GO statistical analyses (GOstats) (25) and DAVID, an online freely available bioinformatic tool (http://david.abcc.ncifcrf.gov/). Moreover, molecular pathways represented by differentially expressed genes were identified from the Kyoto Encyclopedia of Genes and Genomes (KEGG), Panther, and Reactom databases using the DAVID bioinformatic tool. The heat maps and clustering of differentially expressed genes were constructed using Bioconductor (http://www.bioconductor.org/) and GENE-E (http://www.broadinstitute.org/cancer/software/ GENE-E/index.html). Analysis of imprinted genes was performed with the online catalog freely available from the Jirtle Laboratory, Duke University (http://www.geneimprint.com/).

Quantitative real-time polymerase chain reaction. Quantitative real-time polymerase chain reaction (qPCR) was used to validate some of the differentially expressed genes detected in different comparisons. In addition, some of the differentially expressed genes in placenta were investigated in the fetuses of IVP, SCNT, and AI to examine whether the genes affected in placenta could also affected in fetuses. For this, sequence-specific primers were designed using primer 3.0 (http://frodo.wi.mit.edu/primer3/) (Supplemental Table S1).[1] The standard PCR reaction was performed to confirm the specificity of each of the primer. Plasmid DNA from each PCR product was then prepared using pGEM-T Easy Vector Systems (Promega) following the manufacturer's protocol. The plasmids were sequenced using GenomeLab GeXP Genetic Analysis System (Beckman Coulter) to confirm the presence the specific gene product. The serial dilution consisting of 10^1–10^9 copy numbers of molecules was then prepared from each plasmid DNA. Following this, the cDNA samples used for qPCR were synthesized by reverse transcription with equal amounts of total RNA from each placenta and fetus group and fibroblast cells. The qPCR was then performed in a 20 μl reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Munich, Germany), the cDNA samples, and the specific forward and reverse primer in the StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA). At the end of the qPCR reaction, the presence of specific amplification was monitored. The copy number of each gene in each sample was then determined by the relative standard curve method using a serial dilution prepared from each plasmid. The data analysis was performed after normalizing the copy numbers of the target genes against the housekeeping gene, β-actin. The qPCR was performed in three biological and two technical replicates. The data from qPCR were considered for further analysis provided that the slope was between −3.2 and −3.6, while the regression line (R^2) was ≥0.99. We employed Student's *t*-test or least significant difference test procedures to detect differences between the mRNA mean copy numbers between samples. The results were then reported as *n*-fold expression of the gene in target sample relative to the reference sample. Differences with $P < 0.05$ were considered as significant.

RESULTS

Placental gene expression profile dysregulation in SCNT embryo-derived pregnancies. To uncover genes that were abnormally expressed in SCNT pregnancies at *day 50* of the bovine gestation period, we compared the transcriptome profile of placentas derived from pregnancies established after the transfer of embryos produced by SCNT with the AI groups. For this, the pregnancies were generated by AI, IVP, and

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

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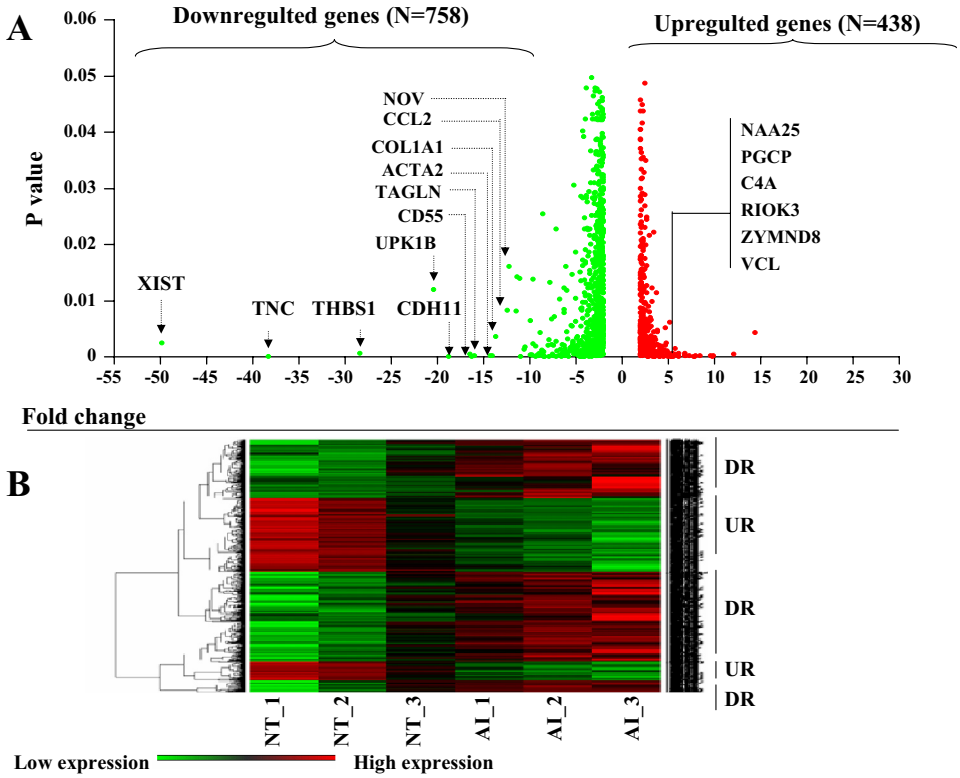


Fig. 1. Transcriptome profile differences between placentas derived from somatic cell nuclear transfer (SCNT) and artificial insemination (AI) pregnancies. *A*: fold change and *P* value distribution of differentially expressed genes (DEG). *B*: heat map showing the expression pattern of DEGs within and between biological replicates. NT_1, NT_2, and NT_3 represent biological replicates in SCNT-derived placenta, while AI_1, AI_2, and AI_3 represent biological replicates in AI-derived placenta. DR, downregulated; UR, upregulated.

SCNT until *day 50* of gestation. Thus, the pregnancy rate to *day 50* of gestation was 66, 30, and 30% in AI, IVP, and SCNT groups, respectively. To investigate the pattern of gene expression in placenta of each pregnancy, we used three placentas from the AI, IVP, or SCNT pregnancy groups. After hybridization to GeneChip Bovine Genome Array, the genes whose patterns of expression were most distinctive in SCNT or IVP were identified with a linear model described in the LIMMA pipeline and Bioconductor packages. The results have shown that a total of 1,196 gene transcripts to be dysregulated (fold change ≥ 2, $P \leq 0.05$, and FDR ≤ 0.2) in SCNT placenta compared with the AI groups (Fig. 1). From those, the expression of 438 genes was upregulated, while the expression of 758 genes including 13 putatively imprinted genes (Table 1) was downregulated in SCNT placenta. Furthermore, 26% of the upregulated genes displayed 3- to 14-fold higher expression compared with the AI groups. The first top 19 upregulated genes were dominated by uncharacterized express sequence tags (ESTs) followed by *N*-alpha-acetyltransferase 25 (*NAA25*), plasma glutamate carboxy peptidase (*PGCP*), complement component 4A (*C4A*), RIO kinase 3 (*RIOK3*), zinc finger (*ZMYND8*), and vinculin (*VCL*) (Fig. 1*A*). The list of top upregulated genes with $\geq$ 2.5-fold change is provided in Supplemental Table S2. Similarly, 35% of downregulated genes exhibited 3- to 49-fold mRNA reduction in SCNT-derived placenta groups. Some of the highly reduced genes (with 11- to 49-fold) include inactive X-specific transcripts (*XIST*), tenascin C (*TNC*), thrombospondin 1 (*THBS1*),

Table 1. *List of dysregulated putatively imprinted genes in SCNT compared with AI*

Gene Name	Gene Symbol	FC	*P* Value	FDR
X (inactive)-specific transcript	XIST	−49.85	0.002	0.049
CD81 molecule	CD81	−9.89	0.000	0.031
Decorin	DCN	−4.49	0.000	0.015
AXL receptor tyrosine kinase	AXL	−4.02	0.001	0.041
Sarcoglycan, epsilon	SGCE	−3.26	0.014	0.091
Transmembrane protein 60	TMEM60	−2.81	0.004	0.057
GNAS complex locus	GNAS	−2.65	0.010	0.082
Mesoderm specific transcript homolog (mouse)	MEST	−2.63	0.000	0.028
Phosphohistidine phosphatase 1	PHPT1	−2.45	0.007	0.068
Histocompatibility (minor) 13	HM13	−2.42	0.035	0.139
Copper metabolism (Murr1) domain containing 1	COMMD1	−2.41	0.003	0.054
Small nuclear ribonucleoprotein polypeptide N	SNRPN	−2.05	0.026	0.121
Interleukin 4 receptor	IL4R	−1.99	0.007	0.068
Delta-like 1 homolog	DLK1	3.75	0.011	0.086

SCNT, somatic cell nuclear transfer; AI, artificial insemination; FC, fold change; FDR, false discovery rate. Negative numbers indicate downregulation in SCNT placenta.

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uroplakin 1B (*UPK1B*), CD55 molecule (*CD55*), transgelin (*TAGLN*), and actin (*ACTA2*) (Fig. 1*A*). The list of genes whose mRNA was reduced by ≥ 2.5-fold is provided in Supplemental Table S3.

Further detailed characterization of the dysregulated genes reveals that the SCNT placenta was associated with simultaneous downregulation of several gene clusters. That cluster of genes includes the cathepsins (CTSS, CTSL2, CTSK, CTSC, CTSB, CTSA), sorting nexins (*SNX9, -7, -3, -10, -31*), CD molecules (*CD302, CD320, CD55, CD81, CD99*), mitochondrial ribosomal, peroxiredoxin (PRDX6, PRDX4, PRDX3), mediator complexes (*MED6, MED4, MED30, MED19*), the collagens families, transmembrane clusters (*TMEM70, -60,-59, -47, -43, -183, -67, -165, -159, -157, -150, -14, -120, -119*), and ATPases (*ATP6, ATP5, ATP2, ATP1*) (Fig. 2).

In addition to clustering, the chromosomal distribution of the dysregulated genes was investigated using GeneChip Bovine Genome Array annotation data (http://www.affymetrix.com/Auth/analysis/downloads/na32/ivt/Bovine.na32.annot.csv.zip). For this, first we analyzed the chromosomal distribution of all genes available on the GeneChip Bovine Genome Array. The first nine chromosomes with higher number of mapped genes were 19, 3, 7, 5, 10, 18, 11, 2, and 1, while the last nine chromosomes with less mapped genes were X, 9, 14, 12, 26, 20, 28, 24, and 27. Following this, analysis of the chromosomal distribution of differentially expressed genes shows that genes mapped to chromosomes X, 1, 27, 10, 26, and 9 to be strongly affected, while genes located on chromosomes 7, 19, 25, 17, and 24 were less affected in SCNT placenta.

