Reproduction Advance Publication first posted on 12 December 2014 as Manuscript REP-14-0392

1 **Cyclooxygenase and prostaglandins in somatic cell populations of the testis**

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17 **Running head**

18 Role of prostaglandins in the testis

19

20 **Abstract**

21 Prostaglandins (PGs) are synthesized through the action of the rate-limiting
22 enzyme cyclooxygenase (COX) and further specific enzymes. The development of
23 *Cox*-deficient mice in the 1990s gave insights into the reproductive roles of PGs.
24 Female *Cox*-knockout mice were subfertile or infertile. Interestingly, fertility was not

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affected in male mice deficient in *Cox,* suggesting that PGs may not be critical for the functioning of the testis. However, this conclusion has recently been challenged by observations of important roles for PGs in both physiological and pathological processes in the testis.

The two key somatic cell types in the testis, Leydig and Sertoli cells, express the inducible isoenzyme COX2 and produce PGs. Testicular COX2 expression in these somatic cells is regulated by hormonal input (FSH, PRL and testosterone) as well as by IL1ß. PGs modulate steroidogenesis in Leydig cells and glucose uptake in Sertoli cells. Hence, the COX2/PG system in Leydig and Sertoli cells acts as a local modulator of testicular activity, and consequently may regulate spermatogenic efficiency.

In addition to its expression in Leydig and Sertoli cells, COX2 has been detected in the seminiferous tubule wall, and in testicular macrophages and mast cells of infertile patients. These observations highlight the possible relevance of PGs in testicular inflammation associated with idiopathic infertility.

Collectively, these data indicate that the COX2/PG system plays crucial roles not only in testicular physiology (i.e. development, steroidogenesis, spermatogenesis), but more importantly in the pathogenesis or maintenance of infertility status in the male gonad. Further studies of these actions could lead to new therapeutic approaches to idiopathic male infertility.

Introduction

Prostaglandins (PGs) are bioactive lipid substances derived from arachidonic acid.

48 Arachidonic acid is generated from phospholipid hydrolysis catalyzed by combined
49 phospholipase A2 (PLA_2) and cyclooxygenase or lipoxygenase activities.
50 Arachidonic acid can also be generated from diacylglycerol (DAG) by the action of
51 a diacylglycerol lipase (Harnett & Goodrigde 2005).
52 PGs, which are found in most tissues and organs, are produced by almost all
53 nucleated cells. They were discovered in the 1930s and named *prostaglandins*
54 because they were originally thought to be prostatic products (Goldblatt 1933, Von
55 Euler 1935).
56 PGs are involved in a diversity of physiological and pathological systems such as
57 regulation of inflammatory and immune responses, cell growth, intraocular
58 pressure, calcium movement, contraction and relaxation of vascular smooth
59 muscle cells, aggregation and disaggregation of platelets, glomerular filtration rate
60 in the kidney, sensitivity of spinal neurons to pain, body temperature in response to
61 fever and parturition (Narumiya 2007).
62 The biosynthetic pathway of PGs is initiated when cyclooxygenase (COX)
63 catalyzes two sequential reactions, cyclooxygenation of arachidonic acid to PGG
64 and a subsequent peroxidation in which PGG is reduced to PGH. The resulting
65 PGH is converted to other bioactive PG isomers by the action of synthases and
66 ketoreductases, reactions of dehydration, and non-enzymatic isomerization (Fig. 1;
67 Simmons *et al.* 2004, Frungieri *et al.* 2006). The majority of the biologically active
68 PGs belong to series 2, characterized by the presence of two double bonds in the
69 hydrocarbon structure (Simmons *et al.* 2004, Frungieri *et al.* 2006).
70 COX, the rate-limiting enzyme of PG biosynthesis, is also known as prostaglandin
71 H synthase (PGHS) or prostaglandin endoperoxide synthase (PTGS). COX is

