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Infertility Diagnosis has a Significant Impact on the Developing Blastocyst’s Transcriptome

Running title: Infertility diagnosis impacts blastocyst transcriptome

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Abstract

Infertility is a disease that affects 1 out of 6 reproductive-age couples. The causes are diverse including, but not limited to, polycystic ovaries (PCO), male factor (MF), and when all sources have been ruled out, the couple is defined as having unexplained infertility. While each etiology is distinct, they are all typically associated with lower implantation potential and poorer pregnancy outcomes. The aim of this study was to characterize the global transcriptome of human blastocysts, from patients presenting with specific infertility etiologies, to elucidate novel biological pathways that may influence downstream implantation. Surplus, cryopreserved, day 5 blastocysts of transferrable quality were donated with Institutional Review Board (IRB) approval and patient consent for transcriptome microarray analysis. The human blastocyst transcriptome contained 13,136 annotated genes with the most significant alteration observed for blastocysts derived from infertile PCO patients. 869 genes were differentially expressed in PCO blastocysts, 348 in MF blastocysts, and 473 in blastocysts from unexplained etiology compared to fertile, donor controls (P<0.05; >2-fold). Validation utilizing real-time quantitative PCR was performed on genes belonging to enriched pathways including: BCL2L10, HSPA1A, HSPA1B, ATF3, FGF9, LEFTY1, LEFTY2, GDF15, INHBA, AJAP1, CDH9, and LAMA4 (P<0.05; >2-fold). Functional annotation of biological and molecular processes revealed both similarities, as well as differences, across the infertility groups. All etiologies displayed transcriptome alterations in signal transducer activity, receptor binding, reproduction, cell adhesion, and response to stimulus. However, blastocysts from PCO patients were also enriched for apoptosis while MF blastocysts displayed enrichment for cancer processes. Blastocysts from couples with unexplained infertility were enriched for pathways related to various disease states which included mTOR and adipocytokine signaling. In conclusion, underlying patient infertility diagnosis is reflected in the blastocyst transcriptome, which may then impact developmental competence and implantation outcomes. Ongoing research could
result in the development of new laboratory or clinical therapies, improving patient diagnosis and management.

**Keywords:** polycystic ovaries, male factor, unexplained infertility, transcriptome, gene expression
The World Health Organization (WHO) estimates that 1 out of 6 couples struggle with infertility and the origins are equally distributed between male and female. There are many different causes of infertility including, among others, polycystic ovaries (PCO) and male factor (MF). Infertility can be the result of a variety of problems ranging from genetic to hormonal and even environmental. When all known sources have been ruled out, the couple is defined as idiopathic or unexplained.

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in women of reproductive age and a major cause of female factor infertility [Sirmans and Pate, 2013]. It is the result of hormonal imbalances, typically excess androgen production, which lead to rare or irregular ovulation [Krishnan and Muthusami, 2017]. Unlike PCOS, women diagnosed with polycystic ovaries (PCO) do not have a metabolic condition but have ovaries with abnormally high follicle counts and can still possess hormonal imbalances. PCO is far more common than PCOS, affecting anywhere from 20-30% of the population and the causes are largely unknown [Koivunen et al., 1999]. PCO patients are often infertile due to anovulation, thereby requiring assisted reproductive technologies (ART) to conceive. High miscarriage rates are associated with this infertility phenotype, as well as decreased fertilization after IVF, suggesting poorer quality oocytes and embryos [Hardy et al., 1995].

Male factor infertility, which is almost always defined as abnormal semen analysis based on WHO guidelines, is solely responsible for 20-30% of human infertility and is a contributing factor in half of all couples presenting for ART [Agarwal et al., 2015]. Problems with sperm production can originate from many different factors including hormonal, environmental, and even on a physical level within the testicle, causing problems with the seminiferous tubules. These tubules contain the Sertoli cells that act as nourishment for developing germ cells and are the location for spermatogenesis. Poor semen parameters have been shown to result in
delayed and failed fertilization, as well as compromised embryo development and quality (Janny and Menez, 1994; Ron-el et al., 1991).