Along with gene clustering and chromosomal distribution, we performed gene enrichment analysis of dysregulated genes to uncover biological processes and molecular functional alteration that have been occurred in SCNT using GO statistical analyses (GOstats) and the DAVID microarray analysis tool. Accordingly, 32 biological processes, which can be classified into five categorical groups, were found to be downregulated in SCNT placenta (Fig. 3). These include biological processes related to tissue and organ development (lung development, muscle and bone development), blood formation and circulation (vessel development, blood circulation, circulatory system process, angiogenesis), extracellular matrix (ECM) (extracellular organization, collagen fibril organization), hormone regulation (steroid hormone, estrogen hormone), and cell cycle (cell proliferation, apoptosis, and cell adhesion). Since biological processes are the sum of several events performed by one or more molecular functions (http://www.geneontology.org/GO.doc.shtml), the molecular function of the dysregulated genes was also investigated by gene enrichment analysis. Thus, significantly overrepresented molecular functions including protein binding, heparin binding, ECM and collagen binding, carbohydrate and lipid binding, growth factor binding, NAD or NADH binding, oxireductase activity, and calcium ion binding were downregulated in SCNT placenta (Fig. 4). This function may be related to the location of the genes where most of them were found to be localized in extracellular and intracellular regions of the cell, lysome, vacuoles, cytoplasm vesicle, and mitochondria (Fig. 5).

In addition to the biological and molecular functional category, pathway enrichment analysis using KEGG, Panther, and Reactom molecular pathways evidenced the dysregulation of 17 molecular pathways including focal adhesion, hemostasis, integrin signaling pathway, lysosome, angiogenesis, ECM-receptor interaction, platelet-derived growth factor (PDGF) signaling, TGF-β signaling pathway, and pyruvate metabolism (Table 2). Moreover, the majority of the genes involved in those pathways were found to be downrgulated in SCNT compared with AI-derived placenta groups (Table 2).

Transcriptome profile dysregulation in SCNT placenta was partly due to a transcriptome reprogramming error. To investigate whether the dysregulated genes in SCNT placenta were

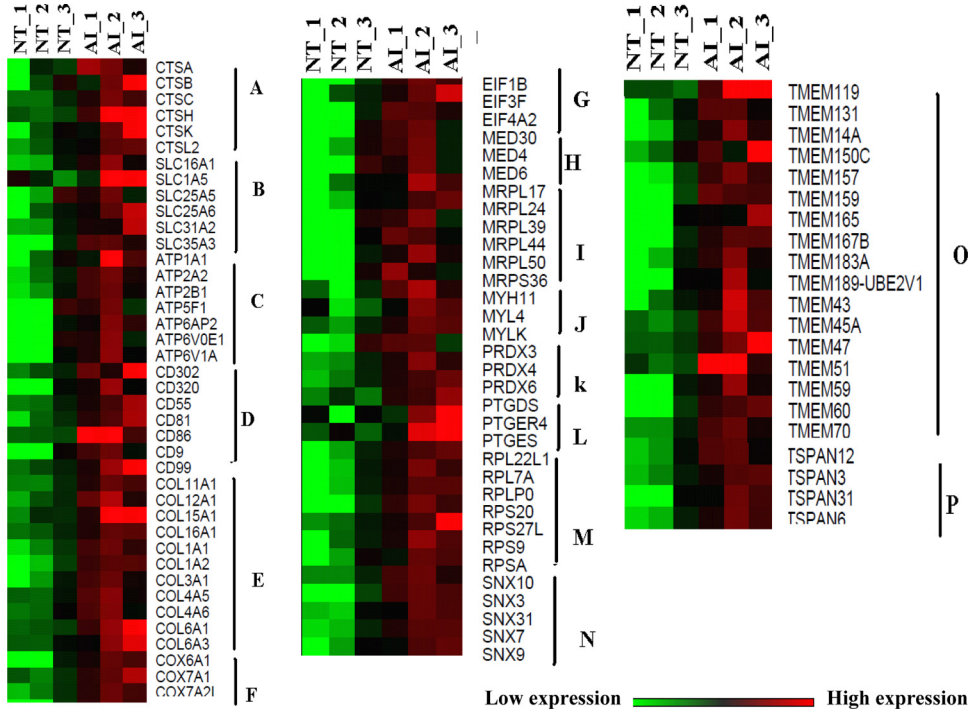
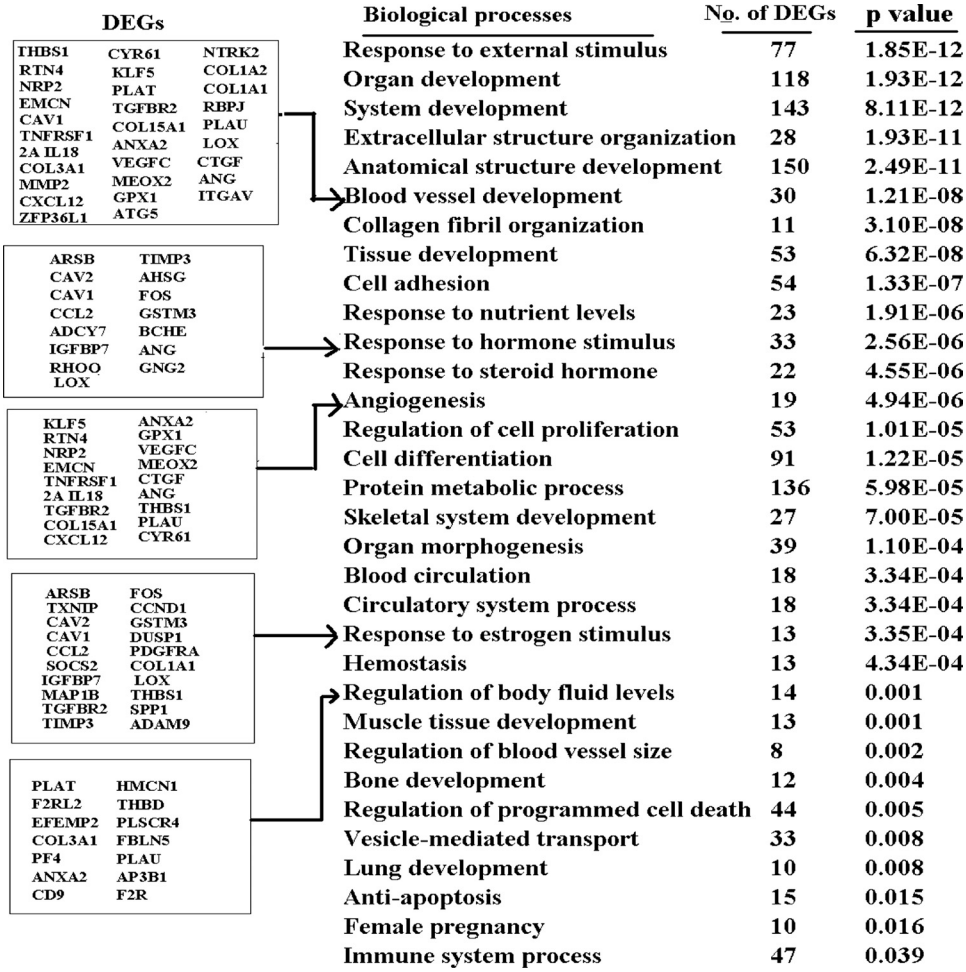


Fig. 2. Gene clusters differentially expressed between placenta samples derived from SCNT and AI pregnancies. *A*: cathepsins, *B*: solute carriers, *C*: ATPases, *D*: CD molecules, *E*: collagens, *F*: cytochrome c oxidases, *G*: eukaryotic elongation factors, *H*: mediator complexes, *I*: mitochondrial ribosomal proteins, *L*: prostaglandins, *M*: ribosomal proteins, *N*: sorting nexins, *O*: transmembrane proteins, *P*: teraspanins. NT_1, NT_2, and NT_3 represent biological replicates in SCNT-derived placenta while AI_1, AI_2, and AI_3 represent biological replicates in AI-derived placenta.

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Biological processes	No. of DEGs	p value
Response to external stimulus	77	1.85E-12
Organ development	118	1.93E-12
System development	143	8.11E-12
Extracellular structure organization	28	1.93E-11
Anatomical structure development	150	2.49E-11
Blood vessel development	30	1.21E-08
Collagen fibril organization	11	3.10E-08
Tissue development	53	6.32E-08
Cell adhesion	54	1.33E-07
Response to nutrient levels	23	1.91E-06
Response to hormone stimulus	33	2.56E-06
Response to steroid hormone	22	4.55E-06
Angiogenesis	19	4.94E-06
Regulation of cell proliferation	53	1.01E-05
Cell differentiation	91	1.22E-05
Protein metabolic process	136	5.98E-05
Skeletal system development	27	7.00E-05
Organ morphogenesis	39	1.10E-04
Blood circulation	18	3.34E-04
Circulatory system process	18	3.34E-04
Response to estrogen stimulus	13	3.35E-04
Hemostasis	13	4.34E-04
Regulation of body fluid levels	14	0.001
Muscle tissue development	13	0.001
Regulation of blood vessel size	8	0.002
Bone development	12	0.004
Regulation of programmed cell death	44	0.005
Vesicle-mediated transport	33	0.008
Lung development	10	0.008
Anti-apoptosis	15	0.015
Female pregnancy	10	0.016
Immune system process	47	0.039

Fig. 3. Biological processes overrepresented in genes downregulated in placenta derived from SCNT compared with AI pregnancies.

due to a transcriptome reprogramming error, the gene expression profile of the SCNT placenta was compared with its donor cell lines (fibroblasts). The result showed that, including 212 ESTs, a total of 5,090 gene transcripts were differentially expressed between the two groups, which is ~50% of the total probe sets submitted to the LIMMA analysis. This indicates the presence of a wider gene expression difference between SCNT placenta and its donor cells. In addition, the mRNA level of pregnancy-associated proteins (*PAG1, -2, -3, -4, -5, -6, -8, -9, -10,-17, -18, -19, -20,* and PAG21), placenta-expressed transcript 1 protein (*PLET1*), and placental growth factor (*PGF*) were upregulated in SCNT placenta, whereas the fibroblast growth factor intracellular binding protein (FIBP), fibroblast growth factor 2 (FGF2), fibroblast activation protein (*FAP*), and insulin-like growth factor 2 (IGF2) were increased in the donor cells. Following this, the genes detected as differentially expressed in SCNT vs. AI and SCNT vs. donor cell were merged to identify the common differentially expressed genes. The results show that from 1,196 aberrantly expressed in SCNT compared with AI, 963 genes were also differentially expressed between SCNT placenta and its donor cells. Of these, the expression level of 398 genes was increased, whereas the expression level of 555 gene transcripts was downregulated in SCNT compared with both AI and its donor cells. Thus, the dysregulation of 963 genes in SCNT placenta was not related to transcriptome reprogramming error. However, 233 gene transcripts that were dysregulated in SCNT did not show significant mRNA expression level alteration compared with its donor cells. We hypothesized that this may be due to the fact that these genes are expressed equally in fibroblast and placenta or the alteration of these genes in SCNT relative to AI was due to transcriptome reprogramming error during embryogenesis. Thus, to get insight about the first notation, we opt to compare the expression pattern of those genes in AI placenta groups relative the SCNT donor cells. Accordingly, from 233 genes, 123 genes were not significantly differentially expressed between AI placentas and the SCNT donor cells, but 110 gene transcripts including *XIST, UPK1B, PLAC8, UPK1A, RARRES1, FMR1NB, BRB,* and *PAG15* were differentially expressed between AI and SCNT donor cells (Fig. 6). Thus, from a total of 1,196, dysregulated genes, altered expression of 110 (9%) genes in SCNT placenta was triggered due to transcriptome reprogramming error. The gene enrichment analysis showed that the majority of these genes were found to reside on cytoplasm, extracellular region, and plasma membrane. The functional annotation also indicates that these genes were found to be involved in developmentally relevant biological process including anatomical structure development, system development, tissue and organ development, hemostasis, and regulation of body fluid levels (Fig. 7). At the molecular level, they were found to be involved in binding of carbohydrate, cytoskeletal proteins, glycosami-

