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1 **The adiponectin agonist, AdipoRon, inhibits**
2 **steroidogenesis and cell proliferation in human luteinized**
3 **granulosa cells**

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author statement

Jérémy Grandhayé Investigation; Data curation, Formal analysis

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Rémy LeGuevel Methodology; Data curation; Formal analysis

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Fabrice Guerif Investigation;

Joëlle Dupont Funding acquisition; Validation; review & editing

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25 **Abstract**

26 During obesity, excess body weight is not only associated with an increased risk of type 2-diabetes, but also
27 several other pathological processes, such as infertility. Adipose tissue is the largest endocrine organ of the
28 body that produces adipokines, including adiponectin. Adiponectin has been reported to control fertility
29 through the hypothalamic–pituitary–gonadal axis, and folliculogenesis in the ovaries. In this study, we
30 focused on a recent adiponectin-like synthetic agonist called AdipoRon, and its action in human luteinized
31 granulosa cells.

32 We demonstrated that AdipoRon activated the adenosine monophosphate-activated protein kinase (AMPK)
33 and peroxisome proliferator-activated receptor alpha (PPAR) signalling pathways in human luteinized
34 granulosa cells. A 25 μ M AdipoRon stimulation reduced granulosa cell proliferation by inducing cell cycle
35 arrest in G₁, associated with PTEN and p53 pathway activation. In addition, AdipoRon perturbed cell
36 metabolism by decreasing mitochondrial activity and ATP production. In human luteinized granulosa cells,
37 AdipoRon increased phosphodiesterase activity, leading to a drop in cyclic adenosine monophosphate
38 (cAMP) production, aromatase expression and oestrogens secretion.

39 In conclusion, AdipoRon impacted folliculogenesis by altering human luteinized granulosa cell function, via
40 steroid production and cell proliferation. This agonist may have applications for improving ovarian function
41 in metabolic disorders or granulosa cancers.

42

43

44 **1. Introduction**

45 Excess fatness is associated not only with an increased risk of type 2-diabetes, but also with several
46 pathological processes, such as atherosclerosis, inflammation, cancer or infertility (Park et al., 2011). The
47 obese state accelerates ovarian follicular development, decreases oocyte quality, and contributes to polycystic
48 ovary syndrome (PCOS) (Gelsomino et al., 2019).

49 It is accepted that adipose tissue is a fat storage organelle, but in the past 20 years, adipose tissue has
50 been shown to secrete numerous hormones, therefore its role in the endocrine system is unequivocal. The
51 hormones secreted by adipocytes are called adipokines, and include leptin and adiponectin (Ahima and Lazar,
52 2008). Adiponectin is a 30 kDa multimeric protein, comprising 244 amino acid residues (Nishida et al., 2007),
53 and is the most abundant secretory product of white adipose tissue (Kadowaki et al., 2006). It binds two
54 seven-transmembrane domain receptors, called AdipoR1 and AdipoR2, and activates the adenosine
55 monophosphate-activated protein kinase (AMPK) pathway, and via AdipoR2, the peroxisome proliferator-
56 activated receptor alpha (PPAR α) pathway (Yamauchi and Kadowaki, 2008; Okada-Iwabu et al., 2013; Wang
57 et al., 2017).

58 AdipoRon, a recently developed adiponectin receptor agonist, binds and activates both AdipoR1 and
59 AdipoR2. In mice fed a high-fat diet, AdipoRon improves insulin resistance and glucose intolerance, via
60 AMPK activation (Okada-Iwabu et al., 2013). Similar to adiponectin, it also improves many metabolic
61 processes, including glucose metabolism, insulin sensitivity, lipid metabolism (Choi et al., 2018), attenuates
62 postischaemic cardiomyocyte apoptosis (Zhang et al., 2015), and tumour suppression both *in vitro* and *in vivo*,
63 through adiponectin receptor-dependent mechanisms (Akimoto et al., 2018; Malih and Najafi, 2015).

64 As described, metabolic disorders that lead to alterations in fertility, impact severely on granulosa
65 cells. Indeed, in growing mammalian follicles, the oocyte is surrounded by several layers of granulosa and
66 thecal cells. Granulosa cells communicate to oocytes via gap junctions, and provide environmental status and
67 various energy substrates to nurse oocytes (Eppig, 2004; Su et al., 2009). Moreover, granulosa cells produce
68 steroids such as oestrogens, which are important sex steroid hormones.

69 In the last decade, several studies have shown that adiponectin signals may affect ovarian activity
70 (Barbe et al., 2019; Chabrolle et al., 2009; Lagaly et al., 2008). Both receptors are expressed by ovarian cells
71 (Maillard et al., 2010), and adiponectin enhances the secretion of progesterone and estradiol in the presence of
72 follicle-stimulating hormone (FSH) or insulin growth factor-1 (IGF-1) (Chabrolle et al., 2009; Pierre et al.,
73 2009). In mice, mutated adiponectin alters steroid production and follicular development (Cheng et al., 2016).

74 The adiponectin concentrations in follicular fluids (FF) have been found significantly lower in both lean and
75 obese women with polycystic ovary syndrome when compared to non-PCOS counterparts (Artimani et al.,
76 2016). These data raise the hypothesis of the use of this new pharmaceutical drug, AdipoRon, in the treatment
77 of female infertility associated to metabolic perturbation. Recently, it was shown that AdipoRon exerts anti-
78 proliferative and pro-apoptotic effects on specific human high grade epithelial ovarian cancer cell lines
79 (Ramzan et al., 2019). However, none or little information are available about the action of this kind of drug
80 in granulosa cells, which are the cells which communicate directly with oocyte and help in its maturation..

81 In this study, we investigated the role of AdipoRon on human luteinized granulosa cells (cell line and
82 primary cells), and performed different assay to evaluate its impact on viability, cell proliferation, metabolism
83 and in particular on steroidogenesis, which is an important role of granulosa cells to control fertility.

84

85 2. Materials and Methods

86 2.1. Isolation of human primary luteinized granulosa cells

87 Human luteinized granulosa cells were collected from pre-ovulatory follicles during oocyte retrieval for
88 *in vitro* fertilisation (IVF). The ovarian stimulation procedures used here were previously reported by **(Roche**
89 **et al., 2016)**. Ethical approval for this study was obtained from the Research Ethics Board of the “Centre
90 Hospitalier Régional et Universitaire” (CHRU) Bretonneau, and written informed consent was obtained from
91 all patients. No patients received any monetary compensation for participating in the study, and all patients
92 provided written informed consent before use of their cells. Primary human granulosa cell preparations were
93 donated by women (36 ± 4 years) undergoing IVF. The causes of infertility were mechanical or male factor
94 infertility, without any known endocrinopathy, such as PCOS or hyperprolactinaemia. The PCOS diagnosis
95 was established according to the Rotterdam criteria (oligomenorrhea and/or anovulation, clinical and/or
96 biochemical signs of hyperandrogenism, and polycystic ovaries on ultrasonography scans, and exclusion of
97 other aetiologies that mimic the PCOS phenotype) **(Rotterdam ESHRE/ASRM-Sponsored PCOS**
98 **Consensus Workshop Group, 2004)**.

99 The cell recovery protocol was described by **(Roche et al., 2016)**. Cells were maintained in *in vitro*
100 cultures in McCoy 5A medium (Sigma-Aldrich, Saint-Quentin Fallavier, France) supplemented with 20
101 mmol/L Hepes, penicillin (100 IU/ml), streptomycin (100 mg/ml), L-glutamine (3 mM), 0.01% bovine serum
102 albumin (BSA), 0.1 $\mu\text{mol/L}$ androstenedione, 5 mg/L transferrin, 20 $\mu\text{g/L}$ selenium, and 5% foetal calf serum
103 (FCS).

104 2.2. Cell culture

105 The KGN cell line is a human cell line, derived from an ovarian carcinoma of granulosa cells, from a 63-
106 year-old Japanese menopausal woman in 1994 **(Nishi et al., 2001)**. KGN has preserved some differentiation
107 criteria, such as the presence of the Follicle Stimulating Hormone (FSH) receptor, sensitivity to FSH, and
108 secretion of steroids. A mutant KGN cell line expressing DsRed was created after infection with a lentivirus
109 (pLV-tTRKRAB-red was a gift from Didier Trono (Addgene plasmid # 12250 ; <http://n2t.net/addgene:12250> ;
110 RRID:Addgene_12250, Watertown, USA) **(Wiznerowicz and Trono, 2003)**.

111 Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) F12 medium (Sigma, l'Isle
112 d'Abeau Chesnes, France), supplemented with L-Glutamine (2 mM final), antibiotics (100 IU/ml
113 Streptomycin/Penicillin, Sigma, Saint-Louis, USA), and (10% FCS), at 37°C in 5% CO₂. Cells were cultured
114 at the following concentrations; 1×10^6 cells/T75 flask, 0.5×10^6 cells/T25 flask for western blots or qRT-

115 PCR analysis, or for 96-well plates, 5×10^3 cells/well for immunocytochemistry, and 1×10^4 cells/well for
116 assays). The medium was changed every 48 h.

117 Cells were exposed to AdipoRon (Sigma Saint-Louis, USA) at two concentrations: 2.5 μM and 25 μM or
118 Nutlin (5 μM , Sigma Saint-Louis, USA) to inhibit the proliferation or a recombinant human adiponectin (1
119 $\mu\text{g/ml}$, Sigma Saint-Louis, USA), or the PPAR ligand (rosiglitazone, Sigma Saint-Louis, USA). AdipoRon at
120 25 μM generates significant effects on the adiponectin pathway (**Fairaq et al., 2017**). Cell surfaces were
121 analysed using ImageJ software (NIH, Bethesda, Maryland, USA).

122 *2.3 Transient transfection of granulosa cells*

123 After 24 h culture, KGN cells were transfected with 250 ng/well of a vector expressing PPAR Response
124 Elements (PPREs), driven by the firefly luciferase gene (PPRE-Luc) (**Blanquart et al., 2002**), and 25 ng
125 renilla luciferase plasmid to normalise transfection efficiencies (Promega, Madison, USA). Transfections
126 were performed using polyethylenimine (PEI) transfection reagent (Sigma Saint-Louis, USA) as described
127 previously (**Raymond et al., 2011**). The transfection medium was removed after 3 h, and cells were incubated
128 in fresh medium with appropriate AdipoRon concentrations for 60 h. Then, cells were lysed according to
129 manufacturer's instructions (Luciferase assay protocol, Promega, Madison, USA), and both firefly and renilla
130 luciferase activities were quantified on a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA).
131 Each treatment combination was assayed in triplicate, from three independent cultures.

