Blastocyst gene expression correlates with implantation potential

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Objective: To investigate the role of the blastocyst in implantation failure. This study examined trophectoderm (TE) gene expression relative to pregnancy outcome.
Design: Retrospective experimental study.
Setting: Nonprofit research foundation.
Animal(s): Six-week-old BDF1 female mice.
Intervention(s): Hatching blastocysts underwent trophectoderm biopsy before single blastocyst transfer (one per uterine horn).
Main Outcome Measures: Blinded gene expression analysis was performed on biopsied TE cells by quantitative real-time polymerase chain reaction (Q RT-PCR). Healthy placenta and absorption sites were biopsied on day 16 of fetal development for comprehensive transcriptome analysis with validation by Q RT-PCR.
Result(s): Compared with blastocysts that resulted in healthy fetal development, blastocysts that failed to implant (negative) showed decreased B3gnt5 and Eomes gene expression, while blastocysts that resulted in spontaneous pregnancy loss (absorption) displayed decreased Wnt3a and Eomes gene expression. Comprehensive transcriptome analysis of biopsied absorption sites and healthy placenta revealed distinct gene expression signatures, with 5,918 significantly altered transcripts (greater than twofold). The predominantly altered pathways associated with spontaneous pregnancy loss were the complement and coagulation cascades.
Conclusion(s): This study revealed for the first time that individual blastocyst gene expression profiles correlate with outcome, including successful implantation and pregnancy loss. (Fertil Steril® 2010;■:■–■. ©2010 by American Society for Reproductive Medicine.)

Key Words: Gene expression, blastocyst, implantation, pregnancy loss

Implantation is a complex and intricate process involving extensive biological changes through a detailed network of crosstalk between the blastocyst and uterus. Equally as important to the potential success of implantation are uterine remodeling for receptivity and the generation of an implantation-competent blastocyst (1). The blastocyst is hypothesized to play an active role during the initial stages via its secreted paracrine and juxtacrine factors that stimulate hatching, apposition, and adhesion (2).

Several signaling molecules and pathways have been found to be important during the implantation process including leukemia inhibitory factor (3) and heparin-binding EGF-like growth factor (4). Nevertheless, the complete molecular dialogue and the order of events leading to the attachment of a competent blastocyst to a receptive uterine luminal epithelium, in particular the role of the blastocyst, still remain poorly understood. It would appear from assisted reproductive technologies (ART) that a considerable portion of implantation failure is attributed to the blastocyst, not the uterus, since it is not uncommon to observe embryo loss and nonviable implantation alongside healthy fetal development. Characterization of the various molecular pathways involved in the intricate blastocyst-uterine crosstalk is critical to our understanding of implantation failure and early pregnancy loss.

Embryonic genome activation is pivotal to the production and secretion of the molecules involved in the embryo-maternal dialogue. Studies of mouse embryos have described two major transient waves of de novo transcription crucial to ongoing development (5). The first wave coincides with the activation of the embryonic genome at the 2-cell stage, while the second wave transpires during the 4-cell to 8-cell stage. This second wave is believed to drive the dramatic morphological changes of compaction and differentiation into the two cell lineages of the blastocyst (5). The hypothesis is that viable blastocysts will have a precise gene expression profile after accurate and complete embryonic genome activation, characterizing their ability to successfully implant and establish healthy fetal development. Two previous studies, human and bovine, have tested this hypothesis with pooled samples and identified unique blastocyst genetic markers for the establishment of pregnancy (6, 7). However, the analysis of pooled samples in both studies may not accurately reflect the potential of the individual sample to predict outcome owing to variability that exists between individual blastocysts.

The objective of this study was to directly investigate, for the first time, the individual blastocyst state of gene activity in defining the success and outcome of implantation. The ability to quantitatively predict viable implantation will have considerable importance in clinical ART practice. In addition, the molecular characterization of spontaneous pregnancy loss and healthy placental development may reveal differential transcription, thereby further contributing to our understanding of miscarriage.

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MATERIALS AND METHODS

Blastocyst Culture

Five- to six-week-old BDF1 female mice (C57BL/6 x DBA; Charles River Laboratories, Wilmington, MA) were superovulated with 5 IU pregnant mare's serum gonadotropin followed 48 hours later by 5 IU of hCG. Zygotes were collected after mating and group cultured (10 embryos per 20 μL microdrop) in sequential media (Vitrolife, Englewood, CO) under oil at 37°C, 6% CO_2, and 5% O_2. Embryos were scored according to Gardner et al. (8).

