

Dormancy and activation of human oocytes from primordial and primary follicles: Molecular clues to oocyte regulation

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28 **Abstract**

29 Study question: Do specific transcriptome dynamics in human oocytes from primordial and
30 primary follicles identify novel pathways in oocyte activation?

31 Summary answer:

32 The transcriptomic profiles in oocytes from primordial and primary follicles, respectively, revealed
33 several new canonical pathways as putative mediators of oocyte dormancy and activation.

34 What is already known: Cellular signalling pathways including PI3K/AKT and AKT/mTOR as well
35 as TGF- β and IGF signalling are known to regulate the primordial-to-primary transition in
36 mammalian follicle development.

37 Study design, size, duration:

38 We performed a class comparison study on human oocytes from primordial (n=436) and primary
39 (n=182) follicles donated by three women having ovarian tissue cryopreserved before
40 chemotherapy.

41 Participants/materials, setting, methods:

42 RNA was extracted from oocytes from primordial and primary follicles isolated by Laser Capture
43 Microdissection, and submitted to the HiSeq Illumina platform. Data mapping, quality control,
44 filtering, and and expression analysis were performed using Tophat (2.0.4), Cufflinks (2.0.2), BWA
45 (0.6.2) and software R. Modelling of complex biological systems was performed using the IPA[®]
46 software. Lastly, qPCR and immunohistochemistry were employed to explore expression and
47 localization of selected genes and products in human ovarian tissue

48 Main results and the role of chance:

49 We found 223 and 286 genes down-regulated and up-regulated, respectively, in the oocytes during
50 the human primordial-to-primary follicle transition ($p < 0.05$ and/or FPKM fold-change > 2). IPA[®]
51 enrichment analysis revealed known pathways ('mTOR Signalling', 'PI3K/AKT Signalling' and
52 'PTEN Signalling') as well as enriched canonical pathways not previously associated with human

53 ovarian follicle development such as ‘ErB Signalling’ and ‘NGF Signalling’ in the down-regulated
54 category and ‘Regulation of eIF4 and P70S6K Signalling’ and ‘HER-2 Signalling in Breast Cancer’
55 in the upregulated group. Additionally, immunohistochemistry on human ovarian tissue explored
56 the intraovarian localisation of VASA, FOXO1 and eIF4E.

57 Limitations, reasons for caution:

58 This is a descriptive analysis and no functional studies were performed. The study was based on a
59 limited number of patients and the experimental design appreciates that natural biological variance
60 in human samples exists. Therefore, qPCR was used to confirm selected genes alongside
61 immunohistochemical stainings.

62 Wider implications of the findings:

63 This study shows, for the first time, a detailed molecular description of global gene transcription
64 activities in oocytes from primordial and primary follicles, respectively. Knowing the global
65 transcription profiles of human oocyte dormancy and activation are important in developing new
66 clinical applications.

67 Study funding/competing interest(s):

68 E.H. Ernst was supported by Health Faculty, Aarhus University and Kong Christian Den Tiendes
69 Fond. KH and SF were supported by an MRC (UK) project grant MR/M012638/1. KLH was
70 supported by grants from Fonden til Lægevidenskabens Fremme, Kong Christian Den Tiendes
71 Fond. KLH and LS were supported by the IDEAS grant from Aarhus University Research
72 Foundation (AUFF).

73

74 **Keywords:** Oocytes from primordial and primary follicles, next generation sequencing,
75 transcriptome, molecular pathway, follicle development, enrichment analysis.

76

77 **Introduction**

78 Activation of primordial follicles is marked morphologically by the primordial-to-primary
 79 transition when the flattened granulosa cells start proliferation and become cuboidal and the oocyte
 80 grows in size. The primordial-to-primary follicle transition is a gradual process in which
 81 intermediate follicles with both flattened and cuboidal granulosa cells can be noted. Maintaining
 82 dormancy of primordial follicles on one hand, and gradual activation towards ovulation on the
 83 other, is a tightly regulated process involving several pathways (Bonnet *et al.*, 2013, Hsueh *et al.*,
 84 2015, Kerr *et al.*, 2013, Kezele and Skinner, 2003, Makker *et al.*, 2014, Oktem and Urman, 2010,
 85 Reddy *et al.*, 2010, Sanchez and Smitz, 2012, Skinner, 2005). Activators (Kit ligand, FGF-2, KGF,
 86 LIF, BMP-4/7/15 and GDF-9) and suppressors (PTEN, Tsc1m/TORC1, FOXO3a, Fox12, p27,
 87 LHX8, FOXL2 and AMH) of the primordial-to-primary follicle transition are known to be
 88 important for its regulation. Conditional ablation of *Foxo3a*, *Pten*, and *Tsc1/2* in oocytes triggers
 89 increased oocyte activation (Adhikari *et al.*, 2009, Adhikari *et al.*, 2012, Reddy *et al.*, 2008). Also,
 90 *in vitro* culture of human ovarian tissue with PTEN inhibition, and AKT stimulation has been
 91 shown to increase primordial follicle activation (Novella-Maestre *et al.*, 2015). Gene expression
 92 studies have been performed on e.g. human fetal ovary (Fowler *et al.*, 2009), human mature
 93 metaphase II oocytes (Grondahl *et al.*, 2010), human oocytes from primordial-primary follicles
 94 (Markholt *et al.*, 2012), human pre-antral follicles (Kristensen *et al.*, 2015), murine embryonic
 95 gonad (Small *et al.*, 2005), mouse ovaries (Herrera *et al.*, 2005) and early follicles (Dharma *et al.*,
 96 2009, Yoon *et al.*, 2006), primordial follicle assembly in rats (Nilsson *et al.*, 2013) and sheep
 97 (Bonnet *et al.*, 2011, Bonnet *et al.*, 2013, Bonnet *et al.*, 2015), which have all contributed to our
 98 current knowledge on transcriptional activity governing oocyte regulation. Previously, studies on
 99 human oocytes were performed on a pool of isolated oocytes from primordial, intermediate and
 100 primary follicles (Markholt *et al.*, 2012) or oocytes in combination with the surrounding granulosa
 101 cells (Kristensen *et al.*, 2015), thus, it has not been possible to specifically dissect gene expression

