Hypoxia-mediated carbohydrate metabolism and transport promote early-stage murine follicle growth and survival

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Makanji Y, Tagler D, Pahne J, Shea LD, Woodruff TK. Hypoxia-mediated carbohydrate metabolism and transport promote early-stage murine follicle growth and survival. Am J Physiol Endocrinol Metab 306: E893–E903, 2014. First published February 25, 2014; doi:10.1152/ajpendo.00484.2013.—Oxygen tension is critical for follicle growth and metabolism, especially for early-stage follicles, where vascularity is limited. Its role and underlying mechanism in the in vitro activation and maturation of immature to ovulatory follicles is largely unknown. In this study, early secondary (110 μm) murine follicles were isolated and encapsulated in alginate hydrogels to replicate the in vivo environment of the growing/maturing follicle. Encapsulated follicles were cultured for 8 days at either 2.5 or 20% O2. Survival (2.6-fold) and growth (1.2-fold) were significantly higher for follicles cultured at 2.5% compared with 20% O2. Using a mouse hypoxia-signaling pathway qRT-PCR array and GeneGo Metacore analysis, we found that direct target genes of the hypoxia-activated HIF1-complex were significantly upregulated in follicles cultured for 8 days at 2.5% compared with 20% O2, including the carbohydrate transport and metabolism genes Slc2a3, Vegfa, Slc2a1, Edn1, Pglk1, Ldha, and Hmox1. Other upregulated genes included carbohydrate transporters (Slc2a1, Slc2a3, and Slc16a3) and enzymes essential for glycolysis (Pfk1, Hmox1, Hk2, Gpi1, Pkl, Pfkp, Aldoa, Gapdh, Pgam1, Eno1, Pkm2, and Ldha). For follicles cultured at 2.5% O2, a 7.2-fold upregulation of Vegfa correlated to an 18-fold increase in VEGFA levels, and a 3.2-fold upregulation of Ldha correlated to a 4.8-fold increase in lactate levels. Both VEGFA and lactate levels were significantly higher in follicles cultured at 2.5% compared with 20% O2. Therefore, enhanced hypoxia-mediated glycolysis is essential for growth and survival of early secondary follicles and provides vital insights into improving in vitro culture conditions.

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Growth and maturation of ovarian follicles requires coordinated activity of various growth factors and hormones that interact with the physical environment of the follicle, including pH, temperature, and oxygen (O2) tension. O2 is critical for aerobic ATP production, in which pyruvate is converted to energy; however, excess O2 generates free radicals and reactive oxygen species, which can damage the oocyte and its precious DNA cargo within the follicle (44). By design, ovarian follicles operate in a moderate hypoxic environment rather than an anoxic one (16). Granulosa cells use relatively less O2 and convert glucose to pyruvate and lactate via the glycolytic pathway; the pyruvate is shunted to oocyte, which uses it and O2 to create ATP via the TCA cycle (8, 13).

The ability to culture follicles in vitro to produce mature, fertilizable oocytes has potential clinical applications in fertility preservation, and yet recapitulating the complexity of follicle growth and metabolism in vitro has been challenging. The alginate-based three-dimensional in vitro follicle culture system has been used to mimic the in vivo physical environment of the ovary, acting as a structural support for the encapsulated follicle. Follicles from rodents (39, 42) and nonhuman primates (41, 43) have been grown to the antral stage using this technology, and alginate-based in vitro follicle culture has been applied to the study of environmental factor effects on follicle growth, antrum formation, steroid production, egg quality, and gene expression. For instance, primate primordial follicles require a rigid physical environment to grow and survive (14). Alginate-encapsulated secondary mouse follicles (>120 μm) grow and mature to produce fertilizable eggs, resulting in live birth (42); however, growth of smaller follicles (<120 μm) requires additional factors and culture conditions. For example, growth and maturation of oocytes within cultured alginate-encapsulated primary follicles (70–80 μm) was enhanced with coculture on a monolayer of mouse embryonic fibroblasts (36) or by culturing multiple primary follicles (>10) together (15). Together, these studies in our laboratory demonstrate that in vitro culture of primordial and primary follicles requires a shift toward the follicles’ native environment.

