Metformin counteracts the effects of FSH on rat Sertoli cell proliferation

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Abstract

Metformin (MET) is one of the most widely used anti-hyperglycemic agents for treating patients with type 2 diabetes and it has started to be used in pediatric population at ages when Sertoli cells are still proliferating. It is well known that follicle-stimulating hormone (FSH) is the major Sertoli cell mitogen. The aim of the study is to investigate a possible effect of MET, which has been shown to have anti-proliferative properties, on FSH regulation of postnatal Sertoli cell proliferation and on the molecular mechanisms involved in this regulation. The present study was performed in eight-day-old rat Sertoli cell cultures. The results obtained show that MET in the presence of FSH increases phosphorylated acetyl-CoA carboxylase and decreases phosphorylated p70S6K levels. Moreover, we show that MET decreases FSH-stimulated Sertoli cell proliferation, and this decrease is accompanied by a reduction in FSH-stimulated *Ccnd1* and *Ccnd2* expression and an increase in cell cycle inhibitor *p21^{Cip}* expression. Altogether, these results suggest that MET can, at least in part, counteract the effect of FSH on postnatal Sertoli cell proliferation.

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Introduction

Sertoli cell proliferation occurs during a restricted period of time. In rats, Sertoli cells proliferate throughout fetal life and up to 15 days of postnatal age. At around 15–18 days of age, the blood–testis barrier is formed, and Sertoli cells keep on differentiating to be able to support full spermatogenesis. In humans, Sertoli cells proliferate during fetal and neonatal periods up to 6 months after birth and at the onset of puberty (Sharpe *et al.* 2003, Rey *et al.* 2016). The final number of Sertoli cells reached during the proliferative periods determines sperm production capacity in adulthood, therefore, is of paramount importance to evaluate possible external factors that may alter Sertoli cell proliferation.

Metformin (MET) is one of the most widely used anti-hyperglycemic agents for treating patients with type 2 diabetes (T2D). In addition to its strong antidiabetic properties, numerous studies have demonstrated the anti-proliferative activity of MET in cancer cells. It has been shown that it inhibits proliferation of glioma, breast, ovary and prostate cancer cells among others (Isakovic *et al.* 2007, Ben Sahra *et al.* 2008, Rattan *et al.* 2011). Despite the latter findings, few studies have analyzed the effect of this drug in non-cancer cells. Particularly in the testis, Tartarin *et al.* (2012) have shown that *in vivo* administration of MET to pregnant mice reduces the number of Sertoli cells in the male fetus. However,

it has not been determined yet if MET has any effect on the hormonal regulation of postnatal Sertoli cell proliferation and if so, the potential mechanisms that may be participating.

Several studies have shown that MET exerts its anti-proliferative action partly by activating AMP-activated protein kinase (AMPK) (Zakikhani *et al.* 2006, 2008). AMPK is a serine/threonine protein kinase that maintains cellular energy homeostasis, which once activated turns on catabolic pathways that generate ATP and turns off pathways that consume ATP (Gowans & Hardie 2014). AMPK is activated in response to ATP depletion leading to an increase in the intracellular AMP:ATP ratio. It is activated allosterically by AMP and by phosphorylation by upstream kinases (Hardie *et al.* 2006). Acetyl-CoA carboxylase (ACC) is one of the best characterized downstream targets of AMPK. Even though it was observed that MET activates AMPK in chicken Sertoli cells (Faure *et al.* 2016), the capacity of the drug to activate AMPK in mammalian testis has not been analyzed yet.

Follicle-stimulating hormone (FSH) regulates Sertoli cell proliferation and differentiation after cessation of mitosis. MAPK, PI3K/Akt and mTORC1/p70S6K pathways have been involved in FSH-stimulated Sertoli cell proliferation (Crépieux *et al.* 2001, Riera *et al.* 2012). We have previously observed that pharmacological activation of AMPK by AICAR and A769662, specific

AMPK activators, leads to a decrease in the activity of mTORC1/p70S6K pathway and in FSH-stimulated Sertoli cell proliferation (Riera *et al.* 2012). As mentioned before, the anti-proliferative effect of MET observed in different cell types is exerted through the activation of AMPK. Thus, we hypothesized that MET might be counteracting the action of FSH on Sertoli cell proliferation.

Proliferation of human immature Sertoli cells occurs at the onset of puberty when the increase in FSH levels induces mitosis of this cell type. This increase in Sertoli cell number leads to a duplication of testicular volume from 2 to 4 mL, clinically reflected in Tanner stage 2 (Rey *et al.* 2016). At this point, it is important to remind that the incidence of both T2D and obesity in children has risen at staggering rates and that MET has started to be used in pediatric population at the time when Sertoli cells are proliferating (Brufani *et al.* 2013, Adeyemo *et al.* 2015, Smith *et al.* 2016). If MET alters postnatal Sertoli cell proliferation in mammalian testis, it should be considered a warning about possible deleterious effects on future fertility when this drug is used in children. The aim of the present study is to investigate a possible effect of MET on FSH regulation of postnatal rat Sertoli cell proliferation and of the possible molecular mechanisms involved, as a first approach to the issue.

