Massive dysregulation of genes involved in cell signaling and placental development in cloned cattle conceptus and maternal endometrium

Fernando H. Biase[a,1], Chanaka Rabel[b], Michel Guillomot[c], Isabelle Hue[c], Kalista Andropolis[b], Colleen A. Olmstead[b], Rosane Oliveira[b], Richard Wallace[b], Daniel Le Bourhis[c], Christophe Richard[c,d], Evelyne Campion[c], Aurélie Chaulot-Talmon[c], Corinne Giraud-Delville[c], Géraldine Taghouti[c], Hélène Jammes[c], Jean-Paul Renard[c], Olivier Sandra[c], and Harris A. Lewin[a,b,e,2]

[a]Institute for Genomic Biology, University of Illinois at Urbana–Champaign, Urbana, IL 61820; [b]Department of Animal Sciences, University of Illinois at Urbana–Champaign, Urbana, IL 61820; [c]UMR Biologie du Développement et Reproduction (BDR), Institut National de la Recherche Agronomique (INRA), École Nationale Vétérinaire d'Alford (ENVA), Université Paris Saclay, 78350 Jouy en Josas, France; [d]Unité Commune d'Expérimentation Animale de Bressonvilliers (UCEA), INRA, 91030 Leudeville, France; and [e]Department of Evolution and Ecology and The Genome Center, University of California, Davis, CA 95616

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2012.

Contributed by Harris A. Lewin, November 7, 2016 (sent for review October 23, 2015; reviewed by Douglas F. Antczak and Thomas E. Spencer)

A major unresolved issue in the cloning of mammals by somatic cell nuclear transfer (SCNT) is the mechanism by which the process fails after embryos are transferred to the uterus of recipients before or during the implantation window. We investigated this problem by using RNA sequencing (RNA-seq) to compare the transcriptomes in cattle conceptuses produced by SCNT and artificial insemination (AI) at day (d) 18 (preimplantation) and d 34 (postimplantation) of gestation. In addition, endometrium was profiled to identify the communication pathways that might be affected by the presence of a cloned conceptus, ultimately leading to mortality before or during the implantation window. At d 18, the effects on the transcriptome associated with SCNT were massive, involving more than 5,000 differentially expressed genes (DEGs). Among them are 121 genes that have embryonic lethal phenotypes in mice, cause defects in trophoblast and placental development, and/or affect conceptus survival in mice. In endometria at d 18, <0.4% of expressed genes were affected by the presence of a cloned conceptus, whereas at d 34, ∼36% and <0.7% of genes were differentially expressed in intercaruncular and caruncular tissues, respectively. Functional analysis of DEGs in placental and endometrial tissues suggests a major disruption of signaling between the cloned conceptus and the endometrium, particularly the intercaruncular tissue. Our results support a "bottleneck" model for cloned conceptus survival during the periimplantation period determined by gene expression levels in extraembryonic tissues and the endometrial response to altered signaling from clones.

somatic cell nuclear transfer | conceptus | placentation | conceptus–maternal communication

In cattle, as in other mammals, exquisitely orchestrated physiological changes of the conceptus and uterus are necessary for a successful pregnancy. Synchronization of the complex events at the time of implantation relies on the timed release of molecular signals from the conceptus and the endometrium. Embryo-derived IFN-τ (IFNT) is the major signal of pregnancy in cattle, preventing luteolysis and regulating the expression of genes that are responsible for promoting local changes in the endometrium to accommodate the conceptus (1–3). In females, progesterone is the major driver of endometrial changes that prepare the uterus for conceptus implantation (4, 5). In addition to IFNT and progesterone, signaling between the bovine conceptus and the endometrium is bidirectional, and involves several pathways that work concomitantly (6) for the successful establishment of pregnancy.

Independent studies have shown that the majority of embryonic losses in cattle occur during the period that spans embryo cleavage until the attachment of the blastocyst to the endometrium (7). The reasons for these losses remain unclear and likely result from several factors, including embryonic lethal genes (8, 9), environmental stressors (7), and endometrial condition (10). Cloning of cattle by somatic cell nuclear transfer (SCNT) induces perturbations in conceptus development and greatly increases the probability of developmental arrest before and after implantation (11, 12). Embryonic and fetal losses substantially reduce the efficiency of SCNT and thus present a significant limitation to the widespread application of the technology for animal improvement. However, the disrupted embryonic and fetal development of cattle clones produced by SCNT has been used as a model to elucidate the mechanisms of embryo loss, the maternal recognition of pregnancy (13, 14), and placental development (15–17). Understanding the mechanism of embryonic and fetal losses in SCNT clones is thus not only practically important but also serves as a window to understanding infertility in cattle and other mammals.

Significance

Cloning cattle by somatic cell nuclear transfer (SCNT) is an agriculturally important technology and is also used as a model system for the study of mammalian development. The SCNT process is inefficient, typically yielding fewer than 10% live offspring. The majority of losses are the result of embryonic death, failure of the implantation process, and development of a defective placenta. A critical period is the implantation window, when survival of the conceptus depends on factors including genetics, epigenetics, and the communication between conceptus and the endometrium. Our study of gene expression in cloned conceptuses and endometrial tissues during the periimplantation period enhances understanding of the mechanisms that lead to pregnancy failure in SCNT cloning. The results have wide implications for cloning of other mammals.

Author contributions: J.-P.R., O.S., and H.A.L. designed research; F.H.B., C. Rabel, M.G., I.H., K.A., C.A.O., R.O., R.W., D.L.B., C. Richard, E.C., A.C.-T., C.G.-D., G.T., H.J., and O.S. performed research; F.H.B. analyzed data; and F.H.B. and H.A.L. wrote the paper.

Reviewers: D.F.A., Cornell University; and T.E.S., University of Missouri–Columbia.

The authors declare no conflict of interest.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE74152).

[1]Present address: Department of Animal Sciences, Auburn University, Auburn, AL 36849.

[2]To whom correspondence should be addressed. Email: lewin@ucdavis.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1520945114/-/DCSupplemental.

Our group has investigated the effects of SCNT cloning on gene expression and on developmental anomalies at different phases of cattle development and in different tissues, ranging from day (d) 7 blastocysts to placentomes that were collected at the term of pregnancy (14, 17–19). The resemblance of gene expression profiles between SCNT-derived blastocysts and those derived by artificial insemination (AI) (18) was the first evidence that the effects of cloning by SCNT could be more severe after blastocyst hatching. At d 18, the beginning of the implantation window, developmental asynchrony between the embryonic disk and the extraembryonic tissue (EET) was shown to be one reason for arrest at implantation (19). In addition, relatively few differentially expressed genes are detected in cloned fetuses (20, 21), and comparison of gene expression in term placentomes collected from pregnancies produced by SNCT, in vitro fertilization, and AI (17) showed that, at later stages of development and at term, placentomes of SCNT-derived pregnancies had an altered transcriptome, regardless of whether pathological morphology was observed (15, 17). These studies demonstrated that the abnormal development of the placenta is the major cause of failure of clones to complete pregnancy after implantation.

In dams carrying cloned conceptuses, gene expression in the endometrium was shown to be differentially responsive to the presence of a cloned conceptus before attachment occurs at d 18 (13) and d 20 (14), indicating altered molecular communication signals at the conceptus–maternal interface. Thus, multiple lines of experimental evidence have led to the conclusion that EET is the origin of the developmental pathologies in placentation of SCNT-derived clones (17, 22–26). Therefore, study of the communication between cloned conceptuses and the endometrium should yield important insights into the mechanisms underlying average losses of ∼60% of cloned embryos before implantation and 80% of fetuses within the first trimester of gestation (27, 28).

