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1 **Infertility Diagnosis has a Significant Impact on the Developing Blastocyst's**
2 **Transcriptome**
3
4 **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

54 result in the development of new laboratory or clinical therapies, improving patient diagnosis
55 and management.
56
57 **Keywords:** polycystic ovaries, male factor, unexplained Infertility, transcriptome, gene
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80 **Introduction**

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

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

98 Male factor infertility, which is almost always defined as abnormal semen analysis based
99 on WHO guidelines, is solely responsible for 20-30% of human infertility and is a contributing
100 factor in half of all couples presenting for ART (Agarwal *et al.* , 2015). Problems with sperm
101 production can originate from many different factors including hormonal, environmental, and
102 even on a physical level within the testicle, causing problems with the seminiferous tubules.
103 These tubules contain the Sertoli cells that act as nourishment for developing germ cells and
104 are the location for spermatogenesis. Poor semen parameters have been shown to result in

105 delayed and failed fertilization, as well as compromised embryo development and quality (Janny
106 and Menezo, 1994, Ron-el *et al.* , 1991).

107 Unexplained infertility is diagnosed in about 15-30% of infertile couples and is difficult to
108 treat due to the unknown underlying etiology (Practice Committee of the American Society for
109 Reproductive, 2006). It is defined as the inability to conceive after 12 months of regular,
110 unprotected intercourse and when all recommended fertility assessments fail to reveal any
111 anomaly (Quaas and Dokras, 2008). Patients can present with varying infertility histories
112 including multiple IVF failures, poor embryo development, as well as lengthy periods of infertility.
113 A retrospective review of 45 studies found that couples with this diagnosis have, on average, a
114 1-4% chance of achieving pregnancy during any given menstrual cycle without utilizing ART
115 (Guzick *et al.* , 1998). Nevertheless, 40-60% will spontaneously conceive within 3 years
116 (depending on the female partner's age) and this rate can increase to as high as 75% with the
117 use of ART (Daniela Galliano, 2015). ART techniques can also potentially help further address
118 the cause of infertility in these patients (i.e. low fertilization rates, embryo fragmentation,
119 abnormal oocytes, etc.) as well as improve time to conception.

120 A fertilized oocyte must not only facilitate the syngamy of the male and female genomic
121 contributions but also undergo a series of cellular divisions before embryonic genome activation
122 is initiated (Fragouli *et al.* , 2013). Both the timing of the activation, as well as the synchrony of
123 genes activated, must be accurately controlled to produce a blastocyst stage embryo that is
124 viable and developmentally competent for implantation to occur (Latham and Schultz, 2001). In
125 the mouse model, studies have observed two waves of embryonic gene transcription, the first
126 corresponding to zygotic genome activation which occurs at the 1-2 cell stage, and the second
127 occurring during the morula-to-blastocyst transition (Hamatani *et al.* , 2004a). While these
128 transcriptional events are similar in the human embryo, the timing is different with the zygotic
129 genome activation occurring at the 4-8 cell stage (Niakan *et al.* , 2012). Any irregularities during
130 this critical time can lead to embryos that are incompetent and unable to implant.

131 The interactions between the blastocyst and the uterus that result in successful
132 implantation are directed by an equally complex molecular dialogue (Fitzgerald *et al.* , 2008).
133 Uterine receptivity has been extensively studied on all molecular levels, including the cross-talk
134 between the embryo and endometrium which is quite extensive and results in an environment
135 ideal for embryo adhesion and placentation (Miravet-Valenciano *et al.* , 2015). It has also been
136 shown that viable mouse embryos have a specific gene expression profile that favors uterine
137 attachment and invasion of the maternal endometrium. Chaen et al. found that ovarian
138 estrogen indirectly coordinates mouse blastocyst adhesion through integrin activation in the
139 blastocyst (Chaen *et al.* , 2012). Additionally, a mammalian model for blastocyst activity has
140 shown that specific molecular signaling directs either blastocyst activation or dormancy,
141 affecting implantation competency (Hamatani *et al.* , 2004b). Our lab has previously reported
142 that differential mouse trophectoderm gene expression following embryo biopsy is associated
143 with murine blastocyst implantation success. Specifically, higher gene expression of *B3gnt5*,
144 *Cdx2*, *Eomes*, and *Wnt3a* were predictive of sustained implantation. In contrast, decreased
145 gene expression of *Eomes* and *Wnt3a* were associated with absorption or pregnancy loss and
146 decreased gene expression of *B3gnt4* and *Cdx2* were observed with negative outcomes (Parks
147 *et al.* , 2011).