Within the ovary, the smaller primordial and primary follicles are abundant in the ovarian cortex, whereas secondary and large antral follicles are found in the medulla (27). Compared with the medulla, the ovarian cortex is less vascularized and thus more hypoxic (27); this suggests that primordial and primary follicles exist in an environment of lower O2 tension (2–5%) compared with larger follicles (30, 31). Therefore, in vitro culture of immature follicles in low O2 tension would also move a step closer to creating a native environment for follicles. In support, rhesus secondary follicles cultured at an O2 tension of 5% compared with 20% O2 had better survival (41). However, the underlying mechanism was not explored. The culture of primordial and primary follicles is still challenging in the field. However, we can get valuable insights from the culture of secondary follicles.

There is little or no consensus on the physiological levels of O2 tension that support ovarian follicle growth and maturation at different stages and locations within the ovary. O2 tensions of 5 or 20% have been used for in vitro oocyte maturation of murine cumulus oocyte complexes (COCs) (2) and bovine COCs (3); however, follicle metabolism that occurs under relatively hypoxic conditions has been poorly characterized. We hypothesized that early-stage follicles would have higher growth and survival rates when cultured under hypoxic conditions that mimic the environment of the ovarian cortex. The culture of primordial and primary follicles is still challenging in the field. However, we can get valuable insights from the...
culture of early secondary follicles at low O2 tension. We examined the physiological response of alginate-encapsulated early secondary (110 μm) mouse follicles to either 2.5 or 20% O2 tension. We determined the effect of hypoxia on follicle growth, survival, and expression of hypoxia-related signaling pathway components over an 8-day culture period. This study not only improves our understanding of follicle metabolism but also brings us closer to optimizing in vitro culture conditions for early-stage follicles.

MATERIALS AND METHODS

Animals

Preweaned female CD1 mice (12 days old) were obtained from Northwestern University’s Center for Comparative Medicine facility (Chicago, IL). Animals were bred and housed in a controlled barrier facility on a 14:10-h light-dark cycle with free access to food and water. All animal experiments were approved by the Northwestern University Institutional Use and Care Committee (2010-1239) in accordance with National Institute of Health (NIH) guidelines.

Hypoxia Chambers

Follicles were cultured in a PLAS Labs (Lansing, MI) hypoxia chamber (Fig. 1). This custom-built chamber has six individual microincubators with adjustable O2, CO2, and temperature. Most studies have utilized 5% O2 in either preantral follicle or embryo culture (38, 41). In this study, 2.5% O2 was selected as our hypoxic condition, and 20% O2 was used for controls to mimic conventional in vitro culture conditions. In our preliminary studies, survival was better at 2.5% compared with 5 or 10% O2 (data not shown).

In vitro follicle culture. Ovarian follicles (100–120 μm, 2-layer early secondary follicles) were isolated mechanically from female CD1 mice and encapsulated individually in 0.25% alginate, as described previously (39, 42). The encapsulated follicles were cultured in 200 μl of growth medium (α-MEM, 3 mg/ml BSA, 1 mg/ml bovine fetuin, 10 mIU/ml FSH, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenite, pH 7.4) at 2.5 or 20% O2, 5 %CO2, and 37°C for 8 days. The follicle cultures were static, meaning that the medium was not changed during the culture period; this differs from conventional follicle culture (42) in which half of the medium is replaced every other day (Fig. 2A). At the end of the culture period, the diameter of each follicle was measured using Image J software (NIH, Bethesda, MD), as described previously (39, 42). Survival of follicles was determined morphologically, as described previously (39, 42). Briefly, round follicles with intact basement and visible round oocyte with prominent germinal vesicle were considered viable, hence surviving. Misshapen follicles with deformed or dark oocytes were considered as nonviable, hence nonsurviving. The culture medium was collected and stored at −80°C for hormone, growth factor, and metabolite assays.

Lactate Assay

The lactate assay was performed using a kit from Sigma-Aldrich (St. Louis, MO). Samples were deproteinized using 10-KDa MWCO spin columns (EMD Millipore, Billerica, MA) prior to the assay. The amount of lactate in the culture medium was determined enzymatically against the L(+)-Lactate kit reference preparation. The sensitivity of the assay was 0.1 μg/ml. Inter- and intra-assay variations were 0.3 and 4.4%, respectively.