In the present study, we aimed to reveal potential causes of early pregnancy failure of clones by using a transcriptomic approach. Two developmental time points were chosen for the collection of EET from conceptuses produced by SCNT and AI at d 18 and at d 34, i.e., the periimplantation period. In addition, endometrial tissues were collected from the same pregnancies to gain a better understanding of the molecular pathways affected by the presence of clones, and how perturbation of those pathways might affect communication between mother and conceptus, which in turn can lead to the failure of pregnancy following implantation. Our findings revealed that the cloning process produces EET with highly variable expression of thousands of genes, several of which have been shown to cause embryonic lethality and placental abnormalities in knockout (KO) mice. For those conceptuses that implant and survive until d 34, gene-expression data indicate that communication and molecular signaling between the conceptus and the endometrium is defective, which in turn can result in pathology of the placenta and pregnancy loss.

Results

Experiment Overview. Cloned conceptuses derived from the same cell line, and paternal half-sibling conceptuses produced by AI, were surgically collected after euthanasia at d 18 and d 34 of gestation. Simultaneously, endometrial samples were collected for all pregnancies. The preimplantation conceptuses collected at d 18 were dissected into embryonic disk and EET; only EET was subjected to analysis. The postimplantation EET collected at d 34 was physically separated into chorion and allantois. Samples of caruncular and intercaruncular tissues of the endometrium were dissected from the uterus at d 18 and d 34. Representative tissue samples are shown in Fig. 1. We sequenced the transcriptome of 87 samples, averaging more than 30.7 million reads per sample, to determine transcript levels of more than 13,000 annotated cattle genes. We focused the analysis on three major components: (*i*) developmental changes in gene expression in d-18 and d-34 EETs and corresponding endometrium from SCNT and AI pregnancies; (*ii*) the effects of SCNT on gene expression in EET and endometrium at d 18 and at d 34; and (*iii*) network analysis of differentially expressed genes in EET and endometria at d 18 and d 34. The large number of analyses requires us to present only a subset of the most interesting results in the main text; the remainder can be found in the *SI Appendix* and Datasets S1–S25. Summaries of all comparisons for EET and endometrial tissues are shown in Table1 and Table 2, respectively.

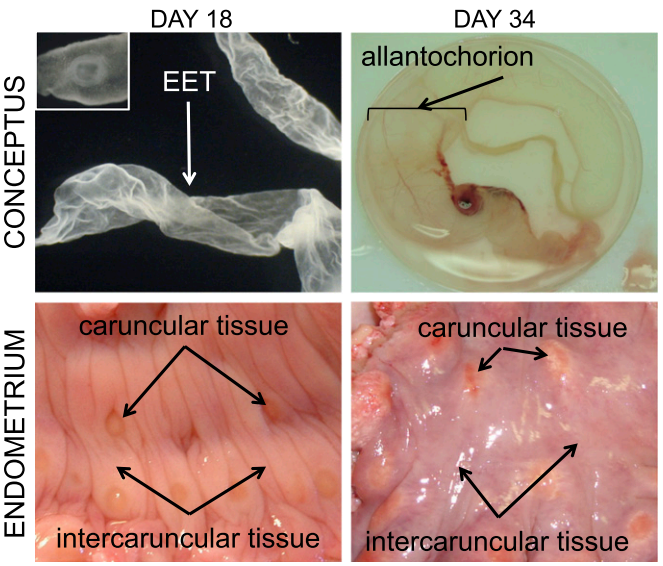

Fig. 1. Representative images of the collected samples.

Developmental Changes in Gene Expression in EET Collected from Pre- and Postimplantation AI Embryos. The expression of >10,000 genes was detected in EETs collected from AI pregnancies at gestation d 18 and chorion and allantois at d 34 (Table 1 and *SI Appendix*). We identified 9,492 genes commonly expressed in all three EETs, which accounted for 91.81%, 86.81%, and 82.47% of the genes expressed in d-18 EET, d-34 chorion, and d-34 allantois, respectively (Fig. 2*A*). Although many common genes were expressed in the three tissues, cluster analysis based on transcript levels revealed distinct gene expression profiles of the d-18 EET (Fig. 2*C* and *SI Appendix*, Fig. S1*A*). Cluster analysis also separated d-34 chorion from d-34 allantois samples ($P < 0.05$), with chorion and allantois being more similar to each other than to d-18 ETT (Fig. 2*C*; further analysis and interpretation is provided in *SI Appendix*). These results illustrate that gene expression levels define the common lineage of EETs and also reflect changes in the developmental patterns of expression of hundreds of genes as the EET differentiates into chorion and allantois after implantation (Table 1 and *SI Appendix*, Tables S1 and S2). The results from AI EET also present a baseline for comparisons with EET from SCNT conceptuses (as detailed later).

Developmental Changes in Gene Expression in Endometrial Tissues from Pre- and Postimplantation AI Embryos. The expression of more than 12,000 protein-coding genes was detected in caruncular and intercaruncular tissues collected from d-18 and d-34 AI-derived gestations (Table 2). Greater developmental changes in gene expression were in intercaruncular tissue (67% DEGs) compared with caruncular tissue (22% DEGs; Table 2). Cluster analysis of gene expression levels of the caruncular and intercaruncular samples distinctly grouped the endometrial samples by day collected ($P < 0.05$; Fig. 3*A* and *SI Appendix*, Fig. S1*B*). At d 18, clustering of intercaruncular and caruncular samples was distinct with the exception

Table 1. Summary of expressed and differentially expressed (FDR ≤ 0.05) genes in EETs

Day	Tissue (no. of samples)	Group (no. of samples)	Expressed genes	DEG			Dataset
				Up	Down	%	
34:18	Chorion(5):EET (5)	AI	10,963	2,512	2,102	42.1	S1
34	Chorion (5):allantois (4)	AI	11,993	2,253	2,616	40.6	S2
34:18	Chorion (7):EET (9)	SCNT	11,221	2,502	1,541	36.0	S3
34	Chorion (7):allantois (5)	SCNT	11,809	2,522	2,367	41.4	S4
18	EET	SCNT (9):AI (5)	10,338	2,826	2,311	49.7	S5
34	Chorion	SCNT (7):AI (5)	10,934	31	21	0.4	S6
34	Allantois	SCNT (5):AI (4)	11,510	175	66	2.1	S7

of one sample, whereas, at d 34, the clustering of caruncular and intercaruncular samples was not distinct. The difference in the number of DEGs in caruncular vs. intercaruncular tissues from d 18 to d 34 correlates with the complex morphological and functional remodeling in intercaruncular tissue during the implantation window (29). An in-depth description of gene expression changes in caruncular and intercaruncular tissues associated with the progression of pregnancy before and after implantation is provided in the *SI Appendix*. As for EET, results for AI endometrial tissues served as baseline for comparisons with SCNT pregnancies (as detailed later).

Associated Effects of SCNT on the EET Transcriptome Before and After Implantation. The effects of SCNT on gene expression in EET were analyzed by comparing transcript levels at gestation d 18 and d 34 in EET of conceptuses produced by SCNT and AI. At d 18, we identified more than 5,000 DEGs associated with SCNT [false discovery rate (FDR) < 0.05], or ∼50% of the total genes expressed (Fig. 2*B* and Table 1; *SI Appendix*, Fig. S1*C* shows unsupervised clustering of DEGs). By contrast, at d 34, 0.4% and 2.1% of genes were differentially expressed in chorion and allantois from clones, respectively (Fig. 2*B* and Table 1). These striking results demonstrate the massive dysregulation of gene expression in preimplantation EET from SCNT conceptuses. However, at d 34, relatively few genes were dysregulated, suggesting that cloned conceptuses with gene-expression patterns more similar to AI-derived conceptuses can successfully implant and begin placentation.