Unexplained infertility is diagnosed in about 15-30% of infertile couples and is difficult to treat due to the unknown underlying etiology (Practice Committee of the American Society for Reproductive, 2006). It is defined as the inability to conceive after 12 months of regular, unprotected intercourse and when all recommended fertility assessments fail to reveal any anomaly (Quaas and Dokras, 2008). Patients can present with varying infertility histories including multiple IVF failures, poor embryo development, as well as lengthy periods of infertility.

A retrospective review of 45 studies found that couples with this diagnosis have, on average, a 1-4% chance of achieving pregnancy during any given menstrual cycle without utilizing ART (Guzick et al., 1998). Nevertheless, 40-60% will spontaneously conceive within 3 years (depending on the female partner’s age) and this rate can increase to as high as 75% with the use of ART (Daniela Galliano, 2015). ART techniques can also potentially help further address the cause of infertility in these patients (i.e. low fertilization rates, embryo fragmentation, abnormal oocytes, etc.) as well as improve time to conception.

A fertilized oocyte must not only facilitate the syngamy of the male and female genomic contributions but also undergo a series of cellular divisions before embryonic genome activation is initiated (Fragouli et al., 2013). Both the timing of the activation, as well as the synchrony of genes activated, must be accurately controlled to produce a blastocyst stage embryo that is viable and developmentally competent for implantation to occur (Latham and Schultz, 2001). In the mouse model, studies have observed two waves of embryonic gene transcription, the first corresponding to zygotic genome activation which occurs at the 1-2 cell stage, and the second occurring during the morula-to-blastocyst transition (Hamatani et al., 2004a). While these transcriptional events are similar in the human embryo, the timing is different with the zygotic genome activation occurring at the 4-8 cell stage (Niakan et al., 2012). Any irregularities during this critical time can lead to embryos that are incompetent and unable to implant.
The interactions between the blastocyst and the uterus that result in successful implantation are directed by an equally complex molecular dialogue \cite{Fitzgerald2008}. Uterine receptivity has been extensively studied on all molecular levels, including the cross-talk between the embryo and endometrium which is quite extensive and results in an environment ideal for embryo adhesion and placentation \cite{Miravet-Valenciano2015}. It has also been shown that viable mouse embryos have a specific gene expression profile that favors uterine attachment and invasion of the maternal endometrium. Chaen et al. found that ovarian estrogen indirectly coordinates mouse blastocyst adhesion through integrin activation in the blastocyst \cite{Chaen2012}. Additionally, a mammalian model for blastocyst activity has shown that specific molecular signaling directs either blastocyst activation or dormancy, affecting implantation competency \cite{Hamatani2004b}. Our lab has previously reported that differential mouse trophectoderm gene expression following embryo biopsy is associated with murine blastocyst implantation success. Specifically, higher gene expression of B3gnt5, Cdx2, Eomes, and Wnt3a were predictive of sustained implantation. In contrast, decreased gene expression of Eomes and Wnt3a were associated with absorption or pregnancy loss and decreased gene expression of B3gnt4 and Cdx2 were observed with negative outcomes \cite{Parks2011}.

There is limited knowledge of the human preimplantation embryo transcriptome and how it correlates to pregnancy outcomes. Jones et al. examined the transcriptome of human trophectoderm biopsies and identified more than 7000 transcripts expressed exclusively in viable blastocysts \cite{Jones2008}. A more recent study performed single-cell RNA sequencing on both human and mouse preimplantation embryos to determine a dataset of genes that are important for pluripotency \cite{Blakeley2015}. Ongoing transcriptome analysis in our lab revealed differential gene expression from blastocysts obtained from PCO women compared with donor controls. Over 800 genes were found to be disrupted in these
PCO blastocysts in addition to 12 altered protein biomarkers, demonstrating a link between patient infertility phenotype and embryo development [Katz-Jaffe et al., 2010].