Trophectoderm Biopsy and Single Blastocyst Transfer

After 96 hours of culture, expanded blastocysts with herniating trophectoderm (TE) cells were biopsied with the Zilos-TK laser (Hamilton Thorne, Beverly, MA). This technique is routinely used in human IVF with minimal impact reported on outcome (9). Biopsied TE cells were coded and placed in extraction buffer (Molecular Devices, Sunnyvale, CA), snap frozen, and stored at −80°C. Post biopsy blastocysts were allowed to recover in embryo glue (Vitrolife) for 4–6 hours before single blastocyst transfer. A single blastocyst was transferred per uterine horn into 6- to 8–week-old recipient female BDF1 mice early on day 4 of pseudopregnancy. On day 16 of fetal development, recipient females were sacrificed to record transfer outcome. All fetuses were removed, weighed, and measured. Placental tissue and nonviable implantation (absorption) sites were biopsied and stored at −80°C. Negative outcome was defined as the absence of either a fetus or an absorption site.

RNA Isolation of Biopsied TE Cells and Reverse Transcription

Total RNA was isolated from the coded biopsied TE cells using the PicoPure RNA Isolation Kit (Molecular Devices) with modifications. Briefly, blinded samples were lysed and bound to a silica-based filter where they were treated with RNase-free DNase I (Qiagen, Valencia, CA) and washed several times before recovered in 20 μL elution solution. Reverse transcription was performed using the High Capacity Reverse Transcription cDNA kit (Applied Biosystems, Foster City, CA) to generate cDNA template for real-time polymerase chain reaction (PCR).

Quantitative Real-Time PCR

Quantitative real-time PCR (Q RT-PCR) was performed blindly on coded samples using the ABI 7300 Real Time PCR System with the Power SYBR Green PCR Master Mix (Applied Biosystems). After a 10-minute incubation at 95°C, amplification was performed for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, then a dissociation stage for 15 seconds at 95°C, 1 minute at 60°C, 15 seconds at 95°C, and 15 seconds at 60°C. The quantification of six genes, Actr3 (GenBank NM_023735) (10), B3gnt5 (GenBank NM_001159407) (11), Cdx2 (GenBank NM_007673) (12), Eomes (GenBank NM_010136) (13), Slc7a5 (GenBank NM_011404) (14), and Wnt3a (GenBank NM_009522) (15), was calculated relative to the comparatively constant level of transcription in every sample of the housekeeping gene, Gapdh. The PCR reaction efficiency recorded R^2 values ≥ 0.9, and the correlation coefficient was calculated to be >0.99. Statistical analysis was performed with REST-2008 software using bootstrap randomization techniques (www.gene–quantification.de/rest.html). Gene expression fold differences with $P<.05$ were considered statistically significant.

Transcriptome Microarray Analysis

Total RNA was isolated from biopsied healthy placenta from viable blastocysts (n = 6) and absorption tissue from nonviable blastocysts (n = 6) using the RNeasy Mini Kit (Qiagen). Biopsies were collected from female mice that showed a viable pregnancy in one horn and pregnancy loss in the opposing horn to reflect uterine receptivity in these females. Total RNA was in vitro transcribed using the MessageAmp II-Biotin Enhanced Kit (Ambion, Austin TX). RNA concentration was evaluated with the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) before fragmentation and hybridization to the Codelink Mouse Whole Genome Bioarray (Applied Microarrays), which contains 34,967 transcripts other than controls.

Microarray results were analyzed using GeneSpring software (Agilent Technologies, Santa Clara, CA) with data normalized per chip to the 50th percentile and per gene to the mean. Student's *t*-test with the Benjamini and Hochberg False Discovery Correction revealed transcripts that were differentially expressed with significance at $P<.05$. Unsupervised hierarchical clustering with Pearson correlation created a condition tree that grouped samples according to a relationship in gene expression profiles. Annotation was performed using EASE (www.ease.com) to identify overrepresented biological processes and biochemical pathways from the differentially expressed genes.