To further understand the possible mechanism(s) of why cloned conceptuses perish before or during the implantation period, we queried the Mouse Genomic Informatics Knockout database (30) for gene KOs that cause lethality before implantation, KOs that disrupt the development of EET after implantation, and KOs that are associated with the development of an abnormal placenta (*SI Appendix*, Table S3). We found 123 DEGs at d 18 in EET that have the KO database annotation term "abnormal extra-embryonic tissue morphology," 121 DEGs annotated with the term "embryonic lethality," and 14 DEGs annotated as "abnormal embryo implantation" (*SI Appendix*, Table S3). These results indicate that the losses in preimplantation SCNT embryos are likely a result of dysregulation of many critical developmental genes in EET. Further support for this conclusion was gained from gene-network analysis (*SI Appendix*, Fig. S2). Many of the genes down-regulated at d 18 in EET from SCNT conceptuses and annotated in the KO database (54 of 180) were found to encode proteins that can associate into a regulatory network (*SI Appendix*, Fig. S2). Several genes encoding transcription factors were found; *caudal homeobox 2* (*CDX2*) and *snail homolog 1* (*SNAI1*) stand out as a result of their known roles in regulating the development of EET (31, 32).

As described earlier, postimplantation EETs from SCNT conceptuses had far fewer DEGs. However, four genes (*NOS3*, *ESAM*, *RSPO3*, *ERG*) that were down-regulated in d-34 allantois SCNT samples produce abnormal angiogenesis in mouse KOs (*SI Appendix*, Table S3). In contrast, no DEG in the list produced for d-34 chorion had a KO with altered morphology that was directly related to implantation. These data, and the minimal overlap of DEGs at d 18 and d 34 (Fig. 2*B*), further support that only certain embryos with more complete reprogramming patterns are capable of implanting and undergoing further development.

We also performed gene-function analysis on the 52 DEGs expressed in d-34 chorion to identify candidate genes for postimplantation losses. We found these DEGs significantly enriched for the following Gene Ontology (GO) biological processes: "epithelial cell differentiation" and "epithelium development" (FDR ≤ 0.05; Dataset S20). We also analyzed the 241 d-34 allantois DEGs and found significant enrichment for the following GO biological processes: "cell adhesion," "biological adhesion," "positive regulation of inflammatory response," "positive regulation of response to external stimulus," "regulation of acute inflammatory response," and "regulation

Table 2. Summary of expressed and differentially expressed (FDR ≤ 0.05) genes in endometrial tissues

Day (no. of samples)	Tissue (no. of samples)	Group (no. of samples)	Expressed genes	DEG			Dataset
				Up	Down	%	
34 (5):18 (5)	CAR	AI	12,742	1,293	1,483	21.79	S8
34 (5):18 (5)	ICAR	AI	12,860	4,363	4,257	67.03	S9
34 (7):18 (9)	CAR	SCNT	12,779	3,426	2,277	44.62	S10
34 (7):18 (9)	ICAR	SCNT	12,736	2,050	1,631	28.90	S11
18	ICAR (5):CAR (5)	AI	12,740	1,186	952	16.78	S12
34	ICAR (5):CAR (5)	AI	12,465	756	369	9.03	S13
18	ICAR (9):CAR (9)	SCNT	12,796	1,925	1,849	29.49	S14
34	ICAR (7):CAR (7)	SCNT	12,915	774	543	10.20	S15
18	CAR	SCNT (9):AI (5)	12,733	11	37	0.38	S16
18	ICAR	SCNT (9):AI (5)	12,781	17	9	0.20	S17
34	CAR	SCNT (7):AI (5)	12,630	75	6	0.64	S18
34	ICAR	SCNT (7):AI (5)	12,707	2,300	2,384	36.86	S19

CAR, caruncular tissue; ICAR, intercaruncular tissue.

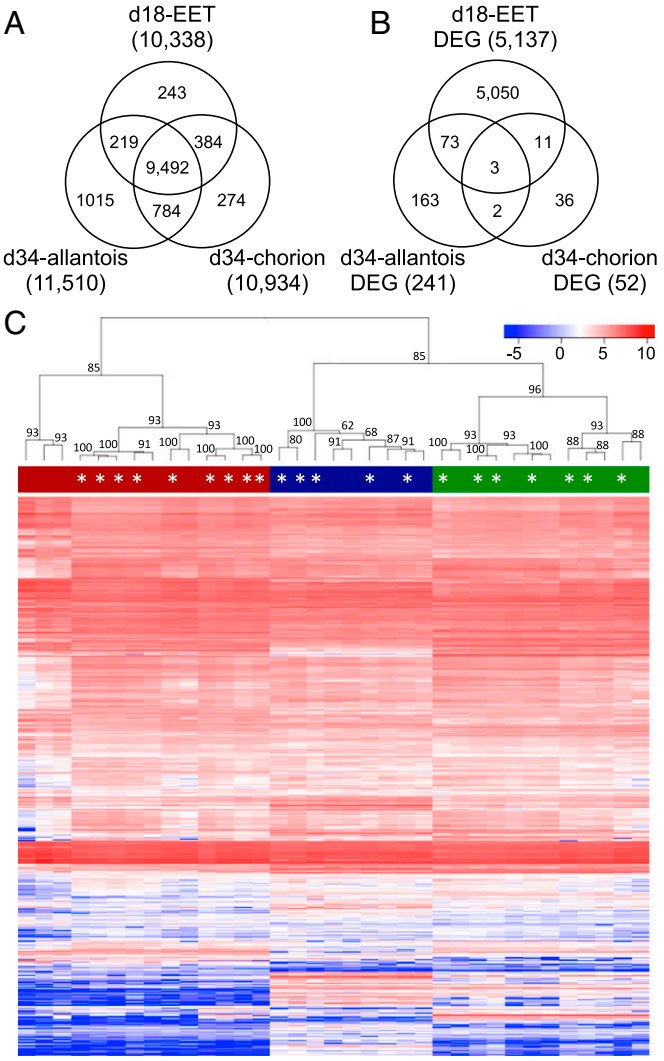

Fig. 2. Genes expressed in EET during the periimplantation period: (*A*) overlap of genes expressed in each tissue in AI- and SCNT-derived conceptuses and (*B*) overlap of differentially expressed genes between SCNT and AI samples; (*C*) unsupervised clustering of all allantois samples collected from conceptuses. Color code for sample dendrogram: red, d-18 EET; green, d-34 chorion (CHO); and blue, d-34 allantois (ALL). Asterisks indicate SCNT samples. Expression data presented as Log2(RPKM + 1). The numbers on the nodes are the bootstrap probabilities (as percentage) of cluster certainty.

of inflammatory response" (FDR ≤ 0.05; Dataset S21). The nonessential functions of most of these genes in development, as determined from KO studies, can explain in part the lower rate of losses postimplantation. Furthermore, these results explicitly link fetal survival to adaptations of the placenta and the transcriptional activity of fetally derived placental tissues.