72 present in two distinct isoforms, type 1 and type 2, encoded by separate genes
73 (Smith & Langenbach 2001, Simmons *et al.* 2004). COX1, commonly known as the
74 constitutive isoform, is found in most cell types, while COX2, the inducible form,
75 appears to be expressed only during early stages of cell differentiation or
76 replication, in response to varying stimuli such as cytokines and mitogenic factors.
77 COX2 expression has been described in physiological and pathological processes
78 including inflammation, angiogenesis, bone absorption, gastric ulceration, kidney
79 diseases, brain disorders and female genital tract disorders (Katori & Majima
80 2000). Furthermore, COX2 is over-expressed in many types of cancer, including
81 breast, colon, lung and prostate cancers (Harris 2009).
82 Depending on the biological process, COX isoenzymes can act individually, in
83 concert, or in cases where one isoenzyme is lacking, in a compensatory manner
84 (Smith & Langenbach 2001). Recently, new variants of COX have been
85 discovered, such as *COX3* and *PCOX1,* splice variants that affect the coding
86 region of COX1, as well as a number of alternatively polyadenylated transcripts of
87 *COX* and single nucleotide polymorphisms (SNPs) (Simmons *et al.* 2004). *COX*
88 variants and mutants are likely to yield altered or expanded biological function.
89 DP, EP, FP, IP and TP are serpentine plasma membrane-localized prostanoid
90 receptors that bind PGD, PGE, PGF, PGI and thromboxane, respectively. In
91 addition, several prostanoids, of which 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ2) is the
92 most potent, may activate the peroxisome proliferator-activated receptor gamma
93 (PPARγ) members of the steroid/thyroid family of nuclear hormone receptors,
94 which act as transcription factors and may thus directly influence gene transcription
95 (Simmons *et al*. 2004, Narumiya 2007).

96

97 **Cyclooxygenase and prostaglandins in the human testis**

98 In the 1990s, the development of *Cox1* and *Cox2* deficient mice yielded insights
99 into the reproductive roles of PGs. Whereas female *Cox2* knockout mice are
100 infertile, those deficient in *Cox1* have difficulties with parturition but produce litters
101 with normal weight. In contrast, fertility is not affected in male mice deficient in
102 *Cox1* or *Cox2* (Langenbach *et al*. 1999). These early reports suggested that PGs
103 may not be critical to testicular function. However, this view has recently been
104 challenged by novel observations. It has been reported that paracetamol and some
105 nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin
106 induce endocrine disturbances in the human fetal testis capable of interfering with
107 testicular descent (Mazaud-Guittot *et al.* 2013). Furthermore, PGD2 influences
108 male germ cell differentiation in the fetal mouse testis (Moniot *et al.* 2014), and it
109 has been proposed that the hematopoietic PGD2 synthase participates in the
110 SOX9 nuclear translocation necessary for the process of Sertoli cell differentiation
111 (Rossitto *et al.* 2014).

112 PG receptors have been described in Leydig cells (i.e. EP1, DP, FP, TP and
113 PPARγ receptors) (Walch *et al.* 2003, Schell *et al*. 2007, Frungieri *et al.* 2006,
114 Kowalewski *et al.* 2009, Pandey *et al.* 2009), Sertoli cells (e.g. EP1, EP2, EP3,
115 EP4, DP, IP, FP and PPARγ receptors) (Ishikawa & Morris 2006, Winnal *et al.*
116 2007, Kristensen *et al.* 2011, Matzkin *et al*. 2012) and the seminiferous tubule wall
117 (PPARγ receptors) (Frungieri *et al.* 2002a). DP prostanoid receptors have also
118 been detected in germ cells of the fetal mouse testis (Moniot *et al.* 2014), whereas

