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Novel molecular mechanisms involved in hormonal regulation of lactate production in Sertoli cells.

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Short title: FSH and bFGF regulate *Pfkfbs* and *Pdks* expression

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ABSTRACT

The aim of the study was to analyze molecular mechanisms involved in follicle-stimulating hormone (FSH) and basic Fibroblast Growth Factor (bFGF) regulation of lactate production in rat Sertoli cells. The regulation of pyruvate availability, which is converted to lactate, could be a mechanism utilized by hormones to ensure lactate supply to germ cells. On one hand, the regulation of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) expression could result in increased glycolysis, while an increase in pyruvate availability may also result from a lower conversion to acetyl-CoA by negative regulation of pyruvate dehydrogenase complex (PDC) activity by phosphorylation. Sertoli cells cultures obtained from 20-day-old rats were used. Stimulation of the cultures with FSH or bFGF showed that FSH increases *Pfkfb1* and *Pfkfb3* expression while bFGF increases *Pfkfb1* mRNA levels. Additionally, we observed that FSH-stimulated lactate production was inhibited in the presence of a PFKFB3 inhibitor, revealing the physiological relevance of this mechanism. As for the regulation of PDC, analysis of pyruvate dehydrogenase kinase (*Pdk*) expression showed that FSH increases *Pdk3* and decreases *Pdk4* mRNA levels while bFGF increases the expression of all *Pdks.* In addition, we showed that bFGF increases phosphorylated PDC levels and that bFGF-stimulated lactate production is partially inhibited in the presence of a PDK inhibitor. Altogether, these results add new information regarding novel molecular mechanisms involved in hormonal regulation of lactate production in Sertoli cells. Considering that lactate is essential for the production of energy in spermatocytes and spermatids, these mechanisms might be relevant in maintaining spermatogenesis and male fertility.

INTRODUCTION

Sertoli cells are essential for the normal development of spermatogenesis, with follicle-stimulating hormone (FSH) playing a pivotal role in the regulation of its function. Sertoli cells are also under autocrine and paracrine control, which result from multiple and complex interactions between the different testicular cells (Gnessi *et al*., 1997). Among the peptides involved in paracrine control, basic fibroblast growth factor (bFGF), which is mainly produced by germ cells, has been observed to regulate many Sertoli cell functions. It has been shown that in these cells bFGF modulates transferrin release (Han *et al.,* 1993), plasminogen activator inhibitor-1, *cFos* and *Jun B* expression (Smith *et al.,* 1989; Le Magueresse-Battistoni *et al*., 1998) and estradiol, glutathione and lactate production (Schteingart *et al.,* 1999; Riera *et al*., 2002; Gualtieri *et al.,* 2009).

Carbohydrate metabolism in the testis presents some unique characteristics. Sertoli cells actively metabolize glucose but the majority of it is converted to lactate (Robinson & Fritz 1981; Grootegoed *et al*., 1986). On the other hand, spermatocytes and spermatids are unable to use glucose for their energetic metabolism and prefer lactate as an energy source (Jutte *et al*., 1981; Mita & Hall 1982). These observations have led to the conclusion that one of the most important Sertoli cell nurse functions is to provide lactate for the production of energy in germ cells (Boussouar & Benahmed 2004). In this context, the mechanisms that regulate lactate production in Sertoli cells are relevant to the maintenance of spermatogenesis and male fertility.

It has been previously demonstrated that FSH and bFGF increase lactate production in Sertoli cells and that several molecular mechanisms are involved (Mita *et al.* 1982; Riera *et al*., 2001, 2002; Meroni *et al*., 2002; Galardo *et al*., 2008). Among the mechanisms which may contribute to increased lactate production by FSH and bFGF, the regulation of pyruvate

availability has not yet been analyzed. Such an increase in pyruvate availability may result from an augmentation in the glycolytic flux and/or from a lower conversion to acetyl-CoA, caused by the negative regulation of pyruvate dehydrogenase complex (PDC) activity.

6-phosphofructo-1-kinase (PFK1) is the enzyme that catalyzes the major regulatory step in the glycolytic pathway. Several allosteric sites are present in PFK1, which turn on and off the enzymatic activity. Among the allosteric regulators, fructose 2,6-biphosphate (Fru-2,6-P2) is the most potent activator of PFK1 and undoubtedly increases glycolytic flux (Hue & Rider 1987). The levels of Fru-2,6-P2 are regulated by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB), which catalyzes both synthesis and degradation of Fru-2,6-P2. In mammals, the isoforms of PFKFB are encoded by four separate genes; *Pfkfb1-4*, which are characterized by their tissue expression pattern and by the particular ratio of kinase to phosphatase activity (Rider *et al*., 2004). It has been known for a long time that phosphorylation plays a pivotal role in the regulation of PFKFB activity (Hue & Rider 1987). More recently, it has been demonstrated that up-regulation of the expression of different isoforms of PFKFBs results in an increase of glycolytic flux in different cell types (Marsin *et al*., 2002; Moon *et al*., 2011; Novellasdemunt *et al.*, 2012). In the testis, it has been observed that PFKFB3 is expressed in Sertoli cells and that PFKFB4 is present in germ cells (Gómez *et al*., 2009). The expression of others PFKFBs and the possible hormonal regulation of their expression have not yet been studied in Sertoli cells.

As previously mentioned, another mechanism contributing to increased pyruvate availability, which in turn leads to increased lactate production, is a decreased conversion of the ketoacid into acetyl-CoA. The PDC is responsible for the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA. As a matter of fact, PDC links glycolysis with the tricarboxylic acid cycle and like many other rate-limiting enzymes, it is tightly regulated. A

reversible phosphorylation / dephosphorylation cycle is the mechanism responsible for the regulation of PDC activity (Holness & Sugden 2003). Phosphorylation of PDC by pyruvate dehydrogenase kinase (PDK) causes inactivation of the complex. Such inhibition can only be reversed by pyruvate dehydrogenase phosphatase (PDP) that removes phosphate from PDC. In this context, the relative activities of PDKs and PDPs will determine the proportion of PDC in its active form and consequently the levels of pyruvate committed to the tricarboxylic acid cycle (Kolobova *et al*., 2001; Sugden & Holness 2006). To date, four isoforms of PDK (PDK1–4) and two isoforms of PDP (PDP1–2) have been identified. These isoforms display unique tissue distribution and varied kinetic and regulatory properties (Bowker-Kinley *et al*. 1998; Huang *et al*., 1998). We have recently observed that different isoforms of PDKs and PDPs are present in Sertoli cells (Regueira *et al*., 2014) but their possible hormonal regulation, particularly by FSH and bFGF, has not yet been analyzed.

The aim of the present study was to investigate whether FSH and bFGF can regulate the expression of *Pfkfbs* and/or the levels of phosphorylated PDC by means of *Pdks* and *Pdps* expression. A role for these molecular mechanisms in lactate production in Sertoli cells in order to ensure nutrient supply for germ cell development is postulated.

MATERIALS AND METHODS

Twenty-day-old Sprague–Dawley rats were obtained from an animal care unit (Animal Care Laboratory, Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina). Animals were killed by CO_2 asphyxiation according to protocols for animal laboratory use following the principles and procedures outlined in the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Ethical Commitee from the Instituto de Biología y Medicina Experimental (Ref.: CE 011/2015, IByME).

Human recombinant bFGF was purchased from Invitrogen (Life Technologies Argentina, Buenos Aires, Argentina). Ovine FSH (NIH-oFSH-S-16) was obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD. PFKFB3 inhibitor, 3PO, was purchased from Calbiochem (EMD Millipore Corporation, Chicago, IL, USA). Tissue culture media, dichloroacetate (DCA) and all other drugs and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Sertoli cell isolation and culture

Sertoli cells from 20-day-old Sprague-Dawley rats were isolated as previously described (Meroni *et al*., 2002). Briefly, decapsulated testes were digested with 0.1% w/v collagenase and 0.006% w/v soybean trypsin inhibitor in Hanks' balanced salt solution for 5 min at room temperature. Seminiferous tubules were saved, cut and submitted to 1 M glycine-2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 min at room temperature to remove germinal cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium,

supplemented with 20 mM HEPES, 100 IU/ml penicillin, 2.5 μg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 μg/ml transferrin, 5μg/ml insulin, 5 μg/ml vitamin E and 4 ng/ml hydrocortisone. Sertoli cells were cultured in 6-, 24- or 96-multiwell plates (5 μg DNA/cm^2) at 34ºC in a mixture of 5% CO_2:95% air.