Associated Effects of SCNT Conceptuses on the Transcriptome of Endometrial Tissues Before and After Implantation. The presence of SCNT-derived conceptus in the uterus caused changes in the expression levels of more genes in caruncular and intercaruncular tissues at d 34 than at d 18 of the pregnancy (Table 2). A comparison of SCNT- and AI-derived gestations identified fewer than 1% of genes that were differentially expressed in d-18 caruncular tissues, d-18 intercaruncular tissue, and d-34 caruncular tissue, whereas ∼37% of the genes were differentially expressed in d-34 intercaruncular tissue (Table 2). Principal component analysis of DEGs in endometrial tissues at d 18 and d 34 clearly distinguished SCNT from AI pregnancies (*SI Appendix*, Fig. S1*D*). In SCNT pregnancies, the intercaruncular and caruncular tissue collected at the same gestation day shared more DEGs than the same tissues at different gestation days (Fig. 3*B*). In dams bearing SCNT clones, the DEGs in d-34 caruncular tissue were significantly enriched for the GO molecular function terms "hormone activity" and "receptor binding" (FDR ≤ 0.05; Dataset S22), whereas, in intercaruncular tissue, there was no significant enrichment in GO terms. These results indicate that the endometrium can distinguish the presence of a cloned conceptus, and that intercaruncular tissue is the principal responding area of the endometrium.

Network Analysis of Conceptus–Maternal Signaling Affected by Cloning. We used the Metacore software suite to identify gene networks, protein–protein interactions, and autocrine or paracrine signaling pathways that may be affected by SCNT cloning. Because the release of IFNT has been considered the major embryonic signal for pregnancy recognition (33), we analyzed IFN pathways in depth. At d 18, four paralogs of the IFN family were down-regulated in d-18 EET SCNT samples (*IFNT*, *IFNT2*, *IFNT3*, and *IFN-tau-c1*; FDR ≤ 0.05; *SI Appendix*, Fig. S3*A*). In the endometrium, two IFNT receptors (*IFNAR1* and *IFNAR2*) were expressed in caruncular and intercaruncular tissues. Among the eight DEGs associated with SCNT cloning in both endometrial tissue types at d 18, three IFN-responsive genes (*CXCL9*, *CXCL10*, and *IFI47*) were down-regulated in caruncular and intercaruncular samples (*SI Appendix*, Fig. S3*B*). These results indicate that the presence of a cloned embryo in the uterus affects a key regulatory system involved in signaling between the conceptus and the endometrium that promotes implantation (2).

A search for transcripts encoding signaling molecules affected by SCNT at the conceptus–maternal interface identified 38 DEGs in d-18 EET that code for ligands with corresponding receptors expressed in the endometrium (Fig. 4*A* and Dataset S23). There was no significant enrichment of genes in any functional category; however, GO annotations revealed 34 DEGs involved in biological processes important to the establishment of pregnancy, such as "regulation of apoptosis," "vasculature development," "neurogenesis," "positive regulation of cell differentiation," "Wnt receptor signaling pathway," and "gland development."

Among the DEGs in chorion at d 34, complement component 3 (*C3*), slit homolog 2 (*SLIT2*), and von Willebrand factor (*VWF*) code for proteins that have cognate receptor genes expressed in the endometrium (Dataset S24). The genes *C3* and *VWF* were up-regulated by more than fivefold in d-34 chorion SCNT compared with AI samples, and are associated with GO functions "response to stress" and "response to external stimulus," "positive regulation of developmental growth," and "placenta development." The gene *SLIT2*, known to be related to the suppression of epithelial growth and to conceptus survival at later stages of pregnancy (34), was down-regulated (0.47 fold) in d-34 chorion SCNT compared with AI samples.

In d-34 endometrial tissues, 56 DEGs code for ligands with receptors in chorion or endometrium (Dataset S25). Thirty-four of those ligands can transduce signals to the chorion (Fig. 4*B*). Among those ligands, several are encoded by genes whose expression was affected by the cloning process in intercaruncular tissue and are associated with essential functions related to the establishment of pregnancy, including "immune response," "cell adhesion," "regulation of growth," and "chemotaxis" (Fig. 4*B*). Most of the genes that are related to the GO functions "gland development," "response to estrogen stimulus," "angiogenesis," and "mesenchymal cell differentiation" were down-regulated in intercaruncular tissue. The proteins encoded by these genes function in autocrine signaling and are important for uterus remodeling.

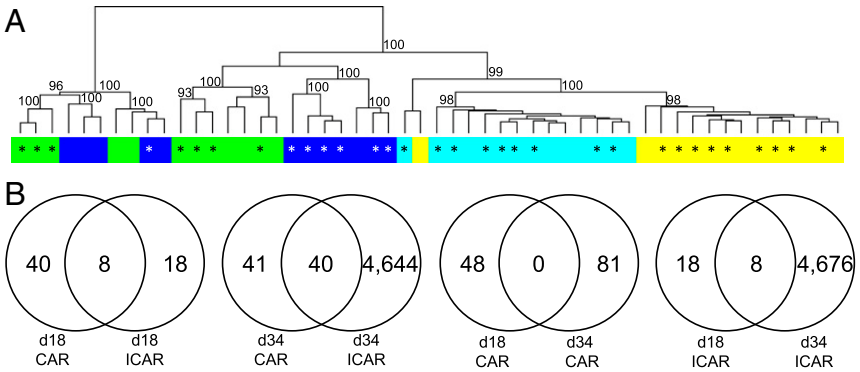

Fig. 3. Genes expressed in endometrial tissues: (*A*) clustering of the endometrial samples and (*B*) overlap of DEGs between SCNT and AI groups for each tissue and time point. Color code for dendrogram: light blue, d-18 intercaruncular tissue (ICAR); yellow, d-18 caruncular tissue (CAR); dark blue, d-34 intercaruncular tissue; and green, d-34 caruncular tissue. Asterisks indicate SCNT samples. The numbers on the nodes are the bootstrap probabilities of cluster certainty (as percentage).

Validation of RNA-seq Results. We selected seven genes of known physiological importance to the implantation to validate the RNA-seq results; five genes were associated with down-regulation of IFNT signaling in clones on gestation d18 (*IFN.tau.c1*, *IFTN2*, *CXCL9*, *CXCL10*, and *IFI47*), and two genes were up-regulated in d-34 chorion from cloned samples (*C3* and *IL6*). The results obtained by quantitative PCR for the genes tested were consistent with the results using RNA-seq (cosine correlation = 0.89; $P < 0.001$), thus providing a validation of the analysis (*SI Appendix*, Table S5 lists full list of tissues and comparisons).

Discussion

The main objective of our study was to understand the molecular mechanisms underlying the failure of most SCNT clones to establish successful pregnancies. We focused on analyzing the transcriptome of conceptuses and paired endometria at two specific