The objective of the present study was to further explore the global transcriptome of human blastocysts from patients with differing infertility etiologies, specifically PCO, male factor, and unexplained infertility, to uncover novel biological pathways associated with their infertility that may influence downstream implantation outcomes. These findings will further our understanding of the impact of infertility diagnoses on the embryonic molecular signature at the time of implantation, and may lead to refined lab-based and clinical approaches for improving IVF outcomes.

Materials and Methods

Human blastocysts

Surplus, cryopreserved, anonymous, human blastocysts from IVF patients with specific infertility diagnoses were donated with Institutional Review Board (IRB) consent. All embryos were considered to be transferable quality with a grade of 3BB or better on day 5 of embryo development [Gardner and Schoolcraft, 1999]. Either slow freezing or vitrification protocols were used to cryopreserve the blastocysts [Kuwayama, 2007; Veeck et al., 2004] which were grouped according to a single distinct infertility diagnosis: n=50 young donor oocyte controls with no male factor infertility; n=50 polycystic ovaries (PCO); n=50 male factor infertility (MF); and n=50 unexplained infertility (UE). Every blastocyst used in this study came from a different patient (female <38 years old, male <40 years old) and all patients had successful pregnancies from the same IVF cohort as the blastocyst used for research. Patients diagnosed with PCO had polycystic ovaries confirmed by ultrasound but did not have any endocrine or metabolic abnormalities, as determined by androgen levels, fasting glucose and insulin levels, and oral glucose tolerance testing. MF infertility patients were all diagnosed based on WHO guidelines as oligoasthenoteratozoospermia with sperm concentration <15 million/ml, motility <32%, and
<4% normal morphology. UE infertility was defined following a negative fertility workup which included normal semen analysis, normal ovarian reserve testing, and normal uterine assessment with no prior failures or missed abortions.

**Blastocyst thaw and RNA isolation**

Blastocysts were either thawed or warmed using routine laboratory procedures, with an overall 95% survival rate. Blastocysts in each distinct infertility diagnosis group were pooled (n=25 per pool, 2 pools per group) and RNA was isolated using the PicoPure RNA Isolation Kit (ThermoFisher Scientific, Grand Island NY) per the manufacturer’s instructions with minor modifications. Briefly, blastocysts were lysed in 10ul of Extraction Buffer before adding 1 volume of 70% ethanol and binding to a silica-based membrane. Samples were then washed and on-column deoxyribonuclease treated (Qiagen, Valencia CA) prior to elution in 20ul and storage at -80°C.

**Microarray hybridization**

Isolated RNA from each group was reverse transcribed, amplified, and labeled using the LowInput QuickAmp Labeling Kit (Agilent Technologies, Santa Clara CA). Quantification and quality of total RNA was performed using the High Sensitivity RNA ScreenTape on a 4200 TapeStation System (Agilent Technologies). Quantification and specific activity of labeled cRNA was determined using the NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific). 600ng of cRNA was then applied to the SurePrint G3 Human Gene Expression Microarray containing 50,599 biological features (Agilent Technologies) per the manufacturer’s instructions and hybridized in a rotating oven for 17 hours at 65°C. Arrays were washed and then scanned using a DNA Microarray Scanner C (Agilent Technologies). Feature Extraction software was utilized to extract gene expression data (Agilent Technologies).

**Real-time quantitative PCR Validation**

RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). cDNA was diluted 1:5 in nuclease-free water and Real-time
quantitative PCR (RT-qPCR) was performed for validation of specific differentially expressed genes identified from the transcriptome analysis. Absolute expression was quantified relative to a standard curve using slope and PCR efficiencies and normalized to a stable housekeeping gene, GAPDH. Briefly, Power SYBR® Green PCR Master Mix (ThermoFisher Scientific) was combined with 5uM primer mix and 5ul diluted cDNA for a total volume of 25ul. The reaction was incubated at 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute with a final dissociation stage for melt curve analysis.