Materials and methods

Materials

MET was purchased from Sigma-Aldrich. Ovine FSH (NIH-oFSH-S-16) was obtained from the National Hormone and Pituitary Program, NIDDK, Bethesda, MD, USA. Tissue culture media and all other drugs were purchased from Sigma-Aldrich.

Sertoli cell isolation and culture

Sertoli cells from eight-day-old Sprague–Dawley rats were isolated as previously described (Riera *et al.* 2012). Animals were housed and used according to the guidelines recommended by the National Institute of Health and approved by the Institutional Ethical Committee. Animals were killed by cervical dislocation. Briefly, testes were removed, decapsulated and incubated in culture medium containing 0.03% w/v collagenase and 0.003% w/v soybean trypsin inhibitor, for 5 min at room temperature. Culture medium consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 0.1% w/v BSA, 100 IU/mL penicillin, 2.5 µg/mL amphotericin B and 1.2 mg/mL sodium bicarbonate. After the initial dispersion, seminiferous tubules were sedimented and supernatant was discarded to remove interstitial cells. Seminiferous tubules were treated with 1 M glycine-2 mM EDTA (pH 7.4) to remove peritubular cells. Tubules washed with culture media and recovered by sedimentation were treated with collagenase for a second time. Tubules were incubated for 10 min at room temperature with a solution of 0.03% w/v collagenase and 0.003% w/v soybean trypsin inhibitor and 0.03% w/v DNase. The resultant Sertoli

cell suspension, collected by sedimentation, was resuspended in the culture medium described earlier without BSA and with the following additions: 10 µg/mL transferrin, 5 µg/mL insulin, 5 µg/mL vitamin E and 4 ng/mL hydrocortisone. Sertoli cells were cultured in 6- or 24-multiwell plates and in 8-chamber Permanox slides (5 µg DNA/cm²) at 34°C in a mixture of 5% CO₂:95% air.

Culture conditions

Sertoli cells were allowed to attach for 48 h in culture medium mentioned earlier. A medium change was performed at this time (day 3) with fresh medium without insulin. As indicated in figure legends, treatments with different doses of MET in the absence or presence of 100 ng/mL of FSH were started on day 3. This concentration of FSH has been previously shown to elicit maximal biological response (Meroni *et al.* 2002). Cells harvested on day 4 were used to evaluate BrdU incorporation, to count cell number or to perform RT-qPCR analysis. For Western blot analysis, cells maintained under Basal conditions until day 4 and treated as indicated in figure legends were used.

Cell extracts and Western blot analysis

Sertoli cells cultured in six multiwell plates were washed once with PBS at room temperature. Then, 200 µL PBS containing 2 µL protease inhibitor cocktail Sigma-Aldrich (P-8340), 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 50 nM okadaic acid and 2 mM PMSF were added to each well and cells were disrupted by ultrasonic irradiation. A 200 µL volume of 2× Laemmli buffer was added and thoroughly mixed. Proteins were resolved in 5% or 10% SDS-PAGE in a Mini Protean 3 Cell (Bio-Rad). After SDS-PAGE, gels were equilibrated in transfer buffer for 10 min and electrotransferred at 100V for 60 min onto PVDF membranes (Hybond-P; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) using a Mini Trans-Blot Cell (Bio-Rad). Membranes were probed with commercial antibodies that recognize the phosphorylated forms of p70S6K (Thr389) and ACC (Ser79), with antibodies against the non-phosphorylated form of these proteins (Cell Signaling Technology) and with antibodies against Cyclin D1 (Abcam), Cyclin D3 and AKT (Cell Signaling Technology). 1:1000 dilutions of primary antibodies were used. The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image (Scion Corporation, Frederick, MD, USA) software.

BrdU incorporation

Sertoli cell monolayers cultured in eight-chamber Permanox slides were incubated with BrdU (10 µM) for the last 24 h of culture. Subsequently, cells were washed with PBS and fixed with methanol:acetic acid (3:1) for 30 min for immunocytochemistry. DNA was denatured with 70% ethanol and 0.2 M NaOH for 3 min followed by cold 70% v/v and absolute ethanol (1 min each), allowing the plate to dry. Endogenous peroxidase and nonspecific sites were blocked with H₂O₂ in 3% methanol and horse serum in PBS for 30 min, respectively. Finally, cells were incubated with monoclonal BrdU antibody (1/200; Dako) in wet chamber at 4°C overnight.

Immunoperoxidase staining was performed according to the manufacturer's recommended protocol for universal-labeled streptavidin biotin/system horseradish peroxidase (Vectastain Elite ABC HRP Kit; Vector Laboratories, Burlingame, CA, USA). Nuclei were counterstained with hematoxylin, and over 2000 cells were counted. The percentage of proliferating (BrdU-positive) Sertoli cells was calculated as (BrdU-positive cells/total Sertoli cells) × 100.

Cell count assay

Sertoli cell monolayers cultured in 24-multiwell plates were washed twice with PBS and then cells were recovered by trypsinization. Cell suspension was centrifuged for 5 min at 400g and the cell pellet was recovered. Cells were resuspended in fresh medium and 0.4% w/v trypan blue was added. Cells that excluded the colorant – viable – and those that resulted stained – non-viable – were counted in a Neubauer chamber. Results are expressed as means ± s.d. of triplicate incubations.

