Endometrium as an early sensor of in vitro embryo manipulation technologies

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Implantation is crucial for placental development that will subsequently impact fetal growth and pregnancy success with consequences on postnatal health. We postulated that the pattern of genes expressed by the endometrium when the embryo becomes attached to the mother uterus could account for the final outcome of a pregnancy. As a model, we used the bovine species where the embryo becomes progressively and permanently attached to the endometrium from day 20 of gestation onwards. At that stage, we compared the endometrial genes profiles in the presence of an in vivo fertilized embryo (AI) with the endometrial patterns obtained in the presence of nuclear transfer (SCNT) or in vitro fertilized embryos (IVF), both displaying lower and different potentials for term development. Our data provide evidence that the endometrium can be considered as a biological sensor able to fine-tune its physiology in response to the presence of embryos whose development will become altered much later after the implantation process. Compared with AI, numerous biological functions and several canonical pathways with a major impact on metabolism and immune function were found to be significantly altered in the endometrium of SCNT pregnancies at implantation, whereas the differences were less pronounced with IVF embryos. Determining the limits of the endometrial plasticity at the onset of implantation should bring new insights on the contribution of the maternal environment to the development of an embryo and the success of pregnancy.

bovine | implantation | microarray | nuclear transfer

In mammals, the implanting embryo establishes permanent physical connections with the endometrium through a fine-tuned and synchronized cross-talk necessary to support the feto-placental development and health throughout gestation (1, 2). Recent findings have demonstrated that perturbations of the maternal physiology during the peri-conceptional period (e.g., maternal diet) lead to impaired health during adulthood (3). Modifications of preimplantation embryo conditions, using assisted reproductive technologies (ART) or somatic cell nuclear transfer (SCNT) have been associated to developmental abnormalities and postnatal consequences such as the large offspring syndrome in animals (LOS) (4–6). Hence, very early alterations of maternal or embryo environment may affect the quality of the embryo-endometrium dialogue that subsequently lead to pregnancy failure or affect pregnancy with postnatal detrimental consequences.

To clarify the sequence of events leading to these observations, high-throughput analyses have been undertaken and they have revealed altered or aberrant gene expression profiles in bovine embryos and placentomes derived from ART or SCNT pregnancies (7–10). Studies have thus focused on embryo or placenta but they have neglected the endometrium whereas the contribution of this microenvironment is decisive at implantation. Using cattle as an animal model, we have postulated that embryo manipulations may affect the endometrium physiology very early. Using pregnancies with different final issues, we tested our hypothesis and analyzed gene profiles at the very early steps of implantation. In ruminants, the structure of the endometrium differs from other mammalian species with the presence of caruncles (C) and intercaruncular areas (IC), both being essential for supporting pregnancy. Our recent transcriptome data have shown that analyzing these 2 areas is necessary for a better understanding of the implantation process (11). In the current study, gene expression profiles demonstrated the differential physiological response of the endometrium in IVF or SCNT pregnancies, with a profound impact on several biological functions including metabolism and immune response in SCNT pregnant animals. We propose the endometrium as an early biological sensor of pregnancy, whose limits in plasticity remain to be determined. We suggest that early alterations of endometrial physiology can in turn affect the development of the conceptus therefore taking part to the final success of pregnancy.

Results

Gene Expression Profiling of Caruncles (C) or Intercaruncular areas (IC) in AI Compared with SCNT or IVF-ET Pregnancies. Using a 13,257 elements bovine oligoarray and a dye-switch loop design, gene expression patterns were established in either C or IC areas by comparing IVF-ET or SCNT pregnancies with AI (total number of microarrays: 40). Only the females with morphologically normal embryos were considered for analyses at day 20 of pregnancy. Each AI pregnant animal had 1 embryo and each SCNT pregnant female had 2 embryos. In IVF-ET pregnancies, 3 females had 2 embryos and 2 females had 1 single embryo. The number of embryos did not affect the global expression profiles in this type of pregnancy. When the FDR threshold was set at 0.05, only 9 differentially expressed genes (DEG) were found in the IVF-ET vs. AI comparison in the C areas and none in the IC. When the FDR threshold was set at 0.20, 118 DEG were found in the C areas that were classified into biological functions (Table S1) and only 5 DEG in the [IC] areas.