119 functional PPARγ and PGE receptors have been found in sperm (Schaefer *et al.*
120 1998, Santoro *et al.* 2013).
121 PGs, mainly those from the PGE and 19-hydroxy-PGE series, are present in
122 human seminal plasma. Several reports have claimed that there is a correlation
123 between PG levels in semen and otherwise unexplained male infertility (Kelly
124 1978). The lipocalin and hematopoietic PGD2 synthase is also detected in seminal
125 plasma and its concentration is lower in oligozoospermic than in normozoospermic
126 men (Tokugawa *et al.* 1998). PGs in human seminal plasma are mostly secreted
127 from the seminal vesicles. Nevertheless, testicular secretions also contribute up to
128 5 percent of the composition of the semen (Thibodeau & Patton 2012).
129 Data from our group revealed that COX is not detectable by immunohistochemistry
130 in normal adult human testes without morphological abnormalities. However, the
131 inducible isoenzyme COX2 is expressed by several cell types in testicular biopsies
132 of men with impaired spermatogenesis and infertility (Frungieri *et al*. 2002a, Welter
133 *et al*. 2011). They include Leydig cells, Sertoli cells and cells of the tubular wall that
134 present an altered morphology (Figs. 2 and 3; Schell *et al.* 2008, Matzkin *et al.*
135 2010). COX2 was also found in testicular immune cells, namely mast cells and
136 macrophages (Matzkin *et al.* 2010, Welter *et al.* 2011, Rossi *et al.* 2014).
137 Similarly, Hase *et al*. (2003) did not detect COX expression in the normal human
138 testis, but described induction of COX1 and COX2 in testicular cancer. Additionally,
139 lipocalin and hematopoietic PGD2 synthases are expressed in testes from patients
140 with impaired spermatogenesis (Schell *et al*. 2007).
141 These data suggest that in pathological situations the human testis is capable of
142 synthesizing PGs. In this regard, we recently described the presence of the PG

143 metabolite, 15d-PGJ2, in biopsies of patients suffering from idiopathic infertility
144 (Kampfer *et al*. 2012).
145 Overall, the wide distribution of PG receptors and synthesizing enzymes in the
146 testis emphasizes the plethora of functions and potential key roles exerted by
147 these bioactive lipid substances on testicular development, steroidogenesis, sperm
148 maturation and male fertility.
149 Physiological studies cannot be performed using human testicular biopsies. In the
150 search for an adequate model, our laboratory turned to the Syrian hamster. It was
151 chosen as the experimental model because the exposure of male adult animals to
152 less than 12.5 h of light per day for 3–4 months results in a severe testicular
153 regression with morphological features resembling those seen in biopsies of
154 patients suffering from hypospermatogenesis and germ cell arrest. For instance,
155 seminiferous tubules in photoperiodically regressed hamster testes contain mostly
156 Sertoli cells, spermatogonia and a few primary spermatocytes (Fig. 4; Sinha Hikim
157 *et al.* 1988, Rossi *et al.* 2014).
158

159 **Cyclooxygenase and prostaglandins in Leydig cells**

160 We initiated the investigation of COX expression in Syrian hamster testes, and
161 although COX1-immunoreactive cells were not detected, immunoperoxidase
162 staining revealed the presence of COX2 in the cytoplasm of interstitial cells
163 showing the characteristic punctate chromatin pattern of Leydig cells in
164 peripubertal, pubertal and adult hamster testes. Surprisingly, testicular expression
165 of COX2 was barely detectable when adult hamsters were exposed to light
166 deprivation conditions (Frungieri *et al.* 2006). Thus, although testes from regressed

167 hamsters are histologically similar to biopsies of infertile patients, they are deficient
168 in COX2 expression a typical feature of Leydig cells in the pathological human
169 testis. This discrepancy may imply that PGs play distinctly different roles in testes
170 of different species (Frungieri *et al.* 2006). Thus, COX2 and PGs may have a
171 biological relevance in the pathogenesis or maintenance of infertility states in men.
172 Conversely, considering that COX2 levels are much more abundant in Leydig cells
173 of reproductively active hamsters than in testes of photoperiodically-regressed
174 animals, we propose that PGs could act as physiological mediators involved in the
175 modulation of steroidogenic cell function in seasonal breeders.
176 In contrast to our observations in testes of reproductively active hamsters, we
177 failed to detect COX2 by immunohistochemistry in testes from other species (i.e.
178 Rhesus monkeys, pigs, BALBc mice, Wistar rats, Sprague Dawley rats) (Frungieri
179 *et al.* 2006). However, Parillo *et al.* (2011) have recently described COX
180 immunoreactivity in Leydig cells of the alpaca *Lama pacos.* Furthermore, some
181 authors (Wang *et al.* 2005, Balaji *et al.* 2007, Winnall *et al.* 2007) have reported
182 COX2 expression in mouse and rat Leydig cells using more sensitive assays such
183 as western blot, quantitative PCR and enzyme activity assays. These data allow us
184 to speculate about the existence of species-specific levels of COX2 expression in
185 Leydig cells which may be explained by the evolutionary divergence in testicular
186 coding sequences (Oduru *et al.* 2003) and/or the existence of a marked variation
187 between different species in the regulation of the hypothalamic-pituitary-testicular
188 axis by hormones and local factors (Lincoln 2000).
189 Revisiting the issue of COX2 expression in hamster Leydig cells, this isoenzyme is
190 detected mainly in pubertal and adult reproductively active hamsters with increased