132 *2.4. Colony formation assay*

133 1.5×10^3 KGN cells were seeded into each well of a 6-well plate, and treated with AdipoRon (2.5 and 25
134 μM). The cells were allowed to form colonies for up to two weeks, with media replaced every 48 h. Colonies
135 were stained (1% crystal violet in 35% methanol), and colony surfaces were quantified using ImageJ software
136 (NIH, USA).

137 *2.5. Wound-healing assay*

138 KGN cells were seeded at 5×10^5 cells per well into 12-well culture dishes, and were grown to
139 confluence. Then using a sterile tip, a linear scratch was generated in the cell monolayer. The cells were
140 washed three times with PBS to remove floating cells, and medium was changed and supplemented with 2.5
141 or 25 μM AdipoRon. Three different representative fields of the initial scratch, and at three different times:
142 4h, 8h and 24h were recorded. The distances of cell migration were calculated by subtracting the distance
143 between the wound edges at 4h, 8h, and 24 h from the distances measured at 0 h. The data were analysed
144 using NIH Image J (NIH, USA). Experiments were performed three times for each sample.

145 *2.6. Cell cycle analysis using flow cytometry*

146 KGN cells were cultured for two days, followed by serum starvation for 6 h to synchronise cells, and
147 then subjected to treatments for 48 h. After treatment, cells were harvested, washed in 1 x phosphate buffered
148 *saline* (PBS), centrifuged and incubated in buffer that lysed cells and marked DNA containing 0.01%
149 trisodium citrate, 1% Nonidet P-40, 16 µg/ml propidium iodide and 250 µg/ml RNase A. Cells were analysed
150 using flow cytometry (MoFlo Astrios^{EQ}, Beckman Coulter, USA). Twenty thousand events were collected per
151 sample, and the percentage of cells in each cell cycle phase was assessed using MultiCycle software for
152 Windows (Phoenix, Flow System, USA).

153 2.7. Immunofluorescence

154 Analysis of Bromodeoxyuridine (BrdU) incorporation, and mitotracker intensity were performed using
155 the High Content Screening (HCS) imaging system.

156 For BrdU analysis, cells were labelled for the last 24 h of culture in 10 µM BrdU (Sigma, Saint-Louis,
157 USA). Cells were fixed in 4% Paraformaldehyde (PFA)/PBS, and BrdU positive cells were immunostained
158 after indirect immunofluorescence, as described by (Migliorini et al., 2002). For the mitotracker, cells were
159 incubated for 30 min at 37°C with 200 nM mitotracker Orange CM-H2TMRos (Invitrogen, Fisher Scientific,
160 Illkirch, France).

161 Briefly, fixed cells were incubated in 1 x PBS/0.1 M glycine for 15 min at room temperature. This was
162 to saturate aldehyde groups. Cells were then permeabilised in 0.1% Triton X-100 (w/v) in 1 x PBS for 15 min,
163 and nonspecific binding sites were blocked in 2% BSA/1 x PBS for 15 min. Cells were incubated for 60 min
164 at room temperature with an antibody against BrdU (clone Bu33, Sigma, l'Isle d'Abeau Chesnes, France) or
165 p53 (clone 1C12, Cell Signalling Technologies) or rabbit or mouse IgG used as negative controls and
166 purchased from Sigma. All primary antibodies were diluted at 1:100 in 1% BSA/1 x PBS. After primary
167 antibody incubation, cells were washed three times in PBS and incubated for 45 min at room temperature with
168 goat anti-rabbit or anti-mouse IgG Alexa Fluor® 488 antibodies (diluted at 1:500 in 1% BSA/1 x PBS,
169 Invitrogen, Fisher Scientific, Illkirch, France). Cells were counterstained with Hoechst 33342 (Sigma), and
170 examined using standard immunofluorescence microscopy (phalloidin staining) on the ArrayScan® VTI HCS
171 Reader (Thermo Fisher Scientific - Cellomics, Pittsburgh, PA, USA). Analyses were performed in triplicate,
172 on three different cultures. A minimum of 3×10^3 cells was analysed per well.

173 2.8 Cell viability

174 Cell viability was evaluated using the Cell Counting Kit-8 (CCK8, Sigma Aldrich, Saint Quentin-
175 Fallavier, France). Cells were previously dispensed into sterile 96 wells cell culture plats to have about 4,000

176 cells per well. Twenty-four hours later, cells adhering to the plat were stimulated with increasing
177 concentrations of ligands for at least 24 h. Supernatant was then removed and replaced with DMEM medium
178 containing 100 μ L of CCK8 10%. After 3.5 h incubation at 37°C in water-saturated atmospheres with 5%
179 CO₂, the absorbance at 450 nm was measured using a microplate reader. The optic density obtained were then
180 related to a standard range previously defined.

181 *2.9. Cell apoptosis*

182 Cell apoptosis was assessed by caspase-3 cleavage activity. Caspase activity measurements were
183 performed using the commercially available Caspase-Glo™ 3/7 assay kit (Promega, Madison, USA),
184 according to manufacturer's instructions. Cells were seeded in a white 96-well culture plate at a density of $1 \times$
185 10^4 cells per well, and treated with different concentrations of AdipoRon (2.5 or 25 μ M) for 48 h. Then, a
186 volume of Caspase-Glo® 3/7 assay reagent equal to the volume in each well was added, and incubated for 30
187 min in the dark to stabilise the luminescent signal. The plate was assayed on a Luminoskan Ascent microplate
188 reader, (VWR International, France) to record luminescence.

189 *2.10. Oxidative stress analysis*

190 The ROS-Glo™ H₂O₂ assay kit (Promega, Madison, USA) was used to analyse oxidative stress in cells
191 after 48 h exposure to different concentrations of AdipoRon (2.5 and 25 μ M). Assays were performed
192 according to manufacturer's instructions. Briefly, after treatment, cells were stressed with hydrogen peroxide
193 (H₂O₂) substrate solution for 3 h, then incubated for 20 min with ROS-Glo™ detection solution in the dark to
194 stabilise the luminescent signal. The plate was assay on a Luminoskan Ascent microplate reader, (VWR
195 International) to record luminescence

196 *2.11. Metabolites*

197 ATP and cAMP concentrations were measured using the CellTiter-Glo™ ATP assay kit and cAMP-
198 Glo™ assay kit (Promega, Madison, USA), respectively, according to manufacturer's instructions. Cells were
199 seeded in a white 96-well culture plates at a density of 1×10^4 cells per well, and treated with different
200 concentrations of AdipoRon (2.5 or 25 μ M) for 48 h.

201 Lactate and pyruvate concentrations in the culture medium were measured using Lactate Assay Kit
202 (Sigma Saint-Louis, USA) and Pyruvate Assay Kit (Cliniscience, Nanterre, France) respectively, according to
203 manufacturer's instructions. (Sigma, Clinisciences, Nanterre, France). Cells were seeded in a white 96-well
204 culture plates at a density of 1×10^4 cells per well, and treated with different concentrations of AdipoRon (2.5
205 or 25 μ M) for 48 h. The results of each assay were normalised per 1×10^4 cells.

206 2.12. *Glucose uptake assay*

207 Glucose uptake was measured using the Glucose Uptake-Glo™ assay (Promega, Madison, USA)
208 according to manufacturer's instructions. Cells were seeded in white 96-well culture plates at a density of $1 \times$
209 10^4 cells per well, and treated with different concentrations of AdipoRon (2.5 or 25 μ M) for 48 h. After
210 treatment, the medium was removed and cells were incubated in 2-deoxyglucose (2-DG) for 10 min. Cells
211 were further incubated with 2DG6P detection reagent for 60 min in dark. 2-DG uptake was measured on the
212 Luminoskan Ascent microplate reader, (VWR International) to record luminescence.

213 2.13. *Phosphodiesterase assay*

214 Cyclic nucleotide phosphodiesterase activity measurements were performed using the luminescent *PDE-*
215 *Glo*™ phosphodiesterase assay (Promega, Madison, USA) according to manufacturer's instructions.
216 Experiments were performed on KGN protein extracts, incubated with increasing AdipoRon concentrations
217 for 15 min.

218 2.14. *Steroid analysis*

219 Estradiol levels were determined by enzyme-linked immunosorbent assay (ELISA) kit according to
220 manufacturer's instructions (No. 501890, Cayman Chemical Company, Ann Arbor, MI, USA). Other steroids
221 (testosterone and dihydrotestosterone) levels were determined by liquid chromatography–mass spectrometry
222 (LC-MS/MS), as described by Meunier *et al.* (**Meunier et al., 2015**).

223 2.15. *Western immunoblotting*

224 KGN or primary granulosa cells were lysed and exposed to three repeated freeze/thaw cycles in lysis
225 buffer. Protein concentrations in supernatants were determined using a calorimetric assay kit (DC assay kit;
226 Uptima Interchim, Montluçon, France), and equal protein concentrations (80 μ g) were electrophoresed on
227 SDS-PAGE under reducing conditions, and transferred to nitrocellulose membranes (Schleicher & Schuell,
228 Ecquevilly, France), as described (**Tartarin et al., 2012**). Membranes were incubated at 4°C overnight with
229 the following antibodies: phospho- α AMPK (Thr172), proliferating cell nuclear antigen (PCNA), phospho-akt
230 (ser473), Akt, PTEN, p53, Cyclin D3 (antibodies #2535, #2586, #4058, #9272, #9559, #2524, #2936, Cell
231 Signalling Technologies), 3 β hydroxysteroid dehydrogenase (3 β HSD), p21 and p27 (antibodies #37-2, #F-5
232 and #F-8, Santa Cruz, CA, USA), vinculin (clone hVIN-1, Sigma), steroidogenic acute regulatory protein
233 (StAR) and aromatase antibodies (ab58013, ab18995, Abcam, Cambridge, UK). All antibodies were used at
234 a 1:1000 dilution, except 3 β HSD antibodies which were diluted at 1:500. Signals were detected by enhanced
235 chemiluminescence (Amersham Pharmacia Biotech, Orsay France) and band densities were quantified using

236 ImageJ software (NIH, USA). Intensity signals were expressed in arbitrary units after normalisation to an
237 internal standard (total protein or vinculin), corresponding to the average of three different experiments.

238 2.16. Mitochondrial DNA analysis

239 The relative number of mitochondrial DNA (mtDNA) copies in KGN cells was assessed using
240 quantitative real-time PCR (qPCR). Nuclear DNA was used as a standard. For quantification, primers for
241 mtDNA, and primers for nuclear DNA (ACTB, EEF1A1, GAPDH), were used (Supplemental Table). Each
242 reaction was performed in duplicate and included a negative control (without DNA). Cycle threshold (Ct)
243 values were calculated automatically, and analysed using CFX Manager™ Software (version 3.1). Relative
244 mtDNA copy numbers were calculated using the geometric means of ACTB, EEF1A1 and GAPDH signals
245 (Bijak et al., 2017; Vandesompele et al., 2002).