Immunoassays

Inhibin A ELISA. The inhibin A ELISA from Diagnostics Systems Laboratory (DSL; Webster, TX) was used as described previously (22, 23). The assay uses a βA-subunit antibody (E4) as the capture antibody and an α-subunit antibody (R1) as the label. Inhibin A from the kit calibrated against inhibin A (91/624) was used as the reference preparation. The sensitivity of the assay was 5.2 pg/ml. Inter- and intra-assay variations were 7.5 and 7.1%, respectively.

Inhibin B ELISA. The inhibin B ELISA was performed as described (22, 23) using the DSL kit. The assay uses a βB-subunit antibody (C5) as the capture antibody and an α-subunit antibody (R1) as the label. Inhibin B included in the kit was used as the reference preparation. The sensitivity of the assay was 37.1 pg/ml. Inter- and intra-assay variations were 5.7 and 7.0%, respectively.

Anti-Mullerian hormone ELISA

The anti-Mullerian hormone (AMH) ELISA was run using a kit from DSL per the manufacturer’s instructions. The sensitivity of the
assay was 0.2 ng/ml. Inter- and intra-assay variations were 5.8 and 7.9%, respectively.

**Estradiol ELISA**

Estradiol ELISAs were performed using a kit from Calbiotech (Spring Valley, CA). In this competition assay, binding of the samples was compared with a reference preparation. The sensitivity of the assay was 3.5 pg/ml. Inter- and intra-assay variations were 7.8 and 3.0%, respectively.

**Mouse VEGFA ELISA**

Mouse VEGFA ELISAs were run using ELISA kit reagents from R & D Systems (Minneapolis, MN). The ELISA recognizes both isoforms of mouse VEGFA (VEGF120 and VEGF165). The sensitivity of the assay was 1.4 pg/ml. Inter- and intra-assay variations were 1.4 and 7.0%, respectively.

**RT² Profile PCR Arrays**

The mouse hypoxia-signaling pathway qRT-PCR array (Qiagen, Hilden, Germany) was utilized to determine the gene expression differences between follicles cultured in vitro at 2.5 and 20% O₂. RNA extraction, cDNA synthesis, and qRT-PCR array were performed per the manufacturer’s instructions. Follicles in each group were removed from alginate beads on day 8 of culture by incubating in 10 mIU/ml alginate lyase (Sigma-Aldrich, St. Louis, MO). The follicles were then flash-frozen in liquid nitrogen in extraction buffer and stored at −80°C. RNA was extracted using RNaseasy Micro Kit (Qiagen), and cDNA was generated using pathway-specific primers to enrich for mouse hypoxia-signaling pathway genes. Genomic DNA was also eliminated in this step. Amplified cDNA was added to RT² SYBR Green qPCR master mix (Qiagen), and 10 μl was added to a 384-well plate, with each well containing predispersed gene-specific primer sets. Included on the plate were one genomic DNA control, three reverse transcription controls, and three positive PCR controls. The plate was sealed and loaded onto an ABI 7900 HT thermal cycler. ABI 7900 HT SDS software version 2.3 (Applied Biosystems, Foster City, CA) was used to determine the threshold cycle (C_T) values for all genes on the qPCR array. Using the RT² Profiler PCR Array Data Analysis software version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.sphp; Qiagen), the C_T values were normalized against the average C_T of housekeeping genes (Actb, B2m, Gusb, and Hsp90ab1 expression) included on the plate to generate ΔC_T (∆C_T). The 2^−ΔC_T method (21) was used to determine the fold change in gene expression. Fold change (2^−ΔC_T) was the normalized gene expression (2^−ΔC_T) in follicles cultured at 2.5% O₂ divided by the normalized gene expression (2^−ΔC_T) in the follicles cultured at 20% O₂. The array analysis was based on four biological replicates for each group.

**GeneGo Metacore Analysis**

Fold change in gene expression and P values from the qRT-PCR array were entered into the web-based GeneGo Metacore software (https://portal.genego.com/cgi/data_manager.cgi; Thomson Reuters, New York, NY) to identify the biological processes affected by differences in hypoxia-regulated genes in follicles cultured in 2.5% O₂.