148 There is limited knowledge of the human preimplantation embryo transcriptome and how
149 it correlates to pregnancy outcomes. Jones et al. examined the transcriptome of human
150 trophectoderm biopsies and identified more than 7000 transcripts expressed exclusively in
151 viable blastocysts (Jones *et al.* , 2008). A more recent study performed single-cell RNA
152 sequencing on both human and mouse preimplantation embryos to determine a dataset of
153 genes that are important for pluripotency (Blakeley *et al.* , 2015). Ongoing transcriptome
154 analysis in our lab revealed differential gene expression from blastocysts obtained from PCO
155 women compared with donor controls. Over 800 genes were found to be disrupted in these

156 PCO blastocysts in addition to 12 altered protein biomarkers, demonstrating a link between
157 patient infertility phenotype and embryo development (Katz-Jaffe *et al.* , 2010).
158 The objective of the present study was to further explore the global transcriptome of
159 human blastocysts from patients with differing infertility etiologies, specifically PCO, male factor,
160 and unexplained infertility, to uncover novel biological pathways associated with their infertility
161 that may influence downstream implantation outcomes. These findings will further our
162 understanding of the impact of infertility diagnoses on the embryonic molecular signature at the
163 time of implantation, and may lead to refined lab-based and clinical approaches for improving
164 IVF outcomes.
165
166 **Materials and Methods**
167 ***Human blastocysts***
168 Surplus, cryopreserved, anonymous, human blastocysts from IVF patients with specific
169 infertility diagnoses were donated with Institutional Review Board (IRB) consent. All embryos
170 were considered to be transferable quality with a grade of 3BB or better on day 5 of embryo
171 development (Gardner and Schoolcraft, 1999). Either slow freezing or vitrification protocols
172 were used to cryopreserve the blastocysts (Kuwayama, 2007, Veeck *et al.* , 2004) which were
173 grouped according to a single distinct infertility diagnosis: n=50 young donor oocyte controls
174 with no male factor infertility; n=50 polycystic ovaries (PCO); n=50 male factor infertility (MF);
175 and n=50 unexplained infertility (UE). Every blastocyst used in this study came from a different
176 patient (female <38 years old, male <40 years old) and all patients had successful pregnancies
177 from the same IVF cohort as the blastocyst used for research. Patients diagnosed with PCO
178 had polycystic ovaries confirmed by ultrasound but did not have any endocrine or metabolic
179 abnormalities, as determined by androgen levels, fasting glucose and insulin levels, and oral
180 glucose tolerance testing. MF infertility patients were all diagnosed based on WHO guidelines
181 as oligoasthenoteratozoospermia with sperm concentration <15 million/ml, motility <32%, and

182 <4% normal morphology. UE infertility was defined following a negative fertility workup which
183 included normal semen analysis, normal ovarian reserve testing, and normal uterine
184 assessment with no prior failures or missed abortions.

185 ***Blastocyst thaw and RNA isolation***

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

194 ***Microarray hybridization***

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

205 ***Real-time quantitative PCR Validation***

206 RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit
207 (ThermoFisher Scientific). cDNA was diluted 1:5 in nuclease-free water and Real-time

208 quantitative PCR (RT-qPCR) was performed for validation of specific differentially expressed
209 genes identified from the transcriptome analysis. Absolute expression was quantified relative to
210 a standard curve using slope and PCR efficiencies and normalized to a stable housekeeping
211 gene, *GAPDH*. Briefly, *Power* SYBR® Green PCR Master Mix (ThermoFisher Scientific) was
212 combined with 5uM primer mix and 5ul diluted cDNA for a total volume of 25ul. The reaction
213 was incubated at 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15
214 seconds and 60°C for 1 minute with a final dissociation stage for melt curve analysis.

215 ***Statistical analysis***

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

224 ***Ethical approval***

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

227

228 **Results**

229 ***The blastocyst transcriptome according to infertility diagnosis***

230 The overall human blastocyst transcriptome contained 33,587 gene transcripts which
231 included numerous splicing variants and isoforms, revealing 13,136 annotated genes. PCA and
232 unsupervised hierarchical clustering distinguished each of the four blastocyst groups by their
233 transcriptomes (Figure I). The most significant transcriptome variation was observed in

234 blastocysts derived from infertile PCO patients. Compared to donor controls, significant
235 differences in transcription (>2 fold; $P<0.05$) were observed for 869 genes in PCO blastocysts,
236 348 genes in MF blastocysts, and 473 genes in blastocysts from couples with unexplained
237 infertility (Figure II). Both upregulation and downregulation were observed in each group: PCO
238 = 647 increased, 222 decreased; MF = 143 increased, 205 decreased; UE = 305 increased, 168
239 decreased (Table I).