Statistical analysis

Transcript analysis was performed using GeneSpring software (version 7, Agilent Technologies), including principal component analysis (PCA), unsupervised hierarchical clustering, one way ANOVA and unpaired t-test with Benjamini-Hochberg correction (significance at P<0.05). qPCR analysis was performed with REST 2009 software (Qiagen) which uses the correction for exact PCR efficiencies with mean crossing point deviations between sample and control groups to determine an expression ratio that is tested for significance by a Pair Wise Fixed Reallocation Randomization Test. Significance was defined as P<0.05.

Ethical approval

All human participants were consented and this study was approved by an institutional review board.

Results

The blastocyst transcriptome according to infertility diagnosis

The overall human blastocyst transcriptome contained 33,587 gene transcripts which included numerous splicing variants and isoforms, revealing 13,136 annotated genes. PCA and unsupervised hierarchical clustering distinguished each of the four blastocyst groups by their transcriptomes (Figure I). The most significant transcriptome variation was observed in
blastocysts derived from infertile PCO patients. Compared to donor controls, significant
differences in transcription (>2 fold; P<0.05) were observed for 869 genes in PCO blastocysts,
348 genes in MF blastocysts, and 473 genes in blastocysts from couples with unexplained
infertility (Figure II). Both upregulation and downregulation were observed in each group: PCO
= 647 increased, 222 decreased; MF = 143 increased, 205 decreased; UE = 305 increased, 168
decreased (Table I).

Functional annotation of PCO blastocysts was performed using DAVID
[https://david.ncifcrf.gov] which revealed significant differences in gene ontology including: cell
communication, differentiation and adhesion, reproduction, transcription factor activity,
regulation of apoptosis, receptor binding, signal transducer activity, and response to hormone
stimulus. Pathway analysis identified enriched biological processes with altered transcripts in
PCO vs. control blastocysts (P<0.05) including gap junction proteins and genes involved in p53
signaling, calcium signaling, TGF-beta signaling, histidine metabolism, and apoptosis (Table I).

Transcriptome analysis of MF blastocysts resulted in some similar gene ontology
differences in relation to PCO blastocysts that included: signal transduction, regulation of
apoptosis, cell adhesion, reproduction and receptor binding. Unique differences were also
observed for MF including: response to stress, regulation of growth, and protein dimerization
activity. Pathway analysis of MF vs. control blastocysts revealed enrichment in TGF-beta, ErbB,
B cell receptor and GnRH signaling (Table I).

Functional annotation of UE blastocysts also had similar outcomes to PCO blastocysts in
signal transducer activity, receptor binding, cell differentiation, adhesion and morphogenesis,
reproduction, and response to stimulus, among others. Unique differences for UE included:
oxidoreductase activity, protein dimerization activity, and monoxygenase activity. Pathway
analysis of UE vs. control blastocysts had some similarities (TGF-beta signaling and focal
adhesion) compared to the other two groups but many more differences including affected
pathways: Type I diabetes, antigen processing, leukocyte migration, autoimmune thyroid disease, systemic lupus erythematosus, mTOR signaling, and adipocytokine signaling (Table I).

**Microarray Validation**

RT-qPCR was used to validate the microarray data by investigating the expression levels of genes involved in stress response, apoptosis, cell growth and adhesion, and embryonic development. qPCR results confirmed a significantly higher expression of the stress sensing protein activating transcription factor 3 (ATF3) in PCO blastocysts compared to donor controls (P<0.05) and lower levels of anti-apoptotic oocyte-inherited gene (BCL2L10) (P<0.05) and the heat shock proteins HSPA1A and HSPA1B (P<0.05; Figure III). Blastocysts from patients with MF infertility displayed an increased expression of growth differentiating factor 15 (GDF15) (P<0.05) and the cell proliferation regulator, INHBA (P<0.05; Figure IV) as observed in the microarray data. Additionally, reduced expression was validated in MF blastocysts for fibroblast growth factor 9 (FGF9) (P<0.05), and left-right determination factors 1 and 2 (LEFTY1, LEFTY2) (P<0.05; Figure IV). Three genes were also confirmed to have reduced expression in blastocysts with UE infertility as observed in the microarray data: Adherens Junctions Associated Protein 1 (AJAP1), cadherin 9 (CDH9), and Laminin Subunit Alpha 4 (LAMA4) (All P<0.05; Figure V).