191 circulating concentrations of LH, PRL and androgens (Frungieri *et al*. 2006,
192 Matzkin *et al.* 2009, Matzkin *et al*. 2012). On the other hand, in adult hamsters
193 exposed to a short-day photoperiod and also in prepubertal hamsters, testicular
194 COX2 is barely detected, coinciding with low serum concentrations of LH, PRL and
195 androgens (Frungieri *et al*. 2006, Matzkin *et al.* 2009, Matzkin *et al*. 2012). These
196 results suggest that some hormones (LH, PRL and/or androgens) could be
197 involved in the regulation of testicular COX2 expression and PG production.
198 The unique expression of PGD synthase in adult Leydig cells had already been
199 described (O'Shaughnessy *et al.* 2002, Schell *et al.* 2007). However, to our
200 knowledge, the potential role of COX2 as a marker of mature active Leydig cells
201 during cell development has not previously been suggested.
202 *In vitro* experiments performed in Leydig cells purified from reproductively active
203 adult hamsters incubated in the presence or absence of LH/hCG and testosterone,
204 and with or without the addition of bicalutamide (a pure non-steroidal antiandrogen)
205 to the incubation medium showed an up-regulation of COX2 expression and
206 PGF2α production. This LH action is not derived from a direct mechanism but
207 rather from its stimulatory role in testosterone synthesis (Matzkin *et al.* 2009). In
208 fact, testosterone effects in hamster Leydig cells are exerted via androgen
209 receptors (Matzkin *et al.* 2009). The classical mechanism of testosterone action
210 involves binding of this steroid to the cytoplasmic androgen receptor, translocation
211 of the newly formed complex into the nucleus, its binding to specific DNA
212 regulatory elements and finally, gene transcription regulation. In addition to this
213 classical pathway, there is growing evidence indicating that androgens can trigger
214 cellular processes through rapid, non-genomic mechanisms (Foradori *et al.* 2008).

215 In this context, the stimulatory effect of testosterone on COX2/PGF2α in hamster
216 Leydig cells takes place via a non-classical mechanism that involves
217 phosphorylation of the extracellular signal-regulated kinase isoforms 1 and 2
218 (ERK1/2) (Matzkin *et al.* 2009).
219 On the other hand, PRL also mediates up-regulation of COX2 expression and
220 stimulation of PGD2 and PGF2α production in hamster Leydig cells through
221 activation of p38-MAPK and JAK2 (Matzkin *et al*. 2012). Post-translational
222 modifications of the PRL molecule including glycosylation, tyrosine sulfation,
223 phosphorylation and deamination, may well represent a key mechanism for
224 creating diversity in the biological actions of this hormone (Sinha 1992). In
225 particular, pituitaries from reproductively active hamsters contain PRL charge
226 analogues with isoelectric points (pI) of 5.16, 4.61 and 4.34. The exposure of adult
227 hamsters to a short-day photoperiod of 6 h light per day results in a decline in PRL
228 pituitary levels and in the presence of less acidic PRL charge analogues with a pI
229 of 5.44. Interestingly, the more acidic PRL charge analogues present in the
230 pituitaries of reproductively active hamsters strongly induce COX2 expression in
231 hamster Leydig cells. By contrast, the less acidic analogues detected in the
232 pituitaries of regressed animals have no effect (Matzkin *et al*. 2012). The
233 stimulatory effect of more acidic PRL charge analogues on COX2 expression in
234 hamster Leydig cells takes place through a mechanism that involves the pro
235 inflammatory cytokine IL1ß (Matzkin *et al*. 2012). It has been shown that IL1ß
236 induces COX2 expression and PGD2 and PGF2α production in mouse TM3 Leydig
237 cells (Matzkin *et al*. 2010). The expression of the IL1R1 functional receptor of IL1ß