102 in oocytes from primordial and primary follicles, respectively.

103 The ability to induce or inhibit recruitment of dormant follicles into the growing pool and support
104 their complete development *in vivo* and/or *in vitro* could help to address the scarcity of oocytes
105 available for assisted reproductive technologies. This could help women with low ovarian reserve
106 that is either physiological (age) or pathological (such as polycystic ovarian syndrome (PCOS) and
107 premature ovarian insufficiency (POI) (Bilgin and Kovanci, 2015, Franks and Hardy, 2010, Franks
108 *et al.*, 2008, Qin *et al.*, 2015), and women following autotransplantation after fertility
109 cryopreservation. Insight in the molecular pathology associated with the above mentioned
110 conditions could potentially support research in female infertility.

111 In this study we aimed to characterize and describe the transcriptome dynamics specifically
112 associated with human oocytes from primordial and primary follicles, respectively. Using stringent
113 statistical limits, we explored stage-specific, consistently expressed genes (SSCEG) and
114 differentially expressed genes (DEG), revealing stage-specific enrichment and dynamics in
115 molecular pathways. Using qPCR analysis, we confirmed DEG down-regulated (*FOXO1*, *PLCG1*
116 and *IRF6*) and upregulated (*EIF4E* and *MDM2*) as well as an equally expressed SSCEG (*RPS14*)
117 correlated with the RNA seq. data. We further interrogated the localization of three selected
118 proteins (VASA, FOXO1 and EIF4E) in human ovarian tissue, and found their expression in
119 coherence with the sequencing-generated expression profiles of their transcripts.

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125

126 **Materials and Methods**

127

128 **Participants**

129 Oocyte samples were obtained from ovarian cortical tissue procured from three patients who
 130 underwent unilateral oophorectomy prior to gonadotoxic treatment for a malignant disease
 131 (unrelated to any ovarian malignancies). Patients were normo-ovulatory, with normal reproductive
 132 hormones, and had not received ovarian stimulation with exogenous gonadotropins. All procedures
 133 were carried out in accordance with relevant guidelines and regulations, and The Central Denmark
 134 Region Committees on Biomedical Research Ethics and the Danish Data Protection Agency
 135 approved the study. Written informed consent was obtained from all participants before inclusion.
 136 Patients consented to the research conducted. In subjects undergoing oophorectomy, a small piece
 137 of the ovarian cortex is used for evaluating the ovarian reserve and for research purposes (Danish
 138 Scientific Ethical Committee Approval Number: KF 299017 and J/KF/01/170/99) (Schmidt *et al.*,
 139 2003). Ovarian cortical tissue, collected at a random time point in the menstrual cycle, was
 140 cryopreserved and categorised as normal by morphological assessment (Rosendahl *et al.*, 2011).
 141 Following cryopreservation, one small piece of ovarian cortex from each patient was randomly
 142 chosen for the current study. Until use, the cortical sample was stored in liquid nitrogen (-196°C),
 143 as previously described (Andersen *et al.*, 2008, Schmidt *et al.*, 2004).

144

145 **Laser Capture Micro-dissection (LCM).**

146 From each of the three patients, the following types of isolation were made a) pools of pure
 147 primordial oocytes, and b) pools of pure primary oocytes, (**Table 1**).

148

149 Details on RNA isolation, library preparation and sequencing, mapping and statistical analysis,
 150 bioinformatics, qPCR, and immunohistochemistry are available in Supplementary Information.