**Discussion**

This study highlighted that the human blastocyst transcriptome is significantly impacted by the type of patient infertility diagnosis (PCO, MF, and UE). All three of the infertility diagnoses shared transcriptome alterations, with PCO blastocysts displaying the greatest transcriptome variation. An altered blastocyst transcriptome has the potential to impact overall developmental competence, contributing to the infertility observed in patients with these etiologies.
The expression of genes involved in stress response and apoptosis were significantly different in PCO blastocysts compared to donor controls, suggesting a PCO environment has a significant impact on the developing blastocyst’s transcriptome, including alterations in stress signaling pathways and the regulation of apoptosis. These findings are consistent with those of Wang et al, who reported differential expression of 650 transcripts in the ovaries of women with PCOS compared to normal ovaries and found similar alterations in pathways involved in stress response, apoptosis, and regulation of transcription [Wang et al., 2014]. A higher expression of ATF3 and lower expression of BCL2L10, HSPA1A and HSPA1B in PCO blastocysts was observed in this study compared with donor controls. ATF3, a stress sensor, increases p53 protein levels and transcription of p53-responsive genes that result in either cell arrest and DNA repair or apoptosis [Yan et al., 2005], thereby maintaining DNA integrity. In the developing embryo, highly regulated apoptotic events are critical for embryo homeostasis and survival. The BCL2 proteins are both anti- and pro-apoptotic; BCL2L10 is an anti-apoptotic oocyte-inherited transcript and elimination of BCL2L10 accelerates oocyte death [Guillem in et al., 2009]. HSPA1A and B are involved in embryonic genome activation and decreased expression has been observed in mammalian arrested embryos [Le Masson and Christians, 2011] [Pan et al., 2014]. Likewise, gene expression analyses of oocytes from PCOS women also revealed reduced expression in these heat shock proteins [Wood et al., 2007]. Decreased fertilization rates after IVF, as well as a higher risk of miscarriage are associated with the PCO infertility diagnosis. Altered expression levels of each of these genes in PCO may disrupt the normal balance of apoptosis in the pre-implantation embryo, with downstream consequences for implantation and developmental outcomes.

Blastocysts derived from MF infertility were significantly altered for TGF-beta and ErbB signaling pathways which are crucial during cell growth and proliferation. GDF15 is a gene belonging to the TGF-beta superfamily and plays a role in regulating inflammatory and apoptotic pathways. The increased expression observed for GDF15 in MF blastocysts is associated with
numerous disease states including inflammation and oxidative stress. Likewise, INHBA, which
encodes the same TGF-beta superfamily of proteins, was also found to have increased
expression in MF blastocysts. It is a negative regulator of gonadal stromal cell proliferation,
thus excess expression would lead to inappropriate decreases in cell proliferation which could
negatively impact implantation potential. Decreased gene expression in MF blastocysts was
observed for FGF9, LEFTY1, and LEFTY2. FGF9 is involved in many biological processes
including embryo development, cell growth, and morphogenesis. It has been found to be
required for stimulating Erk1/2 activation in differentiating spermatagonia [Tassinari et al.,
2015]. LEFTY proteins are critical in sustaining pluripotency and implicated in differentiation of
embryonic stem cells [Khalkhali-Ellis et al., 2016]. Inactive LEFTY has been shown to result in
embryos that become entirely mesoderm and fail to develop [Hamada et al., 2002]. Poor
sperm parameters in MF patients are correlated with fertilization failure and compromised
embryo quality and development. Decreases in the expression of these genes could severely
impact embryo developmental competence, which is crucial for implantation.