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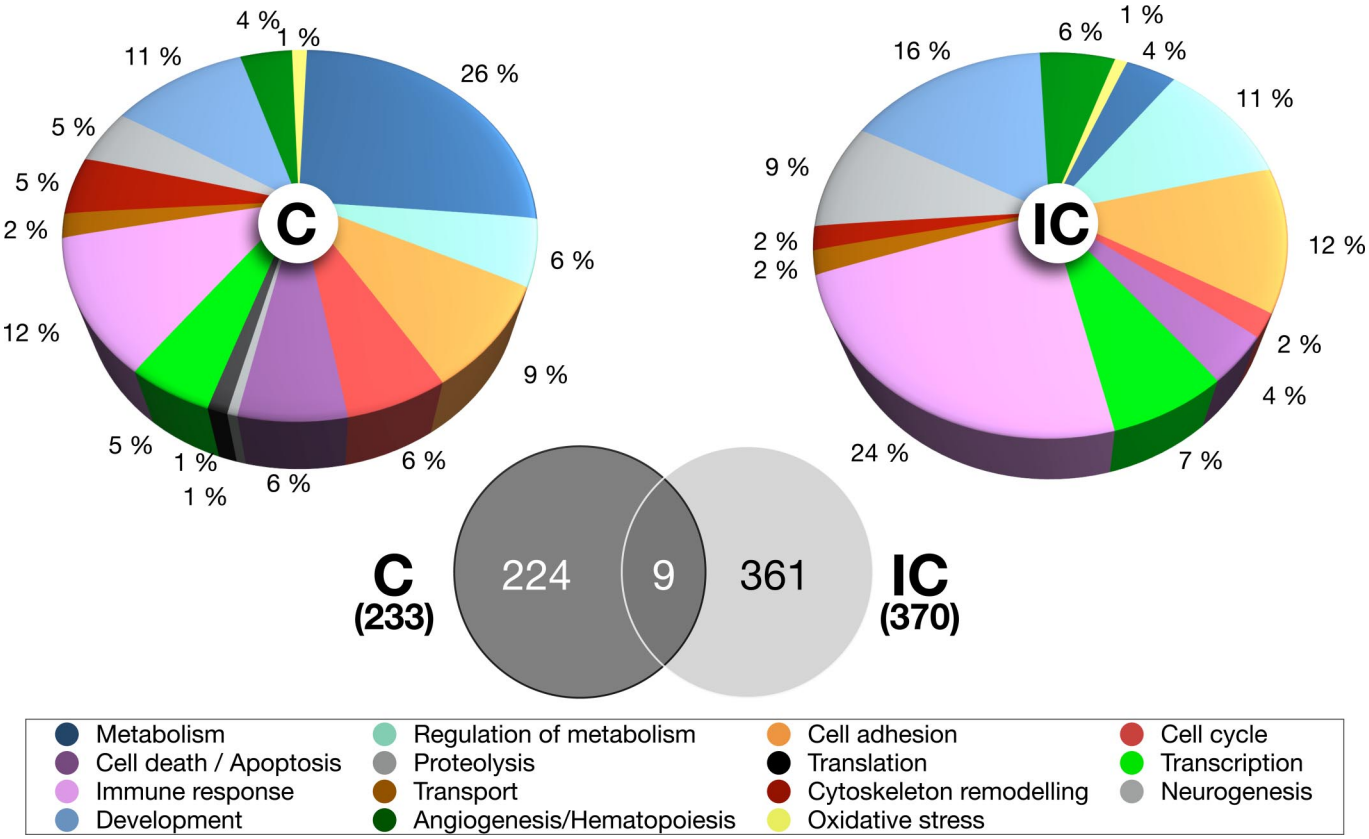

Fig. 1. Analysis of the bovine microarray data for the AI versus SCNT comparison in endometrial caruncles [C] and intercaruncular [IC] areas at implantation. (*Upper*) Gene Ontology classification of the AI versus SCNT differentially expressed genes (DEG) in [C] or [IC] areas. Multiple naming is possible. (*Lower*) Venn diagram showing the AI versus SCNT DEG common or specific to [C] or [IC] endometrial areas. The total number of DEG for C or IC is indicated in parentheses.