246 2.17. Imaging of ex-vivo organs

247 Leg muscle from embryonic chicks at day 15 were injected with 2×10^6 KGN-DsRed cells in PBS, in
248 addition to 2.5 or 25 μ M AdipoRon. Muscles were cultured in inserts (porosity: 0.4 μ m) (VWR, Radnor,
249 USA) at 37°C in 5% CO₂ for 72 h. After this period, injected muscles were analysed with a "In Vivo Imaging
250 System" (IVIS Spectrum, Perkin Elmer, Waltham, USA), with auto exposure epi-illumination settings to
251 record images, according to manufacturer's instructions. Living Image 4.5.2 software (Perkin Elmer)
252 facilitated apparatus set up, data acquisition and data analysis.

253 2.18. Statistical analysis

254 The GraphPad Prism® software (Version 8, La Jolla, CA, USA) was used for all analyses. Data were tested
255 for homogeneity of variance by Bartlett's test and for normal distribution by Shapiro-Wilk test. One-way
256 ANOVA was performed with Tukey-Kramer multiple comparisons tests. Mann-Whitney tests were performed
257 where variances were unequal. Values were deemed significant when *p < 0.05, **p < 0.01 and ***p < 0.001.

258

259 3. Results

260 3.1. Activation of AMPK and PPAR by AdipoRon in human luteinized granulosa cells

261 Under our experimental conditions, AdipoRon significantly increased AMPK phosphorylation in
262 primary human granulosa cells (Figure 1A, $p < 0.01$). In KGN cells transfected with a PPRE reporter vector,
263 AdipoRon (25 μM) significantly enhanced PPRE promoter activity at the same level as recombinant human
264 adiponectin (1 $\mu\text{g/ml}$), or rosiglitazone after 24 h stimulation (Figure 1B, $p < 0.05$).

265 3.2. AdipoRon reduces human luteinized granulosa cell viability

266 The first phenotype observed in KGN cells with optic microscope was a modification of the cells
267 morphology associated with reduction in cell surface of KGN cells exposed to 25 μM AdipoRon
268 (approximately 50%, $p < 0.001$), in comparison to untreated cells (Figure 2A, $p < 0.001$). Also, KGN
269 granulosa cell viability were assessed by CCK-8, TUNEL and Annexin V assay. A 2.5 μM AdipoRon
270 exposure for 48 h was enough to significantly decrease cell viability (Supplemental Data 1, $p < 0.05$). The
271 caspase 3 cleavage activity, which is a marker associated with cell death, was also analyzed and shown that
272 activity was significantly increased at 2.5 μM and 25 μM AdipoRon concentrations in KGN (Figure 2B, $p <$
273 0.05) and primary granulosa cells (Figure 2B, $p < 0.01$). In addition, we observed a significantly higher
274 oxidative status, due to elevated Reactive Oxygen Species (ROS) production in primary human luteinized
275 granulosa cells, at both AdipoRon concentrations (Figure 2C, $p < 0.05$).

276 3.3. AdipoRon impacts on different cell characteristics in both cell types

277 AdipoRon effects on cell proliferation were evaluated by BrdU incorporation, colony formation and
278 scratch assay. In KGN cells, AdipoRon (25 μM) reduced BrdU incorporation to approximately 48%, and
279 increased the p53-positive cells (Figure 3A, $p < 0.01$, Supplemental Data 1). In primary human luteinized
280 granulosa cells, cell proliferation was decreased to approximately 27% after 25 μM AdipoRon exposure, with
281 11% BrdU positive cells, when compared to 15% BrdU positive cells in untreated controls (Figure 3B, $p <$
282 0.05). In parallel, KGN cells were seeded at 1.5×10^3 cells/well in plate 6 wells and the colonization of KGN
283 was measured after 2 weeks of treatment. The surface of KGN cell colony seeded at low density was reduced
284 to approximately 20% for 2.5 μM ($p < 0.05$) and 40% for 25 μM ($p < 0.001$, Figure 3C).

285 The speed of repair process in case of a wound healing test was slower after 25 μM AdipoRon
286 supplementation, with a speed of 95 $\mu\text{m}/24$ h after 25 μM AdipoRon exposure versus 160 $\mu\text{m}/24$ h for
287 untreated cells (Supplemental Data 2). Similarly, in an *ex-vivo* experiment, 25 μM AdipoRon limit the volume
288 of tumour progression induced by injection of Ds-Red KGN cells into chick embryo muscle (Supplemental
289 Data 3). To determine how AdipoRon impacted cell proliferation, we investigated cell cycle progression by

290 flow cytometry. We observed a significant accumulation of KGN cells in G₁ phase upon 25µM AdipoRon
291 treatment ($p < 0.001$, Figure 4), accompanied by decreases in cells in G₂ phase ($p < 0.001$, Figure 4).
292 However, the number of KGN cells in S phase did not vary according to the treatments (Figure 4).

293 Cell cycle arrest was also associated with decreased expression (approximately 40%) of the cell cycle
294 progression proteins, Cyclin D3 and PCNA by western blotting, (2.5 µM AdipoRon, $p < 0.05$ and 25 µM
295 AdipoRon, $p < 0.01$, Figure 5), and an increase in expression of the CDK inhibitors, p21 and p27 (25 µM
296 AdipoRon, Figure 5, $p < 0.05$).

297 Because Akt is involved in proliferation and steroid production in granulosa cells (Brandmaier et al.,
298 2017; Vara et al., 2004), we analysed the active form of Akt and its phosphatase PTEN by
299 immunohistochemistry and western blotting. We observed that KGN cells exhibited a decrease in Akt
300 phosphorylation (Ser 473) ($p < 0.001$, Figure 6A), which was associated with higher levels of phosphatase
301 PTEN after a 48 h (25 µM) AdipoRon exposure ($p < 0.001$, Figure 6B).

302 3.4. *AdipoRon affects mitochondria activity*

303 Because AdipoRon was developed to control different metabolic processes, and to improve insulin
304 sensitivity (Okada-Iwabu et al., 2013), we observed increased glucose uptake in primary human luteinized
305 granulosa cells, after AdipoRon exposure (KGN: $p = 0.06$, primary cells: $p < 0.05$, Figure 7A). In KGN cells,
306 the analysis of mtDNA content has allowed to show a lower mtDNA content after 25µM AdipoRon exposure
307 showing a reduced mitochondrial activity ($p < 0.01$, Figure 7B). Moreover, mitochondrial activity
308 (represented by mitotracker intensity) was decreased in staining intensity after 25 µM AdipoRon exposure,
309 when compared to untreated cells ($p < 0.05$, Figure 7C). Additionally, ATP production was significantly
310 reduced ($p < 0.01$, Figure 7D). In contrast, lactate production was higher after 25 µM AdipoRon treatment, in
311 comparison to untreated cells (KGN: $p < 0.05$, primary luteinized cells: $p < 0.01$, Figure 7E).

312 3.5. *AdipoRon reduces steroid production*

313 In granulosa cells, a direct consequence of dysfunctional mitochondria activity is the production of
314 steroid hormones. To investigate steroidogenesis, we investigated StAR expression, a cholesterol transporter,
315 and two enzymes involved in steroid production, 3β-HSD and aromatase, in human primary granulosa cells.
316 StAR expression was not affected at the protein level by AdipoRon (Figure 8). However, a decrease in 3β-
317 HSD and aromatase protein levels was observed (3β-HSD: $p = 0.08$, aromatase: $p < 0.001$, Figure 8) and led
318 to a decrease in androgen precursors and estradiol secretion measured after adding dehydroepiandrosterone

319 (DHEA) (testosterone: $p < 0.05$, DHT: $p < 0.01$, estradiol: $p < 0.01$) (Figure 9) showing a decrease in steroid
320 production.

321 Decreased enzyme expression was associated with lower cAMP levels after AdipoRon (25 μM)
322 treatment (Supplemental Data 4A). This was due to increased phosphodiesterase activity which transformed
323 cyclic adenosine monophosphate (cAMP) to 5' adenosine monophosphate (5'AMP) (Supplemental Data 4B).
324 5'AMP is an important metabolite that activates the protein kinase A (PKA) pathway and is involved in
325 steroid enzyme expression (Gyles et al., 2001).

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327 4. Discussion

328

329 We sought to understand the effects of the adiponectin synthetic agonist, AdipoRon on two main
330 functions in human luteinized granulosa cells, i.e. steroidogenesis and cell proliferation, which are involved in
331 folliculogenesis (Drummond, 2006). For the first time, we have shown that AdipoRon reduced estradiol and
332 androgen secretion via the expression of enzymes involved in steroid production like aromatase or 3 β -HSD, in
333 human granulosa cells. Moreover, in both our cell models, AdipoRon induced a cell cycle arrest in G₁ phase,
334 through the Akt/PTEN pathway which is involved in granulosa cell functions (Makker et al., 2014) (Figure
335 10).

336 Human primary luteinized granulosa cells appear to be more sensitive to AdipoRon compared to
337 KGN cells. AdipoRon 2.5 μ M is sufficient to decrease cell proliferation, ROS production and
338 metabolic change as measured by ATP content and glucose uptake. Despite a similar phenotype
339 described with the KGN granulosa cell line, this requires a 10 times more concentrated dose of
340 Adiporon (25 μ M) to obtain the same results.

341

342 AdipoRon was developed to treat diabetes and obesity (Okada-Iwabu et al., 2013), by activating
343 AdipoR1, AdipoR2 and key proteins involved in their signalling, the AMPK kinase and PPAR pathways
344 playing an important role in cells such as than cell survival and energy production. Adiponectin receptors,
345 AMPK and PPARs are expressed in ovarian cells including granulosa cells, in different species (Chabrolle et
346 al., 2007; Komar et al., 2001; Pierre et al., 2009; Tosca et al., 2005). In human luteinized granulosa cells, we
347 observed that AMPK and PPAR pathways were activated by AdipoRon (2.5 and 25 μ M), suggesting that both
348 pathways are stimulated by this drug, such as adiponectin.

349 AdipoRon exerts anti-proliferative and pro-apoptotic effects on different cancer cell lines (Kim et al.,
350 2010; Ramzan et al., 2019), however these models are mainly epithelial in nature, thus they do not have the
351 specificity of granulosa cells to switch from proliferative cells, to highly differentiated, steroid hormone
352 secreting cells. In terms of cell proliferation, we observed similar effects from BrdU analyses, migration, and
353 colony assay experiments even with human primary luteinized granulosa cells which are low proliferative
354 cells. Moreover, AdipoRon administration reduced the volume occupied by human granulosa cancer cells,
355 when injected into embryonic chick muscle tissue. By observing a strong effect on cell proliferation, we
356 focused on a few proteins linked to the cell cycle. Consequently, for granulosa cells, AdipoRon treatment

357 induced reductions in Cyclin D3, p53 and the cyclin dependent kinase inhibitors, p21 and p27. In other type
358 cancer such as breast cancer models, AdipoRon has been shown to have an effect on stopping the cell cycle
359 by downregulating Cyclin D (Fairaq et al., 2017; Ramzan et al., 2019). This cell cycle arrest induced by p21
360 and p27 expression was in agreement with other studies (Gelsomino et al., 2019; Huang et al., 2018).