Real-time PCR (RT-qPCR)

Total RNA was isolated from Sertoli cells cultured in six multiwell plates with TRI reagent (Sigma-Aldrich) according to the manufacturer's recommendations. The amount of RNA was estimated by spectrophotometry at 260nm. Reverse transcription was performed as previously described (Riera et al. 2012). Real-time PCR was performed using Step One Real-Time PCR System (Applied Biosystems). Table 1 shows the specific primers used to analyze *Ccnd1-d3*, *Ccne1-e2*, *p21^{Cip}*, *p27^{Kip}*, *c-Myc*, *Actb* and *B2m* expression. Amplification was carried out as recommended by the manufacturer: 25 µL reaction mixture contained 12.5 µL of SYBR Green PCR Master mix (Applied Biosystems), the appropriate primer concentration and 1 µL of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The data were normalized to *Actb* and *B2m*.

The amplification program included the initial denaturation step at 95°C for 10min, 40 cycles of denaturation at 95°C for 15s and annealing and extension at 60°C for 1min. 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 $\Delta\Delta C_t$ method was used to calculate the relative gene expression.

Statistical analysis

All experiments were run in triplicate and repeated four times. Results were expressed as mean ± s.d. One-way ANOVA followed by Tukey–Kramer test using GraphPad InStat, version 3.01 (GraphPad Software) was performed. Probabilities <0.05 were considered statistically significant.

Results

Effect of MET on P-ACC and P-p70S6K levels in Sertoli cells

As mentioned in the introduction, MET activates AMPK in different cell types. To check whether this activation also occurs in mammalian testis, rat Sertoli cell cultures were incubated for variable periods of time (2, 4, 6 and 24 h) with 10 mM MET or for 24 h with variable doses of MET (0.5, 1 and 10 mM). Phosphorylated ACC (P-ACC) levels, which reflect AMPK activation, were evaluated. Figure 1A (left) shows that 10 mM MET produced a time-dependent increase in P-ACC levels that is statistically significant in 4-, 6- and 24-h incubation periods. Figure 1A (right) shows that all doses tested of MET increase P-ACC levels. Given our previous observation that the activation of AMPK in Sertoli cells led to a decrease in the activity of mTORC1/p70S6K pathway (Riera et al. 2012), we further evaluated the effect of MET

Table 1 Rat-specific primers sets for analysis by RT-qPCR.

Gene	Accession number	Primer sequence	Product length (bp)
Cyclin D1 (<i>Ccnd1</i>)	NM_171992.4	5'-CTACCGCACAACGCACTTTC-3' 5'-AAGGGCTTCAATCTGTTCTG-3'	85
Cyclin D2 (<i>Ccnd2</i>)	NM_022267.1	5'-CTGACCAAGATCACCCACAC-3' 5'-CTCTTAGACGGAAGTCTGAAG-3'	99
Cyclin D3 (<i>Ccnd3</i>)	NM_012766.1	5'-GAAACCACACCCCTGACTATTG-3' 5'-AGGTCCCACTTGAGCTTCC-3'	113
Cyclin E1 (<i>Ccne1</i>)	NM_001100821.1	5'-ACAGCTTATTGGGATTTCAGC-3' 5'-GGAGCAAGCACCATCAGTAAC-3'	103
Cyclin E2 (<i>Ccne2</i>)	NM_001108656.1	5'-AGCCAGACTCTCCACAAGAAG-3' 5'-ATTCCTCCAGACAGTACAGGTG-3'	145
<i>p21^{Cip}</i> (<i>Cdkn1a</i>)	NM_080782.3	5'-GTCTTGCACTCTGGTGTCTCA-3' 5'-GCACTTCAGGGCTTTCTCTT-3'	156
<i>p27^{Kip}</i> (<i>Cdkn1b</i>)	NM_031762.3	5'-TTCGACGCCAGACGTAAAC-3' 5'-TTCAATGGAGTCAGCGATATG-3'	126
MYC proto-oncogene (<i>c-Myc</i>)	NM_012603.2	5'-CACCAGCAGCGACTCTGAA-3' 5'-GACCCGTGACTCGGACCTCTT-3'	102
β -actin (<i>Actb</i>)	NM_031144.3	5'-TGGCACCACACTTTCTACAAT-3' 5'-GGTACGACCAGAGGCATACA-3'	189
Beta-2 microglobulin (<i>B2m</i>)	NM_012512.2	5'-CGTGATCTTTCTGGTGCTTG-3' 5'-GAGGTGGGTGGAAGTGAAC-3'	143

Figure 1 Effect of metformin on P-ACC and P-p70S6K levels in Sertoli cells. Sertoli cell monolayers were maintained under Basal conditions or incubated for variable periods of time with 10 mM metformin (MET; A and B left panel). Additionally, Sertoli cells were incubated with variable doses of metformin for 24 h (A and B right panel). Western blot analysis was performed utilizing antibodies for P-ACC, T-ACC, P-p70S6K and T-p70S6K. The upper panels show a representative experiment out of three. The lower panels show pooled data of three independent experiments indicating the fold variation in phosphorylation (ratio of P-ACC to T-ACC and of P-p70S6K to T-p70S6K in each sample) relative to Basal. Results are expressed as means $\pm$ S.D. * $P < 0.05$; ** $P < 0.01$ vs Basal. A full colour version of this figure is available at <https://doi.org/10.1530/REP-18-0233>.

on phosphorylated p70S6K (P-p70S6K) levels. **Figure 1B** (left) shows that 10 mM MET produced a time-dependent reduction in P-p70S6K levels. **Figure 1B** (right) shows that the decrease in P-p70S6K levels in 24-h incubation period was only observed with a 10 mM dose of MET.