In the SCNT vs. AI comparison, 233 DEG were found in the C areas (first 100 DEG described in Table S2). Sixty-five genes displayed a differential expression of 2-fold or greater. In SCNT pregnancies, 112 DEG were up-regulated as *TKDP3* (11.24-fold), *TKDP1* (9.39-fold), *TKDP4* (7.46-fold) and *PAG12* (3.78-fold). In AI pregnancies, 121 genes were up-regulated as *ADAMDEC1* (5.83-fold), *BOLA-DQA5* (5.78-fold), *BOLA-DQB1* (3.66-fold) and *IL6R* (3.38-fold). Ingenuity pathway analysis (IPA) showed 22 biological function categories with 5 genes or more signicantly regulated ($P < 0.01$; Table S3) including "oxidative phosphorylation," "mitochondrial dysfunction," "ubiquinone biosynthesis," "antigen presentation pathway," "fructose and mannose metabolism" and "purine metabolism." Six canonical pathways with 3 genes or more were significantly regulated ($P < 0.05$; Table S4).

In the IC areas, the analyses of the SCNT vs. AI comparison showed 370 DEG with 188 genes displaying a differential expression of 2-fold or greater (first 100 DEG described in Table S2). In AI pregnancies, 142 genes were found to be up-regulated such as *JSP.1* (29.34-fold), *F9* (19.51-fold) and *ZNF347* (10.36-fold) whereas 230 genes were found to be up-regulated in SCNT pregnancies including *C11ORF34* (36.48-fold), *FABP3* (23.40-fold), *MGC155145* (15.99-fold). The distribution of the DEG showed 33 IPA Bio functions with 5 terms or more significantly regulated ($P \leq 0.01$; Table S3) including "cell-to-cell signaling and interaction," "cellular movement," "cellular growth and proliferation," "cellular assembly and organization" and "cell death." Twenty canonical pathways were found significant ($P < 0.05$; Table S4) including "tight junction signaling," "hepatic fibrosis/hepatic stellate cell activation," "acute phase response signaling" or "neuregulin signaling." Only 9 genes were found to be common between the C and the IC expression profiles in the SCNT vs. AI comparison.

The DEG found in the C or the IC expression profiles were classified according to their biological functions, using GeneGo Metacore software (Fig. 1). "Metabolism" was the most represented biological function in the C areas (26%) whereas in the IC areas only 4% of DEG was classified into this biological function. The most affected class in the IC areas was immune response (24%) whereas it reached only 12% in the C areas.

Expression Profiles of Caruncles (C) Versus Intercaruncular Areas (IC) in AI, IVF-ET, or SCNT Bovine Pregnancies at Implantation. To uncover subtle differences in the endometrium reaction as recently published, the C vs. IC expression profiles were analyzed in AI, IVF-ET and SCNT pregnancies at implantation. A total of 453 DEG (84 DEG >2-fold), 595 DEG (127 DEG >2-fold), and 478 DEG (287 DEG >2-fold) were found for AI, IVF-ET, and SCNT pregnancies, respectively (Fig. 2; first 100 DEG described in Table S5). The comparison between AI, IVF-ET and SCNT pregnancies, using a Venn diagram indicated that 40% (239 genes) and 47% (225 genes) of the DEG in IVF-ET and SCNT pregnancies were shared with AI pregnancies. A total of 153 DEG were found to be common between AI, IVF-ET and SCNT pregnancies.

The DEG were then classified according to their biological functions, using the GeneGo Metacore software (Fig. 2). Every biological function was represented in each type of pregnancy (AI, IVF-ET and SCNT) with a similar percentage except "metabolism" (8% in IVF-ET vs. 16% and 18% in AI and SCNT respectively), "immune function" (3% in SCNT vs. 18% in AI and 16% IVF-ET),), "neurogenesis" (3% in SCNT vs. 8% in AI