**Statistical Analyses**

All statistical data analyses were performed using GraphPad Prism Software, version 5 (Carlsbad, CA). Data are presented as means ±
Table 1. Growth, survival, secreted growth factors, hormones, and metabolites of early secondary follicles cultured at 2.5 or 20% O₂

<table>
<thead>
<tr>
<th></th>
<th>O₂ (%)</th>
<th>2.5% O₂</th>
<th>20% O₂</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 follicle diameter, μm²</td>
<td>20% O₂</td>
<td>117</td>
<td>112 ± 1.0 (110–115)</td>
<td>114</td>
</tr>
<tr>
<td>Day 8 follicle diameter, μm²</td>
<td>20% O₂</td>
<td>71</td>
<td>189 ± 4.0 (181–197)</td>
<td>55</td>
</tr>
<tr>
<td>Survival (% of Day 0)</td>
<td>20% O₂</td>
<td>9</td>
<td>76.1 ± 3.2 (68.8–83.5)</td>
<td>11</td>
</tr>
<tr>
<td>Inhibin A, pg/ml</td>
<td>20% O₂</td>
<td>18</td>
<td>101.5 ± 17.7 (64.2–138.8)</td>
<td>11</td>
</tr>
<tr>
<td>Inhibin B, pg/ml</td>
<td>20% O₂</td>
<td>47</td>
<td>330.9 ± 39.9 (250.5–411.4)</td>
<td>23</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
<td>20% O₂</td>
<td>24</td>
<td>446 ± 85 (270–622)</td>
<td>14</td>
</tr>
<tr>
<td>AMH, ng/ml</td>
<td>20% O₂</td>
<td>53</td>
<td>5.63 ± 0.33 (4.95–6.30)</td>
<td>20</td>
</tr>
<tr>
<td>VEGFA, pg/ml</td>
<td>20% O₂</td>
<td>17</td>
<td>62.7 ± 9.9 (41.6–83.8)</td>
<td>9</td>
</tr>
<tr>
<td>Lactate, μg/ml</td>
<td>20% O₂</td>
<td>21</td>
<td>53.3 ± 5.3 (42.3–64.3)</td>
<td>20</td>
</tr>
</tbody>
</table>

Values are means ± SE (95% confidence interval). O₂, oxygen; NS, not significantly different; AMH, anti-Müllerian hormone. *Follicles were cultured at either 2.5 or 20% O₂, 5% CO₂, 37°C in individual hypoxia chambers. †P values were determined by Student’s t-test; P < 0.05 considered significantly different. ‡Growth or follicle diameter was determined using Image J software. §Survival was determined in 9-11 experiments, 20-40 follicles per experiment per group.
Estradiol levels were slightly lower (1.2-fold, \(P < 0.05\)) in media from follicles cultured at 2.5% O\(_2\) compared with follicles cultured at 20% O\(_2\) (Table 1). Estradiol level and follicle diameter were more strongly correlated in the media from the 2.5% O\(_2\) group (\(r^2 = 0.3078, P = 0.0049;\) Fig. 4\(E\)) compared with media from the 20% O\(_2\) group (\(r^2 = 0.2147, P = 0.0952;\) Fig. 4\(F\)). For both O\(_2\) tensions estradiol/inhibin B ratios were calculated; at 2.5% O\(_2\) the estradiol/inhibin B ratio was 1.4, and at 20% O\(_2\) the estradiol/inhibin B ratio was 1.9. This would suggest that follicles at 20% O\(_2\) produce slightly more estradiol compared with follicles cultured at 2.5% O\(_2\). 

**AMH Production**

AMH levels were significantly slightly higher (1.3-fold, \(P = 0.0296\)) in the media from follicles cultured at 2.5% O\(_2\) compared with 20% O\(_2\) (Table 1). Follicle diameter and AMH levels in the media did not correlate in either the 2.5% O\(_2\) (\(r^2 = 0.006, P = 0.479;\) Fig. 4\(G\)) or the 20% O\(_2\) (\(r^2 = 0.005, P = 0.686;\) Fig. 4\(H\)) groups. The slightly elevated AMH levels in the 2.5% O\(_2\) group compared with the 20% O\(_2\) group were not related to the 1.3-fold greater diameter of follicles cultured at 2.5% O\(_2\). For both O\(_2\) tensions, the AMH/inhibin B ratios were calculated; at 2.5% O\(_2\) the AMH/inhibin B ratio was 0.017, and at 20% O\(_2\) the AMH/inhibin B ratio was 0.015. These comparisons suggest that follicles cultured at 2.5% O\(_2\) have slightly higher AMH levels relative to granulosa cell mass (inhibin B levels); however, this will need to be validated further.