No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to Sertoli cell cultures using a specific antiserum to smooth muscle α actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5% after 48 h in culture as examined by phase contrast microscopy.

Culture conditions

Sertoli cells were allowed to attach for 48 hrs in the presence of insulin and medium was replaced at this time with fresh medium without insulin. Cells incubated for 24- or 48-hrs with FSH (100ng/ml) or bFGF (30ng/ml) were used to evaluate *Pfkfbs*, *Pdks* and *Pdps* mRNA levels and to determine phosphorylated PDC (P-PDC) protein levels. To evaluate the role of PFKFB3, cells were incubated in the absence or presence of variable doses of 3PO for 48 hrs. The conditioned media were used to evaluate lactate production. To evaluate the role of PDKs, cells were incubated in the absence or presence of variable doses of dichloroacetate (DCA) for 48 hrs. The cells and the conditioned media were used to evaluate P-PDC levels and lactate production respectively.

Reverse Transcription-PCR (RT-PCR)

Testicular tissue and purified Sertoli were utilized to isolate total RNA using TRI Reagent (Sigma-Aldrich) according to the manufacturer's recommendations. The amount of

158 RNA was estimated by spectrophotometry at 260 nm. Reverse transcription (RT) was
159 performed on 2 µg RNA at 42°C for 50 min with a mixture containing 200U MMLV reverse
160 transcriptase enzyme, 125 ng random primers and 0.5 mM dNTP Mix (Invitrogen). The
161 cDNAs enconding *Pfkfb1-3*, *Pdk1-4* and *Pdp1-2* were amplified from 1 µl of the cDNA
162 reaction mixture using specific gene primers (Table 1). PCR was performed with GoTaq DNA
163 polymerase (Promega Corporation, Madison, USA) under the following conditions: initial
164 denaturation at 94ºC for 5 minutes; 35 cycles of 94ºC for 30 seconds; 60 ºC for 30 seconds and
165 extension at 72ºC for 50 seconds followed by 10 minutes at 72ºC. The PCR products were
166 resolved by 2% w/v agarose gel and stained with ethidium bromide.
167
168 **Real-time PCR (RT-qPCR)**
169 Total RNA was isolated from Sertoli cells cultured in 6-multiwell plates with TRI Reagent
170 (Sigma-Aldrich) according to the manufacturer's recommendations. The amount of RNA was
171 estimated by spectrophotometry at 260 nm. Reverse transcription (RT) was performed as
172 mentioned in RT-PCR. Real-time PCR was performed by a StepOnePlusTM Real-Time PCR
173 System (Applied Biosystems, Warrington, UK). Amplification was carried out as
174 recommended by the manufacturer: 25 µl reaction mixture containing 12.5 µl of SYBR Green
175 PCR Master mix (Applied Biosystems), the appropriate primer concentration and 1 µl of
176 cDNA. Table1 shows the specific primers used to analyze *Pfkfb1-3*, *Pdk1-4*, *Pdp1-2* and
177 hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) expression. The relative cDNA
178 concentrations were established by a standard curve using sequential dilutions of a cDNA
179 sample. *Hprt1* was used as reference gene. The amplification program included the initial
180 denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 seg, and
181 annealing and extension at 60°C for 1 min. Fluorescence was measured at the end of each

extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative ΔΔCt method was used to calculate relative gene expression.

Western blot analysis

Cells cultured in 6-multiwell plates were washed once with PBS at room temperature. Then, 200 μl of PBS containing 2 μl of protease inhibitor cocktail (P-8340; Sigma-Aldrich), 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 50nM okadaic acid and 2 mM PMSF was added to each well. Cells collected by scrapping were then placed on ice and disrupted by ultrasonic irradiation. For Western blot analysis, 200 μl of 2X Laemmli buffer (4% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, 0.004% w/v bromophenol blue, and 0.125 M Tris-HCl, pH 6.8) was added and thoroughly mixed. Samples were immersed in boiling water bath for 5 min and then immediately settled on ice. Proteins (40 μg in each lane) were resolved in 10% SDS-PAGE (10% acrylamide/bisacrylamide for the resolving gel and 4.3% acrylamide/bisacrylamide for the stacking gel) in a Mini Protean 3 cell (Bio-Rad, Hercules, CA, USA). After SDS-PAGE, gels were electrotrasferred at 100 V for 60 min onto PVDF membranes (Hybond-P, GE Healthcare Life Sciences, Piscataway, NJ, USA) using a Mini Trans-blot cell (Bio-Rad). Membranes were probed with specific antibodies that recognized the phosphorylated form of pyruvate dehydrogenase complex (P-PDC) (Pyruvate dehydrogenase E1-α subunit (P-Ser293) antibody, Novus Biologicals, Littleton, CO, USA) or total AKT (T-AKT) (AKT antibody, Cell Signaling Technology, Inc., Danvers, MA, USA). A 1:4000 (P-PDC) or 1:1000 (T-AKT) dilutions of primary antibodies were used. Levels of T-AKT were used as loading control. For chemiluminescent detection of the blots a commercial

kit was used (Cell Signaling Technology). The intensities of the autoradiographic bands were estimates by densitometry scanning using NIH Image Software (Scion Corporation, Frederick, MD, USA).

Lactate determination

Conditioned media obtain from cells cultured in 24-multiwell plates were used to determine lactate production. Lactate was measured by a standard method involving conversion of NAD^+ to NADH. The amount of NADH was determined as the rate of increase of absorbance at 340 nm. A commercial kit from Sigma-Aldrich was used.

Other assays

A cell viability test was performed in cells cultured on 96-multiwell using a commercial kit (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega Corporation). DNA was determined as described previously (Riera *et al*., 2002). Protein content was determined by Lowry's assay.

Statistical analysis

All experiments were run in triplicates and repeated three to four times. Results are expressed as means±S.D. One way ANOVA and post hoc analysis using Tukey-Kramer's multiple comparisons test were performed using GraphPad InSat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). P values < 0.05 were considered statistically significant.

RESULTS

Regulation of Pfkfb1-Pfkfb3 mRNA levels by FSH and bFGF

RT-PCR analysis was used to evaluate the expression of three isoforms of PFKFBs, *Pfkfb1-Pfkfb3,* in rat Sertoli cells. Figure 1A showed that Sertoli cells express *Pfkfb1*, *Pfkfb2* and *Pfkfb3*. To evaluate hormonal regulation of their expression, Sertoli cell cultures were stimulated for 24- or 48-hrs with FSH (100 ng/ml) or bFGF (30 ng/ml), doses that have been shown to promote a maximal response in lactate production (Riera *et al*., 2001; 2002). Figure 1B shows that FSH increased *Pfkfb1* and *Pfkfb3* and did not modify *Pfkfb2* mRNA levels. Figure 2 shows that bFGF increased *Pfkfb1* mRNA levels and did not modify *Pfkfb2* or *Pfkfb3* expression.

Participation of PFKFB3 in FSH-stimulated lactate production

To analyze a possible role of PFKFB3 activity in the stimulatory effect of FSH on lactate production, cells were incubated with FSH (100 ng/ml) for 48 hrs in the absence or presence of 3PO, a specific PFKFB3 inhibitor. Figure 3 shows that 3PO decreased FSH-stimulated lactate production while this inhibitor did not modify lactate secretion under basal experimental conditions. A cell viability test performed at the end of the 48-hrs incubation period showed that 3PO had no effect on cell viability (Table 2).