Effect of MET on Sertoli cell proliferation

The effect of MET on Sertoli cell proliferation was next evaluated. Sertoli cell cultures were incubated for 24 h with MET 0.5 or 10 mM. Sertoli cell proliferation was assessed by BrdU incorporation into DNA and by counting cells in a Neubauer chamber. **Figure 2** shows that 10 mM MET significantly diminished BrdU incorporation into DNA. Cell counting in a Neubauer chamber showed that 10 mM MET decreased the number of Sertoli cells (Basal: 1250 ± 72 ; MET 0.5 mM: 1220 ± 96 ; MET 10 mM: $890 \pm 98^*$ number of cells $\times 10^{-2}$ /well; means $\pm$ S.D., $n = 3$

Figure 2 Effect of metformin on Sertoli cell proliferation. Sertoli cell monolayers were maintained under Basal conditions or incubated for 24 h with metformin (0.5 or 10 mM). At the end of the culture period, bromodeoxyuridine (BrdU) incorporation was evaluated by immunocytochemistry. Representative images of Sertoli cells showing immunoreactivity for BrdU (brown) were shown. In the graphic, each bar represents % BrdU-positive cells (2000 cells/group). Results are presented as means $\pm$ S.D. of four independent experiments. * $P < 0.05$ vs Basal. NC, negative control.

* $P < 0.05$ vs Basal), while it did not affect cell viability, as evaluated by a trypan blue exclusion test (Basal: 15 ± 5 ; MET 0.5 mM: 17 ± 7 ; MET 10 mM: 16 ± 8 , number of trypan blue stained cells $\times 10^{-2}$ /well; means $\pm$ S.D., $n = 3$).

Having demonstrated that MET decreased cell proliferation, we next analyzed if MET regulated the expression of cyclins and cell cycle inhibitors. **Figure 3A** shows that 10 mM MET significantly decreased *Ccnd1* and *Ccne1* mRNA levels. Also, this figure shows that MET did not modify the expression

Figure 3 Effect of metformin on *Ccnd1-d3*, *Ccne1-e2*, *p21^{Cip1}* and *p27^{Kip1}* mRNA levels. Sertoli cells were maintained under basal conditions or incubated with variable doses of metformin (0.5, 1 or 10 mM) for 6 h (panel A) or 24 h (panel B). Then total RNA was extracted and RT-qPCR was performed. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal. Results are expressed as means $\pm$ s.d. * $P < 0.05$; ** $P < 0.01$ vs Basal.

of *Ccnd2*, *Ccnd3* and *Ccne3* at any of the doses tested. On the other hand, Fig. 3B shows that 10 mM MET significantly increased *p21^{Cip1}* mRNA levels, and it did not modify the expression of *p27^{Kip1}* at any of the doses studied.

Effect of MET on FSH action

The next set of experiments was designed to analyze whether MET is also able to modulate AMPK and

mTORC1/p70S6K pathways in the presence of FSH, the main Sertoli cell mitogen. To this end, Sertoli cell cultures were maintained under Basal conditions or stimulated for 6 h with FSH (100 ng/mL) in the absence or presence of MET (10 mM). Figure 4 shows that FSH did not decrease the ability of MET to increase P-ACC levels. Also, this figure shows that FSH-stimulated P-p70S6K levels and that the combination with MET significantly decreased the stimulation produced by FSH.

We next explored the potential effects of MET on FSH-stimulated Sertoli cell proliferation. Figure 5 shows that 0.5 and 10 mM MET decreased FSH-stimulated BrdU incorporation into DNA in Sertoli cells.

The next set of experiments was designed to analyze the effect of MET on FSH action on the levels of expression of cell cycle regulators. Figure 6A shows that 10 mM MET decreased FSH-stimulated *Ccnd1* and *Ccnd2* mRNA levels. This figure also shows that FSH produced a slight increase of *Ccne1* and *Ccne2* mRNA levels, which was not statistically significant and that a 10 mM dose of MET decreased it. Figure 6B shows that in the presence of FSH, 10 mM MET produced a significant increase in *p21^{Cip1}* mRNA levels while it did not modify *p27^{Kip1}* mRNA levels. The protein levels of Cyclin D1 and D3 were also evaluated. Similar to the effect on mRNA levels, 10 mM MET decreased FSH-stimulated Cyclin D1 levels, while it did not affect Cyclin D3 levels (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Finally, considering that in some cancer cells, the anti-proliferative effects of MET are accompanied by a reduction in c-Myc expression, that in Sertoli cells FSH increases c-Myc mRNA levels and that this fact has been related to the regulation of FSH-stimulated Sertoli cell proliferation (Lim & Hwang 1995), we decided to evaluate the effect of MET on the expression of this transcription factor. In this respect, Fig. 7 shows that MET did not modify Basal or FSH-stimulated c-Myc mRNA levels.