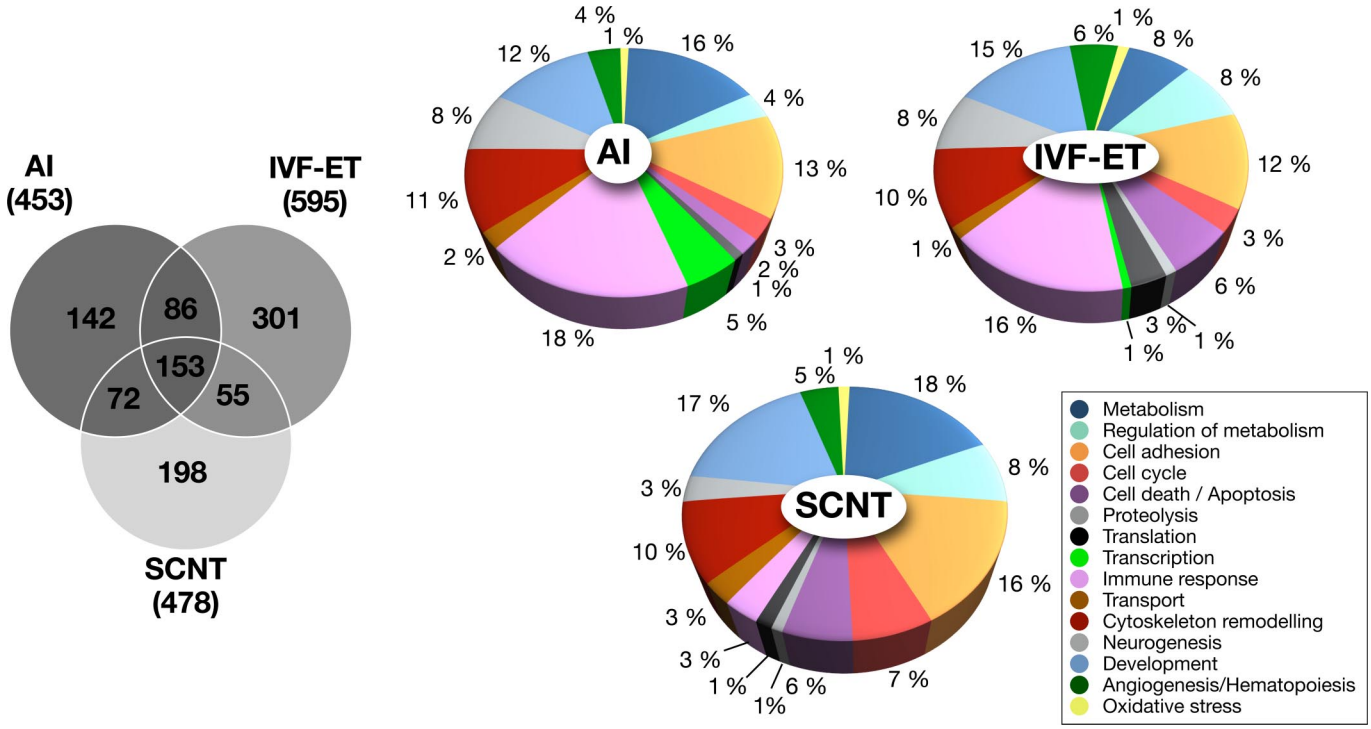

Fig. 2. Analysis of the bovine microarray data for the endometrial caruncles [C] versus intercaruncular areas [IC] paired comparisons in AI, IVF-ET and SCNT pregnancies at implantation. (*Left*) Venn diagram showing the C vs. IC DEG common or specific to AI, IVF-ET and SCNT pregnancies. The total number of DEG for AI, IVF-ET, and SCNT is indicated in parentheses (*Right*) Gene Ontology classification of the C versus IC differentially expressed genes (DEG) in AI, IVF-ET and SCNT pregnancies. Multiple naming is possible.

or IV-ET) and "cell cycle" (7% in SCNT vs. 3% in AI or IVF-ET). Among the C vs. IC DEG specifically found in AI (n = 142) or SCNT (n = 198) pregnancies, 60% of the DEG were related to "metabolism," "regulation of metabolism" (40%) and "immune response" (20%).