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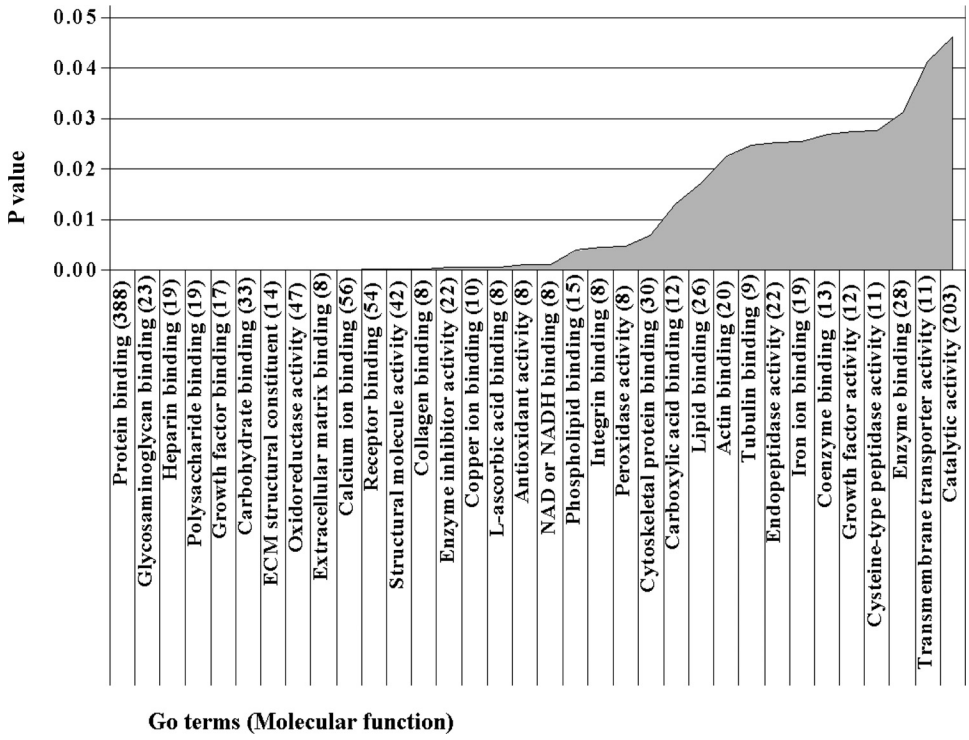


Fig. 4. Molecular functions overrepresented in genes downregulated in placenta derived from SCNT compared with AI pregnancies. Molecular functions with lower *P* values including protein binding, heparin binding, extracellular matrix (ECM) and collagen binding, carbohydrate and lipid binding, growth factor binding, NAD or NADH binding, oxireductase activity, and calcium ion binding were highly significantly overrepresented molecular functions. Numbers in parentheses on *x*-axis indicate the number of downregulated genes.

noglycans, heparins and phospholipids, and thrombin receptor activity (Fig. 7). In addition, these genes were found to participate in several pathways including hemostasis, leukocyte migration, tight junction, complement cascades, and integrin cell surface interactions pathways (Fig. 7).

Placental gene expression profile alteration in in vitro embryo-derived pregnancies. To investigate the presence of transcriptome profile dysregulation in IVP-derived pregnancies at *day 50* of the gestation, the placental gene expression profile of IVP groups was compared with the AI controls. Principal component analysis (PCA) indicated the overall gene expression pattern of IVP to be an intermediate between AI and SCNT (Fig. 8). In addition, the LIMMA analysis revealed the expression of 72 genes to be dysregulated in IVP compared with AI. Among these, the expression level of 29 genes including *PGCP*, *TEX12, ARG2, DIRAS3, ELL3, ZMYND8,* and *C4A* was upregulated, while the expression level of 43 genes including *TNC, TAGLN, CCL2, ACTA2, CXCR7, COL1A1, CTSS,* and *ASPN* was downregulated in IVP placenta groups (Fig. 9). Functional annotation of those genes showed that the genes involved in immune system process, lymphoid organ, and gut development were activated (Fig. 10*A*), while genes involved in blood vessel size formation, organ development, neurogenesis, and neuron differentiation were downregulated in IVP placenta (Fig. 10*B*).

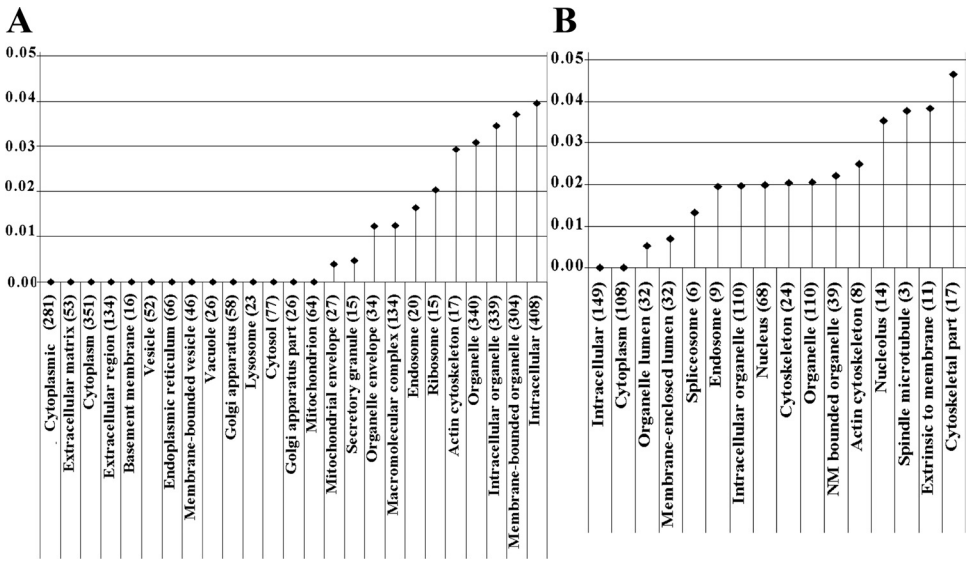


Fig. 5. Cellular components overrepresented in downregulated (*A*) and upregulated genes (*B*) in SCNT-derived placenta. Values on *y*-axis indicate the *P* value and numbers in parentheses on *x*-axis indicate the number of DR or UR genes.

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Table 2. *Molecular pathways containing genes with increased and reduced levels of mRNA in SCNT compared with AI placenta groups*

Pathways	Dysregulated Genes
Focal adhesion	*MYL7, CAV2, CAV1, TNC, COL3A1, ITGB5, ITGAV, COL6A3, COL6A1, PDGFC, THBS1, COL11A1, FN1, SPP1, ROCK1, COL4A6, VEGFC, CCND1, FYN, GSK3B, COL1A2, PDGFRA, RAP1A, COL1A1, MYLK, VCL, DOCK1, BRAF, LAMC1*
Hemostasis	*F2RL2, CAV1, CLU, PF4, APBB1IP, TIMP1, CD9, SLC16A1, APP, ITGAV, GNG2, HSPA5, THBS1, FN1, PLAT, PSAP, SPARC, VEGFC, THBD, FYN, COL1A2, RAP1A, TMSB4X, COL1A1, PLAU, F2R, TTN, CXADR, VCL*
Integrin signaling pathway	*CAV1, COL3A1, RHOQ, ITGB5, VCL, DOCK1, ITGAV, COL6A3, RRAS, COL6A1, COL12A1, COL11A1, FN1, COL15A1, COL16A1, COL4A6, COL4A5, RND3, FYN, COL1A2, RAP1A, COL1A1, LAMC1, BRAF, DOCK5*
Lysosome	*AGA, ARSB, PSAP, LGMN, HEXB,, MANBA, GNS, LAMP1, CTSK, AP1S2, GNPTAB, CTSC, CTSB, CTSH,CTSA,CTSS, CTSL2, AP3B1, GBA*
Angiogenesis	*PRKCI, FZD1, FZD3, JAG1, FRZB, NOTCH2, FOS, SFRP2, GSK3B, PDGFRA, RRAS, PDGFC, RBPJ, PRKCZ, ABR, BRAF, DLK1, APC*
ECM-receptor interaction	*TNC, COL3A1, ITGB5, COL4A6, ITGAV, COL6A3, COL1A2, COL6A1, LAMC1, COL1A1, THBS1, COL11A1, FN1, SPP1*
Integrin cell surface interactions	*TNC, ITGB5, APBB1IP, COL4A5, VCAM1, ITGAV, COL1A2, RAP1A, LAMC1, COL1A1, THBS1, FN1, SPP1*
Signaling by PDGF (platelet-derived growth factor)	*PLAT, COL6A3, COL3A1, COL1A2, PDGFRA, COL6A1, PDGFC, COL1A1, THBS1, COL4A5, SPP1*
Colorectal cancer	*FOS, CCND1, GSK3B, TGFBR2, PDGFRA, FZD1, FZD3, BRAF, SMAD3, APC*
TGF-β signaling pathway	*INHBA, LTBP1, ID2, ROCK1, TGFBR2, SMAD3, DCN, THBS1, BMPR1A, ACVR2B*
Viral myocarditis	*CAV1, CD55, CD86, CCND1, FYN, MYH11, MYH7, CXADR, SGCB*
Axon guidance	*FYN, COL6A3, COL3A1, COL1A2, COL6A1, COL1A1, PRNP, COL4A5*
Blood coagulation	*PLAT, F2RL2, APP, THBD, SERPINF1, ITGB5, PLAU, F2R*
Pyruvate metabolism and TCA cycle	*SDHB, SLC16A1, SDHD, IDH3A, FH, DLD*
Glycine, serine and threonine metabolism	*ALAS2, GATM, PHGDH, PSAT1, DLD, DMGDH*
Citrate cycle (TCA cycle)	*SDHB, SDHD, IDH3A, FH, MDH1, DLD*
Prion diseases	*C1QA, EGR1, FYN, LAMC1, HSPA5, PRNP*

Underlined genes have increased level of mRNA in SCNT compared with AI placenta groups; genes not underlined have reduced level of mRNA in SCNT compared with AI placenta groups.