Figure 4 Effect of metformin in the presence of FSH on P-ACC and P-p70S6K levels. Sertoli cell monolayers were maintained under Basal (B) conditions or incubated with 10 mM metformin in the absence or presence of FSH for 6 h. Western blot analysis was performed utilizing antibodies for P-ACC, T-ACC, P-p70S6K and T-p70S6K. The upper panels show a representative experiment out of three. The lower panels show pooled data of three independent experiments indicating the fold variation in phosphorylation (ratio of P-ACC to T-ACC and of P-p70S6K to T-p70S6K in each sample) relative to Basal. Results are expressed as means $\pm$ s.d. Different letters indicate statistically significant differences ($P < 0.05$).

Figure 5 Effect of metformin on FSH-stimulated Sertoli cell proliferation. Sertoli cells monolayers were maintained under Basal (B) conditions or incubated for 24 h with FSH (100 ng/mL) in the absence or presence of metformin (0.5 or 10 mM). At the end of the culture period, bromodeoxyuridine (BrdU) incorporation was evaluated by immunocytochemistry. Representative images of Sertoli cells showing immunoreactivity for BrdU (brown) were shown. In the graphic, each bar represents % BrdU-positive cells (2000 cells/group). Results are presented as means $\pm$ s.d. of four independent experiments. Different letters indicate statistically significant differences ($P < 0.05$).

Discussion

Sertoli cell proliferation occurs in fetal and postnatal periods up to 15 days of age in the rat. Each Sertoli cell supports a fixed number of germ cells throughout spermatogenesis. Consequently, the final number of Sertoli cells reached during the proliferative periods will determine the number of germ cells that can be maintained and hence define the level of sperm production, an issue with a clear bearing on fertility.

Figure 6 Effect of metformin in the presence of FSH on *Ccnd1-d3*, *Ccne1-e2*, *p21^{Cip1}* and *p27^{Kip1}* mRNA levels. Sertoli cells monolayers were maintained under Basal conditions or incubated with FSH (100 ng/mL) in the absence or presence of metformin (0.5 or 10 mM) for 2 h (*Ccnd1*) or 6 h (*Ccnd2-d3*; *Ccne1-e2*) in panel A and for 24 h (*p21^{Cip1}* and *p27^{Kip1}*) in panel B. Then, total RNA was extracted and RT-qPCR was performed. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal. Results are expressed as means $\pm$ s.d. (* $P < 0.05$ vs Basal; # $P < 0.05$ vs FSH).

MET is the most commonly given oral drug for the treatment of T2D, and it has been recently started to be prescribed in pediatric patients (Jones *et al.* 2002, Brufani *et al.* 2013, Adeyemo *et al.* 2015, Smith *et al.* 2016). The mechanisms through which MET affects cell function have not been fully defined. In this respect, it has been reported that MET acts through AMPK-independent and -dependent mechanisms. In the present study, we observed that MET treatment in Sertoli cells increases P-ACC levels, indirectly showing that activation of AMPK is taking place and that an AMPK-dependent mechanism may be altering cell function. Additionally, it was observed that MET decreases the activity of the p70S6K pathway. These results are in agreement with observations made in several cell lines showing that MET activates AMPK and inhibits p70S6K pathway (Zakikhani *et al.* 2006, Qu *et al.* 2012, White-Al Habeeb *et al.* 2016). The notion that activation of AMPK and decreased activity of p70S6K pathways are related events in Sertoli cells is supported by previous studies demonstrating that AMPK activation by the pharmacological activator A-769662 promotes an inhibition of p70S6K signaling in this cell type (Riera *et al.* 2012).

In the last years, the effect of MET on the regulation of cell proliferation has captured much attention. Specifically, the potential antitumoral effect of this drug has been evaluated. It has been demonstrated that MET causes G1/S phase cell cycle arrest in several

Figure 7 Effect of metformin on c-Myc mRNA levels. Sertoli cells monolayers were incubated under Basal conditions or stimulated for 2 h with FSH (100 ng/mL) in the absence or presence of metformin (0.5 or 10 mM). Then, total RNA was extracted and RT-qPCR was performed. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal. Results are expressed as means $\pm$ s.d. (* P < 0.05 vs Basal).

cancer cells such as human head and neck squamous cell carcinoma (HNSCC) cells, esophageal squamous cell carcinoma (ESCC) cells and breast cancer cells among others (Zhuang & Miskimins 2008, Sikka *et al.* 2012, Cai *et al.* 2015). The results presented herein show that MET decreases BrdU incorporation into DNA and total cell number without modifying cell viability demonstrating that the drug induces an inhibition of rat Sertoli cell proliferation. Our results are in agreement with those presented by Tartarin *et al.* (2012) who observed that *in vivo* administration of MET to pregnant mice showed a reduced number of Sertoli cells in newborn pups. It has also been recently observed that MET decreases the proliferation of Sertoli cells obtained from peripubertal chickens (Faure *et al.* 2016). Altogether, these results suggest that MET can affect Sertoli cell proliferation at different stages of development and consequently can modify final Sertoli cell number reached in adulthood, which may eventually compromise fertility.