361 We demonstrated that AdipoRon increased phosphatase PTEN, which dephosphorylates and inactivates
362 Akt kinase (Vara et al., 2004). The PI3K/Akt/PTEN pathway is a key component in granulosa mediated
363 steroidogenesis and cell proliferation. PTEN dephosphorylates Akt and inhibits G₁ phase progression
364 (Brandmaier et al., 2017; Furnari et al., 1997; Vara et al., 2004), by inducing the expression of p27 (Li and
365 Sun, 1998). Interestingly, Akt phosphorylation levels were increased in mutant mice deficient for
366 adiponectin, and its (adiponectin) overexpression reduced high levels of phosphorylated Akt (Fujishima et al.,
367 2014). In the ovaries, mutated PTEN in granulosa cells upregulated Akt phosphorylation, leading to the
368 development of granulosa cell tumours in 7% of their mouse model (Laguë et al., 2008). To validate this, the
369 authors analysed PTEN and phospho-Akt in human and equine tumours and demonstrated that several
370 granulosa cell tumours also presented abnormal nuclear or perinuclear localisation of phospho-Akt.

371
372 In primary rat granulosa cells, human recombinant adiponectin had no effect on steroidogenesis in the
373 presence or absence of FSH (Chabrolle et al., 2007). However, the exposure of human primary granulosa cells
374 to adiponectin decreased P450 aromatase expression and estradiol synthesis (Tao et al., 2019). The use of
375 short hairpin RNA against adiponectin receptors has allowed the unravelling of pathways and functions of
376 each receptor in cell viability and steroid production. Thus, in KGN cells, AdipoR1 appears to be involved in
377 cell survival, whereas AdipoR2, through MAPK ERK1/2 activation, may be involved in the regulation of
378 steroid production. (Pierre et al., 2009). Elevated lactate production by human granulosa cells could be a
379 factor that decreases estradiol production, via modulation of genes involved in folliculo-luteal transition, such
380 as aromatase which is known as the enzyme which converts testosterone to estradiol as described by
381 (Baufeld and Vanselow, 2018). In our study, lactate was more elevated associated with a dysregulation of
382 steroid production like aromatase, when compared to untreated cells. In addition, steroid enzyme and steroid
383 production dysregulation can disrupt follicle development. On the other hand, several cancers are sensitive to
384 oestrogens (breast cancers, genital tract and ovarian cancers (François et al., 2015)). Granulosa cell tumours
385 comprise approximately 2%-5%, of ovarian cancers, but present the specificity to secrete abnormally
386 hormones (oestrogens, inhibin and Müllerian inhibiting substances). The risk factors for developing granulosa
387 cell tumours include nulliparity, fatness, steroids in oral contraceptives and family cancer history (Li et al.,

388 2018). Approximately 70% of granulosa cell tumour patients present elevated circulating estradiol due to
389 aromatase overexpression, which is known to increase tumour growth and progression in a number of cancers
390 cases (Fleming et al., 2010). The main therapeutic issue with granulosa cell tumours (approximately 20% of
391 patients die from tumour consequences) is the limited success of chemotherapy, therefore surgery remains the
392 main therapeutic approach (Ottolenghi et al., 2007). Thus, in our model, a drop in oestrogen production,
393 associated with reductions in cell proliferation, both *in vitro* and *ex-vivo* induced by AdipoRon, may suggest a
394 therapeutic approach for folliculogenesis, or to limit factor of invasiveness in case of cancer sensitive to
395 steroids.

396 We observed increased glucose uptake by primary luteinized granulosa cells, but not KGN cells.
397 However, we also observed decreased mitochondrial activity and increased lactate production, suggesting a
398 switch to glycolysis. *In vitro*, it has been described that glucose consumption and lactate production rates are
399 gradually increased with follicle development and granulosa differentiation, suggesting that AdipoRon could
400 stimulate differentiation (Boland et al., 1993; Harris et al., 2007). However, in granulosa and cumulus cells, in
401 the case of low ovarian reserves or in aged ovaries, lactate production is increased when compared with young
402 women with normal ovarian reserves (Pacella et al., 2012). Granulosa cells provide nutrients for the
403 development and maturation of follicles and oocytes (Alam and Miyano, 2020). Mitochondria in ovarian
404 granulosa cells generate energy and provide an environment conducive to the synthesis of lipids and steroids,
405 such as androgens and oestrogen, leading to folliculogenesis and good oocytes maturation (Chowdhury et al.,
406 2016; Morohaku et al., 2013; Zhao et al., 2019). In our study, AdipoRon reduced mitochondrial activity
407 probably by decreasing mitochondrial cell numbers, and mitochondrial respiratory activity, leading to a drop
408 in ATP production and ROS accumulation (Dröse et al., 2014; Wang and Hekimi, 2015; Zhao et al., 2019).

409
410 AdipoRon, exhibits antiproliferative and steroid-lowering effects in primary human luteinized granulosa
411 cells. AdipoRon may function by limiting oestrogen production and increasing glucose uptake sensitivity, and
412 could be relevant for treating pathological PCOS issues, or cell proliferation in some cancers that are
413 oestrogen-sensitive. Hence, AdipoRon which has an elevated half-life when compared to endogenous
414 adiponectin, a large molecule with a half-life of 45 to 60 min in the mouse (Halberg et al., 2009), could be
415 more easily adopted as a viable therapeutic strategy to stimulate granulosa cell differentiation for infertility
416 management (Bagnjuk et al., 2019; Khan et al., 2013).

417

418

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678 Legends

679 Figure 1. AdipoRon activates AMPK and PPAR pathways. (A) Western blot analysis of phospho-AMPK-alpha (Thr172)
680 in primary granulosa cells after 1h of AdipoRon exposure (0, 2.5, 25 μ M). Quantification of proteins was shown on the
681 side of the western-blot. Results are representative of 3 independent experiments. (B) KGN human granulosa cells were
682 transfected with the PPRE-Luc plasmid as described in *Materials and Methods*. After transfections, granulosa cells were
683 stimulated for 24 h with AdipoRon (2.5, 25 μ M), positive controls were human adiponectin 1 μ g/ml and 10 μ M of
684 rosiglitazone (n = 6 independent experiments). Luciferase activities were measured in cell lysates and values were
685 expressed in percentage \pm SEM of control, the activity of the unstimulated control being taken as 100%. Values are the
686 mean \pm S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from control conditions.

687 Figure 2. Reduction in viability of granulosa cells after AdipoRon exposure. (A) Representative microscopic fields of
688 KGN cells exposed or not with 25 μ M AdipoRon for 48h. Scale bar = 50 μ m. (right panel), Quantification of cell surface
689 (μ m²) by ImageJ software (B) Quantification of the caspase-3/7 cleaved activity and (C) ROS content in KGN and
690 primary human granulosa cells incubated with increasing concentration of AdipoRon for 48h (n = 3 independent
691 experiments). Data are presented as mean \pm S.E.M. (n = 3 independent experiments). Values are the mean \pm S.E.M. *P
692 < 0.05, **P < 0.01, ***P < 0.001, significant differences from control conditions.

693 Figure 3. AdipoRon decreased proliferation rate in granulosa cells (A) KGN cells were cultured for 48h in presence or
694 absence of AdipoRon or nutlin (5 μ M) as a positive control. Cells were cultured in presence of 10 μ M BrdU during the last
695 24h of the culture. Percentage of BrdU positive cells was analysed by High Content Screening. Results are representative
696 of 3 independent experiments in triplicate with at least 3000 cells counted per well. (B) The percentage of BrdU positive
697 cells was analysed in primary human granulosa cells by High Content Screening. (C) For colony formation assay, cells
698 were plated at a very low density (1.5 \times 10³ cells/well) and 2 weeks later surface occupied by foci were quantified (n = 3
699 independent experiments). Values are the mean \pm S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from
700 control conditions.

701 Figure 4. AdipoRon reduced KGN cell progression (A) Representative graphics of flow cytometry analysis to detect the
702 effect of AdipoRon on G0/G1, G2/M phases of cell cycle. (B) Cells were stimulated for 48h with AdipoRon and were
703 stained with propidium iodide. Percentage of cells in each cell cycle phases measured by flow cytometry. (n = 3
704 independent experiments). Values are the mean \pm S.E.M. ***P < 0.001, significant differences from control conditions.

705 Figure 5. AdipoRon stimulated expression of cell-cycle inhibitors. (A) Western blot analysis of PCNA, Cyclin D3, p21
706 and p27 in KGN cells after 48h of AdipoRon exposure. (B) Quantification of proteins. Results are representative of 3
707 independent experiments. Values are the mean \pm S.E.M. *P < 0.05, **P < 0.01, significant differences from control
708 conditions.

709 Figure 6. AdipoRon inhibited Akt through activation of PTEN. (A) Immunostaining and quantification of the phospho-akt
710 (ser473) and PTEN in KGN cells after 48h of AdipoRon exposure analysed by High Content Screening. Results are

711 representative of 3 independent experiments in triplicate with at least 3000 cells counted per well. IgG was used as
712 negative control. Scale bar = 10 μ M. (B) Western blot analysis of phospho-akt (ser473) and PTEN in KGN granulosa
713 cells. Results are representative of 3 independent experiments. Values are the mean \pm S.E.M. *P <0.05, **P < 0.01,
714 ***P <0.001, significant differences from control conditions.

715 **Figure 7.** AdipoRon mediated alterations on cell metabolism. AdipoRon has changed cell metabolism. (A) Glucose
716 uptake in KGN and human primary granulosa cells was quantified using the Glucose Uptake-Glo Assay after medium
717 change and 30 min incubation with 2DG (2-DeoxyGlucose). (n = 3 independent experiments). (B) mtDNA mitochondria
718 gene expression in KGN cells exposed to AdipoRon for 48h was analyzed by RT-qPCR (n = 6 independent experiments)
719 (C) The mitochondrial membrane potential in KGN cells was analysed by mitotracker dye after 25 μ M AdipoRon
720 exposure for 24h. Intensity of mitotracker was quantified by High Content Screening. (D) Concentration of ATP in
721 granulosa cells (M) per 10 000 cells. (E) Lactate concentration was quantified in the conditioned medium of KGN and
722 human primary granulosa cells after 48h of AdipoRon exposure per 10 000 cells. (n = 3 independent experiments) Results
723 are representative of 4 independent experiments. Values are the mean \pm S.E.M. *p <0.05, **p < 0.01, ***p <0.001,
724 significant differences from control conditions.