Common and exclusively dysregulated genes in SCNT and IVP-derived placenta. Compared with SCNT, the number of dysregulated genes and transcriptome functional alterations was lower in IVP placenta compared with their AI counterparts. However, both SCNT and IVP shared 58 commonly dysregulated genes of which the transcript level of 18 genes including *PGCP, C4A, ZMYND8, ARG2, ELL3, SFXN1, YAP1,* and *TET2* was increased, while the expression level of 40

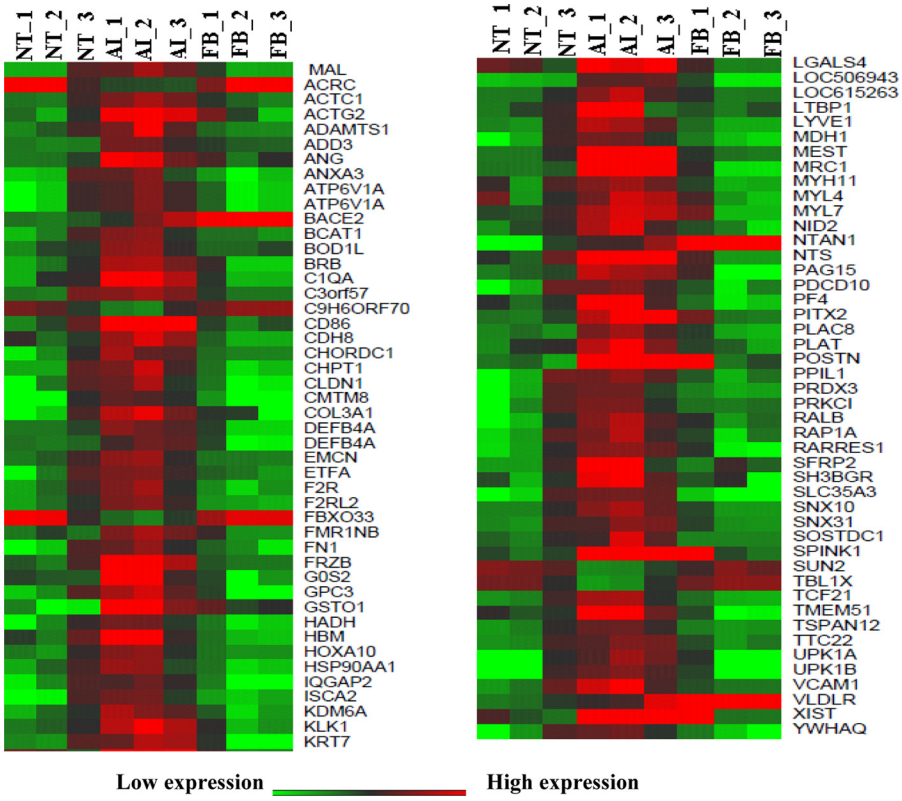


Fig. 6. Heat map depicting the expression patterns of genes dysregulated in SCNT due to transcriptome reprogramming error but maintained similar expression pattern compared with donor cells. NT_1, NT_2, and NT_3 represent biological replicates in SCNT-derived placenta, while AI_1, AI_2, and AI_3 represent biological replicates in AI-derived placenta. FB_1, FB_2, and FB_3 indicate biological replicates in donor cells.

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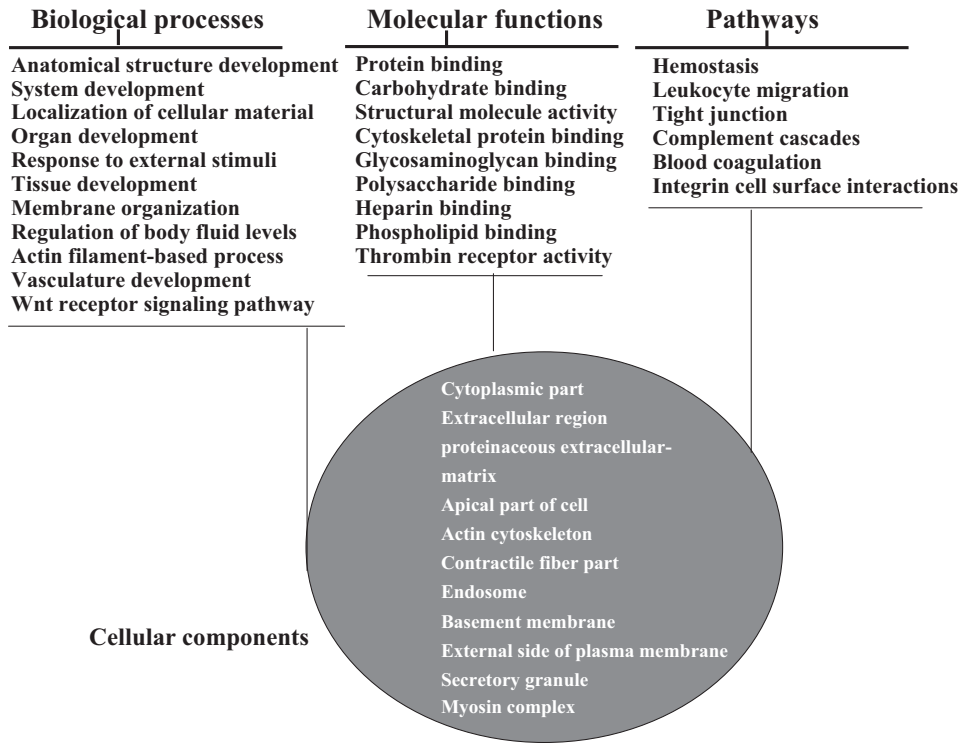


Fig. 7. Biological processes, molecular functions, pathways, and cellular components overrepresented in genes dysregulated in SCNT-derived placenta due to transcriptome reprogramming error.

genes including *TNC, TAGLN, ACTA2, CTSS, CTSH, ACTG2, ASPN*, and *FKBP7* was reduced in both SCNT and IVP compared with AI (Fig. 11, *A* and *B*). Thus, essential transcriptome functional alteration including organ development, immune system, blood vessel development, and extracellular organization was affected in both IVP and SCNT placenta (Fig. 12*A*).

Despite the fact that the SCNT- and IVP-derived pregnancies were established after the transfer of blastocysts developed in the same in vitro culture system, the former is derived from a fusion of somatic cell and an enucleated oocyte. Thus, to evaluate the extent of transcriptome differences between SCNT and IVP pregnancies, we also compared the placental transcriptome profile between these two groups. The results indicated 15 transcripts to be differentially expressed (fold change $>$ 2, $P < 0.001$, and FDR < 0.2) between the two groups. However, PCA (Fig. 8) showed the presence of strong within difference in SCNT placenta unlike in IVP placenta. Because of this, the FDR was 20%, while P was < 0.001. Thus, to overcome underestimation of the results, we opted to select genes with $P < 0.004$, FDR < 0.32, and fold change ≥ 2 followed by qPCR confirmation. According to this analysis, a total of 85 differentially expressed genes were detected between the two placenta groups (Fig. 13), in which the expression of 46 genes including *AGA, LRP11, LOC783192*, *ALDH5A1,* and *CPA1* was upregulated and the expression of 39 genes including *THBS1, ADD3, PLAC8*, *DEFB4A, PON2, SLC10A1, MCM10, MTPN, ASZ1, ITGAV, PRL, BOD1L,* and *DUSP1* was highly reduced in SCNT compared with IVP placentas.

Although both IVP and SCNT embryos shared the same culture conditions during the preimplantation development, IVP became more similar to AI in that both pregnancies were established after transfer of embryos derived from fertilized oocyte. We further investigated the genes dysregulated in SCNT relative to both AI and IVP by overlapping the dysregulated genes identified in SCNT vs. IVP and SCNT vs. AI. Including 19 ESTs, a total of 52 differentially expressed genes were found to be common to both comparisons (Fig. 11, *B* and *C*). From those, the mRNA level of 31 genes including *HSPB3, CPA1, SERPINB6, SFPQ, PLCH2, SUN2,* and *ALDH5A1* was increased, while the mRNA level of 21 genes including *THBS1, PLAC8, ADD3, PON2, ITGAV,* and *H2AFV* was reduced in SCNT compared with both AI and IVP. Functional enrichment analysis showed that those genes were found to be involved in response to stress, apoptotic cell clearance membrane invagination, regulation of lipid transport, and membrane organization (Fig. 12*C*).

Validation of microarray data by qPCR. We randomly selected 24 differentially expressed genes to validate the array results with qPCR. The expression levels of all 24 genes in various comparisons in both microarray and qPCR data are presented in Table 3. As shown in Table 3, we found the qPCR data to be consistent with the microarray data. However, TIPARP and BAG3 showed a reverse pattern in IVP vs. AI and SCNT vs. AI comparisons, respectively.

Quantification of candidate differentially expressed genes in corresponding fetal samples. Following identification of differentially expressed genes in placenta of various pregnancies, we opted to analyze the expression pattern of some of the candidate genes in the corresponding fetal samples. Total RNA was isolated from each individual fetal sample of AI, IVP, and SCNT fetal groups after being crashed and homogenized. After RNA isolation and cDNA synthesis, three samples of each AI, IVP, and SCNT fetuses were subjected to mRNA measurement by qPCR. A total of 11 differentially expressed genes that were validated by qPCR including CSTH, CTSS, HSPB3, PDGFR2, SUN2, TNC, and IGFB7 were analyzed in the fetal samples.