The present study also attempted to uncover the molecular mechanisms that may be involved in MET effects on Sertoli cell proliferation. Cyclins and cell cycle inhibitors tightly control cell cycle progression and the effect of MET on the regulation of their expression has been analyzed in some cell lines. Previous studies have shown a downregulation of cyclin D1 expression in response to MET in MCF7 breast cancer cells and in prostate cancer cell lines (Ben Sahra *et al.* 2008, Zhuang & Miskimins 2008). As for cell cycle inhibitors, it has been demonstrated that MET increases p21^{Cip1} and p27^{Kip1} protein levels in ESCC cells and in hepatocellular

carcinoma (HCC) cell lines (Cai *et al.* 2013, 2015). Our results show that MET decreases *Ccnd1* and *Ccne1* mRNA levels in rat Sertoli cells. In addition, results show that MET promotes a marked increase in the expression of p21^{Cip1}. Altogether, these data strongly suggest that MET may reduce Sertoli cell proliferation through the regulation of the expression of cyclins and/or cell cycle inhibitors.

Despite the fact that it has been observed that MET can inhibit Sertoli cell proliferation in other experimental models (Tartarin *et al.* 2012, Faure *et al.* 2016), none of these studies have analyzed the effect of this drug on the hormonal regulation of cell proliferation. Even though a role for several hormones has been assigned to Sertoli cell proliferation, it is widely accepted that FSH is the major Sertoli cell mitogen (Griswold *et al.* 1977, Archambeault & Yao 2010, Lucas *et al.* 2014). Several signal transduction pathways may participate in FSH stimulation of Sertoli cell proliferation (Orth 1984, Crépieux *et al.* 2001). Particularly, it has been shown that FSH activates mTORC1/p70S6K pathway in order to increase Sertoli cell proliferation (Riera *et al.* 2012). In the present study, we show that even in the presence of FSH, MET can activate AMPK, and it diminishes FSH-stimulated P-p70S6K levels, indicating that MET is able to inhibit at least one of the signaling pathways activated by FSH to promote proliferation of Sertoli cells.

Considering that MET can counteract signaling pathways activated by FSH, possible effects of this drug on FSH-stimulated Sertoli cell proliferation may be expected. In fact, results presented herein show that MET inhibits FSH-stimulated Sertoli cell proliferation. The latter decrease in FSH-stimulated Sertoli cell proliferation may be accounted for by, at least in part, a decrease in FSH-stimulated *Ccnd1* and *Ccnd2* expression and a marked increase in the expression of the cell cycle inhibitor p21^{Cip1}. Upregulation of p21^{Cip1} expression by AMPK activation has been previously linked to the inhibition of proliferation in FSH-stimulated Sertoli cells (Riera *et al.* 2012). It is noteworthy that testosterone, retinoic acid and triiodothyronine – hormones that promote physiological cessation of Sertoli cell proliferation – also increase p21^{Cip1} expression (Buzzard *et al.* 2003).

There is a vast amount of evidence pointing out the important role that c-Myc has on cell proliferation. As mentioned before, FSH increases c-Myc mRNA levels in eight-day-old rat Sertoli cells and that regulation of c-Myc expression has been suggested to be involved in the proliferative effect of FSH (Lim & Hwang 1995, Riera *et al.* 2012). Moreover, studies performed utilizing different cancer cell lines have shown that MET exerts anti-proliferative actions, at least in part, by decreasing c-Myc expression (Mogavero *et al.* 2017, Zhang *et al.* 2017). The above-mentioned observations led us to evaluate a possible effect of MET on the regulation of c-Myc mRNA levels. Our results showing that MET does

not modify Basal- or FSH-stimulated *c-Myc* mRNA levels indicate that the decrease of Sertoli cell proliferation produced by MET may not be related to the expression of this transcription factor.

One of the major obstacles for extrapolating the findings of MET action in cultured cells to the human being as a whole is the difference between MET concentrations used in *in vitro* studies and plasma levels of this drug reached in treated patients which are in the micromolar range. In our study, as in most of the investigations that analyze the effects of MET on cell proliferation, MET concentrations of 0.5–10 mM were necessary to inhibit Sertoli cell proliferation. A recent publication offers insights into what otherwise seems to be a non-physiological effect of MET. Gui *et al.* (2016) analyzed the anti-proliferative effect of MET in different culture media and showed that the presence of pyruvate in the culture medium diminishes the sensitivity to MET by approximately two orders of magnitude. The mechanism involves pyruvate availability as this ketoacid can act as an electron acceptor to regenerate NAD⁺ with the result of a change in the cell dependency to respiratory complex I activity (Gui *et al.* 2016). Considering that our culture medium contains 1 mM pyruvate, this may well explain why only elevated doses of MET inhibit Sertoli cell proliferation.