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

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

253 Functional annotation of UE blastocysts also had similar outcomes to PCO blastocysts in
254 signal transducer activity, receptor binding, cell differentiation, adhesion and morphogenesis,
255 reproduction, and response to stimulus, among others. Unique differences for UE included:
256 oxidoreductase activity, protein dimerization activity, and monooxygenase activity. Pathway
257 analysis of UE vs. control blastocysts had some similarities (TGF-beta signaling and focal
258 adhesion) compared to the other two groups but many more differences including affected

259 pathways: Type I diabetes, antigen processing, leukocyte migration, autoimmune thyroid
260 disease, systemic lupus erythematosus , mTOR signaling, and adipocytokine signaling (Table I).

261 ***Microarray Validation***

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

276

277 **Discussion**

278 This study highlighted that the human blastocyst transcriptome is significantly impacted
279 by the type of patient infertility diagnosis (PCO, MF, and UE). All three of the infertility
280 diagnoses shared transcriptome alterations, with PCO blastocysts displaying the greatest
281 transcriptome variation. An altered blastocyst transcriptome has the potential to impact overall
282 developmental competence, contributing to the infertility observed in patients with these
283 etiologies.

284 The expression of genes involved in stress response and apoptosis were significantly
285 different in PCO blastocysts compared to donor controls, suggesting a PCO environment has a
286 significant impact on the developing blastocyst's transcriptome, including alterations in stress
287 signaling pathways and the regulation of apoptosis. These findings are consistent with those of
288 Wang et al, who reported differential expression of 650 transcripts in the ovaries of women with
289 PCOS compared to normal ovaries and found similar alterations in pathways involved in stress
290 response, apoptosis, and regulation of transcription (Wang *et al.* , 2014). A higher expression of
291 *ATF3* and lower expression of *BCL2L10*, *HSPA1A* and *HSPA1B* in PCO blastocysts was
292 observed in this study compared with donor controls. *ATF3*, a stress sensor, increases p53
293 protein levels and transcription of p53-responsive genes that result in either cell arrest and DNA
294 repair or apoptosis (Yan *et al.* , 2005), thereby maintaining DNA integrity. In the developing
295 embryo, highly regulated apoptotic events are critical for embryo homeostasis and survival. The
296 BCL2 proteins are both anti- and pro-apoptotic; *BCL2L10* is an anti-apoptotic oocyte-inherited
297 transcript and elimination of *BCL2L10* accelerates oocyte death (Guillemin *et al.* , 2009).
298 *HSPA1A* and *B* are involved in embryonic genome activation and decreased expression has
299 been observed in mammalian arrested embryos (Le Masson and Christians, 2011, Pan *et al.* ,
300 2014). Likewise, gene expression analyses of oocytes from PCOS women also revealed
301 reduced expression in these heat shock proteins (Wood *et al.* , 2007). Decreased fertilization
302 rates after IVF, as well as a higher risk of miscarriage are associated with the PCO infertility
303 diagnosis. Altered expression levels of each of these genes in PCO may disrupt the normal
304 balance of apoptosis in the pre-implantation embryo, with downstream consequences for
305 implantation and developmental outcomes.
306 Blastocysts derived from MF infertility were significantly altered for TGF-beta and ErbB
307 signaling pathways which are crucial during cell growth and proliferation. *GDF15* is a gene
308 belonging to the TGF-beta superfamily and plays a role in regulating inflammatory and apoptotic
309 pathways. The increased expression observed for *GDF15* in MF blastocysts is associated with