**Differential Expression of Hypoxia-Signaling Pathway Genes in Follicles Cultured at 2.5% O\(_2\) vs. 20% O\(_2\)**

To determine why we observed better growth and survival of early secondary follicles cultured at 2.5% O\(_2\), we performed a
qPCR array for hypoxia-signaling pathway genes. In the gene expression analyses, follicles cultured at 20% O$_2$ served as the control group. $\Delta$C$_T$ values were calculated and normalized against a panel of housekeeping genes (Actb, B2m, Gusb, and Hsp90ab1; Fig. 5A). Genes that showed more than a twofold, statistically significant difference in expression between the 2.5% and 20% O$_2$ follicle culture groups (Table 2) were divided into the following functional categories: carbohydrate metabolism, DNA repair, apoptosis, angiogenesis, and cell proliferation (Fig. 5B).

Notably, expression of Hif1$\alpha$, which encodes the hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) transcription factor, did not differ significantly in follicles cultured at 2.5 or 20% O$_2$ (1.03-fold, $P = 0.9285$); however, HIF-1$\alpha$ target genes were upregulated. In normoxia, HIF-1$\alpha$ is readily hydroxylated and degraded via the ubiquitin pathway. Under hypoxic conditions, such as that present the ovarian cortex, prolyl hydroxylases are downregulated, thereby preventing ubiquitin-mediated degradation of HIF-1$\alpha$ (17, 24). This permits HIF-1$\alpha$ and HIF-1$\beta$ to form a complex that can translocate to the nucleus to upregulate target genes that in turn regulate processes like angiogenesis, glucose transport, and metabolism (26). Consistent with these observations, we found that the gene encoding the prolyl hydroxylase P4hb was downregulated in follicles cultured at 2.5% compared with 20% O$_2$ (0.63-fold, $P = 0.0107$), and HIF-1$\alpha$ target genes were significantly upregulated (Table 2 and Fig. 6).

VEGFA Production

Our qPCR array analysis revealed a 7.21-fold upregulation of Vegfa gene expression in follicles cultured at 2.5% compared with 20% O$_2$. To validate our array results, VEGFA levels were measured in the day 8 media from cultured follicles in each group. VEGFA levels were 18-fold higher in media from follicles cultured at 2.5% compared with 20% O$_2$ ($P < 0.05$; Fig. 7, A and B, and Table 1). There was significant positive correlation between follicle diameter and VEGFA levels in the culture media from follicles cultured in 2.5% O$_2$ ($r^2 = 0.766, P < 0.0001$; Fig. 7A) but not in media from follicles cultured at 20% O$_2$ ($r^2 = 0.278, P = 0.145$; Fig. 7B).

Carbohydrate Transport and Metabolism Genes are Upregulated in Follicles Cultured at 2.5% Compared With 20% O$_2$

Most of the genes (14 out of 30) upregulated in follicles cultured at 2.5% O$_2$ are involved in carbohydrate transport and metabolism, particularly genes essential for glycolysis that convert glucose into pyruvate and lactic acid (Figs. 5B and 8).
Table 2. Hypoxia signaling pathway-related genes upregulated >2-fold in follicles cultured for 8 days at 2.5% O2 compared with 20% O2