Regulation of Pdks and Pdps mRNA levels by FSH and bFGF

RT-PCR analysis was used to evaluate the expression of *Pdks* (*Pdk1-Pdk4*) and *Pdps* (*Pdp1-Pdp2*) in Sertoli cells. Figure 4A shows that Sertoli cells express all isoforms of *Pdks* and *Pdps*. We then evaluated if FSH and bFGF were able to regulate their expression. With that in mind, Sertoli cell cultures were stimulated for 24- or 48-hrs with FSH (100 ng/ml) or

bFGF (30 ng/ml). Figure 4B shows that FSH increased *Pdk3*, decreased *Pdk4* and did not modify *Pdk1* and *Pdk2* mRNA levels. Additionally, Figure 4C shows that FSH did not modify *Pdp1* or *Pdp2* mRNA levels. On the other hand, Figures 5A and 5B respectively show that bFGF increased mRNA levels of all *Pdks* isoforms and did not modify *Pdp1* or *Pdp2* mRNA levels.

Regulation of P-PDC levels by FSH and bFGF

Considering that FSH and bFGF induced a differential regulation of *Pdks* expression, we decided to analyze a possible effect of these hormones on the levels of phosphorylated PDC. For this purpose, Sertoli cell cultures were stimulated for 24- or 48-hrs with FSH (100 ng/ml) or bFGF (30 ng/ml) and the levels of P-PDC were analyzed by Western blot. Figure 6 shows that bFGF produced an increase in P-PDC levels and that FSH did not modify them.

Participation of PDKs in bFGF-stimulated lactate production

The observed concomitant increase in *Pdks* expression and P-PDC levels promoted by bFGF led us to examine the possible role of PDK activity in the increase in lactate production elicited by this hormone. To achieve this goal, cells were incubated with bFGF (30 ng/ml) for 48 hrs in the absence or presence of the PDK inhibitor, DCA. Figure 7 shows that DCA (10 mM) partially decreased bFGF stimulation of lactate production. DCA did not modify lactate secretion under basal experimental conditions. A cell viability test performed at the end of the 48-hrs incubation period showed that DCA had no effect on cell viability (Table 2).

DISCUSION

The process of spermatogenesis and consequently male fertility are dependent upon the somatic cells that are present in the testis. Although, Leydig cells are essential because of androgen production, Sertoli cells are absolutely necessary in order to provide an adequate and protected environment within the seminiferous tubules. Germ cells situated beyond the blood testis barrier need to rely on Sertoli cell production of factors that fuel their metabolism. In this respect, it has been shown that lactate is the final product of glycolysis in Sertoli cells and that this metabolite is used by germ cells as an energy substrate. In addition to its energetic function, Erkkila *et al.* (2002) have shown that lactate inhibits male germ cell apoptosis in human testis and they have proposed that this metabolite may be regarded as a potential compound for optimizing *in vitro* methods involving male germ cells for assisted reproduction. Furthermore, it has been observed that lactate regulates the expression of genes involved in its own metabolism in cultured rat germ cells (Galardo *et al.,* 2014). The importance of lactate for normal spermatogenesis was highlighted in a report showing that spermatogenesis in adult cryptorchid testis is improved by intratesticular infusion of lactate (Courtens & Ploen 1999). Altogether, these data suggest that the provision of adequate levels of lactate is a key Sertoli cell function regarding germ cell development.

As mentioned in the introduction, it has been previously shown that FSH and bFGF increase lactate production by regulating several molecular mechanisms in Sertoli cells; among them, glucose transport mediated by the glucose transporter 1, lactate dehydrogenase (LDH) activity and subunit A of LDH (*LdhA)* expression (Riera *et al*., 2001; 2002; Meroni *et al*., 2002; Galardo *et al*., 2008; 2010). In the present study we intended to unravel additional molecular mechanisms that may be involved in the hormonal regulation of Sertoli cell lactate production.

297 It has been known for quite a long time that cancer cells present a characteristic
298 metabolism. In this context, Warburg's studies (Warburg *et al*., 1927) have shown that glucose
299 metabolism results in a high lactate accumulation, despite adequate oxygen availability. This
300 metabolic pattern, known as the "Warburg effect", is commonly associated with malignant
301 transformation and is characterized by high glycolytic rates associated with reduced
302 mitochondrial oxidation. While looking for mechanisms that may be participating in the
303 Warburg effect, stimulation of glycolysis and inhibition of the conversion of pyruvate to
304 acetyl-CoA mediated by PDC inactivation have been demonstrated (Chesney 2006; Lu *et al*.,
305 2008; McFate *et al*., 2008; Newington *et al*., 2011). Even though Sertoli cells do not
306 proliferate after puberty, these cells in the testis present similar metabolic characteristics to
307 those observed in cancer cells. Consequently, Sertoli cells have been recently proposed as a
308 useful model to revisit the "Warburg effect" (Oliveira *et al*., 2015).

309 It has been previously shown that several molecular mechanisms participate in the
310 hormonal regulation of lactate production; however, no experimental evidence for the
311 hormonal regulation of glycolytic flux and/ or PDC activity in Sertoli cells has been obtained
312 so far. It is also well known that glycolysis is regulated by slowing down or speeding up
313 certain steps in the pathway. PFK1 catalyzes the tightly controlled rate-limiting step in
314 glycolysis. The bifunctional enzyme PFKFB catalyzes the production of Fru-2,6-P2, a potent
315 allosteric activator of PFK1. In the present study, we evaluated *Pfkfbs* isoform expression and
316 their possible hormonal regulation in Sertoli cells. Our results show that Sertoli cells express
317 *Pfkfb1*, *Pfkfb*2 and *Pfkfb*3 isoforms. Additionally, FSH increases *Pfkfb1* and *Pfkfb3* mRNA
318 levels and bFGF increases the expression of *Pfkfb1*. The relative kinase to phosphatase
319 activities vary among the different PFKFB isoenzymes (Rider *et al.,* 2004). In this context, it

has been shown that PFKFB1 has similar kinase and phosphatase activities while PFKFB3 has a 100-fold higher kinase than phosphatase activity, indicating that this isoform mainly acts as a kinase that serves to maintain elevated Fru-2,6-P2 levels (Sakakibara *et al*., 1997; Ros & Schulze 2013). In this respect, it has been demonstrated that up-regulation of *Pfkfb3* expression is accompanied by increments in glycolytic flux and also in lactate production in different cell types (Marsin *et al*., 2002; Atsumi *et al*., 2005; Ando *et al*., 2010). The physiological relevance of *Pfkfb*3 regulation by FSH was revealed by the observation that 3PO, a specific PFKFB3 inhibitor, inhibits FSH-stimulated lactate production.

As mentioned in the introduction, PDKs, which phosphorylate and inhibit PDC, and PDPs, which dephosphorylate and activate the complex, participate in the regulation of PDC activity. Our results show that Sertoli cells express all isoforms of *Pdks* (*Pdk1-Pdk4*) and *Pdps* (*Pdp1-2*). It has been postulated that a distinct tissue-specific expression of PDKs and PDPs isoforms evolved to satisfy tissue-specific metabolic requirements (Gudi *et al*., 1995; Rowles *et al*., 1996; Bowker-Kinley *et al*., 1998). PDC complex has three specific serine residues [Ser-293 (site1); Ser300 (site2); Ser232 (site 3)] that can be phosphorylated. All four PDKs phosphorylate site 1 and site 2 while only PDK1 phosphorylates site 3. It is worthy to note that the phosphorylation of a single site results in complete inactivation of PDC (Korotchkina & Patel 2001; Randin *et al*., 2009). On the other hand, PDP1 and PDP2 can indistinctly dephosphorylate all sites and, as expected, it has been observed that the reactivation of PDC is slower when the three serine residues are phosphorylated (Korotchkina & Patel 1995; Karpova *et al*., 2003). In this context, it has been postulated that cells expressing all isoforms of PDKs, which potentially phosphorylate all sites of PDC, can maintain PDC in an inactive form for prolonged periods of time (Korotchkina & Patel 2001). Supporting this hypothesis, it has been

observed that PDC is less active in astrocytes, which express all isoforms of PDKs, than in neurons, which only express PDK2 and PDK4 (Halim *et al*., 2010). Considering that Sertoli cells express all isoforms of PDKs, it is tempting to speculate that these cells maintain PDC in an inactive form thus inhibiting the entrance of pyruvate into the tricarboxylic acid cycle. This latter hypothesis gains further support with the pioneering studies on the metabolism of glucose in Sertoli cells. These studies show that Sertoli cells actively metabolize glucose but that rather a small proportion of this sugar is oxidized via the tricarboxylic acid cycle (Robinson & Fritz 1981; Grootegoed *et al*., 1986).