The C vs. IC DEG were classified into IPA Bio-functions (Table S6) and IPA canonical pathways (Table S7). In the AI pregnancies, 38 IPA biological function terms revealed significant association for 5 genes or more ($P \leq 0.01$). Eleven canonical pathways with 3 genes or more were significantly regulated ($P \leq 0.05$): dopamine receptor signaling, complement system, IGF1 signaling, Wnt/β-catenin signaling, chemokine signaling. In the IVF-ET pregnancies, 36 IPA biological function terms with 5 genes or more were found with a significant association ($P \leq 0.01$). Thirteen canonical pathways with 3 genes or more ($P \leq 0.05$) were affected, including IGF1 signaling, PI3K/AKT signaling, Wnt/β-catenin signaling, dopamine receptor signaling and VDR/RXR activation. The C vs. IC comparison in SCNT pregnancies showed 51 IPA biological function terms with a significant association of 5 genes or more ($P \leq 0.01$). Six canonical pathways with 3 genes or more ($P < 0.05$) were affected: *Wnt*/β-catenin signaling, hepatic fibrosis/hepatic cell activation, neuregulin signaling, IGF-1 signaling, complement system and VDR/RXR activation.

Validation of Gene Expression, Using Quantitative RT- PCR. To validate our microarray results, the differential expression of 16 DEG was confirmed by quantitative real-time RT-PCR. The genes were selected in the "cell-to-cell signaling," "cellular growth and proliferation," "immunity" and "molecular transport" biological functions. Seven genes (*C11ORF34*, *EPAS1*, *FABP3*, *FBP1*, *JSP.1*, *PLAC8*, and *RSAD2*) and 4 genes (*CALB3*, *FABP3*, *FBP1*, and *JSP.1*) were selected for the AI vs. SCNT comparison and the AI vs. IVF-ET comparison respectively (Table 1). Twelve genes were selected for the C vs. IC expression profiles (Table 2). On the 34 pairwise comparisons, the RT-PCR quantification confirmed the differential gene expression determined by microarrays for 31 comparisons.

Discussion

Expression profiling analyses have recently reported major changes in the endometrium of rodents and ruminants upon pregnancy (12, 13) or by comparing the impact of a mechanic stimulus with postimplantation embryos (14, 15). Our present microarray data show that the endometrium can react early and specifically to embryo manipulations associated with different potentials of term development. Because of the animal experimental design we used, the endometrial reaction integrated the impact of the conceptus and the various experimental steps that differ between an inseminated female and animals transferred with IVF or SCNT embryos at day 7. When FDR was set to 5%, the number of DEG in the C area was very limited in the AI vs. IVF-ET comparison but it reached several hundred genes in the AI vs. SCNT one. At day 7 of development, Smith et al. (7) reported a low variability of SCNT embryos transcriptome and a higher variation among IVF embryos. This is consistent with our observations because the variability of IVF embryo expression profiles is expected to be correlated with a more variable endometrial reaction. The C vs. IC paired comparison within each animal yielded 3 distinct profiles in AI, IVF-ET or SCNT pregnancies and was very informative for each type of pregnancy including IVF-ET.

In our findings, the physiology of the endometrium was affected qualitatively (biological functions) and quantitatively (gene fold expression) in SCNT pregnant animals when compared with control pregnancies. The identified DEG were distributed in every biological function but "metabolism and regulation of metabolism" and "immune response" appeared more specifically altered in the

Table 1. Quantification of mRNA levels by real-time RT-PCR for genes selected from the caruncles [C] or the intercaruncular areas [IC] expression profiles for the AI *versus* IVF-ET or AI *versus* SCNT pregnancies

Comparison	Gene symbol	Unigene ID	Endometrial area	Microarray fold Δ	Real-time fold Δ	Expression
AI versus IVF-ET	*CALB3*	Bt.390	C	10	2*	IVF-ET
	FABP3	Hs.584756	C	6	1.5*	AI
	FBP1	Hs.494496	C	2.6	100***	AI
	JSP.1	Hs.181244	C	32	1.8	AI
AI versus SCNT	*C11ORF34*	Hs.632097	IC	3890	10***	SCNT
	EPAS1	Bt.45570	IC	47	2*	SCNT
	FABP3	Hs.584756	IC	1412	10***	SCNT
	FBP1	Hs.494496	C	14	9*	SCNT
			IC	22	5*	SCNT
	JSP.1	Hs.181244	IC	2344	7*	AI
	PLAC8	Hs.546392	IC	12	8*	AI
	RSAD2	Hs.17518	IC	151	1. 5*	SCNT