151

152

153 **Results**

154

155

156 **Data preparation and filtration of oocyte transcriptome data**

157 Follicles were precisely staged based on their morphology (**Fig. 1A and Fig. 1B**) and oocytes
 158 stringently excised using LCM, allowing the collection of pure oocytes from primordial (N=3
 159 patients, n=436 oocytes) and primary (N=3 patients, n=182 oocytes) follicles, respectively (Table
 160 1). Collected LCM-samples were subsequently grouped into three main samples for each stage,
 161 making a total of six samples (two from each patient), prior to library preparation and RNA
 162 sequencing (**Fig. 1C and Fig. 2**). RNA sequencing generated on average 77 million reads per
 163 sample (range: 64-110 million reads) that was mapped to the Human Genome (hg19) (average
 164 mapping: 35 million reads, range: 23-65 million reads), using RefSeq genes as template. We log
 165 transformed expression values (**Supplementary data Fig. S1**) to obtain final expression values
 166 ($\log_2(\text{CPMS})$) (Fragments Per Kilo base of Exon per million (FPKM) (**Fig. 1C**).

167

168 **Sorting and enrichment analysis of RNA sequencing data from oocytes from primordial and**
 169 **primary follicles**

170 A total of 11914 transcripts (**Supplementary data, Table SI**) were found expressed in oocytes
 171 from primordial follicles, and 10186 transcripts (**Supplementary data, Table SII**) were noted in
 172 oocytes from primary follicles (**Fig. 2**). Using a stringent one-sample t-test ($p < 0.05$) as statistical
 173 cut off, a list of SSCEG was generated for oocytes from primordial and primary follicles,
 174 respectively, and selected for further analysis (**Fig. 2**). The SSCEG analysis revealed 1099
 175 transcripts (9.22%) (oocytes from primordial follicles, (**bold in Supplementary data, Table SI**)),
 176 and 1046 transcripts (10.27%) (oocytes from primary follicles, (**bold in Supplementary data,**

177 **Table SII**) of the expressed genes that were significantly and consistently expressed in a stage-
 178 specific manner in oocytes from primordial and primary follicles, respectively (**Fig. 2**).

179

180 The SSCEG from oocytes from primordial (1099) and primary (1046) follicles were used to
 181 identify genes that were differentially expressed between the two groups. The DEG analysis
 182 identified 223 genes that were significantly down-regulated during the transition of oocytes from
 183 primordial to primary (highest expression in oocytes from primordial follicles), and 268 genes that
 184 were significantly up-regulated (highest expression in oocytes from primary follicles) (**Fig. 2** and
 185 **Supplementary data, Table SIII**).

186 A heatmap of DEG FPKM data was generated to show the expression for the two different cell-
 187 stages in isolates - and the correlation between stage-specific triplicate isolates (**Fig. 3**).

188 The specific identity of the LCM-isolated oocytes was evaluated by the presence of a number of
 189 oocyte-specific transcripts, and the absence (or low, inconsistent presence) of granulosa cell-
 190 specific transcripts (**Supplementary data, Table SIV**).

191 Our findings were further interrogated by qPCR analysis and compared to RNA seq data, which
 192 confirmed the findings of the tested genes. We found that DEG down-regulated (*FOXO1*, *PLCG1*
 193 and *IRF6*) and upregulated (*EIF4E* and *MDM2*) as well as an equally expressed SSCEG (*RPS14*)
 194 correlated with the RNA seq. data (**Supplementary Fig. S2**)

195

196 The continued IPA[®] generated enrichment analysis of the transcriptomes was performed on the
 197 following four groups.

198 **1)** SSCEG in oocytes from primordial follicles (1099 genes) **2)** SSCEG in oocytes from primary
 199 follicles (1046 genes). **3)** DEG down-regulated (223) or **4)** DEG up-regulated (268) in oocytes
 200 during the primordial-to-primary follicle transition (**Fig. 2** and **Supplementary data, Tables SV-**
 201 **VIII**).

202

203 The IPA[®] software was employed to perform the enrichment analysis. In this enrichment analysis,
 204 three Biological System groups were selected to analyse the transcriptomes; 1) 'Canonical
 205 Pathways' 2) 'Molecular and Cellular Functions' and 3) 'Biological Networks', and only significant
 206 ($p < 0.05$) enrichments are included in the text. For full lists, including non-significant enrichments,
 207 please refer to **Supplementary data, Tables SV-VIII**.

208

209 **Global molecular profiles in oocytes from primordial follicles**

210 In oocytes from primordial follicles, a total of 1099 SSCEG were subjected to IPA enrichment
 211 analysis, with the exception of 14 ENSEMBL-IDs that remained un-mapped or filtered out during
 212 initial data uploading, thus leaving 1085 genes for further analysis.