The ability of nutritional states and hormones to regulate PDC activity through the regulation of *Pdks* and/or *Pdps* expression has been observed in various cell types. In this respect, it has been observed that *Pdk2* and *Pdk4* expression is up-regulated and PDC phosphorylated and inactivated in liver during fasting in order to conserve pyruvate for gluconeogenesis (Wu *et al*., 2000; Jeong *et al*., 2012). Additionally, it has been observed in different cell types that insulin, secreted in a well-fed state, decreases mRNA levels of *Pdk2* and *Pdk4*, and increases mRNA levels of *Pdp1* and *Pdp2* in order to decrease PDC phosphorylation and consequently direct pyruvate into the tricarboxylic acid cycle (Huang *et al*., 2002; Abbot *et al*., 2005; Wang *et al*., 2009). Our results show that FSH increases *Pdk3* and decreases *Pdk4* expression and bFGF increases the expression of all *Pdks*. These results indicate that hormones can differentially regulate the expression of *Pdks* isoforms in Sertoli cells. Additionally, the present study also shows that bFGF, but not FSH, increases the levels of P-PDC in Sertoli cells suggesting that bFGF promotes PDC inactivation as part of the mechanisms participating in the regulation of lactate production. The relevance of PDKs in the regulation by bFGF of lactate production in Sertoli cells is highlighted by the observation that bFGF-stimulated lactate production is diminished in the presence of a PDK inhibitor. It is

367 worth mentioning that in Sertoli cells bFGF increases both *LdhA* mRNA and LDH isoenzyme
368 containing four A subunits (LDH5) levels (Riera *et al*., 2002). This increase in LDH5, which
369 can convert high amounts of pyruvate to lactate, in conjunction with the above-mentioned
370 regulation of P-PDC levels probably converge to increase lactate production in response to
371 bFGF in Sertoli cells.

372 As mention before, spermatogenesis is an intricate process highly dependent on Sertoli
373 cell function, which is under endocrine (FSH and testosterone) as well as autocrine and
374 paracrine control (Parvinen 1982). It has been observed that several Sertoli cell functions vary
375 with the stage of the spermatogenic cycle (Johnston *et al*., 2008). It is known that, minimal
376 FSH binding and FSH-stimulated cAMP production occurs at stages VI to VII (Kangasniemi
377 *et al.,* 1990). As for bFGF, it has been demonstrated that this peptide is localized
378 predominantly in pachytene spermatocytes and its expression becomes more prominent in
379 stage VII through IX of the cycle (Mayerhofer *et al.,* 1991). Moreover, the expression of
380 bFGF receptors in Sertoli cells was more pronounced in stages I-VIII (Cancilla & Risbridger
381 1998). Stage-specific lactate secretion may not be expected as this metabolic product has been
382 shown to be important for the survival of both spermatocytes and spermatids that are present
383 along all stages of the cycle. Based on the above-mentioned observations it is tempting to
384 speculate that the coordinated actions of FSH and bFGF throughout the spermatogenic cycle
385 may ensure the provision of adequate lactate levels to maintain the energy requirements of
386 developing germ cells.

387 In conclusion, results presented herein, which are summarized in Figure 8, add new
388 information regarding molecular mechanisms involved in lactate production in Sertoli cells
389 showing the regulation by hormones of pyruvate availability. The results reinforce the idea
390 that the modulation of metabolic pathways in Sertoli cells is controlled by multiple

components including the action of hormones, the metabolic substrate availability and other endogenous or exogenous factors which will contribute all together to the progression of spermatogenesis to ensure male fertility.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Fig.1 Effect of FSH on *Pfkfbs* mRNA levels in Sertoli cells. (A) Characterization of *Pfkfb1-3* expression in Sertoli cells was performed. Total RNA of rat testis (T) or Sertoli cells (SC) were extracted, analyzed by RT-PCR and visualized by ethidium bromide staining. NT indicates no template control. B) Sertoli cells were incubated for variable periods of time (24 and 48 hrs) with 100 ng/ml FSH. Total cellular RNA was then extracted and RT-qPCRs for *Pfkfb1-3* were performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as mean ±S.D. of four independent experiments. **p<0.01; *p<0.05 versus Basal.

Fig.2 Effect of bFGF on *Pfkfbs* mRNA levels in Sertoli cells. Sertoli cells were incubated for variable periods of time (24 and 48 hrs) with 30 ng/ml bFGF. Total cellular RNA was then extracted and RT-qPCRs for *Pfkfb1-3* were performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as mean±S.D. of four independent experiments. **p<0.01; *p<0.05 versus Basal.

Fig.3 Effect of 3PO on FSH-stimulated lactate production. Sertoli cells were maintained under basal conditions or incubated for 48 hrs with 100 ng/ml FSH in the absence or presence of 3PO (10 μM or 30 μM), a PFKFB3 inhibitor. Lactate was determined in the 48-hrs conditioned media. Results are expressed as mean±S.D. of triplicate incubations in one representative experiment out of three (***p<0.001 vs Basal; #p<0.001 vs FSH).

Fig.4 Effect of FSH on *Pdks* and *Pdps* mRNA levels in Sertoli cells. (A) Characterization of *Pdk1-4* and *Pdp1-2* expression in Sertoli cells was performed. Total RNA of rat testis (T) or

Sertoli cells (SC) were extracted, analyzed by RT-PCR and visualized by ethidium bromide staining. NT indicates no template control. (B and C) Sertoli cells were incubated for variable periods of time (24 and 48 hrs) with 100 ng/ml FSH. Total cellular RNA was then extracted and RT-qPCRs were performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results, *Pdk1-Pdk4* (B) and *Pdp1* and *Pdp2* (C), are expressed as mean±S.D. of four independent experiments. **p<0.01; *p<0.05 versus Basal.

Fig.5 Effect of bFGF on *Pdks* and *Pdps* mRNA levels in Sertoli cells. Sertoli cells were incubated for variable periods of time (24 and 48 hrs) with 30 ng/ml bFGF. Total cellular RNA was then extracted and RT-qPCRs for *Pdk1-Pdk4* (A) and for *Pdp1* and *Pdp2* (B) were performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as mean±S.D. of four independent experiments. *p<0.05 versus Basal.

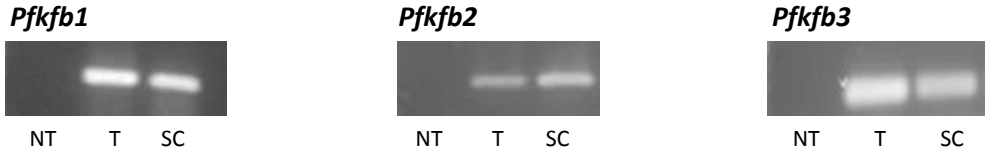
Fig.6 Effects of FSH and bFGF on phosphorylated PDC levels in Sertoli cells. Sertoli cells were stimulated for variable periods (24 or 48 hrs) with 100 ng/ml FSH or 30ng/ml bFGF. Cell extracts were prepared at the designated intervals and utilized for Western blot analysis using antibodies specific for phosphorylated PDC (P-PDC) or total AKT (T-AKT). The upper panels show a representative experiment out of three. The lower panels show pooled data of the three independent experiments performed. Results are expressed as mean±S.D. of the ratio between P-PDC and T-AKT in each sample (*p<0.05 vs Basal)

Fig.7 Effect of DCA on bFGF-stimulated lactate production. Sertoli cells were maintained under basal conditions or incubated for 48 hrs with 30ng/ml bFGF in the absence or presence of DCA (1 mM or 10 mM), a PDK inhibitor. Lactate was determined in the 48-hrs conditioned