Q RT-PCR was performed on additional biopsied healthy placenta (n = 10) and absorption tissue (n = 10) to validate the microarray data. Eleven differentially expressed transcripts from the complement and coagulation cascades were chosen for microarray data confirmation (Table 1). All Q RT-PCR experimental and statistical analyses were performed as described above.

RESULTS

Blastocyst Biopsy and Pregnancy Outcome

A total of 70 hatching blastocysts with equivalent morphology were selected for TE biopsy. On average, 10 (±3) TE cells were biopsied

TABLE 1

Coagulation and complement genes analyzed for validation of microarray transcriptome data.

Gene	GenBank accession no.	Microarray result ($P<.05$)	Q RT-PCR result ($P<.05$)	Validated
A2m	NM_175628	Increased	Increased	Yes
C1qc	NM_007574	Increased	Increased	Yes
C1ra	NM_023143	Increased	No expression differences between the groups	No
C3	NM_009778	Increased	Increased	Yes
Cd55	NM_010016	Increased	Increased	Yes but not significant
F11	NM_028066	Decreased	Decreased	Yes
Serpina1d	NM_009246	Increased	Increased	Yes
Serpina1e	NM_009247	Increased	Increased	Yes
Serpine1	NM_008871	Decreased	Decreased	Yes
Serping1	NM_009776	Increased	Increased	Yes
Tfpi	NM_011576	Decreased	Decreased	Yes

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per hatching blastocyst for individual transcriptome analysis. All blastocysts (100%) survived the biopsy procedure and were individually transferred to recipient pseudopregnant BDF1 females (one per uterine horn). Implantation and fetal development were assessed on day 16 of pregnancy. A total of 28 biopsied blastocysts implanted with viable healthy fetuses on day 16 . The mean weight and crown-rump measurements of the viable, healthy fetuses were 0.2046 g and 11.14 mm, respectively. A total of 12 biopsied blastocysts implanted but were absorbed during pregnancy loss. These nonviable implantation sites were biopsied for transcriptome analysis. The remaining biopsied blastocysts resulted in negative implantation (n = 30). Negative implantations were preferred for analysis when the opposing uterine horn displayed a positive implantation to reflect uterine receptivity in a particular female. The implantation rate for single ET in our BDF1 females was 57.14% (40/70). This is a considerably high implantation rate for a single gestation when the typical litter size for this BDF-1 mouse strain is 8 to 10 pups.

Blastocyst Gene Expression Analysis

Key genes associated with blastocyst developmental competence were selected for blinded gene expression analysis. Each of these genes, Actr3 (TE development), B3gnt5 (cell differentiation and adhesion), Eomes (TE differentiation), Cdx2 (TE differentiation), Slc7a5 (cell growth and differentiation), and Wnt3a (development), were expressed in every individual blastocyst biopsy (n = 44) by Q RT-PCR relative to the internal housekeeping gene, Gapdh. Statistical analysis revealed significant differences in gene expression profiles across these morphologically similar blastocysts, retrospectively corresponding to transfer outcome. Biopsied blastocysts that resulted in successful implantation and healthy fetal development exhibited elevated gene expression of B3gnt5, Wnt3a, and Eomes compared with either the absorption or negative groups ($P<.05$; Fig. 1). The expression profiles of these three genes (B3gnt5, Wnt3a, and Eomes) were able to differentiate among all three transfer outcomes. Blastocysts that resulted in complete implantation failure showed a significant decrease in expression of B3gnt5 and Eomes ($P<.05$; Fig. 1). When blastocysts implanted but were absorbed (nonviable implantation), a significant decrease was observed in Wnt3a and Eomes expression ($P<.05$; Fig. 1). The remaining three genes (Actr3, Cdx2, and Slc7a5) did not display differential expression across the groups (data not shown).