310 numerous disease states including inflammation and oxidative stress. Likewise, *INHBA*, which
311 encodes the same TGF-beta superfamily of proteins, was also found to have increased
312 expression in MF blastocysts. It is a negative regulator of gonadal stromal cell proliferation,
313 thus excess expression would lead to inappropriate decreases in cell proliferation which could
314 negatively impact implantation potential. Decreased gene expression in MF blastocysts was
315 observed for *FGF9*, *LEFTY1*, and *LEFTY2*. *FGF9* is involved in many biological processes
316 including embryo development, cell growth, and morphogenesis. It has been found to be
317 required for stimulating Erk1/2 activation in differentiating spermatagonia (Tassinari *et al.* ,
318 2015). LEFTY proteins are critical in sustaining pluripotency and implicated in differentiation of
319 embryonic stem cells (Khalkhali-Ellis *et al.* , 2016). Inactive LEFTY has been shown to result in
320 embryos that become entirely mesoderm and fail to develop (Hamada *et al.* , 2002). Poor
321 sperm parameters in MF patients are correlated with fertilization failure and compromised
322 embryo quality and development. Decreases in the expression of these genes could severely
323 impact embryo developmental competence, which is crucial for implantation.
324 Important pathways including cell differentiation and morphogenesis, reproduction, and
325 response to stress were affected from blastocysts derived from patients with UE infertility.
326 These pathways affect embryo growth and development as well as cell adhesion and migration.
327 Decreased expression was observed for three genes involved in cell adhesion and migration:
328 *AJAP1*, *CDH9*, and *LAMA4*. *AJAP1* has been observed to be decreased in various cancers and
329 interacts with β-catenin complexes that impact cell cycle function and apoptosis (Zeng *et al.* ,
330 2014). The decreased expression observed in UE blastocysts could have a negative impact on
331 the balance of apoptosis, possibly leading to inappropriate expression of genes that affect
332 cellular invasion. *CDH9* belongs to a family of cell adhesion molecules that regulate
333 morphogenesis and are involved in intracellular signaling pathways (Halbleib and Nelson,
334 2006). These cadherins are responsible for cell-cell adhesion during morula compaction, in
335 addition to playing a role in tissue and organ development (Peyrieras *et al.* , 1983). Decreased

336 expression would inhibit the ability of both early embryo development, as well as later fetal
337 development in utero. *LAMA4* is a laminin that mediates the attachment, migration, and
338 organization of cells into organized tissues during embryonic development. Laminins are vital
339 for organogenesis and have critical functions in several tissues including skin, muscle, and
340 vasculature (Durbeej, 2010). As the etiology of UE infertility is more ambiguous, many adverse
341 outcomes are possible including poor embryo development and IVF failure. The decreased
342 expressed observed in UE blastocysts could have significant consequences to embryo
343 implantation and ongoing development.

344 The similarities between all infertility groups included transcriptome alterations in signal
345 tranducer activity, receptor binding, reproduction, cell adhesion, and response to stimulus.
346 These biological and molecular processes are all inter-related and crucial to embryo
347 development and implantation which are processes characterized by cells that proliferate,
348 migrate, and attach. Receptors are generally transmembrane protein molecules that bind to
349 signaling molecules in response to external stimuli. Once a receptor protein receives a signal,
350 a series of biochemical reactions is initiated which conveys those signals across a cell,
351 triggering changes in cell function or state, known as signal transduction. An example of this is
352 Hedgehog (Hh) proteins which are expressed during vertebrate development. Hh signaling has
353 been observed during embryonic development and has significance during the growth of
354 reproductive tissues including the gonad and uterus (Walterhouse *et al.* , 2003). Cellular
355 adhesion, in which cells interact to attach to a surface, regulates signal transduction and is an
356 essential process for embryo implantation into the uterus lining. It is therefore not surprising
357 that all three infertility diagnoses shared blastocysts with transcriptome alterations in these
358 important biological and molecular processes.

359 The differences between each infertility group were more remarkable when studying
360 their pathway analyses. Blastocysts from women with PCO were enriched for apoptosis. This
361 is in concordance with data published showing that ovaries from women with PCO have

362 abnormal apoptotic activity and folliculogenesis (Cai *et al.* , 2013). On the other hand, signaling
363 pathways from blastocysts with MF infertility were largely involved in cancer processes. Lian et
364 al. also found that infertile men with maturation arrest had hyperactive germ cell proliferation as
365 a result of the inhibition of tumor suppressor *IRF1* by its microRNA, miR-383 (Lian *et al.* , 2010).
366 Interestingly, UE infertility was enriched for pathways involved in mTOR and adipocytokine
367 signaling, both of which are related to various disease states. This could explain some of the
368 difficulties in treating patients with unknown infertility as the cause of their reproductive
369 deficiencies could be the result of anything ranging from environmental to unknown disease risk
370 factors. For example, autoimmune disorders, such as lupus, have been shown to cause a
371 woman's immune system to reject an embryo, thereby preventing implantation into the uterus
372 (Mojarrad *et al.* , 2013).
373
374 **Conclusions**
375 This novel study suggests that underlying patient infertility diagnosis has an impact on
376 the blastocyst transcriptome, modifying genes that may affect developmental competence and
377 implantation outcomes. Ongoing research determining how transcription alterations are linked to
378 inferior pregnancy outcomes for PCO, MF, and UE patients is crucial to improving IVF success.
379 This is especially true for UE patients as a more defined infertility diagnosis could translate into
380 more targeted clinical management. Understanding how different infertility etiologies contribute
381 to embryo viability may also lead to the development of new laboratory and clinical therapies.
382 An example of this type of clinical advancement is the endometrial receptivity array which
383 identifies endometrial receptivity for patients with repeated implantation failure (Ruiz-Alonso *et*
384 *al.* , 2013). Further studies could lead to similar advancements including individualized embryo
385 culture systems and custom stimulation and frozen embryo transfer protocols, thereby
386 improving outcomes for these patients.
387