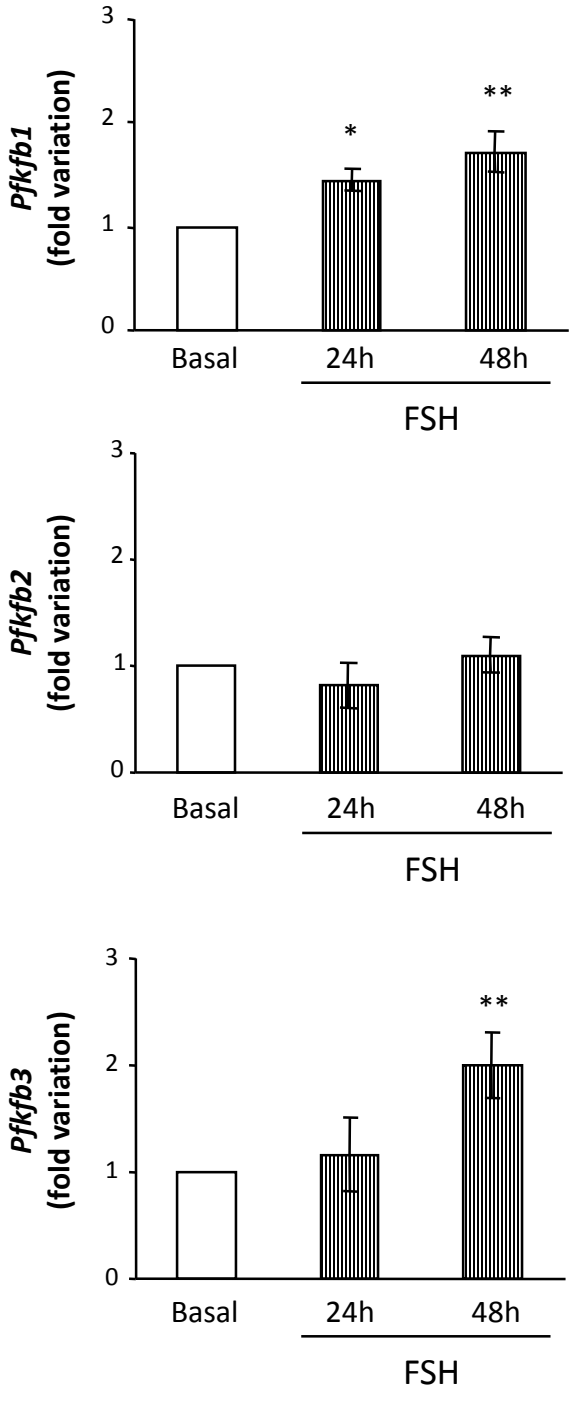
media. Results are expressed as mean±S.D. of triplicate incubations in one representative experiment out of three (*** $p<0.001$ vs Basal; #$p<0.01$ vs bFGF).

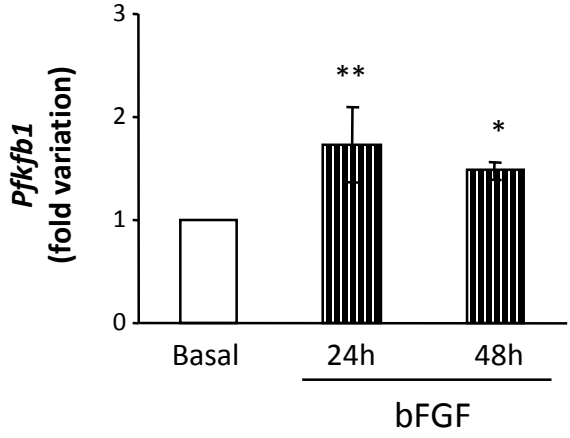
Fig.8 A schematic model of the mechanisms involved in FSH and bFGF-stimulated lactate production in Sertoli cells. See "Discussion" for details.

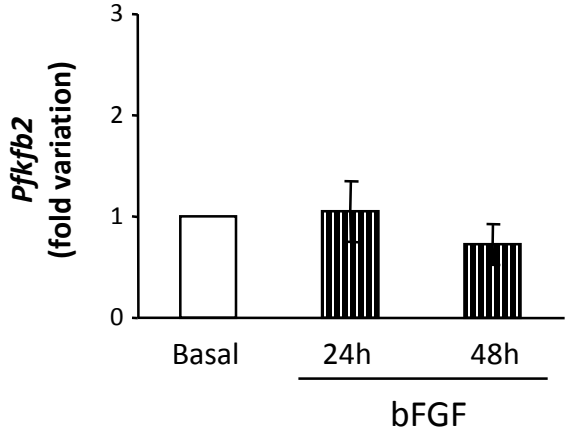
A

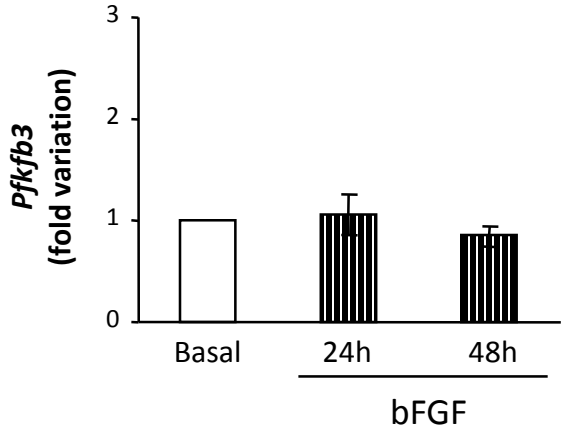

Pfkfb1
NT
T
SC
Pfkfb2
NT
T
SC
Pfkfb3
NT
T
SC

B


Pfkfb1 (fold variation)
3
2
1
0
*
**
Basal
24h
48h
FSH
Pfkfb2 (fold variation)
3
2
1
0
Basal
24h
48h
FSH
Pfkfb3 (fold variation)
3
2
1
0
**
Basal
24h
48h
FSH