Day 16 Placental Transcriptome Analysis

Comprehensive transcriptome analysis of healthy placenta (n = 6) and nonviable implantation sites (n = 6) revealed two distinct transcriptomes. Unsupervised hierarchical clustering created a heat map that clearly and completely separated these two outcome groups (Fig. 2A). Statistical analysis revealed 9,073 transcripts with differential expression, 5,918 displaying a greater than twofold change ($P<.05$); 3,254 transcripts were down-regulated, and 2,664 transcripts were up-regulated in absorptions compared with healthy placental tissue (greater than twofold; $P<.05$). Chromosome mapping of these 5,918 differentially expressed transcripts revealed distribution across all mouse chromosomes (data not shown). Cellular component classification identified 37% of the differentially expressed transcripts to be intracellular, 33% membrane bound, and 30% extracellular. Gene annotation was performed to identify overrepresented categories of biological processes and biochemical pathways among the differentially expressed transcripts. Similarities and differences in overrepresented biological processes and biochemical pathways were observed between up- and down-regulated transcripts in association with spontaneous pregnancy loss (Table 2). The predominantly altered pathways in absorptions were the complement and coagulation cascades, with 26 differentially expressed genes, reflecting the biological significance ($P<.05$).

FIGURE 1

Individual blastocysts of equivalent morphology were transferred after TE biopsy. Q RT-PCR on individual biopsied samples revealed differential gene expression profiles corresponding to implantation outcome. Positive implantation with healthy fetal development displayed increased expression of B3gnt5, Eomes, and Wnt3a (*$P<.05$). Nonviable implantation or pregnancy loss showed significant decrease in gene expression of B3gnt5 and Wnt3a (*$P<.05$). Negative implantation revealed a decrease in gene expression of B3gnt5 and Eomes (*$P<.05$).