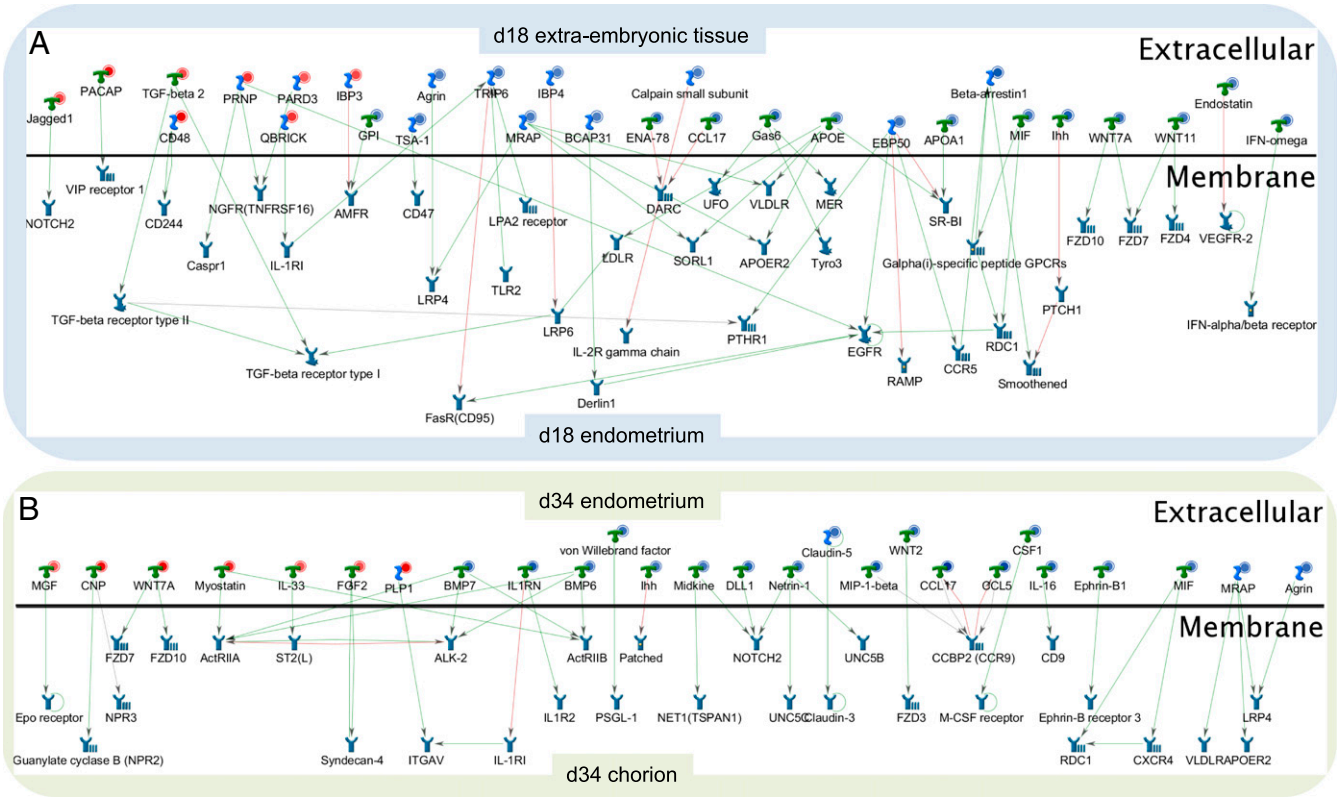

Fig. 4. Gene network models of dysregulated ligand–receptor interactions between EETs and endometrium. (*A*) DEGs in d-18 SCNT EET that encode ligand proteins (above line) and putative receptors expressed in the EET and endometrial tissues (below line). (*B*) DEGs in d-34 endometrium from SCNT pregnancies that encode ligand proteins (above line) and putative receptors in chorion, intercaruncular, and caruncular tissues. The circle at the upper right corner of each object identifies the direction of differential expression. Blue indicates down-regulated and red up-regulated in SCNT samples compared with AI controls. All networks were created from protein interactome data. Ortholog mapping of cattle genes to human or mouse gene identifiers may have led to the appearance of unofficial gene symbols in the figure (e.g., IFNW corresponds to IFNT in cattle). Datasets S23 and S25 provide official gene symbols of the encoded proteins, National Center of Biotechnology Information gene identifiers, gene expression data, and description of potential interactions between proteins.

time points during the periimplantation period, an interval that is critical for a cascade of molecular and cellular events leading to implantation and placental development. We identified several important factors that likely lead to the death of cloned conceptuses, failure of implantation, and failure to develop a normal placenta: (*i*) massive dysregulation of gene expression in EET of d-18 preimplantation conceptuses, including many genes that are known from studies in mice to be critical for implantation and normal development of the trophoblast; (*ii*) failure of appropriate signaling between the conceptus and endometrium; (*iii*) dysregulation of functionally important genes in chorionic tissue and allantois after implantation that are critical for angiogenesis and epithelial cell development and differentiation; and (*iv*) dysregulation of large numbers of genes in allantois and intercaruncular tissue at d 34 (postimplantation) that are involved in hormonal responses, intracellular signaling, and normal growth and development of the placenta.

Preimplantation Losses of Cloned Conceptuses. The cloned conceptuses we collected presented no morphological deficiencies under the light microscope (35), although potential nanostructural alterations (15) were not investigated. As reported by Degrelle et al. (19), the success rate of SCNT is highly dependent of the cell lines used as nuclear donor. In our laboratory, SCNT using the 5538 skin fibroblast cell line produces conceptuses with a high pregnancy rate at d 18 of development (83% in the present study when two blastocysts transferred) compared with other cell lines (19). Thus, gene expression in 5538-derived clones represents a best-case scenario compared with gene expression in other somatic cell lines commonly used for cloning. Cluster analysis (Fig. 2) suggests that it might be possible to distinguish normal from abnormal conceptuses at d 18 (i.e., two outlier AI embryos compared with clustering of most of the SCNT clones), which could lead to improved methods for SCNT by varying conditions during the deprogramming and reprogramming phases (26, 36).

We conducted a detailed functional analysis of the DEGs in EET at d 18 to understand why SCNT clones are more susceptible to death at implantation. The surprisingly large number of DEGs in d-18 EET (>5,000), many with more than threefold differences in expression level, provides strong support for the idea that development of the EET is highly variable in SCNT clones and that this may lead to the death of cloned embryos before implantation. Dysregulated expression of so many genes in the EET at gestation d 18 is also likely to contribute to aberrant gene expression defects in signaling to the endometrium (as detailed later). The annotated functions of these DEGs explain the catastrophic death of cloned conceptuses before and during the implantation process. The best evidence that these DEGs contribute to the death of cloned conceptuses comes from the mouse KO database; 121 DEGs in d-18 EET are associated with embryonic lethality in mice (*SI Appendix*, Table S3). In addition, annotations of 14 DEGs were associated with "abnormal embryo implantation." Thus, although such genomically deranged conceptuses are unlikely to survive much beyond the implantation phase of pregnancy, the relatively large fraction (~72%) of successful pregnancies obtained at d 20 with cell line 5538 (19) suggests that there is significant plasticity in gene expression that can lead to successful implantation.

At d 18, the highly dysregulated gene expression in EET of SCNT clones was not mirrored in the endometrial tissues (Table 2). In d-18 caruncular and intercaruncular tissues, fewer than 0.5% of expressed genes were DEGs. These findings indicate that, at gestation d 18, full recognition of clones by the endometrium has not yet occurred. Although no DEGs were found in common with previous studies performed at d 18 (13) and d 21 (14), possibly as a result of the differences in methodology used, it is noteworthy that approximately one third of DEG in endometrium that were associated with the presence of a cloned conceptus were also differentially expressed as part of conceptus recognition (*SI Appendix*, Table S6). An example of compromised recognition of SCNT conceptuses by the endometrium is the gene encoding the enzyme indoleamine 2,3-dioxygenase-1 (*IDO1*), which is down-regulated in caruncular tissue (fold change, 0.54; FDR < 0.05) and also tended to be down-regulated in intercaruncular tissue (fold change, 0.67; FDR = 0.08). IDO1 is one of the enzymes responsible for catabolizing tryptophan in a pathway essential for maternal immune tolerance toward the conceptus (37, 38). Down-regulation of *IDO1* may be directly linked to the reduced likelihood of implantation success in cloned conceptuses. Our results support prior observations (13, 14) that the health status of the conceptus is associated with the regulation of gene expression in the endometrium during the early stages of pregnancy.