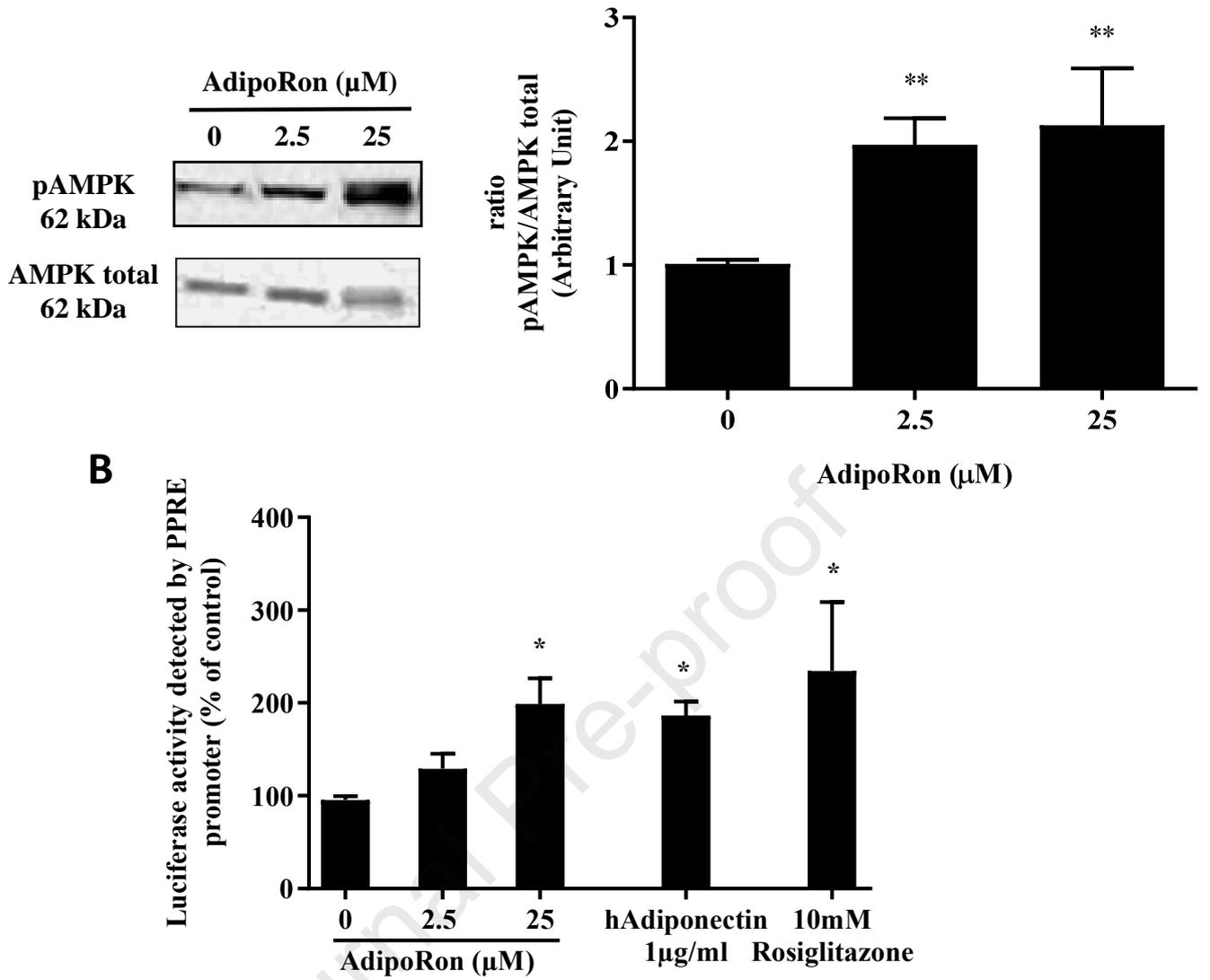
725 **Figure 8.** AdipoRon inhibited expression of proteins involved in steroidogenesis. StAR, 3 β -HSD and aromatase protein
726 expression in primary granulosa cells exposed to AdipoRon for 48h were quantified and normalized to vinculin (n = 3
727 independent experiments). Values are the mean \pm S.E.M. **p < 0.01, ***p < 0.001.

728 **Figure 9.** Steroid production by primary human granulosa cells (A) Human primary granulosa cells were incubated for
729 48h with dehydroepiandrosterone (DHEA) and the following steroids were quantified by mass spectrometry: (A)
730 testosterone (TS), (B) dihydrotestosterone (DHT). (C) Estradiol was measured by ELISA. Values are the mean \pm S.E.M.
731 *P <0.05, **P < 0.01, significant differences from control conditions.

732 **Figure 10.** AdipoRon activity in human granulosa cells: schematic summary of AdipoRon consequences on granulosa cell
733 metabolism, steroid production and cell cycle. cAMP: Cyclic adenosine monophosphate, ATP: Adenosine triphosphate,
734 AC: Adenylate cyclase, E2: estradiol, PDE: phosphodiesterase.