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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

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

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Infertility Diagnosis	# ↑ Genes (P<0.05; >2-fold)	# ↓ Genes (P<0.05; >2-fold)	Enriched Pathways (P<0.05; >2-fold)
PCO	647	222	p53 signaling, TGF-beta signaling, apoptosis, histidine metabolism
MF	143	205	TGF-beta signaling, ErbB signaling, GnRH signaling, B cell receptor signaling
UE	305	168	mTOR signaling, autoimmune thyroid disease, systemic lupus erythematosus, Type I diabetes, and adipocytokine signaling

518

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.

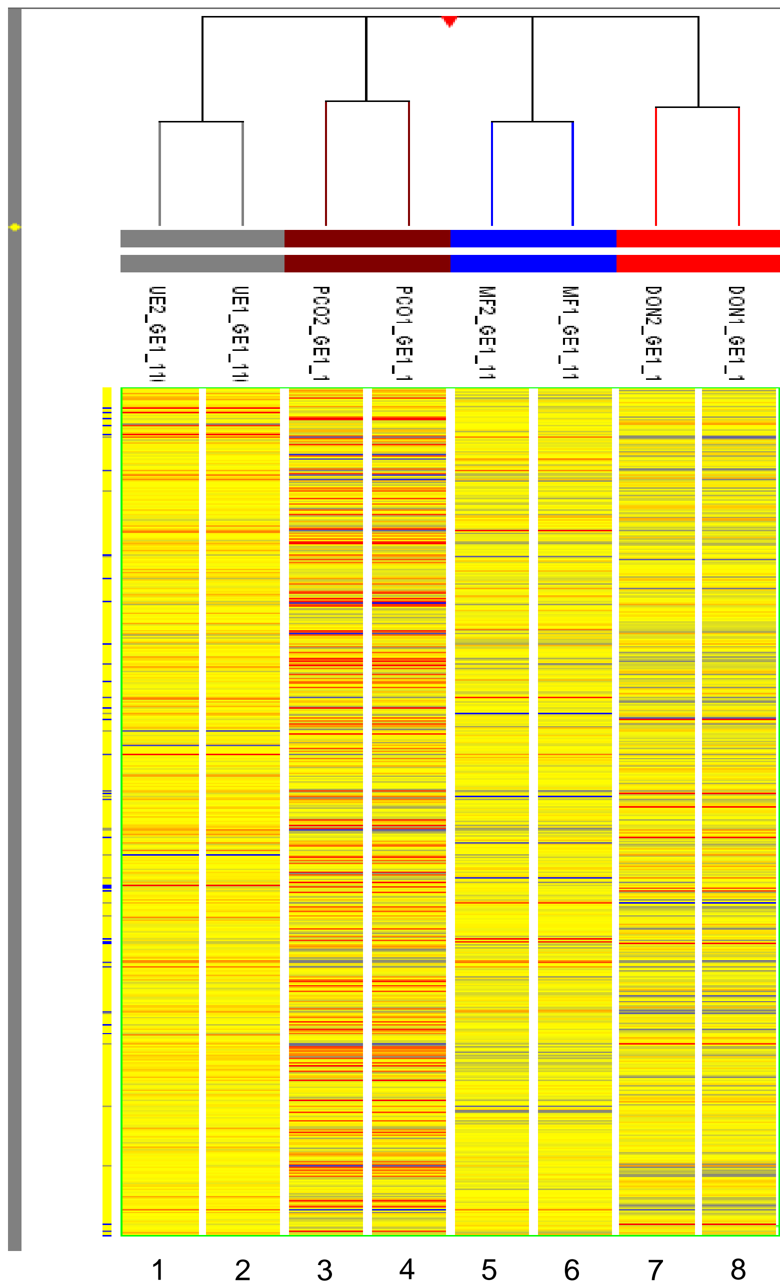


Figure 2. Venn diagram depicting gene overlap of differentially expressed transcripts between the infertility diagnoses groups. P < 0.05; >2-fold; one-way ANOVA and unpaired t-test with Benjamini–Hochberg correction. N = 1385 genes.