Implantation Window. In mammals, implantation proceeds in five stages: hatching from zona pellucida, apposition, attachment, adhesion, and invasion. The invasion of the endometrium by fetal tissue is very limited in ruminants, resulting in a synepitheliochorial structure of the placenta (39). Given the ready access to conceptus tissues and well described developmental processes, ruminants are considered an exceptional model in which to study apposition and attachment during implantation (40). A critical feature of implantation is the coordination of molecular signals with the endometrium during the periimplantation period (1). At d 20, Mansouri-Attia et al. (14) showed that the endometrium was able to transcriptionally sense the presence of a cloned conceptus (14), and that signaling (and other) pathways were likely affected. To extend this study, we analyzed the expression of genes involved in signaling between conceptus and endometrial tissues at and across pre- and postimplantation time points. Although the endometrium is histologically complex, careful excision of tissue excluded the myometrium and standardized samples for comparison. This approach allowed us to explore altered communication between conceptus and endometrium that might lead to the failure of implantation as well as pathologic conditions of the developing placenta and fetus during later stages of pregnancy.

In cattle, IFNT is produced by the mononuclear trophectoderm cells of the elongating conceptus and is the major signal of pregnancy recognition (41, 42). The IFNT protein has antiluteolytic activity (2) and has been shown to have dramatic effects on gene expression in bovine primary endometrial cell cultures (36). The observed down-regulation of genes involved in IFNT signaling at d 18 in EET may disrupt paracrine pathways between EETs and the endometrium and potentially have direct effects on the adhesion of the trophoblast to the endometrium (*SI Appendix*, Fig. S3*C*) (43). In addition to the results for IFNT, data mining revealed 38 ligand-encoding signaling-related genes whose expression at d 18 was affected by the cloning process. Importantly, we also found receptors for these ligands encoded in endometrium at d 18. The catalog of DEG encoding ligand–receptor pairs associated with signaling (Fig. 4 and Datasets S23–S25) is a unique resource for understanding normal and altered gene networks that may cause asynchrony in communication between the conceptus and the endometrium, which in turn could affect development and remodeling of the endometrium. For example, the down-regulation of WNT-related genes (*WNT7A* and *WNT11*) identified at d 18 may delay the remodeling of the endometrial glands during the window of implantation (44). Functional analysis of the transcriptome thus strongly suggests that aberrant signaling between the conceptus and the endometrium at d 18 is likely to have a major effect on implantation and subsequent development of the placenta. These data are consistent with and significantly extend earlier results obtained at d 20 for SCNT clones (14).

Postimplantation Effects of Clones on Placental Development and Pathologic Conditions. In SCNT pregnancies, an additional 30–100% of clones are lost after implantation, depending on the cell line used (27, 28, 45). These losses are typically accompanied by

abnormal placental development, with fewer, enlarged, and edematous placentomes [reviewed by Chavatte-Palmer et al. (12)]. Ruminant placentomes are formed by fetal cotyledons, which consist of allantochorionic villi, and caruncular tissue, which develop from the subepithelial connective tissue of the endometrium. The intercaruncular tissue of the placenta contain the endometrial glands, which synthesize secretions (histotroph) that contains enzymes, cytokines, growth factors, hormones, adhesion proteins, and other molecules that are essential for the development of the conceptus (46). Therefore, we functionally mined the DEGs in EETs (chorion and allantois) after implantation and in the caruncular and intercaruncular tissues at d 34 to gain a better understanding of the mechanisms of fetal mortality following implantation, as well as downstream placental pathologic conditions.

A surprising and informative result was that chorion and allantois of clones at d 34 had few DEGs (0.4% and 2.1%, respectively) relative to d 18. Also, very few DEGs were observed in d-34 caruncular tissue. However, gene expression in d-34 intercaruncular tissue was grossly different in SCNT clones, providing conclusive evidence that it is the intercaruncular tissue that contributes to the major deficiencies in placental function observed in clone-bearing pregnancies. Thus, the effects of SNCT clones on gene expression in endometrium observed after implantation are the result of delayed transcriptional activation arising from earlier communication with the conceptus and/or a direct effect of implantation. This is discussed further later. The dramatic difference in DEGs between pre- and postimplantation SCNT EET suggests that only those conceptuses with relatively normal gene expression patterns will survive past implantation, or, alternatively, the endometrium is able to reprogram gene expression in viable conceptuses that then survive the implantation process. We favor the former explanation given the vast number of dysregulated genes in d-18 EET collected from cloned conceptuses.

Although many SCNT conceptuses survive implantation using cell line 5538, and we show that EETs from clones have gene expression patterns that are more like AI conceptuses at d 34 than at d 18 (Table 1), there are several d-34 DEGs in caruncular and intercaruncular tissues that may have severe consequences on placental development. Previous studies have shown that there are successive losses at d 60 and beyond that reduce the number of live clones born to less than 30% (28). Examination of the few DEGs in caruncular tissue at d 34 revealed four genes (*CSH2*, *NPPC*, *PRP1*, and *PRP4*) that code for hormone-related proteins with somatotropic functions and three genes (*GMFB*, *IL6*, and *THBS4*) that code for growth factors. The up-regulation of these genes in caruncular tissue may contribute to the overgrowth of placentomes in SCNT-derived pregnancies (12, 23, 47). The functions of these genes suggest that angiogenesis, vasodilation, stromal remodeling, and the overrelease of growth modulators are components of the complex mechanism that triggers the maternal contribution to placentomegaly frequently observed in SCNT pregnancies.

In the intercaruncular tissue, we found massive transcriptional changes developmentally and in SCNT pregnancies. Many of the DEGs are associated with epithelial development, thus potentially contributing to the enlarged placentomes that are commonly observed in SCNT-derived placentas (23). The complexity of intercaruncular tissue makes it impossible to discriminate the exact cell type with altered gene expression; nonetheless, the functions of some of those genes offer important clues regarding how the physiology is affected by the presence of a clone. For example, the altered expression of genes that code for potential secreted proteins is a strong indication that the synthesis or delivery of macro- and