Sertoli cells proliferate during distinct periods in life, which include fetal, neonatal life and beginning of puberty in all species. In the rat, the neonatal and pubertal period are superimposed (Sharpe *et al.* 2003). Once they passed these proliferative periods, Sertoli cells remain numerically stable throughout life, and it is widely accepted that this number determines sperm production. Altogether, our findings suggest that MET can counteract, at least in part, the effect of FSH on postnatal rat Sertoli cell proliferation. Bearing in mind that MET is now being used for the therapy of obesity and T2D in children starting at the age of 6 years (Brufani *et al.* 2013, Smith *et al.* 2016), which include ages where Sertoli cells proliferate, attention should be paid to possible alterations in the final number of Sertoli cells in adulthood. A longitudinal study evaluating fertility in these patients would be helpful to gain full confidence on MET treatment in children.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0233>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Adeyemo MA, McDuffie JR, Kozlosky M, Krakoff J, Calis KA, Brady SM & Yanovski JA 2015 Effects of metformin on energy intake and satiety in obese children. *Diabetes Obesity and Metabolism* **17** 363–370. (<https://doi.org/10.1111/dom.12426>)
- Archambeault DR & Yao HH 2010 Activin A, a product of fetal Leydig cells, is a unique paracrine regulator of Sertoli cell proliferation and fetal testis cord expansion. *PNAS* **107** 10526–10531. (<https://doi.org/10.1073/pnas.1000318107>)
- Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auburger P, Tanti JF, Le Marchand-Brustel Y & Bost F 2008 The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene* **27** 3576–3586. (<https://doi.org/10.1038/sj.onc.1211024>)
- Brufani C, Crinò A, Fintini D, Patera PI, Cappa M & Manco M 2013 Systematic review of metformin use in obese nondiabetic children and adolescents. *Hormone Research in Paediatrics* **80** 78–85. (<https://doi.org/10.1159/000353760>)
- Buzzard JJ, Wreford NG & Morrison JR 2003 Thyroid hormone, retinoic acid, and testosterone suppress proliferation and induce markers of differentiation in cultured rat Sertoli cells. *Endocrinology* **144** 3722–3731. (<https://doi.org/10.1210/en.2003-0379>)
- Cai X, Hu X, Cai B, Wang Q, Li Y, Tan X, Hu H, Chen X, Huang J, Cheng J *et al.* 2013 Metformin suppresses hepatocellular carcinoma cell growth through induction of cell cycle G1/G0 phase arrest and p21CIP and p27KIP expression and downregulation of cyclin D1 in vitro and in vivo. *Oncology Reports* **30** 2449–2457. (<https://doi.org/10.3892/or.2013.2718>)
- Cai X, Hu X, Tan X, Cheng W, Wang Q, Chen X, Guan Y, Chen C & Jing X 2015 Metformin induced AMPK activation, G0/G1 phase cell cycle arrest and the inhibition of growth of esophageal squamous cell carcinomas in vitro and in vivo. *Public Library of Science* **10** e0133349. (<https://doi.org/10.1371/journal.pone.0133349>)
- Crépiaux P, Marion S, Martinat N, Fafeur V, Vern YL, Kerboeuf D, Guillouf F & Reiter E 2001 The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. *Oncogene* **20** 4696–4709. (<https://doi.org/10.1038/sj.onc.1204632>)
- Faure M, Guibert E, Alves S, Pain B, Ramé C, Dupont J, Brillard JP & Froment P 2016 The insulin sensitiser metformin regulates chicken Sertoli and germ cell populations. *Reproduction* **151** 527–538. (<https://doi.org/10.1530/REP-15-0565>)
- Gowans GJ & Hardie DG 2014 AMPK: a cellular energy sensor primarily regulated by AMP. *Biochemical Society Transactions* **42** 71–75. (<https://doi.org/10.1042/BST20130244>)
- Griswold MD, Solari A, Tung PS & Fritz IB 1977 Stimulation by follicle-stimulating hormone of DNA synthesis and of mitosis in cultured Sertoli cells prepared from testes of immature rats. *Molecular and Cellular Endocrinology* **7** 151–165. ([https://doi.org/10.1016/0303-7207\(77\)90064-8](https://doi.org/10.1016/0303-7207(77)90064-8))
- Gui DY, Sullivan LB, Luengo A, Hosios AM, Bush LN, Gitego N, Davidson SM, Freinkman E, Thomas CJ & Vander Heiden MG 2016