238 in Leydig cells has been described not only in rodents (hamsters and mice) but
239 also in humans (Matzkin *et al*. 2010, Matzkin *et al*. 2012).
240 The prostanoid receptors DP and FP have been described in both hamster and
241 human Leydig cells (Schell *et al*. 2007, Frungieri *et al.* 2006). Whereas PGD2 has
242 a stimulatory effect on basal testosterone production in hamster Leydig cells
243 (Schell *et al*. 2007), PGF2α exerts an inhibitory effect on the expression of the
244 steroidogenic acute regulatory (StAR) protein and the 17β-hydroxysteroid
245 dehydrogenase (17β-HSD) enzyme, as well as on the synthesis of testosterone
246 induced by hCG/LH (Frungieri *et al.* 2006).
247 It is therefore tempting to assume that, at least in hamster Leydig cells, there exists
248 a regulatory loop in which testosterone induces COX2 expression and PGF2α
249 production. In turn, PGF2α inhibits StAR and 17β-HSD expression and
250 consequently, testosterone production, thereby setting a brake on testicular
251 steroidogenesis (Fig. 5; Frungieri *et al*. 2006, Matzkin *et al.* 2009).
252 In agreement with our findings in hamsters, it has been reported that PGF2α
253 reduced hCG-stimulated testosterone secretion in rat Leydig cells (Romanelli *et al.*
254 1995). Additionally, other authors (Saksena *et al.* 1973, Didolkar *et al.* 1981,
255 Sawada *et al.* 1994) have shown that PGF2α decreases plasma testosterone
256 levels in male rats. On the contrary, injection of PGF2α in male Rhesus monkeys is
257 followed by an abrupt rise in serum testosterone (Kimball *et al.* 1979).
258 Syntin *et al.* (2001) and Wang *et al.* (2005) have described that COX2/PG system
259 represents a potential key factor in the age-related reduction in testosterone
260 production, as up-regulation of COX2 expression in Brown-Norway rats during

261 aging is accompanied by decreased testicular production of testosterone. In this
262 context, COX2 inhibition enhances steroidogenesis and StAR gene expression in
263 MA-10 mouse Leydig cells, whereas its overexpression leads to the opposite
264 (Wang *et al.* 2003). Furthermore, production of testosterone by decapsulated
265 mouse testes is significantly inhibited by adding some PGs (PGA1, PGA2, PGE1)
266 to the incubation medium (Bartke *et al.* 1976). On the other hand, COX2 seems to
267 be involved in aromatase post-translational activation and increased cell
268 proliferation in the rat Leydig tumor cell line R2C (Sirianni *et al* 2009).
269 From the aforementioned data, it is clear that Leydig cells express the inducible
270 isoenzyme COX2 and produce PGs with age-, photoperiodic- and species-specific
271 differences. In addition to its regulation by PRL and IL1ß, COX2 expression is also
272 regulated by testosterone through a non-genomic mechanism. The existence of a
273 COX2/PG system in Leydig cells serves as a local modulator of steroid hormone
274 production.
275