Fold change is expressed in arbitrary units. RPL19 was used as a housekeeping gene. Data are means ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

C and the IC areas, respectively. We found numerous genes involved in ATP production to be altered in SCNT endometrium (e.g., mitochondrial ATP synthases) and cytochrome C oxydases, NADH deshydrogenases and ubiquinol-cytochrome *c* reductases. Furthermore, we observed the up-regulation of glucogenesis enzymes (e.g., *FBP1*) and of several transporters (*SLC2A1*, *SLC35A2*, *FABP3*) in SCNT pregnancies. Some of these genes were also found to be similarly regulated in the IVF-ET pregnancies when compared with the AI ones. Altogether these various factors are involved in the uptake, intracellular metabolism and/or transport of substrates (glucose, mannose, fructose and fatty acid) that will take place in the endometrium at implantation. Because 4 embryos were transferred back to recipient mothers, the higher metabolism detected in the endometrium of IVF-ET or SCNT females could be required to adjust the remodeling of the endometrial tissue to this sudden high number of concepti. In the same time, the higher metabolism and molecular transport in the endometrium requires more energy and affects the substrate homeostasis (e.g., glucose) and the oxidative status at the conceptus-endometrium interface that might become suboptimal for the development of the embryonic annexes then the placentation.

Early failures of pregnancy are associated with placental development abnormalities marked by an unusual vascularisation of extra embryonic tissue (16) and a decreased number of placentomes that are also larger (5). In cattle, placentomes result from the superficial interactions between the endometrial caruncles and the fetal cotyledons (17), whereas the intercaruncular areas are the main source of histotroph (18). Therefore, identifying endometrial genes regulated and involved during early placentation could help in understanding later placental abnormalities. As an illustration, *EPAS1* or *CALB3* were found to be up-regulated in the IC and the C areas of SCNT recipient cows respectively. *EPAS1* (also known as HIF-2 α) is a transcription factor participating to the cellular response to hypoxia, stabilizing the vascular maturation in human angiogenesis (19) and increased with preeclampsia in women endometrium (20). The role of *EPAS1* in the bovine placentation is not yet established but *EPAS1* up-regulation in SCNT pregnancies is in keeping with the increase of *HIF-1*α recently reported in bovine SCNT pregnancies at day 30 (21). Because the success of pregnancy depends on the success of vascularisation in caruncles and placental development, a deregulation of angiogenesis-related factors could contribute to the placental insufficiency reported in SCNT pregnancy. CALB3 is a vitamin D dependant calcium binding protein, found to be localized in maternal caruncular epithelium and trophoblastic binucleated cells of the bovine placenta (22). In every species examined so far, levels of CALB3 expression are high before implantation (23). The increase in the Ca^{2+} mobilization at implantation could perturb the trophoblast adhesion with subsequent consequences on placentation. In addition, our analyses revealed the up-regulation of *PLAC8* and *C11ORF34* in the IC areas of SCNT

Table 2. Quantification of mRNA levels by real-time RT-PCR for genes selected from the caruncles [C] versus intercaruncular areas [IC] expression profiles in the AI, IVF-ET, or SCNT pregnancies

		Caruncles (C) *versus* intercaruncular (IC) areas								
		AI			IVF-ET			SCNT		
Gene symbol	Unigene ID	Microarray fold Δ	Area	PCR fold Δ	Microarray fold Δ*	Area	PCR fold Δ	Microarray fold Δ*	Area	PCR fold Δ
C11ORF34	Hs.632097	3.0	C	2.5*						
CALB3	Bt.390	3.5	IC	4.0*	2.8	IC	1.6*	3.6	IC	2.0*
GJA1	Hs.74471	3.3	C	6.2*	6.7	C	5.2*	5.1	C	3.6*
DECORIN	Hs.653117				1.7	C	2.0*	2.4	C	2.0*
FABP3	Hs.584756	3.3	C	2.0*				3.3	C	1.3
FBP1	Hs.494496	2.0	C	1.6*						
JSP.1	Hs.181244							3.7	C	3.0*
LGALS1	Hs.445351	1.6	C	1.5*	1.4	C	1.3*			
MSX1	Hs.621772	2.3	IC	2.5*	2.3	IC	3.3*	3.3	IC	1.4
MX2	Bt.8143							2.4	C	2.0*
NPY	Hs.1832				1.9	C	4.9*			
PTN	Hs.371249	3.8	C	2.2**	5.0	C	1.5**	5.1	C	1.7*