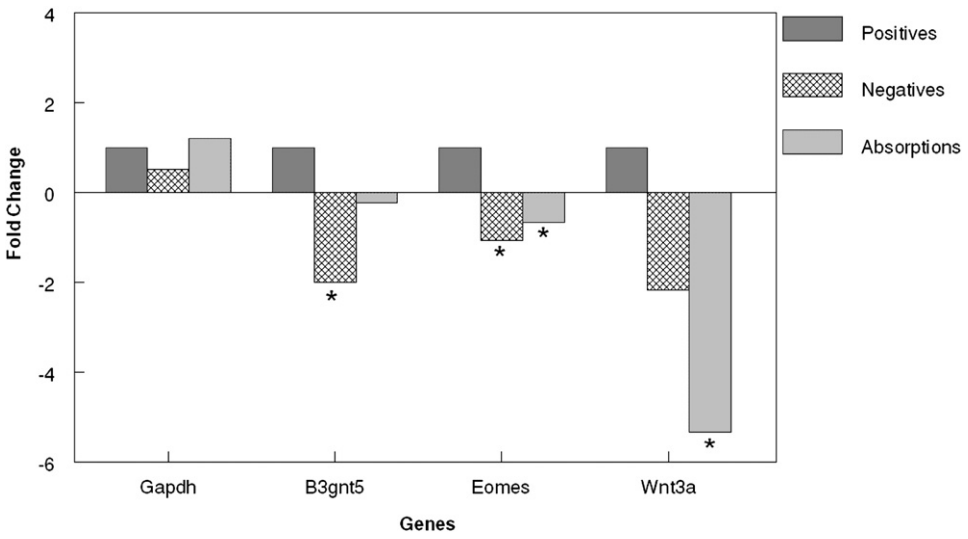

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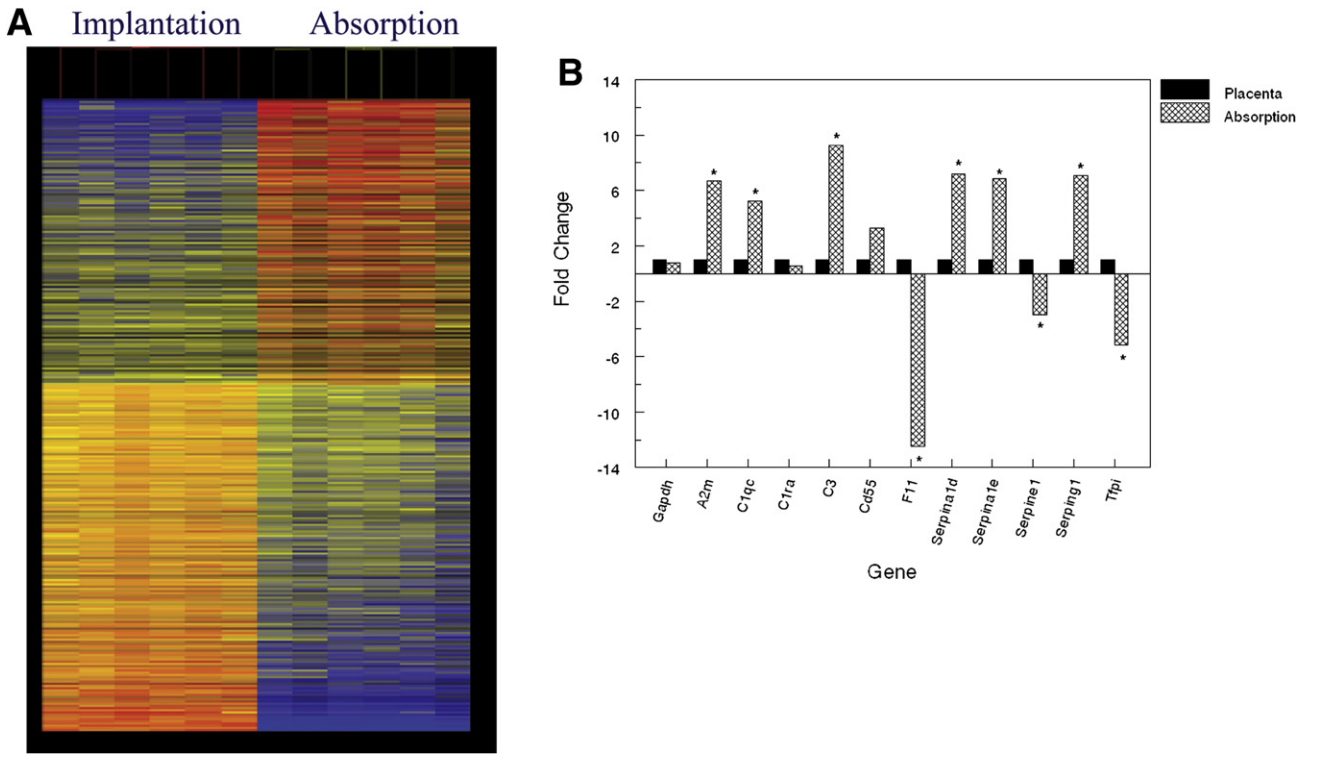
FIGURE 2

(**A**) Unsupervised hierarchical clustering of all microarray samples classified two distinct transcriptomes with complete separation of healthy placental samples from biopsied absorption sites. Normalized gene expression ratios are depicted by color intensity, with the highest expression corresponding to red and the lowest expression corresponding to blue. (**B**) Validation of microarray data using Q RT-PCR examined the expression of 10 altered complement and coagulation cascade genes relative to the housekeeping gene, Gapdh. Differential expression was validated for 9 of the 11 genes analyzed (*$P < .05$).



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Q RT-PCR was performed to validate the microarray results in an additional set of samples, healthy placenta (n = 10) and nonviable implantation sites (n = 10). Ten altered genes from the complement and coagulation cascades were analyzed relative to the housekeeping gene, Gapdh, in each individual sample (Fig. 2B). Three transcripts validated the microarray results and showed decreased gene expression in absorptions, including the coagulation inhibitor Tfp1 ($P<.05$; Fig. 2B; Table 1). Six transcripts displayed increased expression in absorptions, including the key complement factors: C3, C1q, and C2 ($P<.05$), also validating the microarray results (Fig. 2B; Table 1). Only one gene, C1ra, did not confirm the microarray data, with no expression differences observed between the groups (Fig. 2B).

TABLE 2

Overrepresented biological processes and biochemical pathways in association with spontaneous pregnancy loss.

Increased expression in absorption ($P < .05$)	Decreased expression in absorption ($P < .05$)
Defense response (15)	Transport (16)
Development (15)	Transcription (14)
Transport (15)	Development (13)
Cell surface receptor linked signal transduction (13)	Unknown (10)
Transcription (11)	Biosynthesis (9)
Response to stress (9)	Cell proliferation (9)
Proteolysis (9)	Cell surface receptor linked signal transduction (9)
Unknown (7)	Intracellular signaling cascade (7)

Note: Data in parentheses are percents.