213 Top enriched Canonical Pathways (**Table II**) included 'EIF2 Signalling' ($p = 5.99E-40$) with high
 214 expression of genes including several members of the 'Eukaryotic Translation Initiation' group
 215 (*EIF1*, *EIF5*, *EIF2S3*, *EIF3D*, *EIF3F*, *EIF4A2*, *EIF4BP2*, *EIF4G2*), and Ribosomal Protein S6
 216 (*RPS6*) (as well as several others Ribosomal Protein of both type -S, and -L). Moreover,
 217 'Regulation of eIF4 and p70S6K Signalling' ($p = 3.62E-16$), 'mTOR Signalling' ($p = 1.62E-14$) with
 218 high expression of Eukaryotic Translation Initiator 4B (*EIF4B*), Protein Kinase, AMP-Activated,
 219 Alpha 1 Catalytic Subunit (*PRKAA1*, synonym AMPK), RPTOR Independent Companion of
 220 mTOR, complex 2 (*RICTOR*), and *RPS6* were noted. Additionally, 'Cell Cycle: G2/DNA Damage
 221 Checkpoint Regulation' ($p = 3.34E-07$) with, amongst others, consistent expression of Cyclin
 222 Dependent Kinase 7 (*CDK7*), Tumour Protein 53 (*TP53*), Thyroid Hormone Receptor (*TRIP12*),
 223 and Cyclin B1 (*CCBN1*), 'PPAR α /RXR α Activation' ($p = 1.97E-05$), 'Cyclins and Cell Cycle
 224 Regulation' ($p = 2.3E-05$) with consistent expression of Cyclin-Dependent Kinases 2, 6, and 7 was
 225 observed. Finally, top enriched Canonical Pathways further included 'Androgen Signalling'

($p=3.36E-05$), ‘CREB Signalling in Neurons’ ($p=7.4E-03$) and ‘ERK/MAPK Signalling’ ($p=9.3E-03$). The molecular network of ‘ERK/MAPK Signalling’ is shown in **Fig. 4A**.

For detailed information on ‘Molecular and Cellular Functions’, ‘Biological Networks’, and ‘Canonical Pathways’, including which genes were assigned to each group in oocytes from primordial follicle SSCEG, please refer to **Supplementary data, Table SV**.

231

232 **Global molecular profiles in oocytes from primary follicles**

In oocytes from primary follicles, the 1046 significant consistently expressed genes were subjected to IPA enrichment analysis. Here 11 ENSEMBL-IDs remained un-mapped or filtered out during initial data uploading, thus leaving 1035 for further analysis.

Top enriched Canonical Pathways (**Table II**) included ‘EIF2 Signalling’ ($p=4.67E-55$), ‘Regulation of eIF4 and p70S6K Signalling’ ($p=4.76E-22$), ‘mTOR Signalling’ ($p=1.98E-17$), ‘HIPPO Signalling’ ($p=5.2E-07$) with high expression of transcripts belonging to the ‘Protein Phosphatase’ group (*PPP1-CB*, *-CC*, *-R10*, and *PPP-CA*, *-R2A*, *-R2C*, *-R5C*), as well as SMAD family member 2 (*SMAD2*). Further, the following pathways were found enriched ‘Cell Cycle: G2/M DNA Damage Checkpoint Regulation’ ($p=1.33E-06$), ‘Protein Kinase A Signalling’ ($p=4.63E-06$), ‘PI3K/AKT Signalling’ ($p=8.75E-05$) including, amongst others the downstream effector Eukaryotic Translation Initiator 4E (*EIF4E*), and Heat Shock Protein 90AA1, AB1, and B1 (*HSP90-AA1*, *-AB1*, and *-B1*), MDM2 Proto-Oncogene E3 (*MDM2*), Glycogen Synthase Kinase 3 alpha (*GSK3A*), Phosphatidylinositol-4,5-Biphosphate 3-Kinase, Catalytic Subunit Beta (*PIK3CB*), and Forkhead Box 03 (*FOXO3*). Finally, top enriched Canonical Pathways further included the ‘ERK5 Signalling’ ($p=1.21E-04$) and the ‘HIPPO Signalling’ ($p=5.2E-07$) pathways were significantly enriched in primary oocytes. The molecular network associated with the ‘HIPPO Signalling’ pathway is shown in **Fig. 4B**.

250 Detailed information on ‘Canonical Pathways’, ‘Molecular and Cellular Functions’, and ‘Biological
 251 Networks’, including which genes were assigned to each group in oocytes from primary follicles
 252 SSCEG, is provided (**Supplementary data, Table SVI**).

253

254 **Differentially expressed genes between oocytes from primordial and primary follicles**

255 The DEG lists were likewise subjected to IPA enrichment analysis, as described above.

256

257 **Genes down-regulated in oocytes during the primordial to primary transition**

258 Several Canonical Pathways were enriched in the list of downregulated genes (**Table III**) (noted in
 259 bold in the full enrichment listed in **Supplementary Data, Table SVII**). Top Canonical Pathways
 260 included: ‘Leptin Signalling in Obesity’ (p=1.98E-04) including the *PLCG1* gene, ‘ErbB signalling’
 261 (3.04E-03), ‘PPARα/RXRα Activation’ (p=6.77E-04), ‘NGF signalling’ (p=7.75E-03), ‘GNRH
 262 Signalling’ (1.6E-02) with down-regulation of Raf-1 proto-oncogene serine/threonine kinase
 263 (*RAF1*), Adenylate cyclase 9 (*ADCY9*), nuclear factor of kappa light polypeptide gene enhancer in
 264 B-cells 1 (*NFKB1*). Further, ‘Type II Diabetes Mellitus Signalling’ (p=4.33E-02), and ‘PTEN
 265 signalling’ (p=4.8E-02) with down-regulation of *FOXO1* during the primordial-to-primary
 266 transition was found enriched. Information on ‘Molecular and Cellular Functions’, ‘Biological
 267 Networks’, and ‘Canonical Pathways’ are included (**Supplementary data, Table SVII**). The
 268 information on ‘Regulation of eIF4, and P70S6K Signalling’ category and the molecular network
 269 associated with that pathway is shown in **Fig. 5A**.