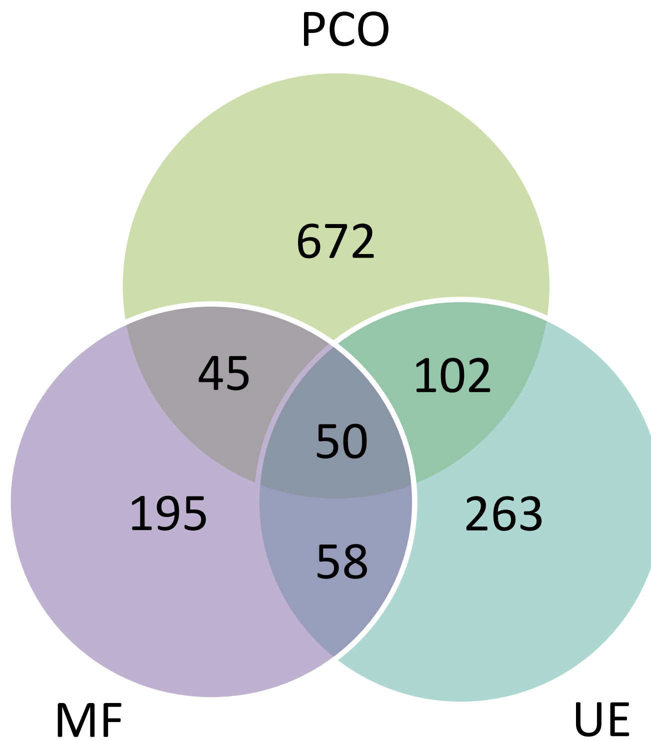


Figure 3. Altered expression of genes involved in apoptosis and stress response in PCO blastocysts. Quantitative PCR (qPCR) was performed to validate expression levels of activating transcription factor 3 (ATF3), BCL2 like 10 (BCL2L10) and heat shock protein family A members 1A and 1B (HSPA1A and HSPA1B) 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.