- Environment dictates dependence on mitochondrial complex I for NAD⁺ and aspartate production and determines cancer cell sensitivity to metformin. *Cell Metabolism* **24** 716–727. (<https://doi.org/10.1016/j.cmet.2016.09.006>)
- Hardie DG, Hawley SA & Scott JW 2006 AMP-activated protein kinase-development of the energy sensor concept. *Journal of Physiology* **574** 7–15. (<https://doi.org/10.1113/jphysiol.2006.108944>)
- Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V, Micic D & Trajkovic V 2007 Dual antglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. *Cellular and Molecular Life Sciences* **64** 1290–1302 (<https://doi.org/10.1007/s00018-007-7080-4>)
- Jones KL, Arslanian S, Peterokova VA, Park JS & Tomlinson MJ 2002 Effect of metformin in pediatric patients with type 2 diabetes: a randomized controlled trial. *Diabetes Care* **25** 89–94. (<https://doi.org/10.2337/diacare.25.1.89>)
- Lim K & Hwang BD 1995 Follicle-stimulating hormone transiently induces expression of protooncogene c-myc in primary Sertoli cell cultures of early pubertal and prepubertal rat. *Molecular and Cellular Endocrinology* **111** 51–56. ([https://doi.org/10.1016/0303-7207\(95\)03543-G](https://doi.org/10.1016/0303-7207(95)03543-G))
- Lucas TF, Nascimento AR, Pisolato R, Pimenta MT, Lazari MF & Porto CS 2014 Receptors and signaling pathways involved in proliferation and differentiation of Sertoli cells. *Spermatogenesis* **20** e28138. (<https://doi.org/10.4161/spmg.28138>)
- Meroni SB, Riera MF, Pellizzari EH & Cigorraga SB 2002 Regulation of rat Sertoli cell function by FSH: possible role of phosphatidylinositol 3-kinase/protein kinase B pathway. *Journal of Endocrinology* **174** 195–204. (<https://doi.org/10.1677/joe.0.1740195>)
- Mogavero A, Maiorana MV, Zanutto S, Varinelli L, Bozzi F, Belfiore A, Volpi CC, Gloghini A, Pierotti MA & Gariboldi M 2017 Metformin transiently inhibits colorectal cancer cell proliferation as a result of either AMPK activation or increased ROS production. *Science Reports* **7** 15992. (<https://doi.org/10.1038/s41598-017-16149-z>)
- Orth JM 1984 The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. *Endocrinology* **115** 1248–1255. (<https://doi.org/10.1210/endo-115-4-1248>)
- Qu Z, Zhang Y, Liao M, Chen Y, Zhao J & Pan Y 2012 In vitro and in vivo antitumoral action of metformin on hepatocellular carcinoma. *Hepatology Research* **42** 922–933. (<https://doi.org/10.1111/j.1872-034X.2012.01007.x>)
- Rattan R, Giri S, Hartmann LC & Shridhar V 2011 Metformin attenuates ovarian cancer cell growth in an AMP-kinase dispensable manner. *Journal of Cellular and Molecular Medicine* **15** 166–178. (<https://doi.org/10.1111/j.1582-4934.2009.00954.x>)
- Rey RA, Campo SM, Ropelato MG & Bergadá I 2016 Hormonal changes in childhood and puberty. In *Puberty*, pp 23–37. Eds P Kumanov & A Agarwal. Springer International Publishing, Switzerland (https://doi.org/10.1007/978-3-319-32122-6_3).
- Riera MF, Regueira M, Galardo MN, Pellizzari EH, Meroni SB & Cigorraga SB 2012 Signal transduction pathways in FSH regulation of rat Sertoli cell proliferation. *American Journal of Physiology: Endocrinology and Metabolism* **302** E914–E923. (<https://doi.org/10.1152/ajpendo.00477.2011>)
- Sharpe RM, McKinnell C, Kivlin C & Fisher JS 2003 Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* **125** 769–784. (<https://doi.org/10.1530/rep.0.1250769>)
- Sikka A, Kaur M, Agarwal C, Deep G & Agarwal R 2012 Metformin suppresses growth of human head and neck squamous cell carcinoma via global inhibition of protein translation. *Cell Cycle* **11** 1374–1382. (<https://doi.org/10.4161/cc.19798>)
- Smith JD, Mills E & Carlisle SE 2016 Treatment of pediatric type 2 diabetes. *Annals of Pharmacotherapy* **50** 768–777. (<https://doi.org/10.1177/1060028016655179>)
- Tartarin P, Moison D, Guibert E, Dupont J, Habert R, Rouiller-Fabre V, Frydman N, Pozzi S, Frydman R, Lecureuil C *et al.* 2012 Metformin exposure affects human and mouse fetal testicular cells. *Human Reproduction* **27** 3304–3314. (<https://doi.org/10.1093/humrep/des264>)
- White-Al Habeeb NM, Garcia J, Fleshner N & Bapat B 2016 Metformin elicits antitumor effects and downregulates the histone methyltransferase multiple myeloma SET domain (MMSET) in prostate cancer cells. *Prostate* **76** 1507–1518. (<https://doi.org/10.1002/pros.23235>)
- Zakikhani M, Dowling R, Fantus IG, Sonenberg N & Pollak M 2006 Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Research* **66** 10269–10273. (<https://doi.org/10.1158/0008-5472.CAN-06-1500>)
- Zakikhani M, Dowling RJ, Sonenberg N & Pollak MN 2008 The effects of adiponectin and metformin on prostate and colon neoplasia involve activation of AMP-activated protein kinase. *Cancer Prevention Research* **1** 369–375. (<https://doi.org/10.1158/1940-6207.CAPR-08-0081>)
- Zhang J, Xu H, Zhou X, Li Y, Liu T, Yin X & Zhang B 2017 Role of metformin in inhibiting estrogen-induced proliferation and regulating ER α and ER β expression in human endometrial cancer cells. *Oncology Letters* **14** 4949–4956. (<https://doi.org/10.3892/ol.2017.6877>)
- Zhuang Y & Miskimins WK 2008 Cell cycle arrest in Metformin treated breast cancer cells involves activation of AMPK, downregulation of cyclin D1, and requires p27Kip1 or p21Cip1. *Journal of Molecular Signalling* **3** 18. (<https://doi.org/10.1186/1750-2187-3-18>)

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