270

271

272 **Genes up-regulated in oocytes during the primordial to primary transition**

273 Several Canonical Pathways were enriched in the list of up-regulated genes (Table III) (noted in
 274 bold in the full enrichment listed in **Supplementary Data, Table SVIII**). Top enriched Canonical

275 Pathways up-regulated during the primordial-to-primary transition included: ‘EIF2 Signalling’
 276 ($p=1.92E-09$), ‘mTOR signalling’ ($p=9.24E-04$) with up-regulation of *EIF4E*, *PIK3CB*, *RPS5*, and
 277 *TSC1*. Also, represented in the genes up-regulated during the primordial-to-primary transition was
 278 the ‘PI3K/AKT signalling’ with transcripts Heat Shock Protein 90 AA1 (*HSP90AA1*), *MDM2*, Ras
 279 homolog enriched in brain (*RHEB*), *EIF4E*, *TP53*, and *TSC1*, which were also assigned to this
 280 pathway. Further, the following Pathways ‘Regulation of eIF4, and P70S6K Signalling’ ($p=3.43E-$
 281 03), ‘HER-2 Signalling in Breast Cancer’ ($p=2.04E-02$), ‘FGF Signalling’ ($p=2.92E-02$), and
 282 ‘Insulin Receptor Signalling’ ($p=3.14E-02$) were up-regulated during the primordial-to-primary
 283 transition.

284 Detailed information on ‘Molecular and Cellular Functions’, ‘Canonical Pathways’, and ‘Biological
 285 Networks’ is included (**Supplementary Table SVIII**). The ‘Regulation of eIF4, and P70S6K
 286 Signalling’ categories, and the molecular networks associated with these pathway are selectively
 287 noted (**Fig. 5B**).

288

289 **Intra-ovarian localisation of VASA, FOXO1, and EIF4E**

290 VASA, the gene product of *DDX4* was selected for immunohistochemical staining (IHC) since
 291 *DDX4* was found to be significantly expressed in both oocyte stages. The IHC showed that the
 292 VASA protein was indeed specifically present in oocytes from primordial and intermediate
 293 follicles, and absent from the surrounding somatic cells (**Fig. 6A**), consistent with the SSCEG
 294 analysis. *FOXO1*, a member of the Forkhead Box family, was selected due to its high expression in
 295 oocytes from primordial follicles, and low expression in oocytes from primary follicles
 296 (**Supplementary data, Table SIII and SIV**) and thus, was significantly down-regulated during the
 297 primordial-to-primary transition (**Supplementary data, Table SV**). IHC showed FOXO1 protein to
 298 be highly present in the nucleus of oocytes from primordial follicles, while staining was weaker and
 299 more diffusely located in the cytoplasm in activated oocytes from primary follicles (**Fig. 6B, C**).

300 The EIF4E protein is an initiator of translation of stored mRNA, and its transcript was low in
301 oocytes from primordial follicles with a significantly higher expression in oocytes from primary
302 follicles (**Supplementary data, Table SV**). IHC using an antibody against the EIF4E protein
303 showed very low signal in oocytes from primordial follicles whilst a stronger signal was observed
304 in the oocyte from primary follicles (**Fig. 6D, E**), and thus, transcript and protein levels appear
305 coupled.

306 **Discussion**

307 The current study is the first that specifically and in detail describe global gene activity in human
 308 oocytes from primordial and primary follicles. We found that while overall some signalling
 309 pathways appear active in both oocytes stages, there are interesting differences, which might reflect
 310 key genes and pathways that are essential for maintaining follicle dormancy and primordial-to-
 311 primary transition regulation.

312 We found enrichment pathways previously reported to be important for dormancy/activation of
 313 resting primordial oocytes such as PI3K/AKT-, and mTOR signalling in both oocyte stages.
 314 Interestingly, we noted several novel factors that may be crucial for sustaining the balance between
 315 oocyte dormancy and activation during oocyte development. In the DEG analysis, we identified
 316 several candidates significantly up-, or down regulated during the primordial-to-primary transition
 317 that may be responsible for maintenance of dormancy or inducing activation, and subsequently,
 318 essential for female fertility.