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DISCUSSION

The embryo-maternal molecular dialogue associated with the stages of implantation, including the order of early events, still remains poorly understood, in particular the role of the blastocyst. This is important not only for ART success but also with regard to pregnancy loss, complications, and even postnatal health. In this study, TE transcription directly predicted an individual blastocyst fate in the uterus. Specific gene expression profiles of three developmental genes (B3gnt5, Wnt3a, and Eomes) characterized the success of implantation and distinguished ongoing healthy fetal development from implantation failure or pregnancy loss after single ET.

The Lc3-synthase gene, B3gnt5, initiates the formation of the lactoseries glycosphingolipids (GSL). GSL are integral components of mammalian cell membranes, subdivided into structural series that

are defined by distinct trisaccharide cores. The lactoseries-derived GSL are believed to participate in cell adhesion during embryogenesis and cell differentiation (16). Disrupting the B3gnt5 gene leads to preimplantation lethality, which potentially is caused by a defect of the lactoseries-derived GSL pathway and possibly by destabilization of other enzymes involved in GSL biosynthesis (11). Our data showed a significant decrease in expression of B3gnt5 in blastocysts that failed to implant (Fig. 1). Taken together, it is probable that there is a role for lactoseries-derived GSL in cell-cell adhesion that may directly contribute to implantation.

Eomesodermin (Eomes) is a T-box transcription factor essential for development of TE differentiation in the mouse blastocyst (12) and specification of the definitive endoderm lineage (13). In vitro studies have shown that Eomes mutants are unable to attach or form trophoblast outgrowths (12) or if they do implant will die around 6.5–7.5 days post conception in utero (13). The blastocysts in our study confirmed these findings and showed decreased Eomes gene expression in conjunction with negative implantation as well as with nonviable implantation.

Wnt proteins are cysteine-rich secreted molecules that regulate cell-cell interactions during embryogenesis and development (17). Canonical Wnt signaling involves Wnt receptor binding, followed by the accumulation and nuclear import of β-catenin to interact with transcription factors influencing expression of specific target genes (18). Wnt/β-catenin signaling is essential for normal blastocyst function and implantation competency (19). Furthermore, uterine Wnt/β-catenin signaling is induced at the putative site of blastocyst attachment just before implantation (20).

Wnt3a has been shown to activate canonical Wnt/β-catenin signaling and is highly expressed throughout the mouse blastocyst using whole-mount in situ hybridization (15). Gene inactivation of Wnt3a reveals initial implantation with early lethality around embryonic day 12.5 (21). Our data were consistent with these studies as gene expression of Wnt3a did not predict negative implantation. Wnt3a was only significantly decreased in blastocysts that implanted but were absorbed, resulting in early pregnancy loss.

Spontaneous miscarriage is a frequent occurrence in human reproduction, including 2%–5% of couples who experience recurrent pregnancy loss. Patient management is contentious, with up to 50% of cases having no identifiable reason. Recent diagnostic approaches have included the investigation of placental function and maternal-fetal immunology. In this study, comprehensive transcriptome analysis of healthy placenta and absorption sites from nonviable blastocysts revealed two distinct tissue types with over 5,918 differentially expressed transcripts ($P<.05$). These transcriptome data most likely reflect the end of the miscarriage process, representing numerous altered biochemical and metabolic pathways.

The most predominant altered pathways observed in these pregnancy losses were the complement and coagulation cascades that act in concert to protect against invading organisms and to initiate inflammation. Appropriate complement inhibition is an absolute requirement for normal pregnancy since it is a proinflammatory, hypercoagulable state. In this fertile mouse model, inappropriate activation of the cascades resulted in differential expression of both activators and inhibitors that were significantly associated with spontaneous miscarriage. This study corroborates previous work that demonstrated a possible causative role for complement activation during pregnancy loss (22). Ongoing strategies to unravel the molecular basis of nonviable implantation may allow development of novel clinical interventions to prevent pregnancy loss.

This study revealed for the first time that individual blastocyst gene expression profiles directly correlate with outcome, including successful implantation and spontaneous pregnancy loss. Consequently, a gene expression profile may describe the state of activity of a blastocyst, thereby revealing the molecular pathways required for successful implantation, including the elucidation of pregnancy loss. Molecular characterization of the intricate blastocyst-uterine crosstalk during implantation, and at the time of spontaneous miscarriage, is relevant to our understanding of these complex processes and could contribute to the potential for quantifying blastocyst competence.

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