Pfkfb1
(fold variation)
3
2
1
0
**
*
Basal
24h
48h
bFGF


Pfkfb2
(fold variation)
3
2
1
0
Basal
24h
48h
bFGF


Pfkfb3
(fold variation)
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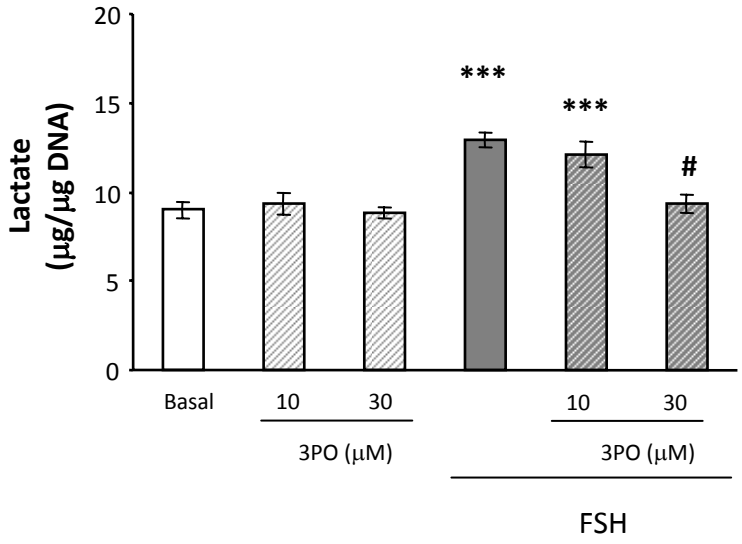
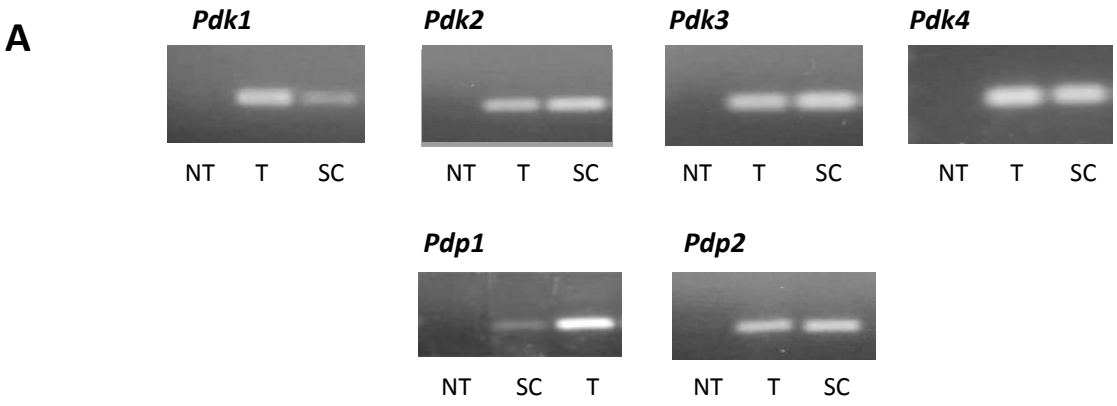
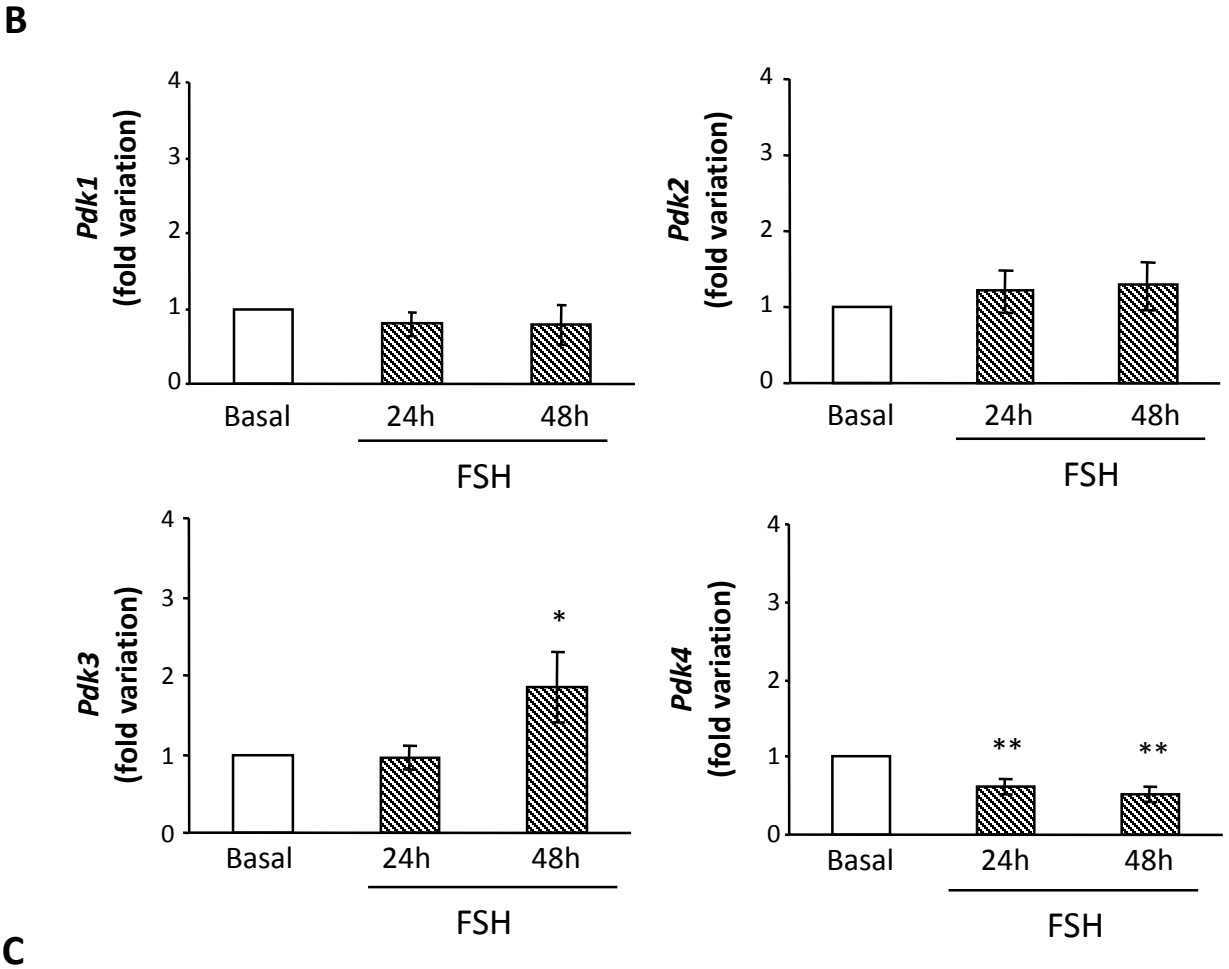
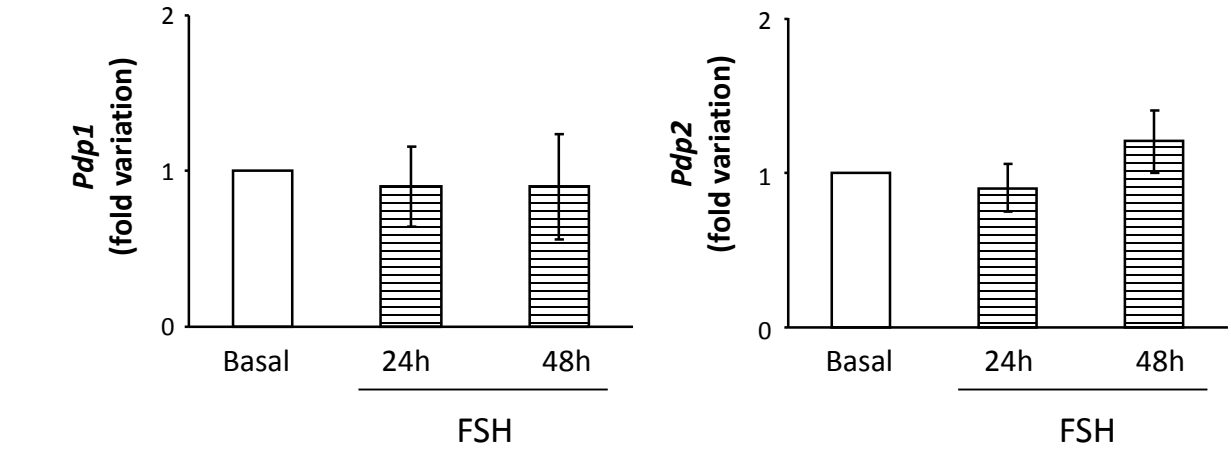
2
1
0
Basal
24h
48h
bFGF