319 We assessed the intraovarian localization of gene products of selected genes as a result of the
 320 presence of their corresponding transcripts in both oocytes (*VASA*), or up-regulated in oocytes from
 321 primordial (*FOXO1*) or primary (*EIF4E*) follicles. We found that these gene transcripts appeared
 322 coupled to the translation product, with VASA present specifically in oocytes and absent from the
 323 surrounding somatic cells. The protein products of *EIF4E* and *FOXO1* followed the expression of
 324 their corresponding mRNA, with the highest detection of FOXO1 in the nucleus of the oocytes
 325 from primordial follicles, and with the highest level of EIF4E in the cytoplasm of oocytes from
 326 primary follicles.

327

328 **Transcriptome characteristics in oocytes from primordial and primary follicles**

329 Below, selected parts of the results are discussed in relation to reproduction, fertility and related
 330 aspects in developmental biology. Most of the selected parts are associated with ‘Canonical

331 Pathways', which can be cross-referenced to both 'Biological Networks' and 'Molecular and
332 Cellular Functions'.

333 We found several pathways enriched in oocytes from both follicular stages, such as 'Estrogen
334 Receptor Signalling', 'Germ Cell-Sertoli Cell Junction Signalling', 'Cyclins and Cell Cycle
335 Regulation' and 'EIF2 Signalling'. The presence of the 'Estrogen Receptor Signalling' category
336 indicates that these sex-steroid hormones are already at play in the primordial follicles probably as a
337 results of intercellular signalling between the somatic cells and the enclosed oocyte (Knight *et al.*,
338 2012).

339
340 Specifically significantly enriched in oocytes from primary follicles were the 'Hippo Signalling'
341 and 'ERK5 Signalling' canonical pathways. The Hippo pathway has emerged as a critical regulator
342 of tissue growth through controlling cellular processes such as cell proliferation, death and
343 differentiation (Hergovich, 2016). A study on mouse oocytes and early embryos found that
344 members of the ERK5 signalling pathway might have an important role in both meiotic and mitotic
345 cell division (Maciejewska *et al.*, 2011). In human oocytes, we found the ERK5 signalling pathway
346 to be highly significantly enriched in the primary oocyte stage, which may indicate that oocytes
347 have begun progression towards the primary stage for meiotic cell division at the later pre-ovulatory
348 stage.

349

350 **Differentially expressed genes during the primordial-primary transition**

351 Noteworthy, 'PTEN Signalling' is down-regulated during the primordial-to-primary transition and
352 thus down-regulated upon activation of oocytes in dormant primordial follicles. This is directly in
353 line with a rodent study that showed global follicle activation upon depletion of *Pten* (Reddy *et al.*,
354 2008). Loss of function of inhibitors of follicle activation, such as Tsc1, PTEN, Foxo3a, p27 and
355 FoxL2 leads to premature activation of the primordial follicles (Adhikari *et al.*, 2009, Castrillon *et*

356 *al.*, 2003, Rajareddy *et al.*, 2007, Reddy *et al.*, 2008). The PTEN signalling pathway controls
 357 follicle activation through the Forkhead transcription factor 3A (FOXO3A). The FOXOs comprise a
 358 family of transcription factors that in the un-phosphorylated state reside in the nucleus of the cell.
 359 Upon exposure to growth factors, especially those that signal via the PTEN/PI3K/AKT pathway,
 360 these transcription factors are phosphorylated and exported from the nucleus and inactivated,
 361 thereby allowing the cell to grow (Kuscu and Celik-Ozenci, 2015). Indeed, genetic ablation of
 362 *Foxo3a* in mice increased the rate of primordial follicle activation (Castrillon *et al.*, 2003), resulting
 363 in oocyte death, early depletion of functional ovarian follicles, and secondary infertility.

364 We found *FOXO3A* to be SSCEG in oocytes from primary follicles, but not in oocytes from
 365 primordial follicles, which is puzzling when considering its role in primordial follicle activation.
 366 However, translational or post-translational modifications of the FOXO3A protein may render it
 367 unable to prevent progression to an oocyte in the primary follicle.

368 Novel to our understanding of human oocyte development, we found *FOXO1* to be significantly
 369 down-regulated during the primordial-to-primary transition, in line with the immunohistochemistry
 370 analysis, where we observed clear nuclear staining of FOXO1 in oocytes from primordial follicles,
 371 and weak cytoplasmic staining in oocytes in primary follicles, indicating nuclear export and
 372 subsequent inactivation upon phosphorylation. These findings suggest that FOXO1 functions in
 373 parallel with FOXO3A as an important downstream effector of the PTEN pathway in
 374 dormancy/activation of human primordial oocytes.