735

Figure 1



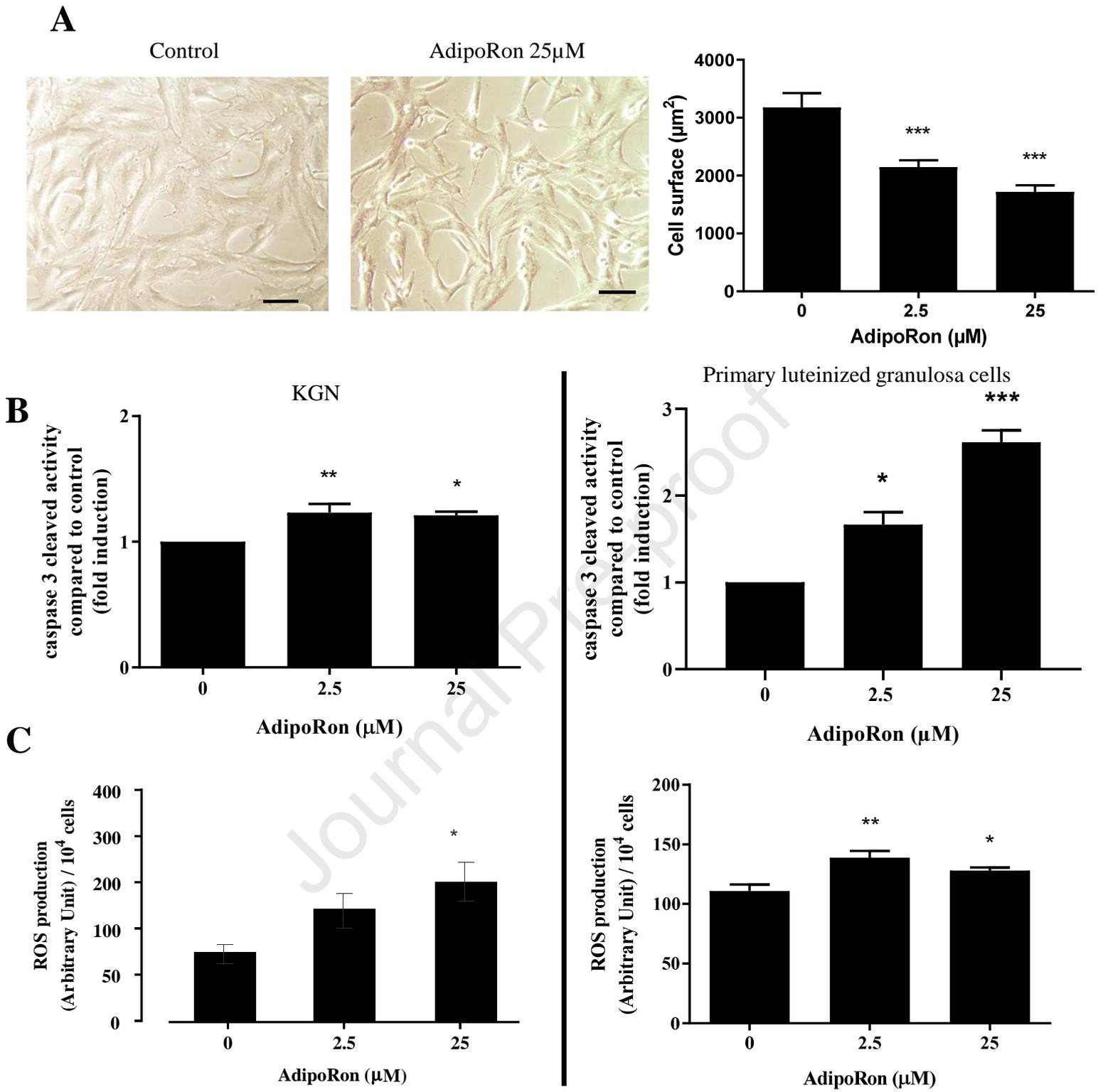


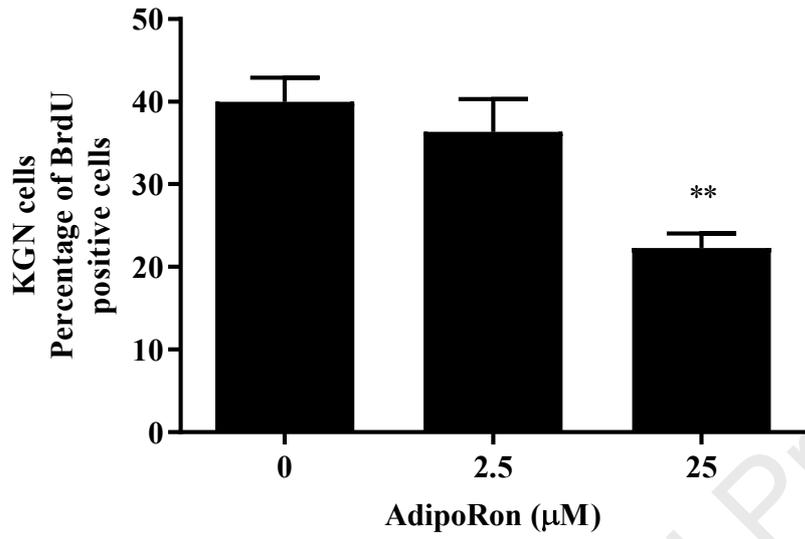
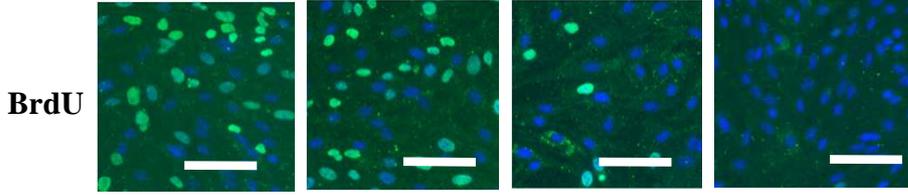
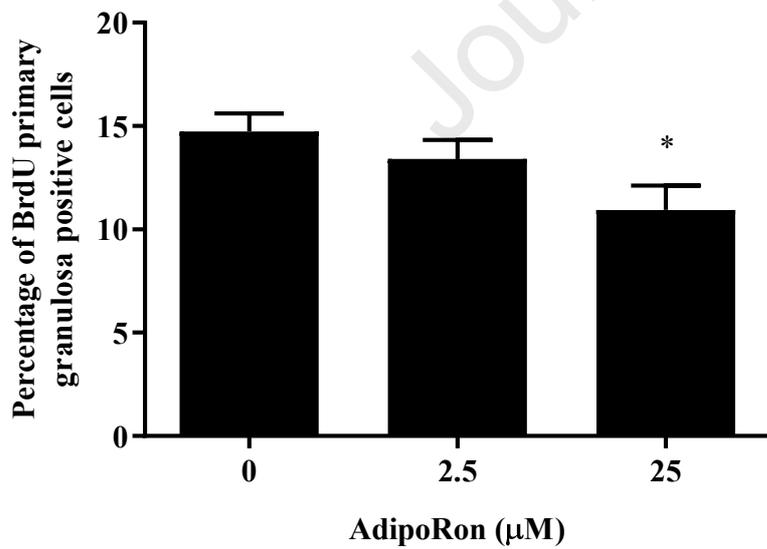
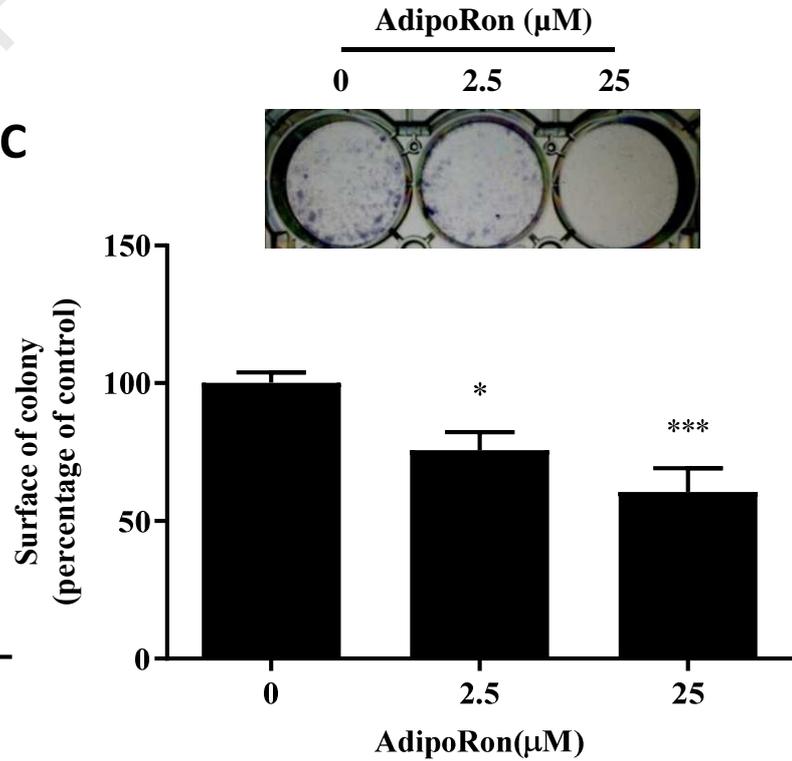
Figure 3**A**Control AdipoRon 2.5 μ M AdipoRon 25 μ M Nutlin 5 μ M**B****C**

Figure 4

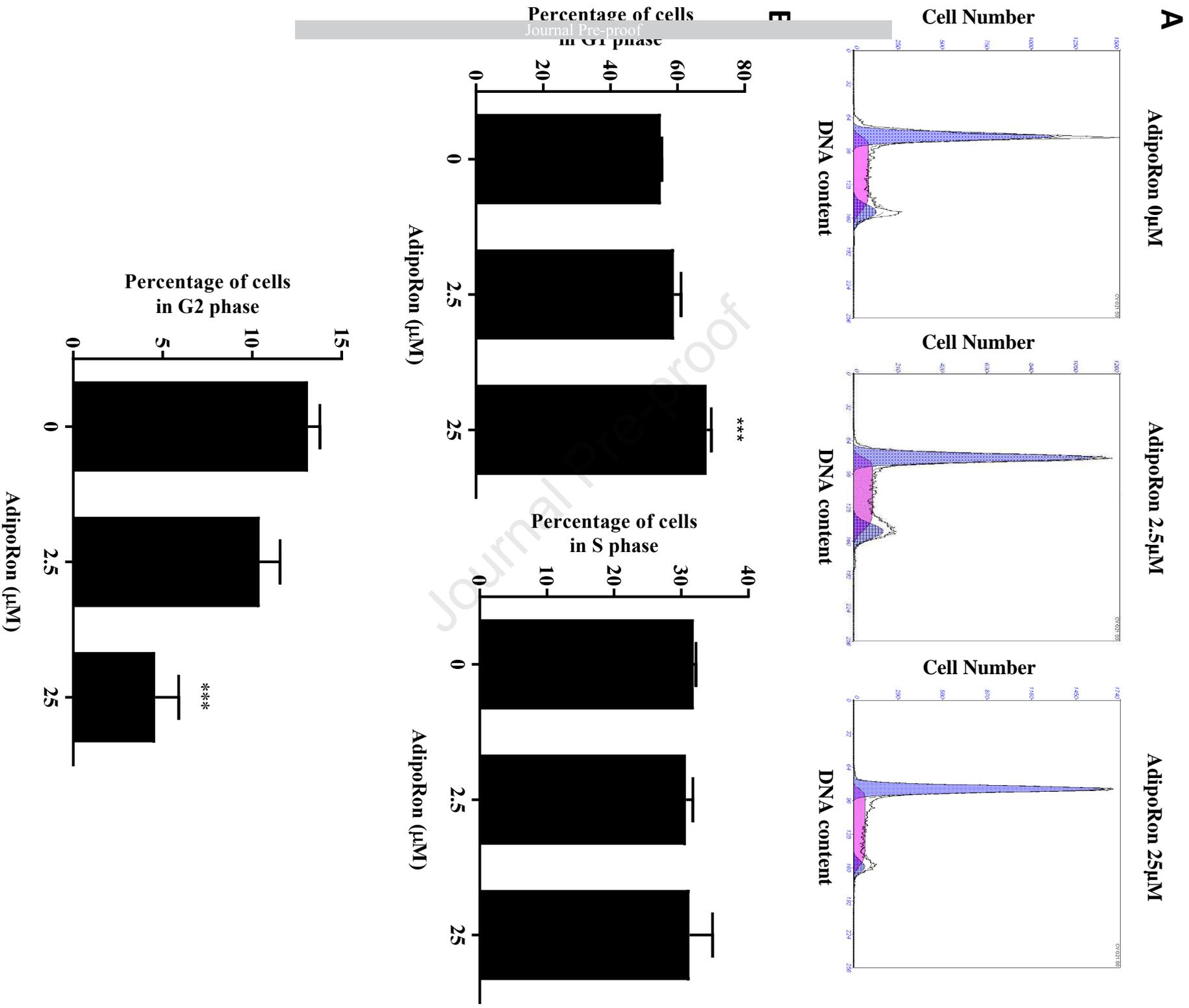


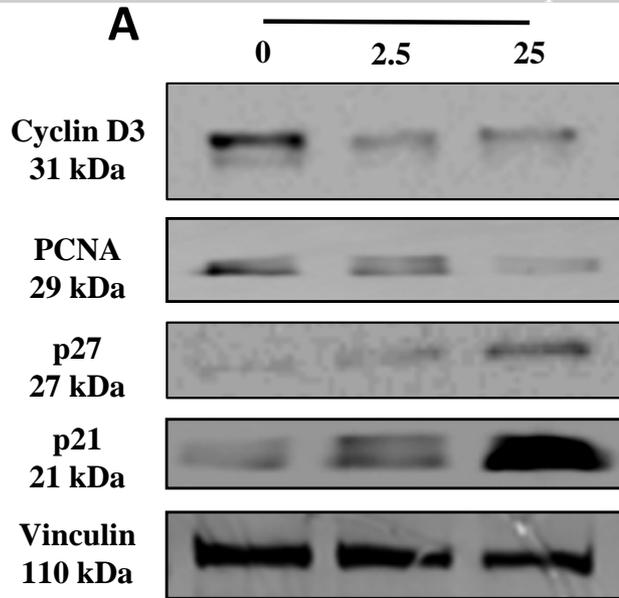
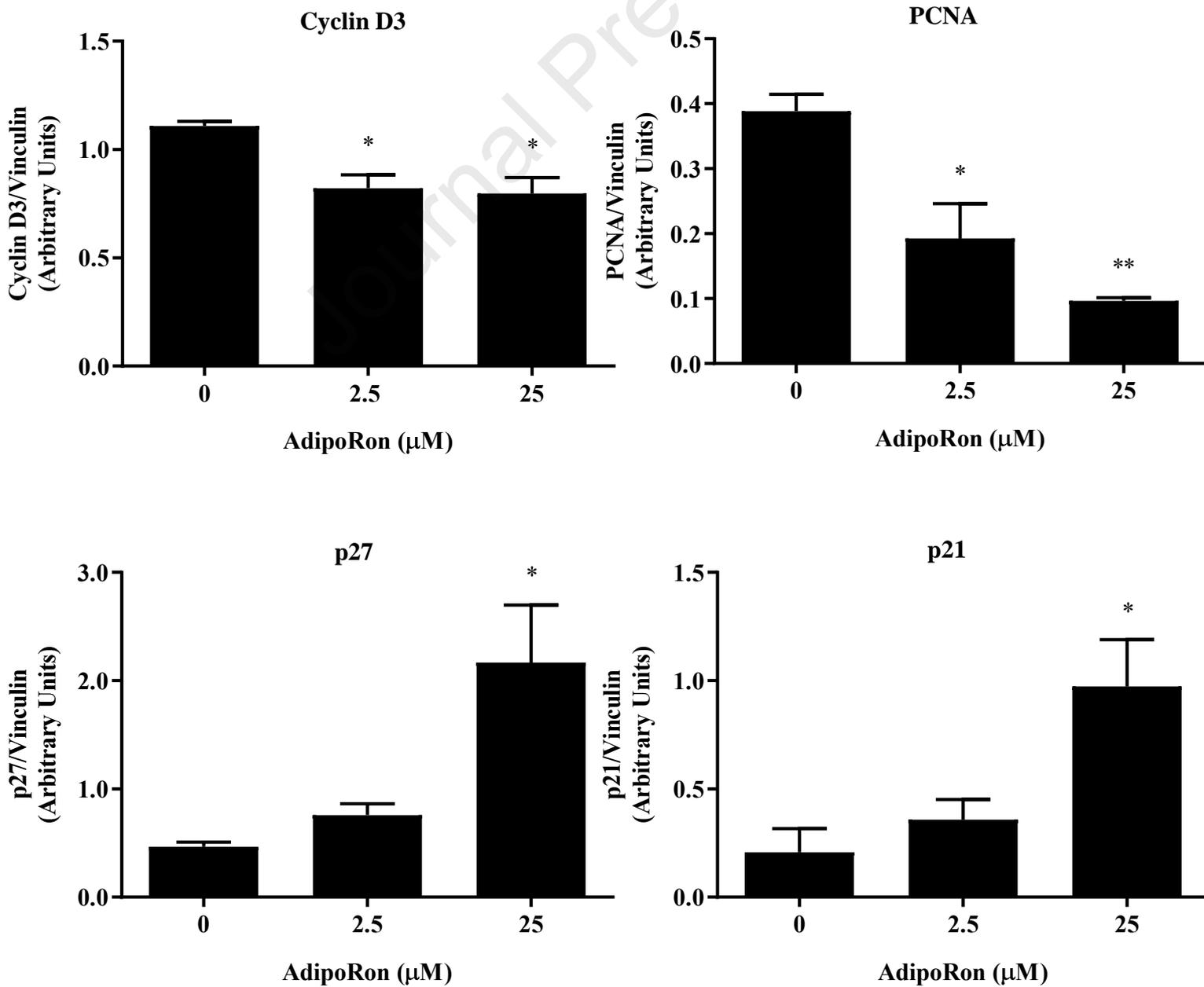
Figure 5**B**