Fold change is expressed in arbitrary units. RPL19 was used as a housekeepinsg gene. Data are means ± SD. *, $P < 0.05$; **, $P < 0.01$.

pregnancies. Both genes were first reported to be placenta-specific (24, 25) but our recent data have clearly indicated their expression in the endometrium at implantation including a very intense staining for *C11ORF34* in the luminal epithelium (11). The biological functions of the orphan *PLAC8* or *C11ORF34* proteins are still undetermined but we assume they are involved in the endometrium-trophoblast interactions. The set of genes described in this section clearly deserve further studies to better understand their contribution to early development of placenta.

In the IC areas of SCNT pregnancies, IPA analyses underlined "cell-to-cell signaling and interaction" and "tight junction signaling" as a highly significant biological function and the most affected canonical pathway respectively. A part of the DEG was found to be up-regulated in SCNT such as *TIMP2*, *IGFBP2*, *SERPING1*, *MUC1* and *-13*, *TNFRSF1B* and *CLDN 4*, and several integrins, such as *ITGAL* or *ITGB4*. In sheep, the increase in tight and adherence junctions at the time of implantation may be required for attachment and adherence of the trophectoderm (26). Therefore, the dysregulation of these genes could be detrimental for the later development by preventing the diffusion of histotroph required for conceptus elongation and survival.

A clear up-regulation of a set of IFN-tau (IFNT) early stimulated genes (ISGs) was noticed in SCNT pregnancies (e.g., *RSAD2*, *OAS1*) whose consequences on the progress of pregnancy are currently difficult to estimate. No significative difference in the *IFNT* transcript level could be detected between AI, IVF and SCNT individual embryos at the peri-implantation period. Nevertheless, 2 intact concepti were harvested in the SCNT pregnancies, leading to a total amount of IFNT protein likely higher than the amount produced by the single conceptus collected in AI pregnancies. The higher content of IFNT protein could thus account for the up-regulation of the ISGs, but the contribution of other factors produced by the SCNT conceptus is not ruled out and will definitely require further investigation. In SCNT pregnancies, the down-regulation of various MHC class I genes (e.g., *JSP.1*) was also detected in the IC areas but the biological meaning is unclear. Overall, these findings raised the question of the long term impact of an early deregulation of the immune function, in keeping with the marked increase of lymphocytes previously reported in the endometrial stroma of SCNT surrogate dams (27). The inadequate or at least altered maternal immune response in pregnant animals involving in vitro manipulated embryos would obviously represent an obstacle for a normal placental development and fetal survival (28). Thorough analyses of the conceptus gene profiles will be required to determine how genome modifications, including genome remodeling upon SCNT procedure, may influence the endometrial immunity.

Collectively, our data showed that the endometrial remodeling occurring at implantation differently affects the C and the IC areas in pregnancies with different potentials of embryo term development. Although additional studies will be necessary to correlate the variation of several hundred genes with pregnancy outcome, our current findings indicate a qualitative and quantitative alteration of major biological functions (e.g., metabolism, immune function), canonical pathways (e.g., oxidative phosphorylation) and expression of individual genes in both C and IC areas of SCNT or IVF-ET compared with the AI control pregnancies. The sum of these modifications reflects the adaptation of the endometrium to the type of pregnancy. It has been proposed that placenta could act as a nutrient sensor according to resources available in the maternal supply line (29) and, in return, could respond to fetal demands (30). We propose that endometrium functions as a very early biological sensor that can fine-tune its physiology in response to embryo or maternal manipulations. This first study strongly suggests that selected endometrial factors could be used as potential biomarkers for discriminating correct from pathological pregnancies. In the future, a major objective will be to determine the degree of the endometrial plasticity and sensing and the limits of its robustness in supporting the development of an embryo. This aspect will require the simultaneous analyses of individual concepti from various origins (AI, IVF or SCNT) and of the recipient endometrium to unravel the contribution of the maternal compartment to the development of the embryo, the issue of pregnancy, and postnatal life.