276 **Cyclooxygenase and prostaglandins in Sertoli cells**

277 Spermatogenesis is dependent upon adequate Sertoli cell function (Griswold
278 1998). The expression of COX, production of PGE2, PGF2α and PGI2, as well as
279 the existence of the prostanoid receptors (i.e. EP1, EP2, EP3, EP4, IP and FP) has
280 been reported in Sertoli cells of immature and juvenile rodents (Ishikawa & Morris
281 2006, Winnal *et al.* 2007, Kristensen *et al.* 2011).
282 Studies are usually limited to Sertoli cells isolated from immature rodents to avoid
283 germ cell contamination during the purification procedure. Consequently, data
284 obtained from adult Sertoli cells are scarce. Because only Sertoli cells,

285 spermatogonia and a few primary spermatocytes are seen in testes of
286 photoperiodically regressed adult Syrian hamsters (Fig. 4; Bartke 1985, Sinha
287 Hikim *et al.* 1988, Rossi *et al.* 2014), this species becomes a useful and available
288 experimental model for isolation of Sertoli cells from adult animals.
289 FSH and testosterone are the two major hormones that act in the testis to regulate
290 spermatogenesis. Sertoli cells transduce signals from FSH and testosterone into
291 the synthesis of factors that are required for spermatogenesis. These actions take
292 place through FSH and androgen receptors located in Sertoli cells (Walker &
293 Cheng 2005, Matzkin *et al.* 2009, Matzkin *et al.* 2012).
294 In recent studies performed on Sertoli cells purified from testes of adult hamsters
295 exposed to a short-day photoperiod, we demonstrated that FSH exerts a
296 stimulatory effect on COX2 expression, as well as on 15d-PGJ2 and PGF2α
297 production through a mechanism that involves ERK1/2 phosphorylation (Matzkin *et*
298 *al*. 2012). Supporting our results, Jannini *et al.* (1994) have shown FSH-stimulated
299 eicosanoid generation dependent upon activation of the COX pathway in immature
300 rat Sertoli cells. Moreover, both stimulatory and inhibitory actions of FSH on
301 ERK1/2 phosphorylation were described in rodent Sertoli cells (Crepieux *et al.*
302 2001, Meroni *et al.* 2004).
303 Testosterone also induces COX2 expression and increases 15d-PGJ2 production
304 in adult hamster Sertoli cells via androgen receptors most likely located on the cell
305 surface (Matzkin *et al*. 2012). The existence of testosterone binding sites in the
306 plasma membrane has been previously reported for Sertoli cells (Fix *et al.* 2004).
307 Using the plasma membrane-impermeable testosterone-BSA, we observed that
308 both COX2 expression and 15d-PGJ2 production are enhanced in adult hamster

309 Sertoli cells, via a non-classical androgen action associated to the activation of the
310 ERK1/2 signalling pathway (Matzkin *et al*. 2012). Supporting these data, members
311 of the MAPK pathway have been shown to form complexes with androgen
312 receptors on molecular scaffolds anchored to the plasma membrane (Pedram *et al.*
313 2007). Moreover, using an immunofluorescence technique, Cheng *et al.* (2007)
314 have found that upon testosterone stimulation of rat Sertoli cells, a population of
315 androgen receptors is localized, in a transient manner, in the plasma membrane.
316 Among Sertoli cell functions that may be important to germ cell development is the
317 provision of adequate levels of energy substrates such as lactate. In this context,
318 the transport of glucose through the plasma membrane is the rate-limiting step in
319 glucose metabolism and, consequently, in lactate production (Riera *et al.* 2001,
320 2009). Glucose enters the cell by carrier proteins called glucose transporters
321 (GLUT). So far, expression of GLUT1, GLUT3 and GLUT8 transporters has been
322 demonstrated in Sertoli cells (Carosa *et al.* 2005, Galardo *et al.* 2008). In adult
323 hamster Sertoli cells, FSH and testosterone induce the uptake of [2,6-^{3}H]-2-deoxy-
324 D-glucose, a non-metabolizable glucose analogue. In accordance with these data,
325 an increased FSH-mediated glucose uptake has been described in immature rat
326 Sertoli cells (Riera *et al.* 2001).
327 The nuclear PPARγ receptor is present in hamster Sertoli cells (Matzkin *et al*.
328 2012), suggesting a potential autocrine action of its natural ligand 15d-PGJ2. In
329 fact, 15d-PGJ2 inhibits glucose uptake in adult hamster Sertoli cells via the nuclear
330 PPARγ receptor (Matzkin *et al*. 2012). The participation of arachidonic acid,
331 precursor in PG biosynthesis, in the regulation of Sertoli cell function has recently
332 been addressed (Meroni *et al.* 2004).