375

376 In the category of up-regulated Canonical Pathways in oocytes from primordial and primary
 377 follicles, notable changes included the ‘mTOR Signalling’ pathway and the ‘PI3K/AKT Signalling’
 378 pathway, already known for their interconnected role in oocyte regulation. A key pathway
 379 implicated in the crucial roles to balance follicle growth suppression, activation and progression is
 380 the PI3K pathway (Kerr *et al.*, 2013). The tuberous sclerosis complex 1 (TSC1) interacts with

PTEN to maintain quiescence, and the mammalian target of rapamycin (mTOR), which is an activator, is negatively regulated by TSC1 (Zheng *et al.*, 2012). In mutant mice lacking the *Tsc1* gene in oocytes, the entire oocyte pool is activated prematurely due to elevated mTORC1 activity in the oocyte, resulting in follicular depletion and leading to premature ovarian failure (Adhikari *et al.*, 2010). We found *TSC1* to be significantly up-regulated during the primordial-to-primary transition which may reflect that even though the oocyte is activated and initiating growth and maturation, some control of cell growth by TSC1 is needed for optimal oocyte development. Other members assigned to the ‘PI3K/AKT signalling’ pathway includes the molecular chaperone Heat Shock Protein 90kDa Alpha (Cytosolic), Class A Member 1 (*HSP90A1*; involved in regulation of cell cycle control and signal transduction), an isoform of the catalytic subunit of phosphoinositide 3-kinase (PI3K), *PIK3CB*, the proto-oncogene *MDM2*, and the tumour suppressor 53 (*TP53*). These findings support the view that control of the human primordial-to-primary transition is governed by complex inhibiting and activating factors maintaining this finely-tuned balance.

Interestingly, we found genes in the groups of the ‘Regulation of eIF4 and P70S6K Signalling’ pathway in both groups of DEG, however, the factors present or absent in each of the DEG groups differ notable in the Molecular Networks, revealing the extensive molecular dynamics even within a single signalling network.

Expression of the *EIF4E* gene that functions in mRNA translation initiation of stored oocyte mRNAs and procreation of meiosis (Henderson *et al.*, 2009), significantly increased during the primordial-to-primary transition. Interestingly, a recent study found that EIF4A could act as an inhibitor for TORC1 upon amino acid starvation, independently of its role in translation initiation (Tsokanos *et al.*, 2016). Maternal mRNA and phosphorylated EIF4EBP1 variants were shown to be associated with the mouse metaphase II spindle (Romasko *et al.*, 2013), and pharmacological blocking of 4E-BP1 phosphorylation using the mTOR inhibitor Torin2, leads to arrest of oocyte maturation at the metaphase I stage (Mayer *et al.*, 2014). It is tempting to speculate that EIF4E may

406 have a similar regulative effect on TORC1 (protein complex that contains at least mTOR and
407 Raptor (regulatory-associated protein of TOR) pathways during the primordial-primary transition,
408 in addition to its role in translation initiation. An interesting approach in this regard was utilized in a
409 pharmacological study in which transient treatment of neonatal and aged mouse ovaries with mTOR
410 stimulators increased activation of primordial follicles (Sun *et al.*, 2015). In cultures of human
411 ovarian cubes, both mTOR and/or PI3K stimulators promoted primordial follicle activation with
412 synergistic effects of combined treatment (Sun *et al.*, 2015). In summary, our data highlight
413 possible roles of pathways not previously associated with early human follicle development.
414

415 **Acknowledgements**

416
417 The authors wish to thank the clinical, paramedical and laboratory team of the Fertility Clinic,
418 Aarhus University Hospital and Lykke-Hartmann laboratory (AU) for scientific discussions. E.H.
419 Ernst was supported by a PhD scholarship from Health Faculty, Aarhus University. This work was
420 further supported by Aarhus University Research Fund (AUFF) (to KLH and LS), and grants from
421 Kong Christian Den Tiendes Fond (to EHE and KLH), Th. Maigaards Eft. Fru Lily Benthine Lunds
422 Fond and Fonden til Lægevidenskabens Fremme (to KLH).

423

424 **Authors' roles**

425 EHE, CYA, LS and KLH conceived the study. EHE performed LCM and IHC experiments. CYA
426 provided samples. SG and PV performed bioinformatics analysis. EHE performed IPA analysis.
427 EHE, MLG and KLH analysed NGS and IPA data. KH and SF supervised IHC. EHE, AH and KLH
428 designed and performed qPCR. EHE and KLH wrote the manuscript. All authors approved the final
429 manuscript.

430

431 **Funding**

432 E.H. Ernst was supported by the Health Faculty, Aarhus University, and Kong Christian Den
433 Tiendes Fond. KH and SF were supported by an MRC (UK) project grant MR/M012638/1. KLH
434 was supported by grants from Fonden til Lægevidenskabens Fremme, Kong Christian Den Tiendes
435 Fond. KLH and LS were supported by the AUFF IDEAS grant from Aarhus University Research
436 Foundation (AUFF).

437

438 **Conflict of interest**

439 The authors declare no conflict of interest.

440

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 558

559 **Figure legends**

560

561 **Figure 1. Experimental design for Laser Capture Microdissection and sequencing**

562 (A-B) Identification, laser cutting and after capture of oocytes from primordial (A) and primary (B)
 563 follicles, revealing the precision in which stage-specific oocytes were isolated. Oocytes from
 564 primordial and primary follicles, as well as granulosa cells (GC) are indicated by bars. Scale bar 50
 565 μm . (C) Oocytes from primordial (N=3 patients; n=185, n=181, n=70 oocytes) and primary (N=3
 566 patients; n=76, n=61, n=45 oocytes) follicles were isolated from three different patients (patient 1-
 567 3). Subsequently, the cap-adhered oocyte was lysed for mRNA isolation, cDNA synthesis, and
 568 RNA sequencing. After sequencing, mapping of the data to the human genome was performed
 569 before annotation. Finally, each gene was normalised by transforming to the $\log_2(\text{CPMS})$.