Important pathways including cell differentiation and morphogenesis, reproduction, and
response to stress were affected from blastocysts derived from patients with UE infertility.
These pathways affect embryo growth and development as well as cell adhesion and migration.
Decreased expression was observed for three genes involved in cell adhesion and migration:
AJAP1, CDH9, and LAMA4. AJAP1 has been observed to be decreased in various cancers and
interacts with β-catenin complexes that impact cell cycle function and apoptosis [Zeng et al.,
2014]. The decreased expression observed in UE blastocysts could have a negative impact on
the balance of apoptosis, possibly leading to inappropriate expression of genes that affect
cellular invasion. CDH9 belongs to a family of cell adhesion molecules that regulate
morphogenesis and are involved in intracellular signaling pathways [Halbleib and Nelson,
2006]. These cadherins are responsible for cell-cell adhesion during morula compaction, in
addition to playing a role in tissue and organ development [Peyrieras et al., 1983]. Decreased
expression would inhibit the ability of both early embryo development, as well as later fetal
development in utero. LAMA4 is a laminin that mediates the attachment, migration, and
organization of cells into organized tissues during embryonic development. Laminins are vital
for organogenesis and have critical functions in several tissues including skin, muscle, and
vasculature \cite{Durbeej2010}. As the etiology of UE infertility is more ambiguous, many adverse
outcomes are possible including poor embryo development and IVF failure. The decreased
expressed observed in UE blastocysts could have significant consequences to embryo
implantation and ongoing development.

The similarities between all infertility groups included transcriptome alterations in signal
transducer activity, receptor binding, reproduction, cell adhesion, and response to stimulus.
These biological and molecular processes are all inter-related and crucial to embryo
development and implantation which are processes characterized by cells that proliferate,
migrate, and attach. Receptors are generally transmembrane protein molecules that bind to
signaling molecules in response to external stimuli. Once a receptor protein receives a signal,
a series of biochemical reactions is initiated which conveys those signals across a cell,
triggering changes in cell function or state, known as signal transduction. An example of this is
Hedgehog (Hh) proteins which are expressed during vertebrate development. Hh signaling has
been observed during embryonic development and has significance during the growth of
reproductive tissues including the gonad and uterus \cite{Walterhouse2003}. Cellular
adhesion, in which cells interact to attach to a surface, regulates signal transduction and is an
essential process for embryo implantation into the uterus lining. It is therefore not surprising
that all three infertility diagnoses shared blastocysts with transcriptome alterations in these
important biological and molecular processes.

The differences between each infertility group were more remarkable when studying
their pathway analyses. Blastocysts from women with PCO were enriched for apoptosis. This
is in concordance with data published showing that ovaries from women with PCO have
abnormal apoptotic activity and folliculogenesis (Cai et al., 2013). On the other hand, signaling pathways from blastocysts with MF infertility were largely involved in cancer processes. Lian et al. also found that infertile men with maturation arrest had hyperactive germ cell proliferation as a result of the inhibition of tumor suppressor IRF1 by its microRNA, miR-383 (Lian et al., 2010). Interestingly, UE infertility was enriched for pathways involved in mTOR and adipocytokine signaling, both of which are related to various disease states. This could explain some of the difficulties in treating patients with unknown infertility as the cause of their reproductive deficiencies could be the result of anything ranging from environmental to unknown disease risk factors. For example, autoimmune disorders, such as lupus, have been shown to cause a woman’s immune system to reject an embryo, thereby preventing implantation into the uterus (Mojarrad et al., 2013).