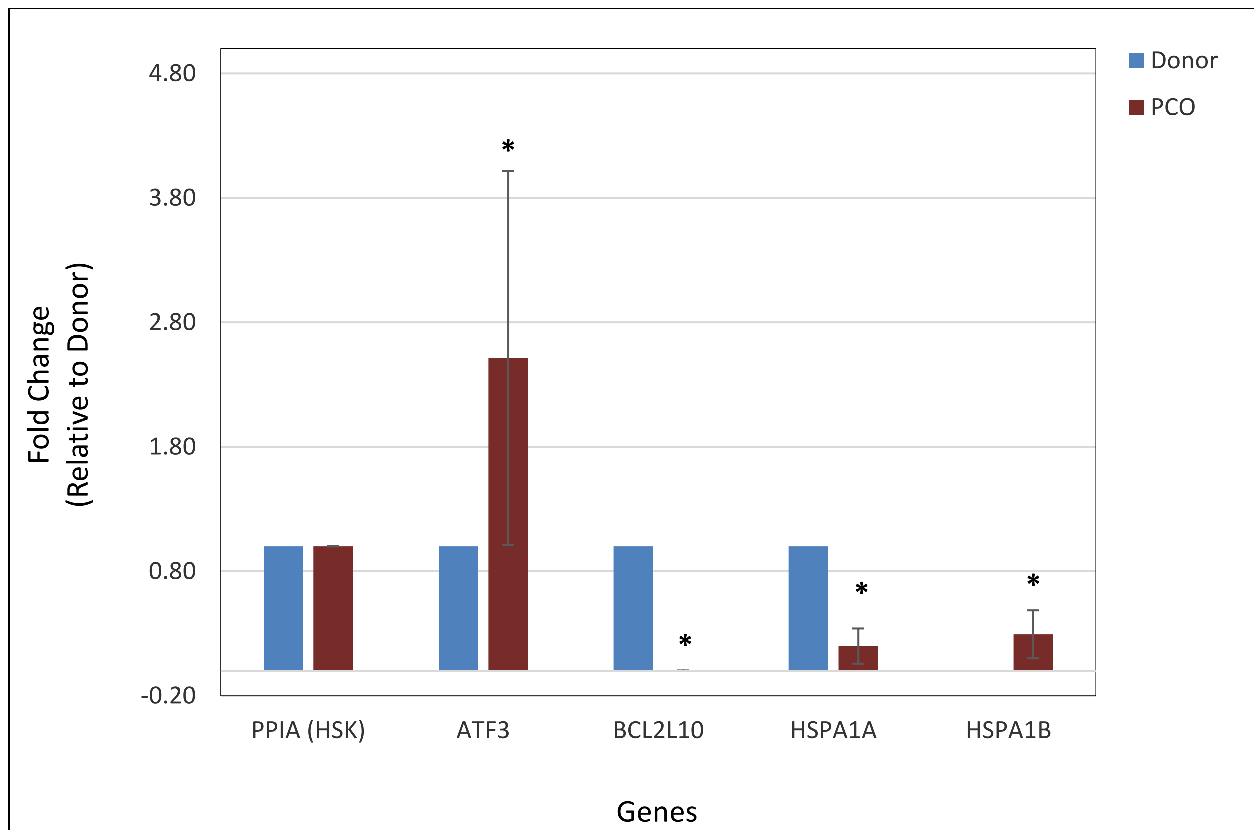
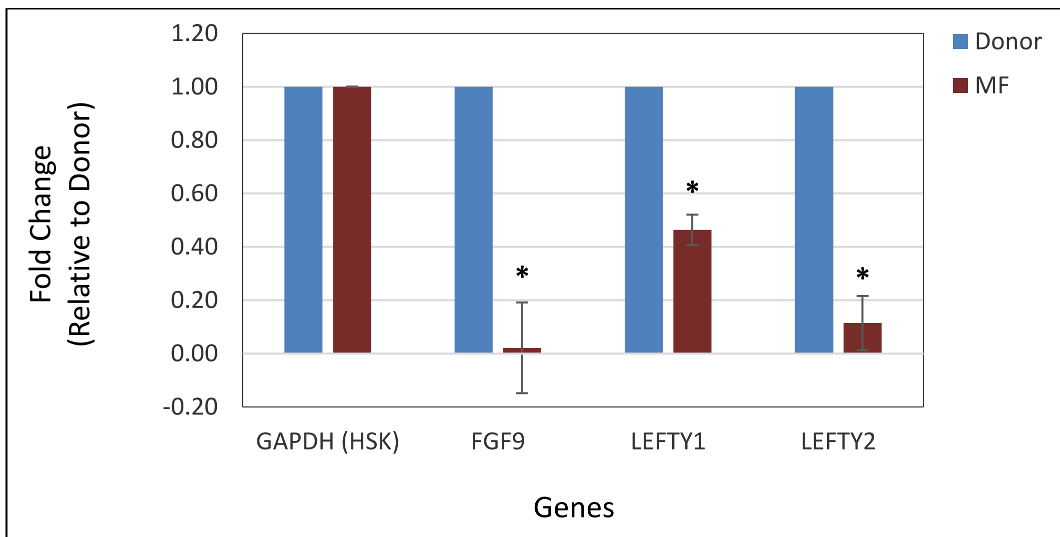


Figure 4. Altered expression of genes involved in cell growth and differentiation in MF blastocysts. qPCR was performed to validate expression levels of growth differentiation factor 15 (GDF15), inhibin beta A subunit (INHBA), fibroblast growth factor 9 (FGF9) and leftright determination factors 1 and 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.

A)



B)