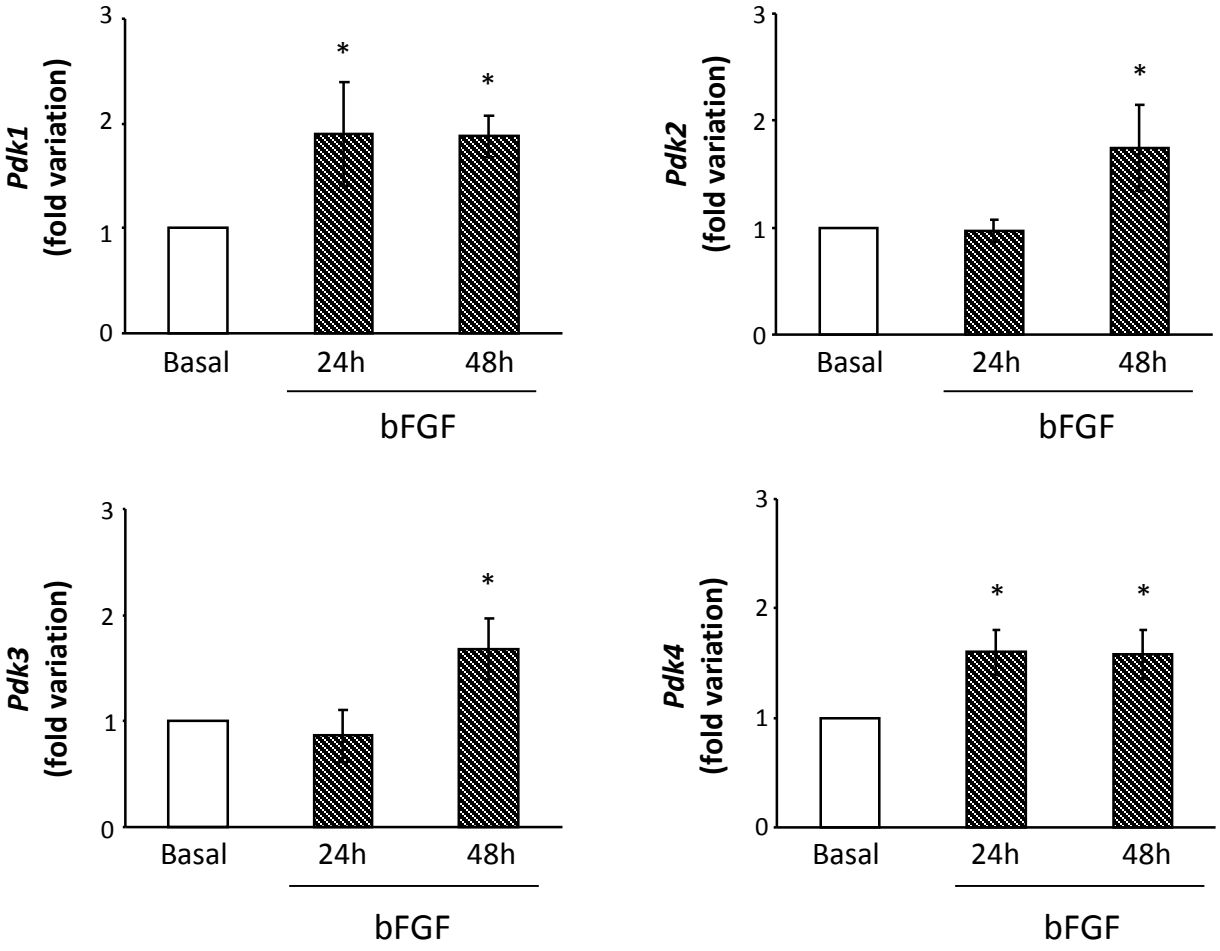

Lactate
(µg/µg DNA)
20
15
10
5
0
***
***
#
Basal
10
30
10
30
3PO (µM)
3PO (µM)
FSH


A
Pdk1
Pdk2
Pdk3
Pdk4
NT T SC
NT T SC
NT T SC
NT T SC
Pdp1
Pdp2
NT SC T
NT T SC

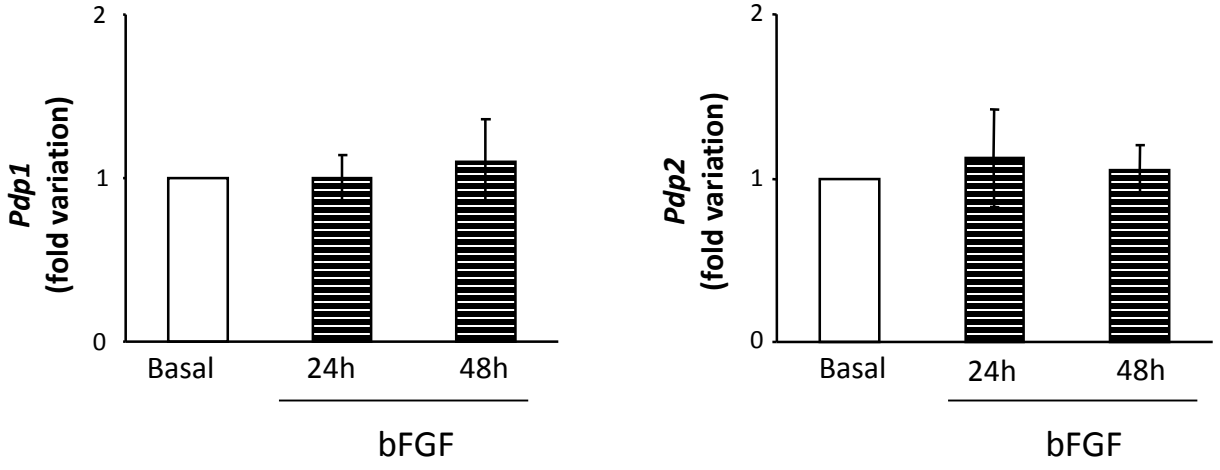

B
Pdk1 (fold variation)
Pdk2 (fold variation)
Pdk3 (fold variation)
Pdk4 (fold variation)
0
1
2
3
4
Basal
24h
48h
FSH
*
**
**

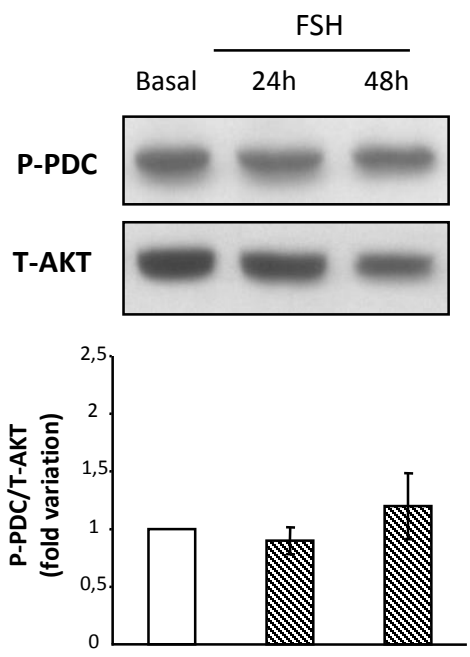

C
Pdp1 (fold variation)
Pdp2 (fold variation)
0
1
2
Basal
24h
48h
FSH

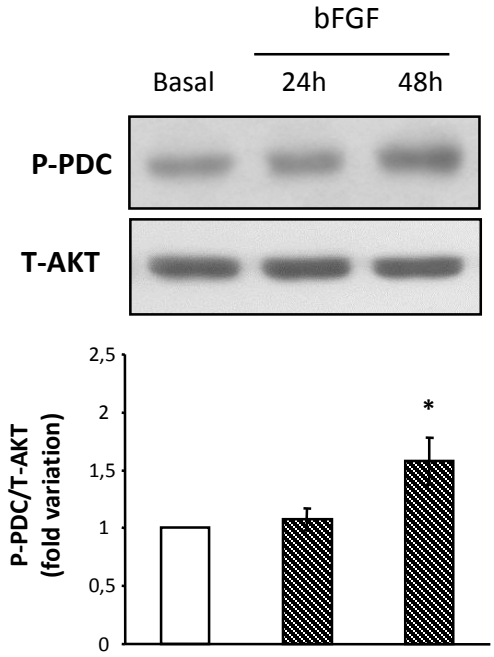
A


Pdk1
(fold variation)
3
2
1
0
*
*
Basal
24h
48h
bFGF
Pdk2
(fold variation)
*
Pdk3
(fold variation)
*
Pdk4
(fold variation)
*
*

B


Pdp1
(fold variation)
2
1
0
Basal
24h
48h
bFGF
Pdp2
(fold variation)