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>Fold Changea</th>
<th>95% CI</th>
<th>P Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ndr1</td>
<td>N-myc downstream regulated gene 1</td>
<td>51.93</td>
<td>(10.38, 93.48)</td>
<td>0.0343</td>
</tr>
<tr>
<td>Lox</td>
<td>Lysyl oxidase</td>
<td>9.95</td>
<td>(3.55, 16.35)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Lgsal3</td>
<td>Lectin, galactose binding, soluble 3</td>
<td>8.84</td>
<td>(1.82, 15.87)</td>
<td>0.0452</td>
</tr>
<tr>
<td>Slc2a3</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 3</td>
<td>8.29</td>
<td>(2.43, 14.15)</td>
<td>0.0464</td>
</tr>
<tr>
<td>Hk2</td>
<td>Hexokinase 2</td>
<td>8.11</td>
<td>(1.89, 14.34)</td>
<td>0.0268</td>
</tr>
<tr>
<td>Vegfa</td>
<td>Vascular endothelial growth factor A</td>
<td>7.21</td>
<td>(2.47, 11.95)</td>
<td>0.0047</td>
</tr>
<tr>
<td>Ankrd37</td>
<td>Ankyrin repeat domain 37</td>
<td>6.08</td>
<td>(2.89, 9.26)</td>
<td>0.0023</td>
</tr>
<tr>
<td>Ptkp</td>
<td>Phosphofructokinase, platelet</td>
<td>5.98</td>
<td>(3.55, 8.42)</td>
<td>0.0029</td>
</tr>
<tr>
<td>Slc2a1</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 1</td>
<td>5.69</td>
<td>(2.76, 8.62)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Edn1</td>
<td>Endothelin 1</td>
<td>4.31</td>
<td>(0.89, 7.73)</td>
<td>0.0228</td>
</tr>
<tr>
<td>Ero1l</td>
<td>ERO1-like</td>
<td>4.30</td>
<td>(2.73, 5.87)</td>
<td>0.0038</td>
</tr>
<tr>
<td>Pdk1</td>
<td>Pyruvate dehydrogenase kinase, isoenzyme 1</td>
<td>4.29</td>
<td>(3.28, 5.31)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Hif3a</td>
<td>Hypoxia inducible factor 3, α-subunit</td>
<td>4.03</td>
<td>(1.92, 6.14)</td>
<td>0.0104</td>
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<tr>
<td>Tpi1</td>
<td>Triosephosphate isomerase 1</td>
<td>3.96</td>
<td>(1.60, 6.32)</td>
<td>0.0010</td>
</tr>
<tr>
<td>Eglh1</td>
<td>EGL nine homolog 1</td>
<td>3.91</td>
<td>(2.39, 5.43)</td>
<td>0.0093</td>
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<tr>
<td>Pgk1</td>
<td>Phosphoglycerate kinase 1</td>
<td>3.77</td>
<td>(1.76, 5.77)</td>
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<tr>
<td>Ier3</td>
<td>Immediate early response 3</td>
<td>3.41</td>
<td>(0.35, 6.48)</td>
<td>0.0088</td>
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<tr>
<td>Mxi1</td>
<td>Max interacting protein 1</td>
<td>3.29</td>
<td>(2.34, 4.23)</td>
<td>0.0008</td>
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<tr>
<td>Ldha</td>
<td>Lactate dehydrogenase A</td>
<td>3.16</td>
<td>(1.55, 4.77)</td>
<td>0.0163</td>
</tr>
<tr>
<td>Ptkl</td>
<td>Phosphofructokinase, liver, B-type</td>
<td>3.15</td>
<td>(2.27, 4.03)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Pkm2</td>
<td>Pyruvate kinase, muscle</td>
<td>3.11</td>
<td>(2.17, 4.06)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Map3k1</td>
<td>Mitogen-activated protein kinase kinase kinase 1</td>
<td>2.88</td>
<td>(1.58, 4.18)</td>
<td>0.0218</td>
</tr>
<tr>
<td>P4ha1</td>
<td>Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), α1-polypeptide</td>
<td>2.74</td>
<td>(1.52, 3.96)</td>
<td>0.0017</td>
</tr>
<tr>
<td>Mif</td>
<td>Macrophage migration inhibitory factor</td>
<td>2.69</td>
<td>(1.83, 3.56)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Hmox1</td>
<td>Heme oxygenase (decycling) 1</td>
<td>2.52</td>
<td>(1.97, 3.08)</td>
<td>0.0012</td>
</tr>
<tr>
<td>Ccn2</td>
<td>Cyclin G2</td>
<td>2.38</td>
<td>(1.31, 3.45)</td>
<td>0.0154</td>
</tr>
<tr>
<td>Gpi1</td>
<td>Glucose phosphate isomerase 1</td>
<td>2.31</td>
<td>(1.52, 3.10)</td>
<td>0.0189</td>
</tr>
<tr>
<td>Bnip3l</td>
<td>BCL2/adenovirus E1B interacting protein 3-like</td>
<td>2.19</td>
<td>(1.50, 2.88)</td>
<td>0.0062</td>
</tr>
<tr>
<td>Gysl</td>
<td>Glycogen synthase 1, muscle</td>
<td>2.02</td>
<td>(1.55, 2.49)</td>
<td>0.0014</td>
</tr>
<tr>
<td>P4hb</td>
<td>Prolyl 4-hydroxylase, β-polypeptide</td>
<td>0.63</td>
<td>(0.44, 0.82)</td>
<td>0.1007</td>
</tr>
</tbody>
</table>

CI, confidence interval. aFold change (2^ΔΔCt) was the normalized gene expression (2^ΔΔCt) in follicles cultured at 2.5% O2 divided by the normalized gene expression (2^ΔΔCt) in follicles cultured at 20% O2. The P values are calculated based on a Student t-test of the replicate 2^ΔΔCt values for each gene in the control group and treatment groups; P values of <0.05 are shown.