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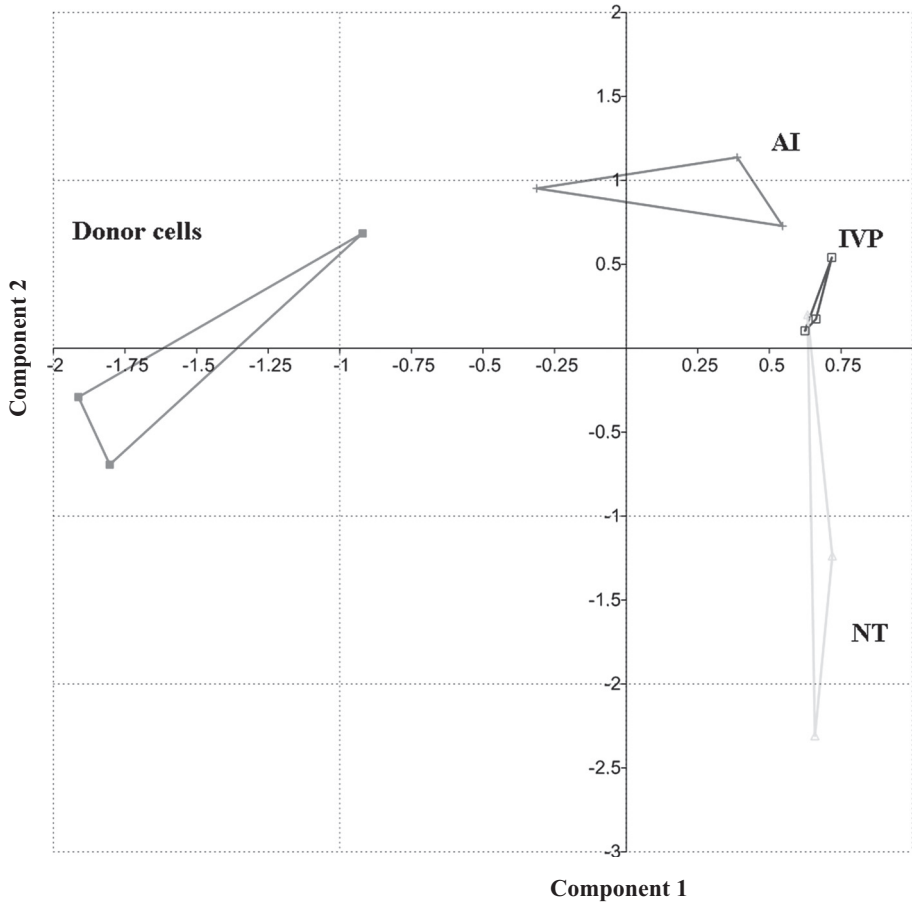


Fig. 8. Principal component analysis showing the similarities and differences in transcriptome profile of in vitro embryo production (IVP), AI, SCNT, and donor cells.

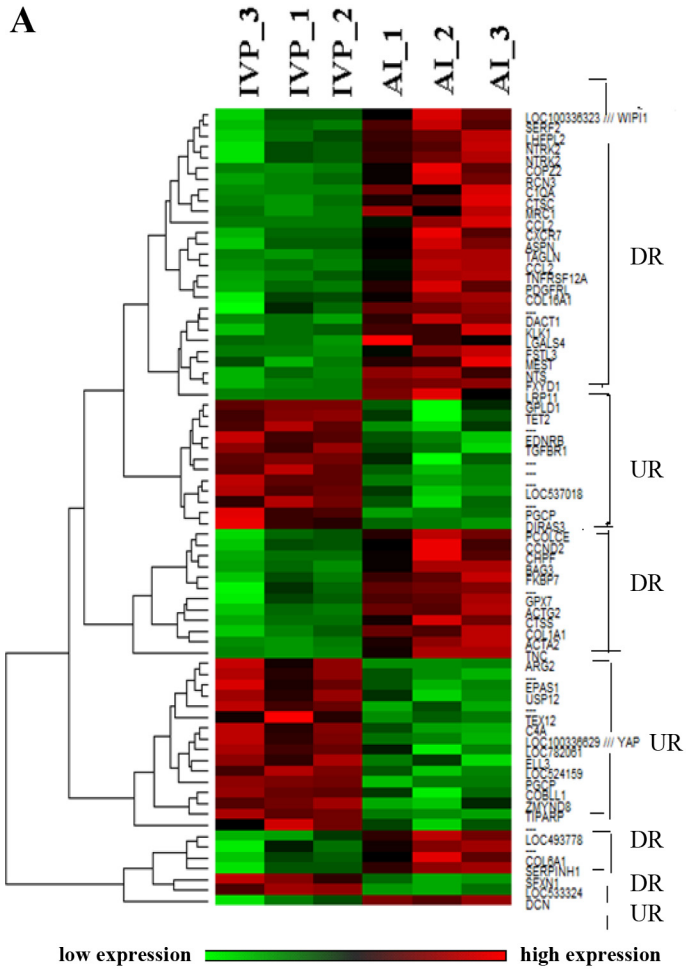
Interestingly, the results indicate that the expression pattern of those candidate genes in the fetuses of differently sourced pregnancies exhibited a similar trend as that of the placenta samples (Fig. 14). This shows that genes that are dysregulated in placenta could also be abnormally expressed in fetus.

DISCUSSION

Understanding the transcriptome landscape of placentas in pregnancies established after the transfer of SCNT and IVP embryos will provide insight into the molecular mechanisms associated with major placental morphological and physiological alterations. Therefore, we conducted this study to identify aberrant placental gene expression at *day 50* of gestation in bovine pregnancies established after the transfer of SCNT- and IVP-derived embryos. For this, we generated AI, SCNT, and IVP pregnancies, and the placentas were recovered after targeted slaughtering of the recipient animals at *day 50* of gestation. To uncover the gene and molecular functional alteration that could be associated with SCNT and IVP pregnancies, we compared the placental gene expression profile in SCNT and IVP with control AI. Thus, the current study has shown higher numbers of dysregulated of genes and molecular functional alterations in placentas derived from SCNT (n = 1,196) than IVP (n = 72) (Figs. 1, 9, 11). Similar patterns of placental gene expression in SCNT- and IVP-derived pregnancies have been reported at *days 174* and *260* of the bovine gestation period (24). Another study also showed hundreds of (n = 500–700) dysregulated genes in SCNT at *day 70* of the gestation period (4). Apart from this, differences in the transcript abundance of embryos derived from SCNT have been evidenced in the blastocyst-stage embryos (80, 118). In fact, interpreting results on the basis of the number of genes requires careful examination and in-depth bioinformatic analysis. Therefore, in this study, functional annotation of the dysregulated genes was performed to provide insight into whether transcriptome alteration in SCNT-derived placenta was associated with culture condition or was an intrinsic property of the tissue. Although dissecting and identifying the intrinsic defects in SCNT-derived pregnancy are a far step from full understanding and requires an in-depth investigation, one speculation addresses the hypothesis that SCNT-derived embryos may carry genetic information that has not been sufficiently reprogrammed during the early stages of embryo genesis. Several reports have documented the genetic and epigenetic reprogramming of SCNT embryos during the preimplantaion embryo development, although some of the reports are conflicting (28, 80, 94, 107). These conflicting data may result when IVP, AI, or the donor cells are used as a reference while measuring the transcriptome reprogramming errors in SCNT-derived embryos. In addition, most of the reports considered pooled embryo samples, which could mask individual variations. Thus, analysis of the individual embryo could be an alternative for better understanding whether the deviation of SCNT transcriptome is associated with faulty transcriptome errors (28). Indeed, in the present study, transcriptome analysis was performed on individual placenta as one biological replicate.

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A



B

Gene symbol	FC	Gene symbol	FC
PGCP	9.1	GPX7	-2.7
TEX12	5.8	FSTL3	-2.8
ARG2	4.7	C1QA	-2.9
DIRAS3	4.6	SERPINH1	-3.1
LOC782061	3.8	CTSC	-3.1
ELL3	3.5	NTRK2	-3.3
ZMYND8	3.2	DCN	-3.3
C4A	3.1	BAG3	-3.5
USP12	3.0	LGALS4	-3.5
LOC100336629	2.9	CCND2	-3.6
TIPARP	2.8	FKBP7	-3.7
TGFBR1	2.8	TNFRSF12A	-3.8
SFXN1	2.7	COPZ2	-3.9
EPAS1	2.7	COL16A1	-3.9
EDNRB	2.5	RCN3	-4.1
LOC533324	2.5	PDGFRL	-4.4
COBLL1	2.5	MRC1	-4.5
GPLD1	2.3	COL6A1	-4.8
TET2	2.2	CTSH	-5.1
LOC537018	2.2	PCOLCE	-5.5
LOC524159	2.1	LRP11	-5.7
DACT1	-2.0	ACTG2	-6.3
LHFPL2	-2.1	ASPN	-6.6
FXYD1	-2.2	CTSS	-6.8
LOC493778	-2.2	COL1A1	-7.1
NTS	-2.3	CXCR7	-8.9
SERF2	-2.3	ACTA2	-9.4
MEST	-2.3	CCL2	-11.6
LOC100336323	-2.4	TAGLN	-13.4
KLK1	-2.4	TNC	-40.7
CHPF	-2.5		

Fig. 9. Placental transcriptome dysregulation in IVP-derived embryos. *A*: heat map showing the expression pattern of dysregulated genes within and between the biological replicates. *B*: list of dysregulated annotated genes and their respective fold change (FC) difference in IVP compared with AI. Positive and negative numbers indicate UR and DR, respectively in IVP compared with AI. IVP_1, IVP_2, and IVP_3 represent biological replicates in IVP-derived placenta, while AI_1, AI_2, and AI_3 represent biological replicates in AI-derived placenta.

Thus, to understand whether the transcriptome dysregulation of SCNT-derived placenta was due to errors in transcriptome reprogramming, we compared the transcriptome profile of SCNT and the donor cells used for the generation of SCNT embryos. The SCNT-derived placenta and its donor cells were found to be different in the expression of >5,000 gene transcripts, which covers nearly 50% of the detected genes in both groups. Moreover, the donor cells were in shortage of placenta-specific gene, and SCNT-derived placentas were in shortage of fibroblast-specific gene expression. This would suggest strong dissimilarities between SCNT-derived placenta and its donor cells, which is indicative of the presence of transcriptome reprogramming in SCNT-derived placenta. Apart from these, DNMT3B, DNMT3A, HDAC1, and HAT1, which are likely to be involved in reprogramming of the somatic cell nuclei (119), were increased in SCNT-derived placenta by 2.3-, 1.7-, 1.4-, and 1.71-fold, respectively, compared with its donor cells. This transcriptome profile difference between the SCNT placenta and the donor cells reflects the degree of transcriptome reprogramming in SCNT-derived placenta. Parallel to these, we have performed chromosomal spreading to understand whether the donor cells exhibited a normal chromosome number and morphology. Although identification and evaluation of the size, shape, and number of chromosomes require careful examination of several cells, the result we got from the donor cells appears normal (Fig. 15). Thus, following this, we attempted to identify genes dysregulated in SCNT placenta due to errors in transcriptome reprogramming. Indeed, 90% (1,086/1,196) of the dysregulated genes in SCNT compared with AI were also found to be altered in SCNT placenta compared with its donor cells, suggesting the dysregulation of these genes was not related to errors in the transcriptome reprogramming. However, 10% (110 of 1,196) dysregulated genes in SCNT-derived placenta did not show significant expression differences compared with its donor cells (Fig. 6), indicating the dysregulation of these genes in SCNT may be associated with errors in transcriptomic reprogramming during embryogenesis. Parallel to this, bioinformatic analysis has indicated these genes were found to be involved in key biological processes and molecular functions that are associated with tissue, organ, and system development (Fig. 7). Thus, dysregulation of these genes may implicate abnormal tissue, organ, or system development in SCNT compared with AI pregnancies. Similarly, Smith et al. (94) suggested the occurrence of reprogramming errors during