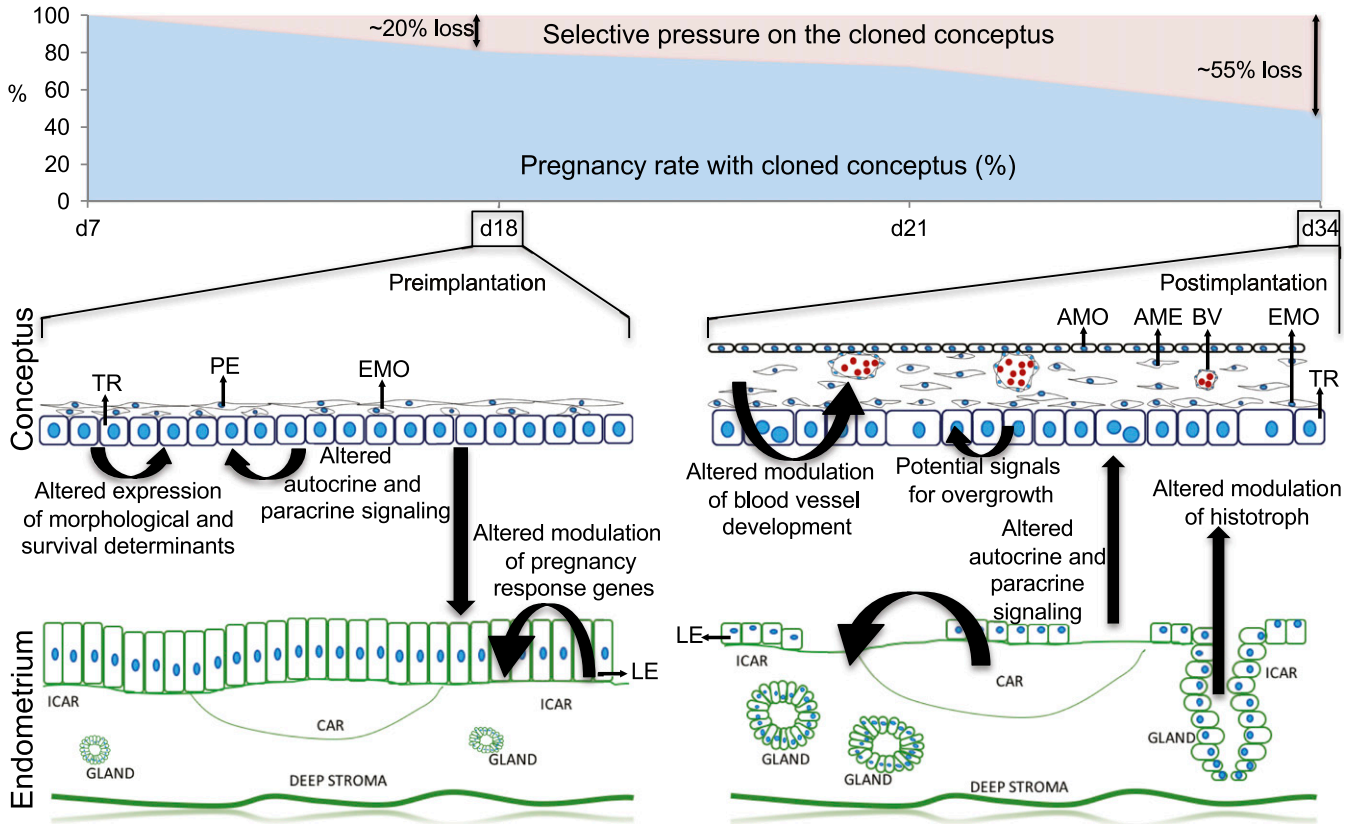

Fig. 5. Hypothetical model of most representative factors for conceptus survival and pathological deficiencies in SCNT-derived pregnancies during the periimplantation period. AME, allantoic mesenchyme; AMO, allantoic mesoderm; BV, blood vessel; CAR, caruncular tissue; EMO, extraembryonic mesoderm; ICAR, intercaruncular tissue; LE, luminal epithelium; PE, parietal endoderm; TR, trophoblast.

micronutrients that will sustain fetal survival are out of balance. Another example is the up-regulation of *FGF2* in intercaruncular tissue, which may contribute to greater migration of trophoblast cells of the SCNT-derived conceptus. These results are consistent with the in vitro results obtained by Yang et al. (48) Thus, after attachment of the clone conceptus, the intercaruncular tissue of the endometrium may be primed for pathologic conditions associated not only with a dysfunctional placenta but also with enlarged organs in the fetus (49).

In the allantois of SCNT-derived conceptuses, most of the DEGs are functionally annotated to be involved in cell adhesion or in the regulation of the inflammatory response. Those genes are likely related to the altered constitution of connective tissue in the allantoic compartment of SCNT-derived conceptuses that develop hydrallantois (23). Hydrallantois is a pathologic condition that is highly correlated with conceptus mortality at midgestation (12). Strikingly, we found four genes (*NOS3*, *ESAM*, *RSPO3*, and *ERG*) down-regulated in d-34 allantois of SCNT clones that are associated with the GO functional term "abnormal angiogenesis" in mouse KO models. These genes are thus candidates for the poor vascularization observed in allantois of some SCNT-derived conceptuses at gestation d 45 (22). Our data suggest that the allantois is one contributor to the abnormal development of the placenta observed in SCNT pregnancies.

Hypothesis for the Survival of SCNT Clones. On the basis of these observations, we propose a model for clone survival during the early and later stages of pregnancy (Fig. 5). We propose that there is a strong selection at the implantation stage in cattle for clones that have been more completely reprogrammed and thus have expression levels of critical developmental genes within a compensatory range. EETs from cloned conceptuses with gene expression levels that do not cause catastrophic effects on normal development or signaling to the endometrium can establish normal or pseudonormal cross-talk with the endometrium. Implantation would thus be the first strong "bottleneck" for the survival of clones, as it is for embryos carrying lethal or semilethal mutations.

Although a significant fraction of SCNT conceptuses may implant, we propose that abnormalities in the expression of genes critical to normal development and maintenance of the placenta eventually cause the SCNT fetuses to go through successive bottlenecks after implantation. These bottlenecks would eliminate most abnormal clones, but some placentas may compensate enough for few clone pregnancies to progress to term despite morphological and physiological abnormalities (17). Thus, even though there are a relatively small number of dysregulated genes at d 34 in EETs and endometrium in SCNT-derived gestations, the numbers of DEGs may again increase as the placenta develops further and additional molecular and cellular functions are required (17).

Our model is strongly supported by the observed progressive loss of clones throughout pregnancy (27, 28), with major postimplantation spikes occurring at d 30 and d 60 of gestation. Losses later in pregnancy are associated with placentomegaly and hydrallantois, which are a result of placental dysfunction. We have previously shown that the placentas of clones at term show severe signs of oxidative stress, abnormal cell growth and proliferation, and cell signaling (17), which could result from dysregulated genes that are compatible with implantation but not with normal placental development. As we have proposed earlier (17, 26), the SCNT pregnancy failures before and early after implantation, as well as at later stages, are likely to be the result of incomplete reprogramming that most strongly affects EET at gestation d 18. This in turn would lead to aberrant communication between the conceptus and endometrium, disrupting normal development and functioning of the placenta, and ultimately yielding the observed high rate of mortality during the SCNT cloning process. Future experiments to validate this model by directly manipulating signaling between conceptus and endometrium will allow for a more precise understanding of the mechanisms of pregnancy failures that occur during the periimplantation period after normal fertilization and SCNT cloning.

Materials and Methods

Clone and AI Pregnancies. Pregnancies initiated by AI and terminated at d 18, and those harboring SCNT-derived cattle conceptuses, were carried at the experimental farm of the Institut National de la Recherche Agronomique (INRA), France. The experiment was conducted in accordance with the rules and regulations of the European Convention on Animal Experimentation. Research with cloned cattle was approved by the INRA Ethics Committee. Gestations initiated by AI and terminated at d 34 were conducted at the University of Illinois at Urbana–Champaign with the approval of the institutional animal care and use committee.

We used AI-derived pregnancies as controls in our experiment because AI is the closest experimental condition to natural breeding and provides a systemic control from natural conception to implantation. We synchronized the estrous of Holstein cows and performed AI with frozen semen from two Holstein bulls (considered as d 0). Gestations were terminated for sample collection at d 18 ($n = 8$) and d 34 ($n = 10$). Gestations were terminated for sample collection at d 18 ($n = 8$) and d 34 ($n = 10$). Pregnancy/conceptus survival rates for AI-derived gestations were 50% and 32% at d 18 and d 34, respectively.

We used SCNT-derived clones to investigate the local communication between conceptus' EET and endometrium in pregnancies prone to pathological placentation. Cloned bovine embryos were produced in vitro using the Holstein cell line 5538 (44) for SCNT following methods described elsewhere (50). Two cloned blastocysts (d 7) were transferred into each estrous-synchronized recipient Holstein heifer, and the pregnancies were terminated at d 18 ($n = 9$) and d 34 ($n = 8$) of gestation. For SCNT-derived gestations, pregnancy rates were 83% and 47% at d 18 and d 34, respectively. As a result of the transfer of two cloned blastocysts to each surrogate, the conceptus survival rate is one half the pregnancy rate. Thus, the survival rates for cloned conceptuses were 41% and 23% at gestation d 18 and d 34, respectively.