Pyruvate is the preferred substrate for ATP production in the oocyte in mice (10, 27) and humans (11). The machinery for glycolytic metabolism is present in oocytes, and both pyruvate and glucose carrier-mediated uptake has been demonstrated in human and mouse oocytes (11, 13); however, oocytes are poor at converting glucose to pyruvate (i.e., glycolysis) (4, 10, 27). Instead, granulosa cells take up glucose and provide pyruvate and other intermediates to the oocyte (12, 35). Our qPCR array analysis revealed that glucose transporter genes Slc2a1 (GLUT1) and Slc2a3 (GLUT3) were upregulated 5.69- and 8.29-fold, respectively, in follicles cultured at 2.5% O2 compared with 20% O2 (Table 2 and Fig. 8). Several other genes essential for converting glucose intermediates to pyruvate were upregulated in follicles cultured at 2.5% compared with 20% O2. A gene encoding a proton-linked monocarboxylate transporter (MCT), Slc16a3 (8.05-fold, P = 0.0030), was also upregulated; MCTs are essential for proton-linked transport of lactate and pyruvate in and out of cells (20).

Lactate Production

Lactate levels were 4.8-fold higher in media from follicles cultured at 2.5% O2 compared with follicles cultured at 20% O2 (P < 0.05; Table 1). There was significant positive correlation between follicle diameter and lactate levels in culture media from follicles cultured in 2.5% O2 (r^2 = 0.865, P < 0.0001; Fig. 7C) and from follicles cultured at 20% O2 (r^2 = 0.570, P = 0.0001; Fig. 7D). The higher lactate production in follicles cultured at 2.5% compared with 20% O2 suggests that follicles are more glycolytic under hypoxic conditions.

DISCUSSION

In this study, we found that early secondary (110 μm) follicles had greater growth and survival when cultured at 2.5%
compared with 20% O2. Follicles cultured at 2.5% O2 had significant upregulation of several hypoxia-mediated HIF-1α-activated downstream target genes, including the carbohydrate transport and metabolism genes Slc2a3, Vegfa, Slc2a1, Edn1, Pkg1, Ldha, and Hmox1. Other upregulated genes were carbohydrate transporters (Slc2a1, Slc2a3, and Slc16a3) and glycolytic enzymes (Pgk1, Hmox1, Hk2, Gpi1, Pfk1, Pfkp, Aldoa, Gapdh, Pgaml, Eno1, Pkm2, and Ldha). Compared with follicles cultured at 20% O2, follicles cultured for 8 days at 2.5% O2 showed a 7.21-fold upregulation of Vegfa gene expression that corresponded to an 18-fold greater level of VEGFA in the culture media. Similarly, a 3.16-fold upregulation of Ldha in follicles cultured at 2.5% compared with 20% O2 for 8 days resulted in a 4.8-fold increase in lactate levels in culture media. Our results suggest that hypoxia-mediated HIF-1α-targeted gene activation promotes glycolysis and perhaps vascularization during early preantral follicle growth, supporting an essential role for hypoxia signaling pathways in the physiological mechanism(s) that controls the growth, survival, and energy metabolism of early ovarian follicles. Follicle morphology and hormone (inhibin A, inhibin B, estradiol, and AMH) profiles were broadly similar in both follicle groups after 8 days in culture, suggesting that although the health of surviving follicles cultured at either 2.5 or 20% O2 is similar, increased carbohydrate metabolism that occurs under hypoxic conditions promotes survival of early-stage follicles.