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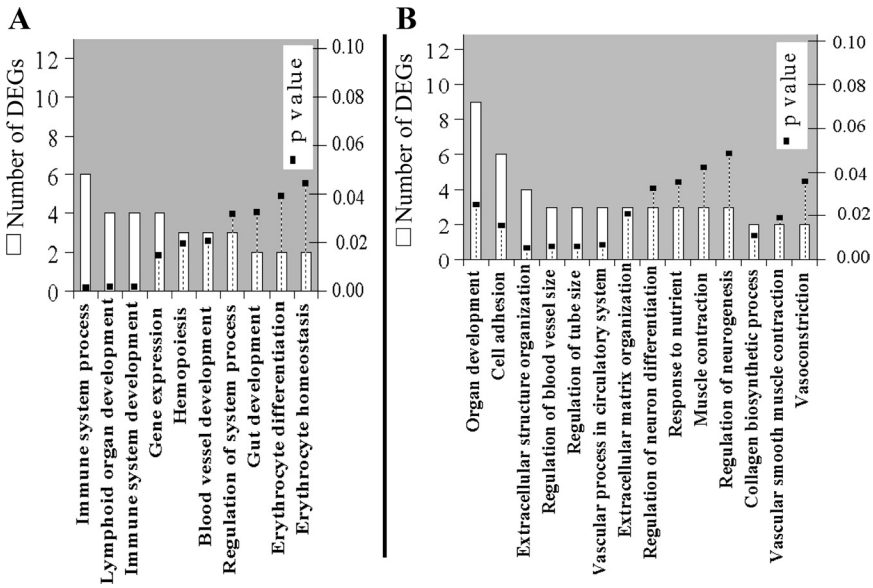


Fig. 10. Biological processes overrepresented in UR (*A*) and DR (*B*) genes in placentas derived from IVP compared with AI-derived pregnancies.

the formation of tissue and organs in SCNT-derived pregnancies. Indeed, genetic and epigenetic modifications arising from errors in transcriptome reprogramming of the donor cell after nuclear transfer are believed to lead to postimplantation lethality and abnormal fetal development (68, 93, 100). However, the mechanism of the transcriptome reprogramming errors in SCNT-derived pregnancies is not clear, though aberrant DNA methylation and histone remodeling (72), which consequently affect telomere maintenance and X chromosome inactivation, could be the possible causes of reprogramming errors in SCNT-derived pregnancies (100).

Apart from comparing them with AI groups alone, we also compared the transcript profile of SCNT-derived placenta with both AI and IVP groups to identify altered genes and gene products that could be unique to the SCNT pregnancies. In fact, the difference between IVP- and AI-derived placenta cannot be ruled out, as these pregnancies were established from blastocysts developed under different culture conditions. However, both groups of pregnancies were established from embryos derived from fertilized oocytes whose nuclear genome integrity is not detached, unlike with the SCNT pregnancies where enucleated oocytes were used for harboring the somatic cell. The current study revealed the dysregulation of 52 transcripts in SCNT-derived placenta compared with both IVP- and AI-derived placenta groups (Fig. 11*A*). For instance, SCNT placenta displayed downregulation of developmentally important genes including thrombospondin 1 (*THBS1*), placenta-specific 8 (*PLAC8*), adducin 3 (*ADD3*), paraoxonase 2 (*PON2*), integrin (*ITGAV*), myotrophin (*MTPN*), aspartylglucosaminidase (*AGA*), and H2A histone (*H2AFV*) compared with both AI- and IVP-derived placenta groups. Although detailed studies are required to draw a conclusion as to whether dysregulation of these genes in SCNT-derived pregnancies is associated with developmental abnormalities, reports published elsewhere appear to support the current results. For instance, thrombospondin-1 (*THBS*1) is implicated in angiogenesis and formation of placental structures (70). Similarly, the importance of PLAC8 in cell proliferation, apoptosis, and embryonic development has been documented (22, 31, 81). Moreover, higher expression of PLAC8 in the spongiotrophoblast layer of the mouse placenta (29) could also suggest the importance of PLAC8 during pregnancy.

The current study hints that 9% of gene expression dysregulation in SCNT-derived placenta is associated with transcriptome reprogramming errors, which suggest that the higher proportion of gene dysregulation in SCNT placenta could be the result of the confounding effect of multidimensional factors including the culture conditions. To explore the effect of the culture condition on the transcriptome profile of SCNT pregnancies, we have investigated the genes commonly dysregulated both in IVP- and SCNT-derived placenta relative to the AI control groups. In line with this, 58 genes were dysregulated both in the SCNT and IVP placenta groups. This in turn resulted in alteration of several transcriptome functions including development and regulation of blood vessel, organ development, and collagen biosynthesis in both pregnancies groups (Fig. 12). Indeed, dysregulation of transcripts related to blood vessel development (angiogenesis) both in SCNT- and IVP-derived pregnancies seems to be interesting. Since the placenta is the organ in which respiratory gases, nutrients, and wastes are channeled between the maternal and fetal systems, and abnormality in this organ could end with abnormal fetal development or termination of the pregnancy. This suggests that sufficient placental blood flow and increased placental vascularity are required for maintaining appropriate placental function (13). Therefore, lack of placental vascularization may lead to placental dysfunction (13, 37, 62), subsequently resulting in loss of pregnancy between *days 30* and *90* of gestation (26, 37).

Apart from angiogenesis, altered expression of genes involved in organ development was evidenced in both SCNT- and IVP-derived placenta. Although we did not record the fetal weight and different organs of the fetuses, the study by Lonergan et al. (57) shows heavier brain, liver, heart, lungs, kidneys, and spleen in cloned fetuses at *day 75* of the gestation period. Similar reports also indicate heavier fetuses and placentas (65) and heavier calves (8) from in vitro-derived pregnancies. Moreover, the presence of abnormal weight and size of fetuses or calves from IVP- and SCNT-derived embryos has

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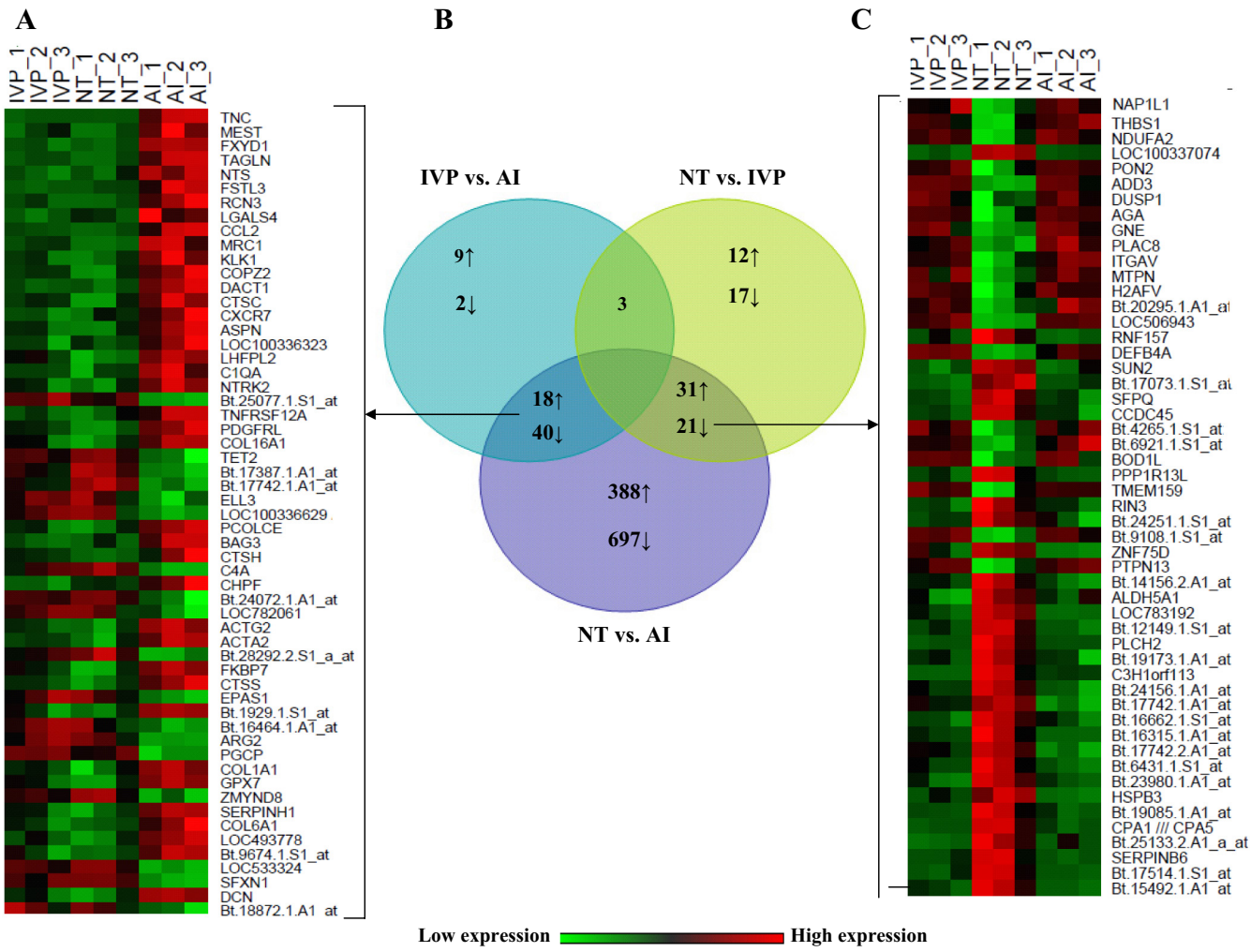


Fig. 11. Overlapped and exclusively dysregulated genes in IVP- and SCNT-derived pregnancies. *A*: expression pattern of commonly dysregulated genes both in IVP and SCNT compared with AI. *B*: number of overlapped and exclusively dysregulated genes in IVP and SCNT placenta. *C*: expression pattern of genes dysregulated in SCNT compared with both AI and IVP placenta groups. The up- and downward arrows indicate the UR and DR, respectively, in SCNT or IVP placenta groups.

been reviewed (26, 114, 116). Therefore, dysregulation of genes involved in organ development both in SCNT and IVP at *day 50* of gestation may hint at abnormal development of organs in both groups. Although we did not give much emphasis to expression alteration in the fetus, quantification of some of the candidate genes in the fetal sample showed a similar trend in expression alteration in the placenta samples (Fig. 14), suggesting alteration in placental gene expression could also be manifested in the fetus. However, further in-depth investigation is required to understand the consequence of aberrant placental transcriptome profile on morphological and physiological characteristics of the developing organs during the different stages of fetal development.