FSH
Basal
24h
48h
P-PDC
T-AKT
P-PDC/T-AKT
(fold variation)
2,5
2
1,5
1
0,5
0


bFGF
Basal
24h
48h
P-PDC
T-AKT
P-PDC/T-AKT
(fold variation)
2,5
2
1,5
1
0,5
0
*

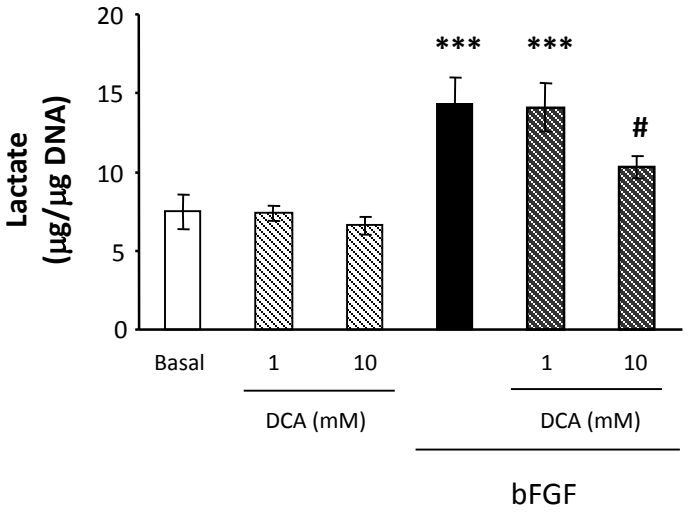

Lactate
(µg/µg DNA)
20
15
10
5
0
***
***
#
Basal
1
10
1
10
DCA (mM)
DCA (mM)
bFGF

Figure 8

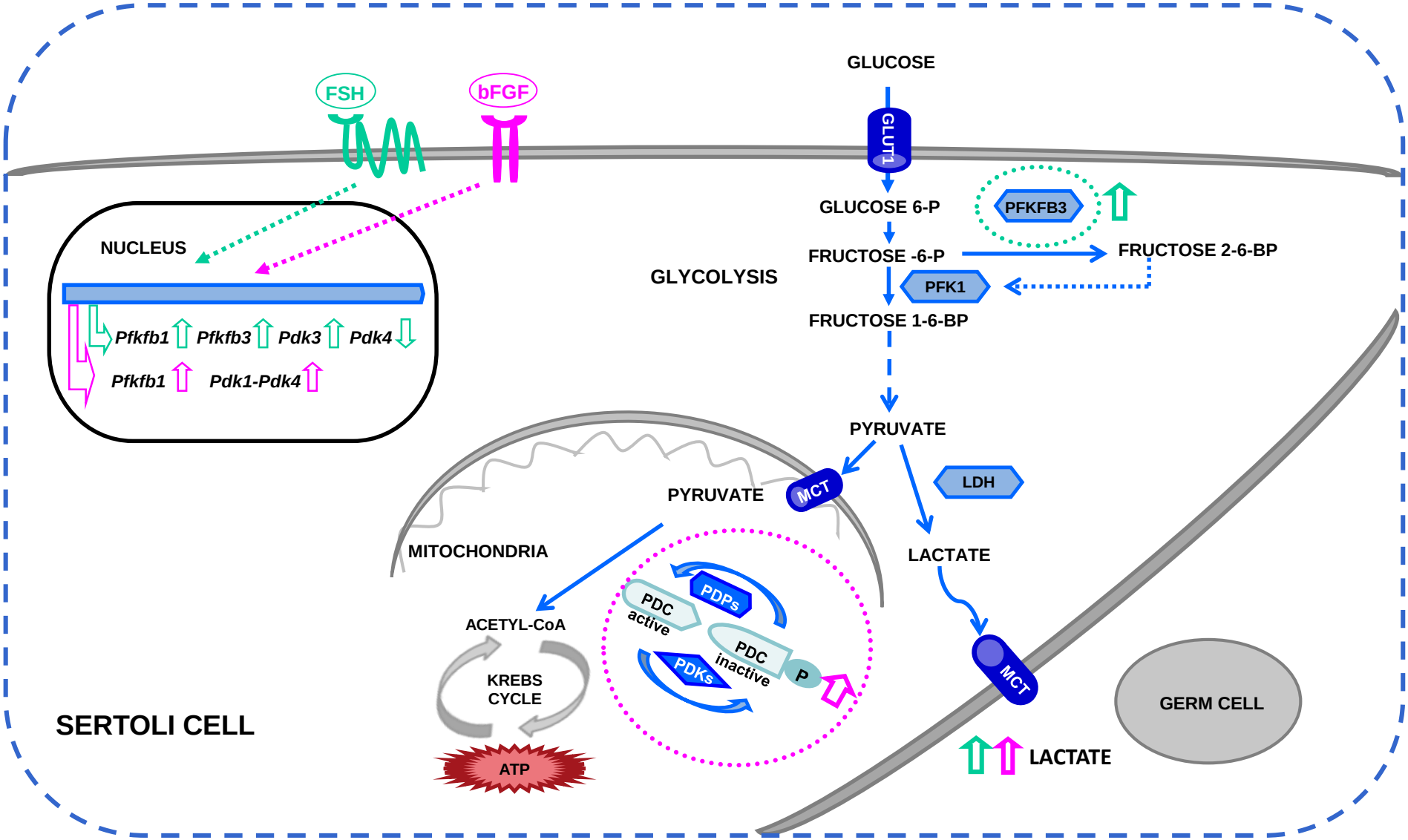

FSH
bFGF
GLUCOSE
GLUT1
GLUCOSE 6-P
PFKFB3
FRUCTOSE -6-P
FRUCTOSE 2-6-BP
GLYCOLYSIS
PFK1
FRUCTOSE 1-6-BP
NUCLEUS
Pfkfb1 Pfkfb3 Pdk3 Pdk4
Pfkfb1 Pdk1-Pdk4
PYRUVATE
MCT
LDH
PYRUVATE
MITOCHONDRIA
LACTATE
PDC active
PDPs
PDC inactive
P
PDKs
ACETYL-CoA
KREBS CYCLE
ATP
MCT
GERM CELL
LACTATE
SERTOLI CELL

Table 1. Rat-specific primers sets for RT-PCR analysis.

Gene	Primer sequence	Accession Number
Pdk1	FWD: 5′-TCCAGGGAGACCTAAAGCTG-3′ REV: 5′-CGTGGTTGGTTCTGTAATGC-3′	NM_053826.2
Pdk2	FWD: 5′-GACCCAGTCTCCAACCAGAAC-3′ REV: 5′-GGGATCAATGCTGCCAATGTG-3′	NM_030872.1
Pdk3	FWD: 5′-GTCGCCGCTCTCTATCAAAC-3′ REV: 5′-AGCCAGTCGCACAGGAAG-3′	NM_01106581.1
Pdk4	FWD: 5′-CGAAGATGCCTTTGAGTGTG-3′ REV: 5′-TGGTGAAGGTGTGAAGGAAC-3′	NM_053551.1
Pdp1	FWD: 5’-CAGGAGAATGTGTGTGTGTCC-3’ REV: 5’-TGGCATCAGAGAACAGTGGTAG-3’	NM_019372
Pdp2	FWD: 5′-AGAGGATTCGCCCAGTGTC-3′ REV: 5′-AAGTGGAGGTGGAGTGTTTTTC-3′	NM_145091.4
Pfkfb1	FWD: 5’-CCATTACTGAGCCCTTTTCAAG-3′ REV: 5’-TGCTACGGACTTCTTCACTGG-3′	NM_012621.4
Pfkfb2	FWD :5’-GTGGTGGCAGTGTTCAAGAG-3′ REV: 5’-TTCCCCGTCCGTCTTCTATC-3′	NM_01033964.1
Pfkfb3	FWD: 5’-CACCCTTCCTGTCCTTTGTTC-3′ REV: 5’-GCATCTTCGTTGCTGTATTCG-3′	NM_057135
Hprt1	FWD: 5’-AGTTCTTTGCTGACCTGCTG-3′ REV: 5′-TTTATGTCCCCCGTTGACTG-3′	NM_012583.2

Table 2: Effect of DCA on Sertoli cell viability.

Sertoli cells were incubated without (Basal), with 3PO (10 or 30 μM) or DCA (1 or 10mM) for 48 hrs. The cell viability assay was performed after this incubation period. Data are expressed as percentage of basal conditions and are presented as mean±SD of quadruplicate incubations in one representative experiment out of three.

	Cell viability (% of Basal)
Basal	100
3PO (10μM)	103±11
3PO (30μM)	109±12
DCA 1mM	98±11
DCA 10mM	92±9

No statistically significant differences were found.