570

571 **Figure 2. Construction of SSCEG and DEG lists**

572 After the sequencing, mapping, annotation and filtering, as illustrated in Fig 1b, the genes (11914
 573 and 10186 from oocytes from primordial and primary follicles, respectively) were filtered to
 574 include transcripts based on t-test, which selected only consistently expressed genes for each oocyte
 575 stage (1099 SSCEG and 1046 SSCEG from oocytes from primordial and primary follicles,
 576 respectively). From those SSCEG genes, DEG genes were identified (268 DEG up-regulated in
 577 oocytes from primordial follicles in comparison to oocytes from primary follicles and 223 DEG
 578 down-regulated in follicles from primordial to primary follicles). For DEG, paired t-test $p < 0.05$
 579 between two sets of triplicates and/or FPKM value fold-change > 2

580

581 **Figure 3. Heatmap representation of DEG analysis.**

582 Heatmap of gene expression in oocytes during the primordial-to-primary follicle transition, The
 583 heatmap only includes 268 upregulated genes (red) and 233 downregulated genes (blue).

584

585 **Figure 4. IPA pathways highlighting trends in oocytes from primordial and primary follicles,**
 586 **respectively.**

587 (A) Pathway analysis (IPA) of the ‘ERK/MAPK Signalling’, based on SSCEG in oocytes from
 588 primordial follicles. Colour intensities are based on p values where high significance is most
 589 intensive in colour (red). (B) Pathway analysis (IPA) of the ‘HIPPO Signalling’, based on SSCEG
 590 in oocytes from primary follicles. Colour intensities are based on p values where high significance
 591 is most intensive in colour (red).

592

593 **Figure 5. IPA pathways highlighting trends in DEG up-regulated or down-regulated from**
 594 **oocytes from primordial to primary follicles.**

595 (A) Pathway analysis (IPA) of the ‘Regulation of eIF4 and P70S6K Signalling’, based on DEG
 596 downregulated in oocytes during the primordial-to-primary follicle transition. Colour intensities are
 597 based on FPKM values where high significance is most intensive in colour (red). (B) Pathway
 598 analysis (IPA) of the ‘Regulation of eIF4 and P70S6K Signalling’, based on DEG up-regulated in
 599 oocytes during the primordial-to-primary follicle transition. Colour intensities are based on FPKM
 600 values where high significance is most intensive in colour (red).

601

602 **Figure 6. Intraovarian distribution of VASA, FOXO1 and EIF4E in human oocytes from**
 603 **primordial and primary follicles.**

604 (A) VASA localized to oocytes in both primordial and primary follicles and presented here is an
 605 example of VASA in primordial oocytes. DAPI staining identifies the nucleus of cells in this slide
 606 and the surrounding flat granulosa cells are characteristic for oocytes from primordial follicles. (B)
 607 FOXO1 localises intensively in the nucleus of the oocyte from primordial follicles, as expected.
 608 Hoechst staining identifies the nucleus of cells in this slide and the surrounding flat granulosa cells

are characteristic for oocytes from primordial follicles. (C) FOXO1 is less intense and less nucleus-associated in oocytes from primary follicles. Hoechst staining identifies the nucleus of cells in this slide and the surrounding cuboidal granulosa cells are characteristic for oocytes from primary oocytes. (D) EIF4E is less intense in the primordial oocytes as expected. Hoechst staining identifies the nucleus of cells in this slide and the surrounding flat granulosa cells are characteristic for oocytes from primordial follicles. (E) EIF4E is more intense in the oocyte from the primary follicle. Hoechst staining identifies the nucleus of cells in this slide and the surrounding cuboidal granulosa cells are characteristic for oocytes from primary follicles. Scale bars; 30 μ m.

617

Supplementary Figure 1. Histograms on log₁₀(CPMS) values.

Data is grouped according to patient and oocyte stage and has undergone log₁₀(CPMS) transformation.

621

Supplementary Figure 2. Real-time quantitative RT-PCR analysis on selected genes confirming the RNA seq data.

Relative qPCR and RNA sequencing expression data of selected genes in oocytes from primordial and primary follicles, respectively. (A) Quantification of relative abundance of *FOXO1*, *PLCG1*, *IRF6*, *EIF4E*, *MDM2*, and *RPS14* transcripts, as indicated (black columns; oocytes from primordial follicles, grey columns; oocytes from primary follicles) (Multiply expression values for *PLCG1* and *FOXO1* $\times 10^{-4}$). All gene expression levels were normalized to *GAPDH* and presented as mean value of triplicate measurements including standard deviations (SD) (B) RNA sequencing data are mean FPKM values.