Figure 6

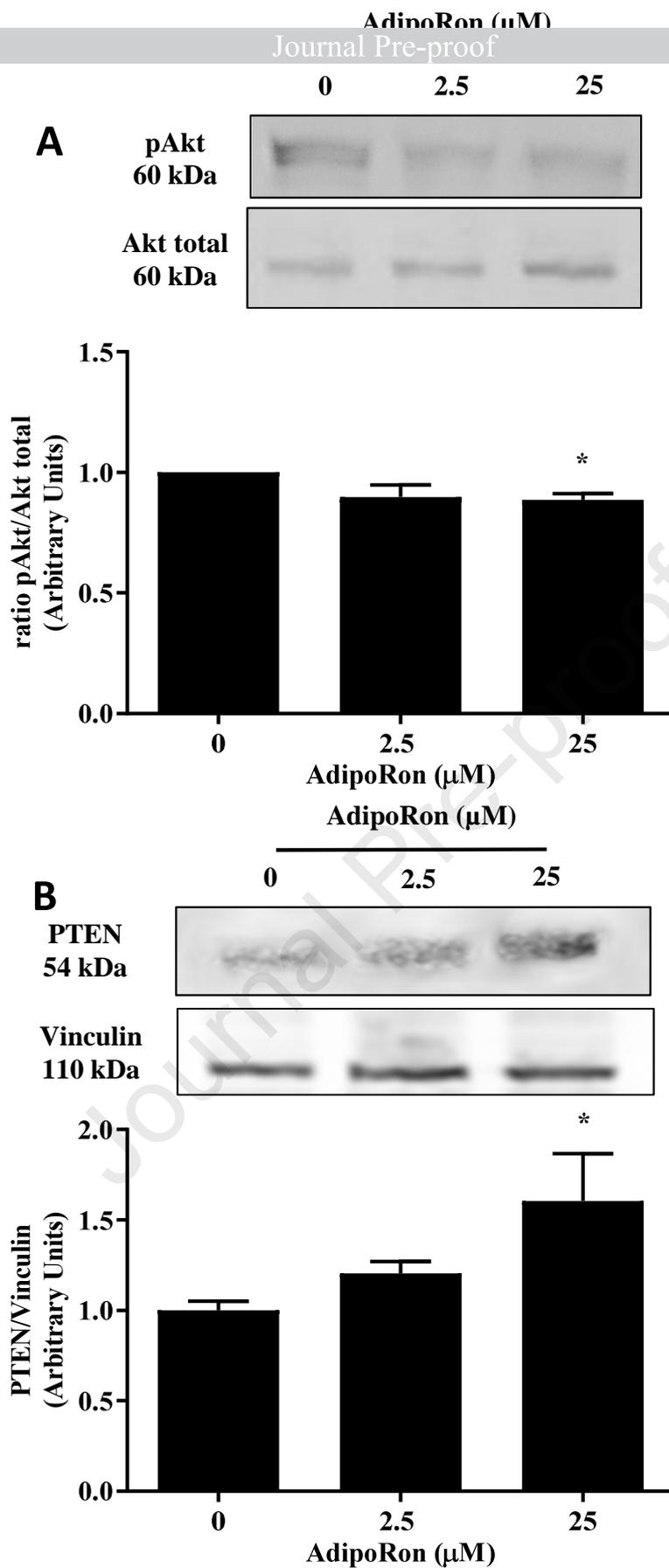


Figure 7**KCN**

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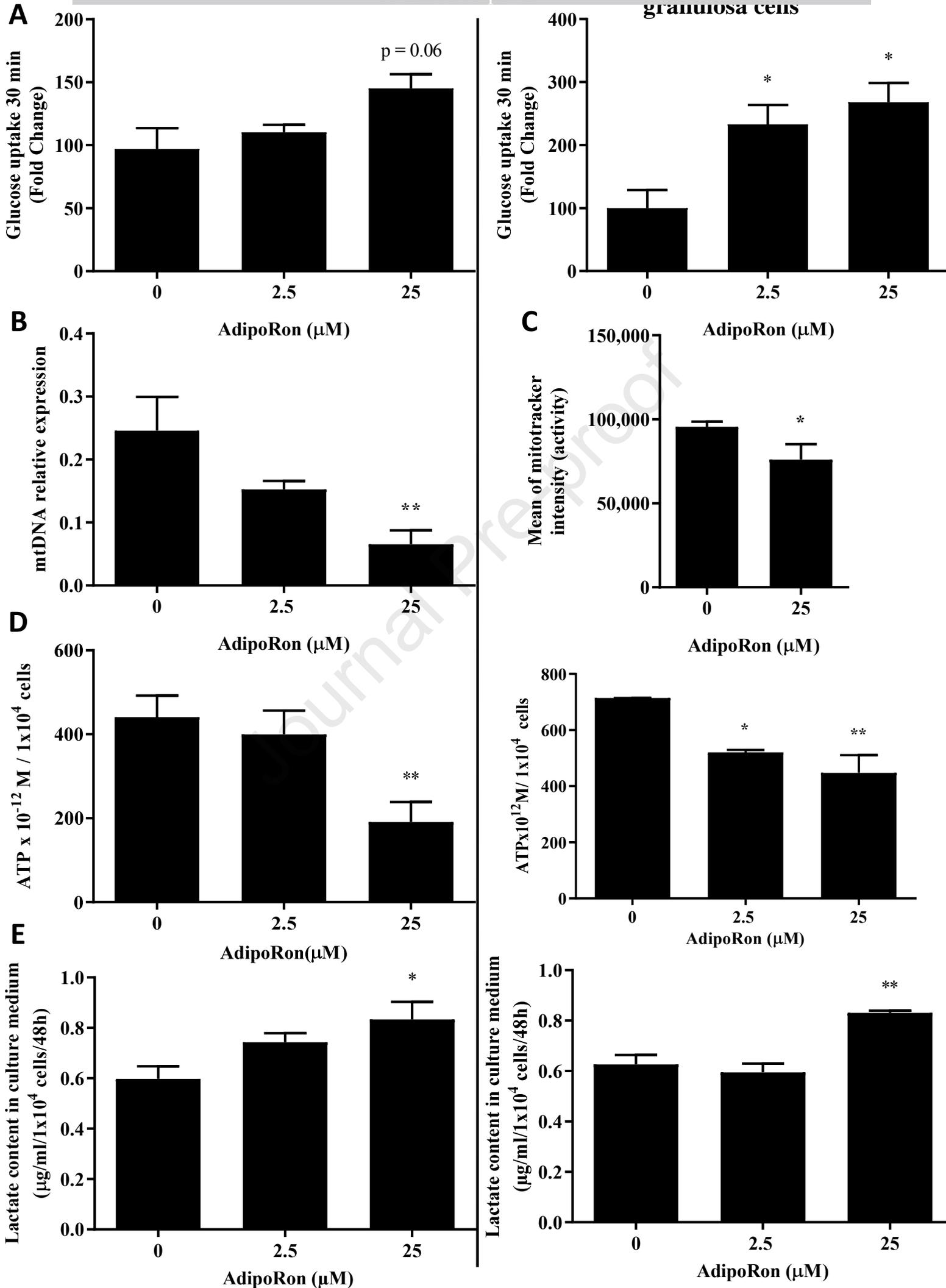
Primary human luteinized**granulosa cells**