Although considerable numbers of transcripts were dysregulated commonly in SCNT- and IVP-derived placentas (Figs. 11, 12), 1,138 (96%) of the affected genes in SCNT-derived placenta were not significantly altered in IVP groups, indicating the presence of distinct sets of genes and molecular functional changes that are unique to SCNT groups. In addition, several gene clusters belonging to the same family were downregulated in SCNT-derived placenta at various levels of fold change (Fig. 2). For instance, the cathepsin cluster (*CTSS, CTSL2, CTSK, CTSH, CTSC, CTSB,* and *CTSA*) were among the clusters of genes that were downregulated in SCNT-derived placenta (Fig. 2*A*). Pathway analysis showed that all these cathepsins were found to be involved in the lysosome pathway (Table 1). Indeed, the exact function of cathepsins in pregnancy establishment is not clear. However, cathepsins are required to regulate ECM degradation and proteolysis (46, 103). Proteolysis is one of the unique functions activated in placenta tissue (59) that ensures favorable conditions for trophoblasts to invade the maternal environment (67). For instance, loss of function of cathepsin B (CTSB) and cathepsin L (CTSL) has been reported to trigger implantation failure in mice (1). Similarly, cathepsin S (CTSS), which was also downregulatd in SCNT-derived placenta, is implicated in immune response, transport of MHC molecules, embryo implantation, and angiogenesis (7, 15, 88, 89). Thus, downregulation of cathepsin genes may suggest the presence of extensive ECM dysregulation in SCNT-derived pregnancies. In addition, the downregulation of 11 collagen genes (COL1A1, -1A2, -3A1, -4A6, -4A5, -6A1,-6A3, -11A1, -12A1, -15A1,-16A1) (Fig.

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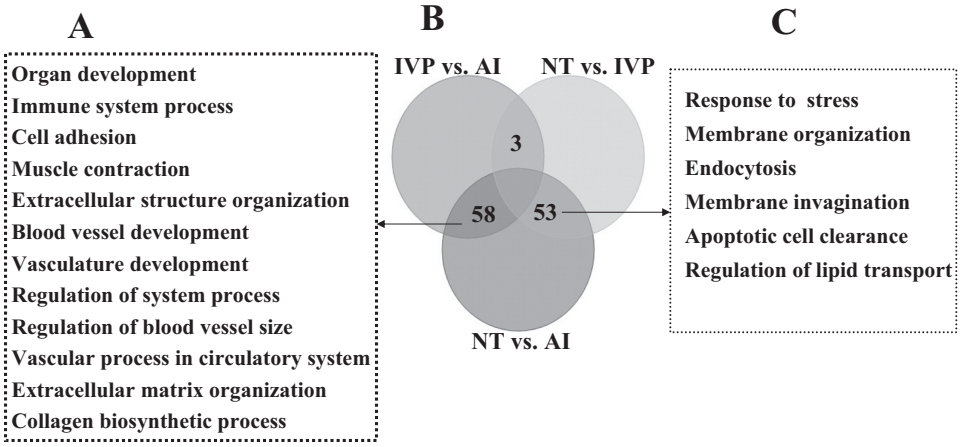
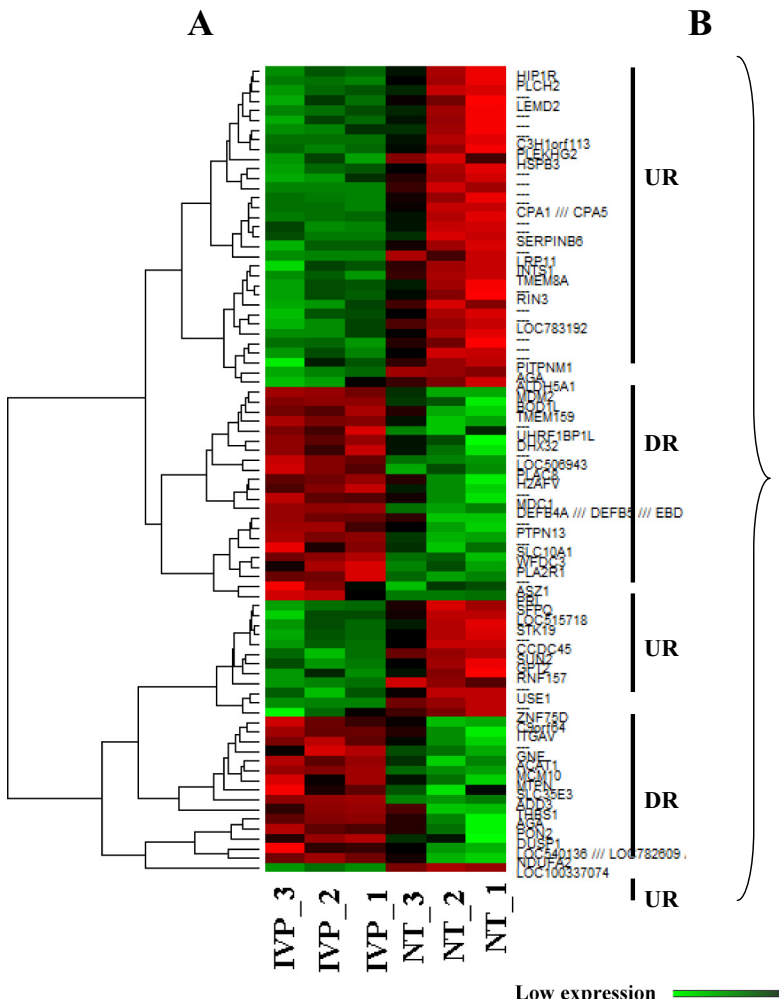


Fig. 12. Overlapped biological processes dysregulated in SCNT and IVP placenta groups. *A*: biological processes dysregulated in both SCNT and IVP placenta groups. *B*: overlapped and exclusively dysregulated genes in IVP and SCNT placenta groups. *C*: biological processes dysregulated in SCNT compared with both AI and IVP.

2*E*) and other genes associated with ECM degradation including tissue inhibitors of matrix metalloproteinase (*TIMP1, TIMP3*) and matrix metalloproteinase (MMP2), tenascin C (*TNC*), fibronectin (*FN1*), follistatins (*FSTL3*, *FSTL1*), and inhibin beta A (*INHBA*) further strengthens the evidence of ECM remodeling dysregulation in SCNT-derived pregnancies. Thus, optimal expression of these genes might be essential for proper fetal-maternal communication, and abnormalities in expression of ECMs may lead to implantation failure (43, 45, 92). Previous reports on TIMP3 (54, 78) and MMP2 (105) support the importance of inhibitors of matrix metalloproteinase (*TIMP1, TIMP3*) and matrix metalloproteinase (*MMP2*)



B

Gene symbol	FC
AGA	4.2
LOC10033707	3.8
LRP11	3.6
LOC783192	3.3
ALDH5A1	3.2
CPA1	3.0
TMEM8A	2.8
HSPB3	2.6
SERPINB6	2.5
C3H1orf113	2.5
LOC515718	2.5
SFPQ	2.4
ZNF75D	2.4
SUN2	2.4
GPT2	2.4
PLCH2	2.4
INTS1	2.3
HIP1R	2.2
RIN3	2.2
CCDC45	2.2
STK19	2.1
LEMD2	2.1
PLEKHG2	2.1
USE1	2.0
PITPNM1	2.0
RNF157	2.0
PPP1R13L	2.0

Gene symbol	FC
EFR3A	-2.0
UHRF1BP1L	-2.0
ACAT1	-2.0
PLA2R1	-2.1
MDC1	-2.1
MDM2	-2.1
NDUFA2	-2.2
DHX32	-2.2
NAP1L1	-2.3
PTPN13	-2.4
GNE	-2.4
WFDC3	-2.4
SLC35E3	-2.4
H2AFV	-2.4
DUSP1	-2.5
BOD1L	-2.5
PRL	-2.6
TMEM159	-2.6
AGA	-2.6
ITGAV	-2.6
ASZ1	-2.6
C9orf64	-2.7
MTPN	-2.8
MCM10	-2.8
SLC10A1	-2.9
PON2	-3.6
DEFB4A	-4.9
PLAC8	-6.8
LOC506943	-10.1
ADD3	-10.4
THBS1	-12.0

Fig. 13. Placental transcriptome profile differences between SCNT and IVP embryo-derived pregnancies. *A*: heat map showing the gene expression patterns within and between biological replicates. *B*: list and FC of annotated differentially expressed genes. Positive and negative numbers indicate UR and DR, respectively, in SCNT compared with IVP.

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Table 3. *Array and qPCR results for selected differentially expressed genes in different comparisons*

Gene Name	Groups	Array Result		qPCR Result	
		FC	*P* value	FC	*P* value
CDH11	SCNT vs. AI	−18.7	0.002	−3.7	0.01
	SCNT vs. FB	−13,6	< 0.000.	−139	0.02
CTSS	SCNT vs. AI	−10.9	< 0.0001	−3.9	0.01
	IVP vs. AI	−6.8	0.0002	−4.5	0.03
C4A5	SCNT vs. AI	−5.0	< 0.0001	−2.7	0.3
HSPB3	SCNT vs. AI	3.4	< 0.0001	4.5	0.05
	SCNT vs. IVP	2.6	< 0.0001	5.4	0.02
	SCNT vs. FB	5.1	< 0.0001	2562	0.01
IGFB7	SCNT vs. AI	−9.7	0.0002	−3.0	0.02
NAA25	SCNT vs. AI	5.8	0.001	1.57	0.32
PDGFRL	SCNT vs. AI	−8.4	< 0.0001	−3.3	0.04
	IVP vs. AI	−4.4	0.0009	−2.7	0.19
PRDX6	SCNT vs. AI	−4.2	0.0002	−25.9	0.17
PGCP	IVP vs. AI	9.1	< 0.0001	9.2	<0.001
SUN2	SCNT vs. AI	2.2	0.0004	2.4	0.01
	SCNT vs. IVP	2.4	0.0001	4.2	0.009
CTSH	SCNT vs. AI	−9.3	0.0017	−3.4	0.029
TNC	SCNT vs. AI	−38.3	< 0.0001	−12.1	0.03
	IVP vs. AI	−40	< 0.0001	−25.9	0.02
	SCNT vs. FB	−222	< 0.0001	−367	0.01
ADD3	SCNT vs. IVP	−10.4	< 0.0001	−2.9	0.01
IGFB6	SCNT vs. AI	−5.5	0.005	−2.7	0.012
GPLD1	IVP vs. AI	2.27	0.0003	3.2	0.43
PAG6	SCNT vs. FB	79.3	0.001	197	0.009
TET2	SCNT vs. AI	2.65	0.0001	9.5	0.03
	IVP vs. AI	2.21	0.0007	9.7	0.0002
	SCNT vs. FB	4.5	< 0.0001	10.84	0.005
BAG3	SCNT vs. AI	−3.2	0.0001	1.3	0.56
	IVP vs. AI	−3.5	< 0.0001	−2.4	0.3
	SCNT vs. FB	−7.8	< 0.0001	−3.2	<0.001
TET3	SCNT vs. AI	3.07	0.001	6.1	<0.001
	SCNT vs. FB	5.6	< 0.0001	10.15	<0.001
PLAC8	SCNT vs. AI	−2.65	0.038	−5.5	0.29
FAB	SCNT vs. AI	−7.8	0.013	−2.11	0.3
	SCNT vs. FB	−122.5	0.0001	−309	0.01
XIST	SCNT vs. AI	−49.9	0.0023	−55	0.05
TIPARP	IVP vs. AI	2.82	0.0001	−4.64	0.26
PAG15	SCNT vs. AI	−9.9	0.0003	−3.0	0.09

FC with negative numbers indicate downregulation in the former compared with the latter, $P \leq 0.05$ considered as significant.

for establishment of pregnancy. Moreover, other ECM-related genes, namely *INHBA*, follistatin, and TNC, are also implicated in placenta development and pregnancy establishment (3, 52, 102). Therefore, downregulation of these genes related to ECM degradation and remodeling in SCNT-derived placenta may result in faulty placenta formation and increased resistance to maternal blood flow.