Importance of Glycolysis to Oocyte Metabolism: A Focus on Glucose and Pyruvate Transporters

Glucose metabolism via glycolysis and the pentose phosphate pathway is essential for production of energy substrates, NADPH, ribose-5-phosphate, and purine precursors (8, 35). Glycolysis produces lactate and pyruvate, which is an essential energy substrate for oocytes and is transported via specialized transporters from granulosa cells to oocytes (28, 33). Glucose transporters GLUT1–14 (SLC2A1–14) are essential for transporting glucose into cells and are essential components of the glycolytic pathway. In addition, pyruvate and lactate are transported by the proton-linked monocarboxylate carrier family (MCT). MCT1–4 (SLC16A1, SLC16A7, SLC16A8, and SLC16A3) are involved in proton-linked lactate and pyruvate transport. Both GLUTs and MCTs have been detected in oocytes (5). Interestingly, there are species differences in the expression of GLUTs and glucose consumption in oocytes; in murine oocytes, glucose uptake is undetectable, but there is a low level of glucose uptake in human (11), porcine (19), and bovine (18) oocytes. Human oocytes express Glut1 but not Glut2–4 (9), whereas nonhuman primate oocytes express Glut4–6 (45).

In this study, we found that follicles cultured at 2.5% O2 had upregulated Slc2a1 (Glut1), Slc2a3 (Glut3), and Slc16a3 (Mct4) compared with follicles cultured at 20% O2, revealing the importance of these transporters in early follicle growth and survival. Downregulation of GLUTs results in decreased intracellular glucose and apoptosis in embryos (6, 25). In a mouse model of type 1 diabetes, a reduction in GLUT-mediated pyruvate production may account for the observed apoptosis of ovarian follicle cumulus cells via the extrinsic pathway (5). In addition, follicles from type 1 diabetic mice exhibit lower connexin 37 and 43 with reduced gap junction communication between granulosa cells and oocytes compared with controls (1, 29).

Carbohydrate Metabolism in Follicles

The energy demands of growing follicles are stage dependent. Primordial follicles at the cortex of the ovary readily
produce lactate and consume twofold more pyruvate than glucose, suggesting that both glycolysis and TCA oxidation occur at this stage (8, 13). Pyruvate is the preferred substrate for energy metabolism in primordial follicles and is attributed to consumption by the oocyte (4, 10, 13), whereas the low levels of glucose consumption and lactate production are attributed to glycolysis by granulosa cells (13). Small antral follicles found at the cortico-medullary interface are more vascularized (27) and demand more pyruvate and O2 (13). Pyruvate oxidation via the TCA cycle is essential for energy demands of a growing oocyte. Interestingly, pyruvate consumption was found to be fourfold higher in primary follicles than ovulatory follicles, adjusted for oocyte volume (13), perhaps because of increased protein and RNA synthesis (32). Mature bovine COCs consume twofold more glucose, O2, and pyruvate compared with preantral and antral follicles (34). In the COC, carbohydrate metabolism shifts from glycolysis to another pathway, i.e., the pentose phosphate pathway, which provides precursors of purine nucleotides and NADPH to the mature oocyte (34, 35).

Improved Growth and Survival of Follicles Cultured at 2.5% O2

Oxygen consumption by murine oocytes is stage dependent and closely linked to carbohydrate metabolism and energy demand at different stages (8, 13). Follicles in this study were cultured using a static culture model (no media refresh) compared with a conventional culture model (media refresh). There is a possibility that static culture may have independent effects on follicle viability and metabolism; however, this would be difficult to discern in this study, as early secondary (110/H9262) follicles also have poor survival rates in conventional culture at 20% O2. The conventional culture method would have introduced additional variables (temperature, CO2) in the hypoxia suite used in this study. In our culture system, early secondary follicles (110 μm) cultured at 2.5% O2 for 8 days had a
significantly higher survival rate compared with follicles cultured at 20% O2. Because the hormone levels produced by follicles cultured at either 2.5 or 20% O2 were similar, indicating that the health of the follicles were similar, the improved growth and survival of follicles at 2.5% O2 can be attributed to enhanced hypoxia-mediated pyruvate production for oxidative energy metabolism. During the early-growth phase, oocytes require more pyruvate (13), and we predict that growth is fueled by hypoxia-mediated glycolysis in vivo. It has also been postulated via mathematical modeling in human (31) and bovine (7) antral follicles that during antrum formation there is a sharp increase in intrafollicular O2, followed by a decline at the preovulatory stage. Therefore, we propose an in vitro culture protocol that starts with low O2 (2–5%) conditions until the preovulatory stage. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. Hum Reprod 12: 2508–2510, 1997.