Sample Collection. Procedures of sample collection and handling were performed as described elsewhere (35). Briefly, at gestation d 18, the uterus was separated from reproductive tract and the ipsilateral horn was flushed with PBS solution. The conceptus was collected and inspected by using a stereomicroscope for separation of embryonic disk and annexes in the immediate vicinity of other EETs (i.e., yolk sac, and amnion folds). At gestation d 18, the EET samples consisted of trophoblast, parietal endoderm, and extraembryonic mesoderm. The development of conceptuses was assessed by the morphology of the embryonic disk and the histology of the EETs, namely trophoblast, parietal endoderm, and mesoderm cells.

At gestation d 34, the isolation of the uterus was followed by a sagittal incision in the ipsilateral horn and separation of the conceptus from the uterine wall. The embryonic body was separated from the EET with the assistance of a stereomicroscope. The EET was separated further into chorion and allantois. We pooled pieces of chorion from the same conceptus for RNA extraction without distinction of cotyledonary regions. Similarly, we pooled pieces of allantois from the same conceptus for RNA extraction.

After the removal of the conceptus, the endometrium was washed several times with PBS solution to confirm that potential loose cells from the chorion were removed before sampling of intercaruncular and caruncular tissues of the endometrium. The EETs were fully separated from the endometrium. In a previous histological classification of these samples (35), there was no evidence that EET was attached to the endometrium surface. Additionally, for each pregnancy, the lumen of the gravid horn was dissected for collection of caruncular and intercaruncular tissues of the endometrium. Samples of endometrium were taken at a depth within 3 mm of the lumen as previously described, thereby not reaching myometrium (14). All tissues were snap-frozen in liquid nitrogen and stored at −80 °C until use.

RNA-seq. For RNA-seq, we used five randomly chosen groups of tissue samples collected from the eight AI gestations terminated at d 18 and the 10 AI gestations terminated at d 34. The sample groups were d-18 EET and d-18 intercaruncular, d-18 caruncular, d-34 chorion, d-34 caruncular, and d-34 intercaruncular tissues. Four randomly chosen samples were used to quantify gene expression in d-34 allantois. All nine samples in each of three tissue groups from SCNT-derived pregnancies were used to quantify gene expression: d-18 EET, d-18 intercaruncular tissue, d-18 caruncular tissue, and d-18 intercaruncular tissue. Seven samples each of d-34 chorion, d-34 caruncular, and d-34 intercaruncular tissues and five samples of d-34 allantois were used for quantification of gene expression

from SCNT-derived pregnancies. The varying numbers of samples from clones used for RNA-seq was a result of variation in sample quantity and quality.

From each sample, total RNA was extracted by using TRIzol reagent (Invitrogen) following a procedure described elsewhere (35). Three micrograms of total RNA was used for the polyA+ library construction using a TruSeq SBS Sequencing Kit, version 3. Sequencing of single-end 100-nt-length reads was conducted by using the HiSeq 2000 system (Illumina). The fastq files were stored in the Gene Expression Omnibus public database (accession no. GSE74152).

Alignment of Sequences. The fastq files were subjected to a custom-built pipeline that consisted of an assessment of sequence quality using the software FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and FASTX-Toolkit 0.0.13 (hannonlab.cshl.edu/fastx_toolkit/index.html). Sequences were aligned to the cattle (*Bos taurus*) genome [Btau4 (51)] using the TopHat software 1.1.4 (52, 53), allowing for two mismatches for each block of 25 nt. The unaligned sequences were trimmed at the 3′ end to 75 nt and realigned; the process was then repeated once more, with unaligned reads trimmed to 50 nt in length. The three files with aligned sequences were merged, and the sequences were filtered to retain those matched to a unique position in the reference genome, with mapping quality greater than 30 and containing up to three mismatches. The filtered alignment file for each sample was subjected to the removal of duplicate reads using Picard tools (picard.sourceforge.net). The retained reads were used for downstream analyses.

Analyses of Differential Gene Expression. Sequence reads were counted according to overlaps on exon models obtained from the ensGene database from the University of California, Santa Cruz (UCSC), genome browser (54). The database was downloaded in January 2011 using R software 3.2.2 (55) and processed using the Bioconductor (56, 57) packages rtracklayer (58), Rsamtools (59), GenomicRanges (60), and GenomicFeatures (60). A set of genes that was not annotated in the Ensembl database but that was annotated in the refGene database from the UCSC genome browser was analyzed in conjunction with the Ensembl annotation. The reads that overlapped to an exon model were used for downstream analyses.

The genes with low expression levels were filtered out when the following criteria were met for all pairwise comparisons: (*i*) the average of reads within a group was less than 50 for both groups and (*ii*) the average reads per kilobase per million reads mapped (RPKM) within a group was less than 0.1 for both groups. For the retained genes, a comparison of the number of reads was performed by using the edgeR package (61). The number of reads in each library were subjected to scaling normalization (62), which was followed by a comparison of two sample groups using a general linear model (63). *P* values were corrected for multiple hypothesis tests by using the false discovery rate (FDR) procedure (64). For each comparison, gene expression levels were considered significantly different when FDR $\leq$ 0.05.

Twinning occurred in two of nine SCNT gestations at d 18. For twins, the tissue of only one randomly selected conceptus was used for gene-expression analyses. There was no twinning in AI-derived pregnancies. Therefore, twinning was not considered as a variable in the statistical model.

GO and Pathway Analysis. GO enrichment analysis was used to obtain a functional view of the DEGs that were associated with the cloning effects and with development. Genes with Ensembl annotation were used for this analysis, which was performed using the GOseq package (65) in R software. For each comparison, the genes with FDR values $\leq$0.05 were used as the test list, and the full complement of genes expressed were used as the background list. The raw *P* values were then corrected by using the FDR procedure, and the GO categories with FDR values $\leq$0.05 considered statistically significant. Molecular signaling across tissues was inferred from gene expression information collected in the present study and a priori knowledge of protein–protein interactions. The protein interaction database and the network models were extracted and built using the MetaCore software from GeneGo (66).

Validation of RNA-seq Results. All samples used for RNA-seq were used for validation of gene expression levels by using quantitative real-time PCR. Ten genes were selected for validation testing on the basis of their relative importance in biological processes. cDNA was synthesized from 600 ng of total RNA by using 200 U SuperScript III Reverse Transcriptase (Invitrogen), 200 ng of oligo$(dT)_{12}$ and 50 ng of random pentadecamers as primers (Invitrogen), 1× first-strand buffer, 5 μM DTT, and 40 U of RNaseOUT (Invitrogen) in a 20-μL reaction following steps recommended by the manufacturer. One twentieth of the reverse-transcribed cDNA was used as the template for the PCR using 1× Power SYBR Green PCR Master Mix (Applied Biosystems) and 100 μM of the specific primers for each target gene (*SI Appendix*, Table S5) in a total volume of 10 μL. Reactions were assayed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) following the parameters recommended by the manufacturer. The data were collected from two technical replicates for an accurate estimation of gene expression levels.

Amplification reactions with cycle threshold less than 35 were used for an estimation of arbitrary relative gene expression levels according to the standard curve method (User Bulletin 2; Applied Biosystems). β-actin was used to normalize gene expression levels, and the average of the normalized gene expression levels between two technical replicates was used for statistical tests. The relative gene expression levels from two groups of samples were compared by *t* test, and the difference was assumed statistically significant when $P < 0.05$.

ACKNOWLEDGMENTS. This work was supported by the United States Department of Agriculture–Agricultural Research Service Grant AG58-1265-7-027 (to H.A.L.), European Program SABRE Grant CT-2006-0162 (to J.-P.R.), and French National Research Agency Grants ANR-06-GANI-003-01 (to I.H.), ANR-09-GENM-012 (to H.J.), and ANR-08-GENM-037 (to O.S.).

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