Materials and Methods

Generation of Pregnant Animals and Sample Collection. All 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.

Cyclic bovine females (Charolais or Holstein) were synchronized by the Crestar method. Artificial insemination (AI) was performed as described in ref. 7. The IVF-ET and SCNT embryos were produced using abattoir oocytes and cultured under the same conditions as reported (31–33). Cultured skin fibroblast cells from the 5,538 genotype (32) were used to derive the SCNT embryos. This genotype leads to clone term development with a 71% pregnancy rate at day 21, similar to IVF-ET but with a 19% pregnancy rate at delivery (34). At day 7, International Embryo Transfer Society quality grades 1 and 2 blastocysts were transferred to the ipsilateral uterine horn to the corpus luteum for IVF-ET or SCNT pregnancies (4 blastocysts per recipient). The day of oestrous was considered as day 0. At day 20 of pregnancy, animals (15 AI, 15 IVF-ET, and 10 SCNT pregnancies) were killed, the uteri were flushed and when present, concepti were observed by microscopy to determine the stage of development (35). Pregnant females whose embryos displayed the expected and correct morphology were selected for subsequent microarray analyses. Endometrial caruncular (C) and intercaruncular (IC) areas were sampled from the uterine horns ipsilateral to the corpus luteum.

Microarray Design and Annotation. A 13,257-element bovine oligonucleotides array (National Center for Biotechnology Information Gene Expression Omnibus accession no. GPL2853) was used to determine the transcript profiles (36). The 70-mer oligonucleotides were selected from ESTs obtained from a placenta, a spleen cDNA libraries (37) and cDNA libraries created from fetal, extra embryonic and endometrial tissues (National Center for Biotechnology Information libraries 15575, 15980, 15992, 15993, 17811, and 16609). The annotation for each selected sequence was updated using human Unigene (build 201), mouse Unigene (build 162), and bovine Unigene (build 83) databases.

Probe Labeling and Microarray hybridizations. Twenty micrograms of RNA samples were converted into aminoallyl-labeled cDNA labeled with a fluorescent dye (Cy5 or Cy3) then hybridized to each microarray. For the AI vs. SCNT -4 AI pregnant animals, 5 SCNT pregnant animals—or the AI vs. IVF-ET -5 AI pregnant animals, 5 IVF-ET pregnant animals—comparisons, the genes profiles were established in the C areas independently from the IC areas, using a dye-switch loop design (38). A total of 40 microarrays were hybridized for this experiment. For the C versus IC paired comparisons, gene expression profiling was established between the C and the IC endometrial areas sampled from the same pregnant animal, using a dye-swap loop design. Five animals were used for each type of pregnancy (AI, IVF-ET or SCNT). A total of 30 microarrays were hybridized for this experiment. The experimental design is summarized in Fig. S1.

After stringency washes to remove unbound cDNA, slides were scanned using a GenePix 4000B scanner (Axon) and features were analyzed with GenePix Pro Version 4.0 software (Axon). The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE14638).

Data Analysis. Data were normalized by a global loess regression (39) followed by a subtraction of the median by block and analyzed using a mixture model that identifies clusters of genes with equal variance (40), using the anapuce R package (www.r-project.org). Statistical significant raw *p*-values were adjusted for multiple comparisons, using the Benjamini–Hochberg procedure that controls the False Discovery Rate (FDR) (41). The level of statistical significance was set at 0.05 for all of the comparisons except for the IVF-ET vs. AI comparison in the C and IC areas where it was set at 0.20.

To facilitate data mining and to analyze the Differentially Expressed Genes (DEG), Metacore (GeneGo, http://trials.genego.com; version 4.5, build 11165) and Ingenuity Pathway Assist (IPA) (www.ingenuity.com; release5.5) softwares were used.

Gene Expression Analysis by Real Time RT-PCR. The primers design (Table S8) and the real-time RT-PCR quantification were carried out as recently described (11), using RNA from the same animals analyzed in microarrays. Real time RT-PCR was performed using the SYBR Green PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems). Values were normalized to the relative amount of RPL19 mRNA. To assess for differential expression, duplicate data were pooled and a mixed model was run on the qPCR values. Genes were declared differentially expressed if the mixed model P value was <0.05.

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