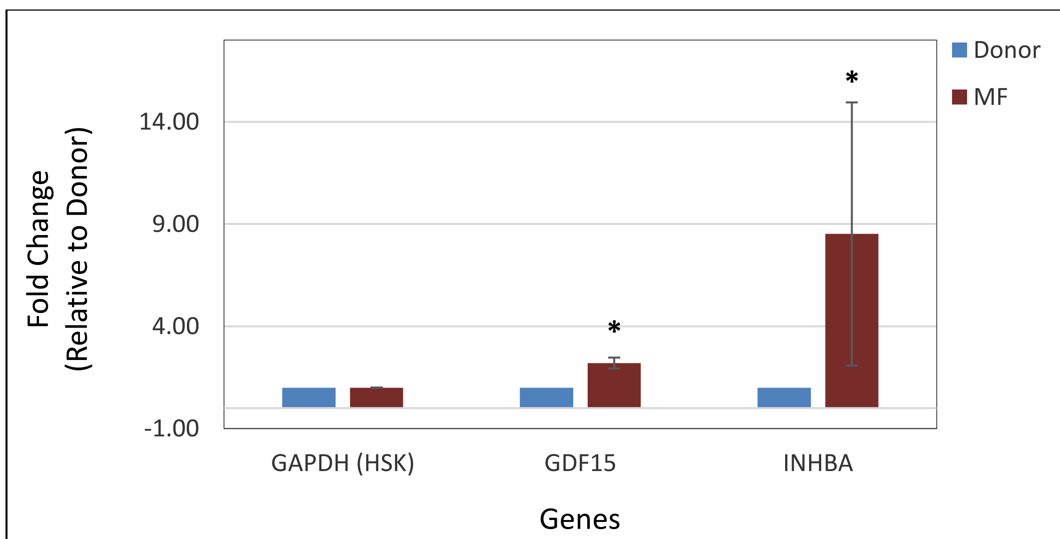


Figure 5. Altered expression of genes involved in cell adhesion and migration in UE blastocysts. qPCR was performed to validate expression levels of adherens junctions associated protein 1 (AJAP1), cadherin 9 (CDH9) and laminin subunit alpha 4 (LAMA4) in donor control and UE blastocysts with GAPDH transcription 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.

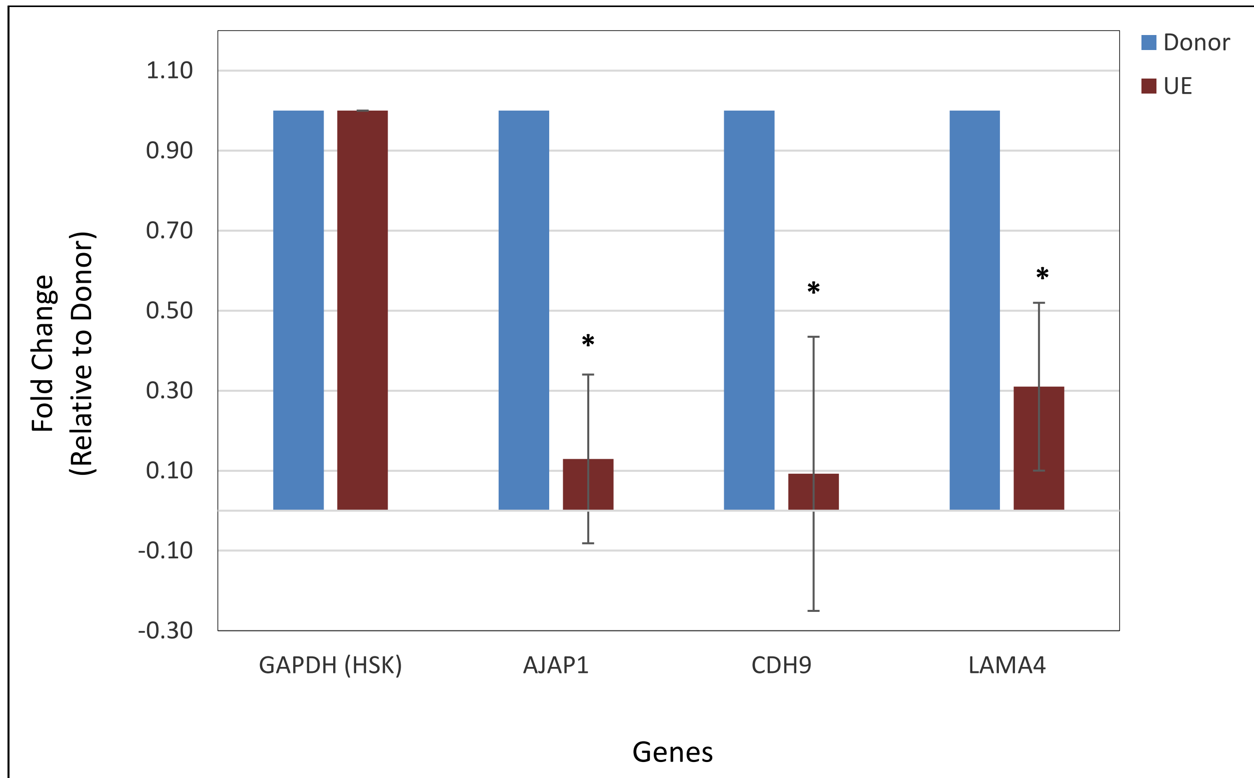


Table 1. Significantly altered transcripts and pathways associated with specific infertility diagnoses

Infertility Diagnosis	# ↑ Genes (P<0.05; >2-fold)	# ↓ Genes (P<0.05; >2-fold)	Enriched Pathways (P<0.05; >2-fold)
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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.