The present study also identified a massive downregulation of transmembrane proteins (Fig. 2*O*). The exact function of this gene cluster in cellular development is unknown. Gene network analysis indicated among the 16 transmembrane proteins, TMEM43 and TMEM165 to be the central genes of the network (Fig. 2), suggesting these molecules could be more relevant than other transmembrane proteins. Previous findings also showed mutation in TMEM43 (LUMA) to be associated with nuclear membrane malfunction (55) and lethality (63) in humans. TMEM43 (LUMA) is a highly conserved protein integral membrane protein that is believed to function as a tetraspanin-like membrane organizer in organizing protein (9). Similarly, the solute carriers (*SLC35A3, SLC31A2, SLC25A6, SLC25A5, SLC1A5,* and *SLC16A1*), which are also integral membrane genes, were downregulated in SCNT placenta. A previous study in our lab (83) also showed the association of SLC with endometrial receptivity as most of the gene in this cluster were found to be enriched in bovine endometrium that resulted in pregnancy and calf delivery after transfer of in vivo-derived embryos. Solute carriers (SLC) are required for transporting of macromolecules within and between cells. For instance, among the differentially expressed SLC, SLC35A3 is required for nucleoside transport (42), SLC31A2 is involved in transporting copper ion (73), SLC25A5 and SLC25A6 are essential for transporting of phosphate and ADP/ATP (71), SLC1A5 is involved in glutamate and neutral amino acid transport (47), whereas SLC16A1 is essential for acetate and propionate transport (34). Thus, downregulation of the transmembrane protein and solute carriers in SCNT-derived placenta may be associated with inefficient trafficking of macromolecules, which consequently affects placenta and fetal development.

In addition to metabolite carrier proteins, the SCNT-derived placenta also displayed underexpression of sorting nexins (*SNX9, SNX7, SNX31, SNX3,* and *SNX10*) (Fig. 2*N*). Sorting nexins are cytoplasmic and membrane-associated proteins believed to be involved in endocytosis, protein trafficking (113),

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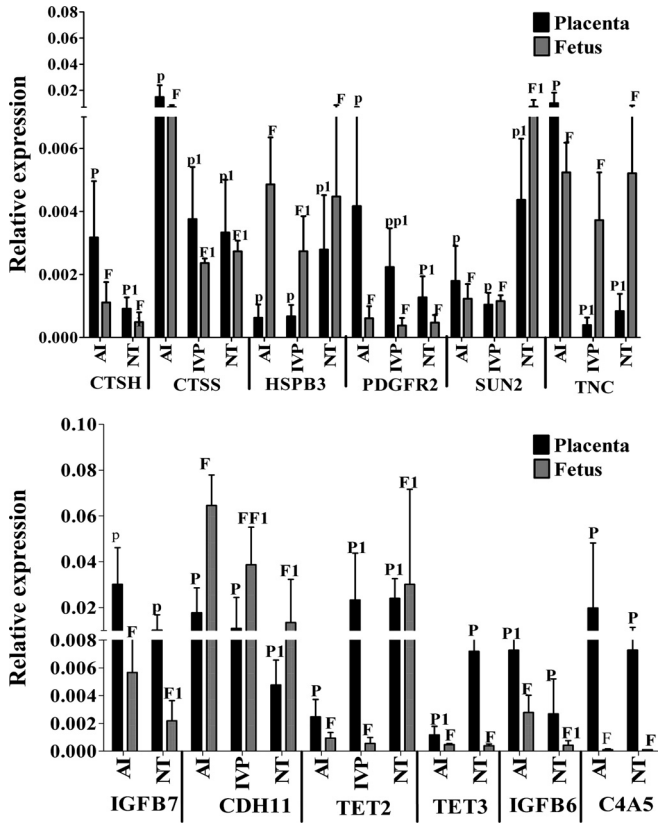


Fig. 14. The expression pattern of DEGs in placental and corresponding fetal samples. P, P1 indicate the significant differences ($P < 0.05$) between the placental samples derived from different source of pregnancies, whereas F, F1 indicate significant differences in the corresponding fetal samples.

and to-term embryo development (86). For example, *SNX3* is essential for membrane trafficking from the early to the recycling endosome (113) and development and function of the central nervous system (66). Similarly, SNX7 is implicated in liver development during embryogenesis (115), whereas SNX9 is reported to be involved in actin remodeling (58), clathrin-mediated endocytosis (97), and membrane remodeling (90). Moreover, literature mining showed that SNX10 is required for osteoclast differentiation (120), left-right patterning of visceral organs (17), and the formation of giant vacuoles in mammalian cells (76). Thus, downregulation of sorting nexins in SCNT-derived placenta may cause an abnormal pattern of organ, faulty actin and membrane remodeling, and malfunction of sorting and trafficking membrane proteins. This was further evidenced as prostaglandins [prostaglandin E synthase (PTGES/PGES), prostaglandin D2 synthase, (PTGDS/ PGD2), and prostaglandin E receptor 4 (PTGER4/EP4)] were downrgulated in SCNT-derived placenta compared with the AI groups (Fig. 2*L*). Prostaglandins are required for normal gestation, homeostasis, and immunity (98, 106). Hence underexpression of prostaglandins genes may cause abnormal pregnancy or termination of pregnancy at any time during the gestation period. For instance, downregulation of PTGES was associated with defects in embryo gastrulation (98), and it is required for the production of PGD2, PGE2, and PGF2 during placental development (74). Similarly, PTGDS has been implicated in the maintenance of pregnancy (82), placenta vasoconstriction (61), and conversion of PGH_2 to PGE_2 (77). Thus, downregulation of prostaglandins in SCNT-derived placenta may in part be associated with cytokines and immune system dysregulation and postnatal difficulties.

One of the limitations in SCNT-derived pregnancies is the ability to use energy and to transport of ions including calcium ions. In normal pregnancies, transport of calcium across the placenta tissue requires energy (87), and a higher concentration of calcium in the fetal circulation may indicate the presence of calcium transport mechanisms to the fetal-facing membrane (27). In line with this, in the current study, ATPases (Fig. 7*C*), which are associated with ATP production and calcium ion transportation, were downregulated in SCNT-derived placenta. A knock-down study on ATP6V1A showed growth retardation, trunk deformation, and loss of internal Ca^{2+} and Na^{+} (39). Similarly, deletion of ATP6AP2 was associated with developmental alterations and early embryonic lethality (91). In addition, downregulation of the ATPases was accompanied by downregulation of cytochrome c oxidases (*COX7A2L, COX7A1,* and *COX6A1*) (Fig. 2*F*), which may further downregulate the mechanism of dioxygen reduction and electron transfer catalyzation (79). Moreover, downregulation of cytochrome oxidases may affect the rate of mitochondrial protons translocation, resulting in lower ATP production in SCNT-derived placenta.

Conclusion

The present study provides significant mechanistic insights into placental transcriptome dysregulation in SCNT- and IVP-derived pregnancies at *day 50* of gestation, using AI groups as a reference control. Our data evidenced a remarkable transcriptome alteration in both SCNT- and IVP-derived placentas. However, gene expression dysregulation was stronger in SCNT than IVP groups. This higher transcriptome profile dysregulation in the SCNT-derived placenta followed by IVP would suggest the degree of placental abnormalities in SCNT- and IVP-derived pregnancies at *day 50* of the gestation period. Moreover, this study has shown that ~9% of the dysregulated genes in SCNT-derived placenta were associated with the transcriptomic reprogramming errors. In addition, the present study has identified dysregulated genes and molecular signatures that were common to both SCNT- and IVP-derived

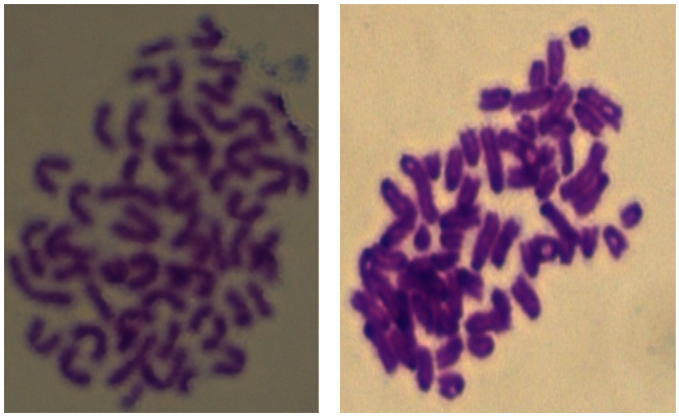


Fig. 15. The chromosomal spreading of the donor cells. Representative pictures of the chromosomal spreading taken from individual cells under ×1,000 magnification.

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placenta. However, the SCNT placenta displayed alterations of several gene and molecular pathways that were not shared by the IVP groups. In addition, the current results present a detail list of developmentally relevant genes that can be used as candidate genes for further investigation of abnormal pregnancies and pregnancy failures in SCNT- and IVP-derived pregnancies in cattle. However, we believe that the results presented here are accompanied by logical speculation, and hence additional in-depth functional studies will be mandatory to reach concrete conclusions. This study contributes extensive and valuable information regarding the placental transcriptome profile changes in bovine SCNT- and IVP derived-pregnancies, which could be associated with the degree of placental abnormality at *day 50* of the gestation period.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.S.-W., M. Hossain, E.H., F.R., and M. Hoelker performed experiments; D.S.-W. and E.T. analyzed data; D.S.-W., D.T., E.T., C.L., and M. Hoelker interpreted results of experiments; D.S.-W. prepared figures; D.S.-W. and D.T. drafted manuscript; D.T., K.S., and M. Hoelker conception and design of research; D.T., M. Hossain, E.T., C.L., K.S., and M. Hoelker edited and revised manuscript; K.S. and M. Hoelker approved final version of manuscript.

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