Figure 8

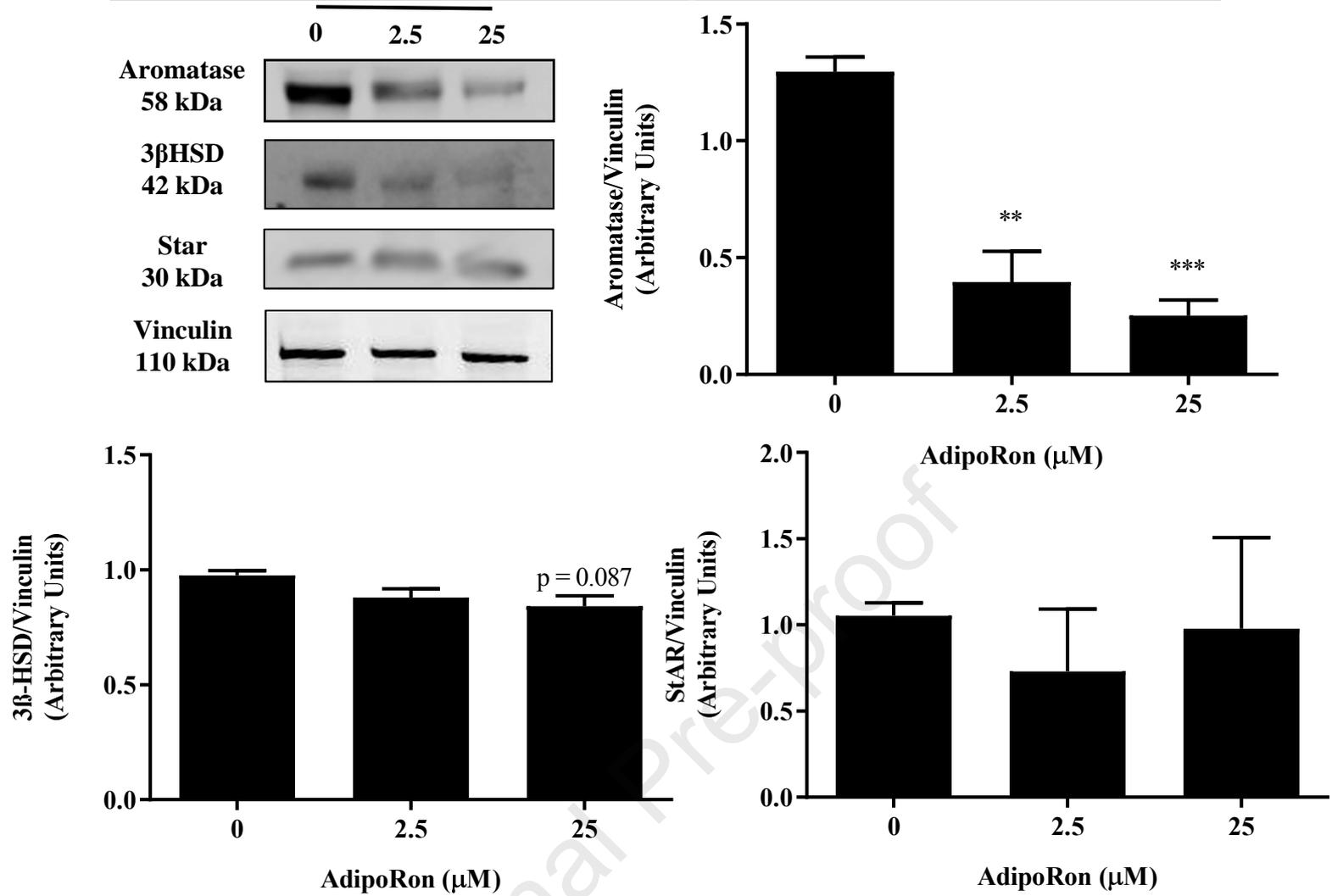
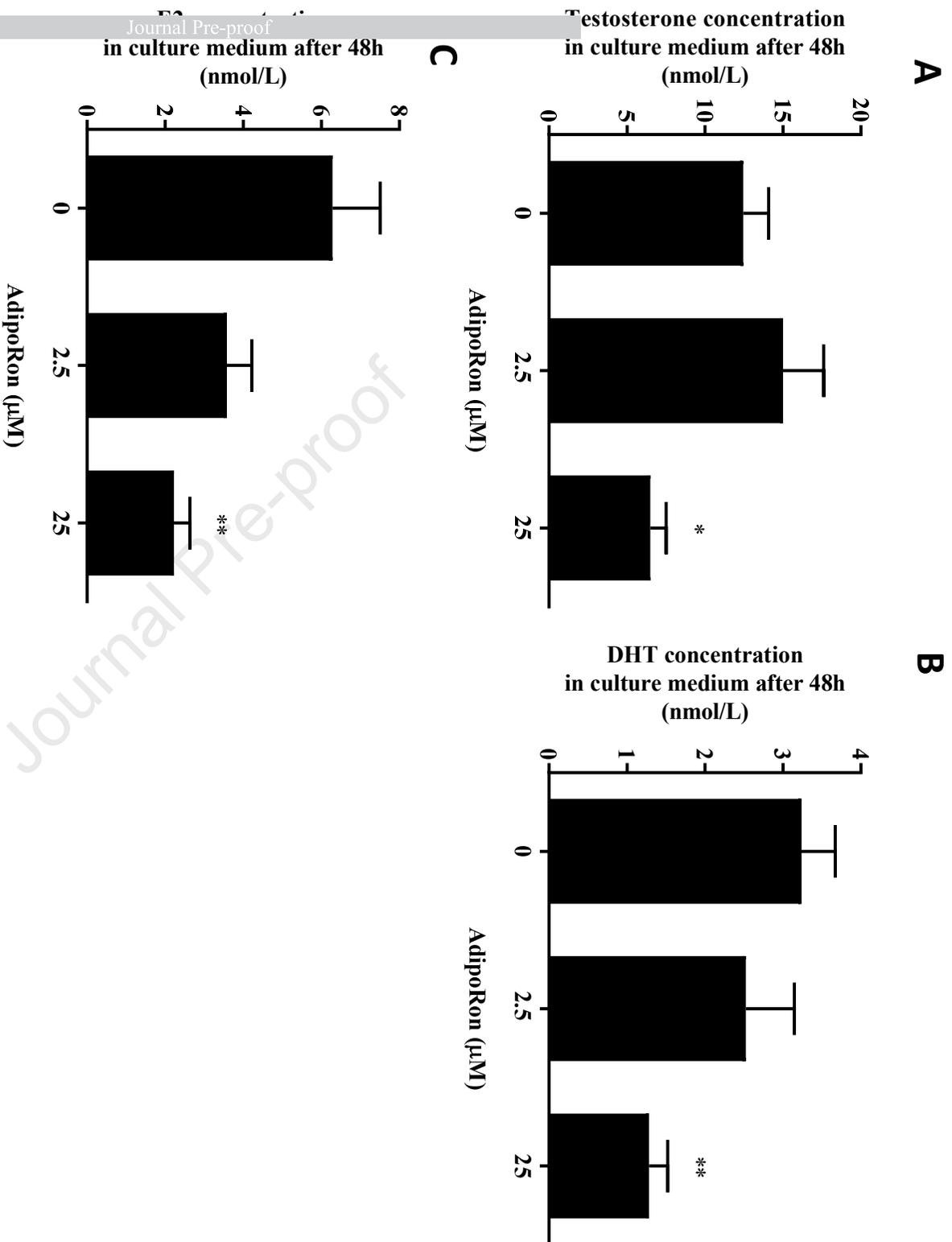
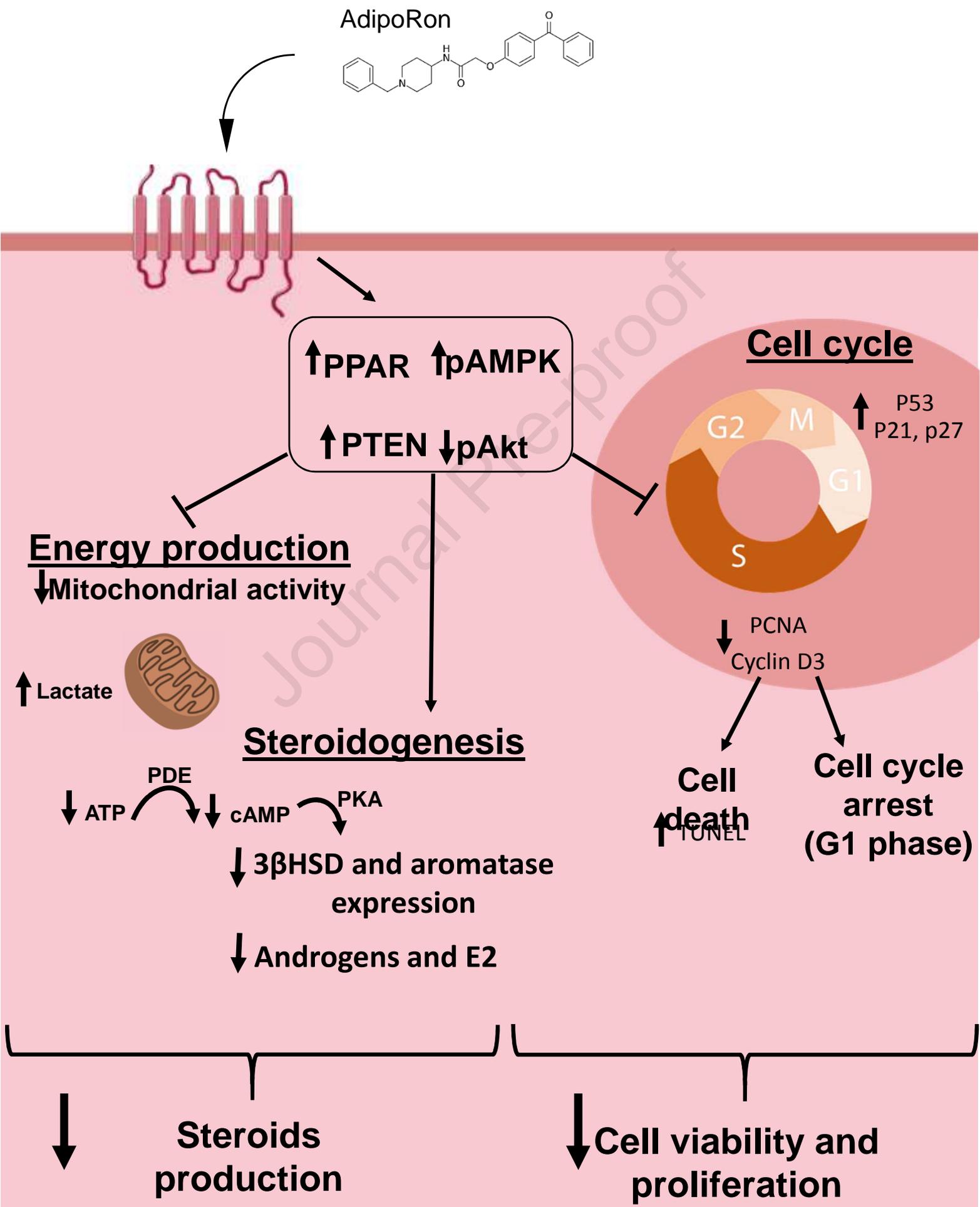


Figure 9





Highlights

The adiponectin pathway is downregulated in infertile women with polycystic ovary syndrome raising the hypothesis of the use of a new pharmaceutical drug called AdipoRon could be of interest in the treatment of female infertility. However, none or little information are available about the action of AdipoRon in granulosa cells, which are the cells nursing the oocyte. In this study, we investigated the role of AdipoRon on human luteinized granulosa cells (cell line and primary cells), and performed different assay to evaluate its impact on viability, cell proliferation, metabolism and in particular on steroidogenesis, which is an important role of granulosa cells to control fertility.

Journal Pre-proof