Conclusions

This novel study suggests that underlying patient infertility diagnosis has an impact on the blastocyst transcriptome, modifying genes that may affect developmental competence and implantation outcomes. Ongoing research determining how transcription alterations are linked to inferior pregnancy outcomes for PCO, MF, and UE patients is crucial to improving IVF success. This is especially true for UE patients as a more defined infertility diagnosis could translate into more targeted clinical management. Understanding how different infertility etiologies contribute to embryo viability may also lead to the development of new laboratory and clinical therapies. An example of this type of clinical advancement is the endometrial receptivity array which identifies endometrial receptivity for patients with repeated implantation failure (Ruiz-Alonso et al., 2013). Further studies could lead to similar advancements including individualized embryo culture systems and custom stimulation and frozen embryo transfer protocols, thereby improving outcomes for these patients.
Acknowledgements

We would like to acknowledge all the physicians and embryologists at the Colorado Center for Reproductive Medicine for their support in this study. We would also like to acknowledge Alyssa Patton for her assistance collecting material in this study.

Authors’ Roles

BRM performed all microarray and RT-qPCR experiments and analysis and took the lead in preparing the manuscript. JCP collected all blastocyst samples for all experiments. DKG and WBS provided critical review of the manuscript. MKJ designed and oversaw the completion of the study. All authors participated in the editing of the manuscript.

Conflict of Interest and Funding

No conflict of interest or outside funding was provided.

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Table I. Significantly altered transcripts and pathways associated with specific infertility diagnoses

<table>
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Figure 1. Differential transcriptome profiles based on infertility diagnosis. Human blastocyst transcriptomes from unexplained infertility (UE) (lanes 1 and 2), polycystic ovaries (PCO) (lanes 3 and 4), male factor (MF) (lanes 5 and 6) and control (lanes 7 and 8). Unsupervised hierarchical clustering clearly separated the transcriptomes of the four groups, with the most significant variation in gene expression observed for the PCO group.
Figure 2. Venn diagram depicting gene overlap of differentially expressed transcripts between the infertility diagnoses groups. P < 0.05; >2-fold; one-way ANOVA and unpaired t-test with Benjamini–Hochberg correction. N = 1385 genes.
Figure 3. Altered expression of genes involved in apoptosis and stress response in PCO blastocysts. Quantitative PCR (qPCR) was performed to validate expression levels of activating transcription factor 3 (ATF3), BCL2 like 10 (BCL2L10) and heat shock protein family A members 1A and 1B (HSPA1A and HSPA1B) in donor control and PCO blastocysts with peptidylprolyl isomerase A (PPIA) transcription as the constant internal reference gene. A significant increase in ATF3 expression was observed, while BCL2L10, HSPA1A and HSPA1B displayed significantly lower expression in PCO blastocysts, compared to donor controls; *P < 0.05; pair-wise fixed reallocation randomization test.
**Figure 4.** Altered expression of genes involved in cell growth and differentiation in MF blastocysts. qPCR was performed to validate expression levels of growth differentiation factor 15 (GDF15), inhibin beta A subunit (INHBA), fibroblast growth factor 9 (FGF9) and left-right determination factors 1 and 2 (LEFTY1 and LEFTY2) in donor control and MF blastocysts with GAPDH transcription as the constant internal reference gene. (A) Expression of FGF9, LEFTY1 and LEFTY2 was significantly lower and (B) GDF15 and INHBA significantly higher in MF blastocysts compared to donor controls; *P < 0.05; pair-wise fixed reallocation randomization test.
Figure 5. Altered expression of genes involved in cell adhesion and migration in UE blastocysts. qPCR was performed to validate expression levels of adherens junctions associated protein 1 (AJAP1), cadherin 9 (CDH9) and laminin subunit alpha 4 (LAMA4) in donor control and UE blastocysts with GAPDH transcription as the constant internal reference gene. All three genes were significantly decreased in expression in UE blastocysts compared to donor controls; *P < 0.05; pairwise fixed reallocation randomization test.
Table 1. Significantly altered transcripts and pathways associated with specific infertility diagnoses

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PCO, polycystic ovaries; MF, male factor infertility; UE, unexplained infertility; TGF, transforming growth factor; ErbB, epidermal growth factor; mTOR, mechanistic target of rapamycin.

Statistical method: one-way ANOVA and unpaired t-test with Benjamini–Hochberg correction.