333 These results therefore have led to the suggestion that testosterone and FSH
334 induce glucose uptake, COX2 expression and 15d-PGJ2 production in Sertoli cells.
335 Subsequently, 15d-PGJ2 acts via the nuclear PPARγ receptor to impair glucose
336 entry. Therefore, the COX2/15d-PGJ2/PPARγ system may serve as a local
337 autocrine modulator of Sertoli cell activity, and consequently of spermatogenic
338 efficiency (Fig. 6).
339 Harmful actions of COX/PGs have also been described in Sertoli cells. Elevated
340 testicular temperature in cryptorchidism decreases the expression of the cystic
341 fibrosis transmembrane conductance regulator (CFTR), resulting in overexpression
342 of COX2 and excessive PGE2 production in rodent Sertoli cells, which in turn leads
343 to further damage of inter-Sertoli cell tight junctions and defective spermatogenesis
344 (Chen *et al.* 2012). In contrast, toxic xenobiotics such as nonylphenol, which is
345 commonly used as a detergent, up-regulates COX2 in TM4 immature mouse
346 Sertoli cells (Liu *et al.* 2014).
347 In summary, Sertoli cells express COX2 and produce PGs in response to FSH and
348 a non-classical mechanism triggered by testosterone. PGs serve as local autocrine
349 modulators of Sertoli cell function, and thus indirectly regulate sperm maturation.
350

351 **Cyclooxygenase and prostaglandins in the seminiferous tubule wall**

352 Depending on the species, the seminiferous tubule wall can be either a simple
353 structure or a rather complex one. For instance, in rodents, the tubular wall is
354 composed of a single cell layer and a tiny extracellular matrix. However, in the
355 human testis, the seminiferous tubule wall is composed of: an internal acellular
356 basal membrane adjacent to the germinal epithelium containing collagen fibers,

357 laminin, glycoproteins and hyaluronic acid, a middle cellular zone made of spindle-
358 shaped and contractile cells (called myoid cells or myofibroblasts) and an external
359 cellular zone consisting of collagen-producing fibroblasts (Pop *et al.* 2011,
360 Mayerhofer 2013). Disturbances in testicular function and decreased or absent
361 spermatogenic activity are associated with a thickening of the seminiferous tubule
362 wall which becomes fibrotically remodeled. Fibroblasts, together with smooth
363 muscle cells, mediate tissue fibrosis and collagen deposition (Mayerhofer 2013).
364 This frequent change is observed irrespective of the causes of male infertility and
365 is regarded as a hallmark of male infertility (Frungieri *et al.* 2002a, Weinbauer *et al.*
366 2010). Different human cellular models have been used to study tubular fibrosis,
367 the involvement of the local COX/PG system and its regulation. For instance, we
368 used human fetal foreskin fibroblast cells (HFFF2) which showed increased COX2
369 protein levels, PG (PGF2α and 15d-PGJ2) production and cell proliferation in the
370 presence of the serine protease tryptase (Frungieri *et al.* 2002a). Tryptase is a
371 mast cell product known to cause proliferation of fibroblasts and fibrosis (Frungieri
372 *et al.* 2002a). The effect of tryptase was tested in HFFF2 because increased
373 numbers of tryptase-immunoreactive mast cells are detected in the seminiferous
374 tubule wall in the testes of infertile men (Meineke *et al.* 2000). Furthermore, the
375 amount of testicular tryptase-immunoreactive mast cells correlates with the fibrotic
376 thickening of the tubular wall in patients with impaired spermatogenesis or Sertoli
377 cell only (SCO) syndrome (Meineke *et al.* 2000). When the COX2 antagonist
378 meloxicam was added to the incubation media, the proliferative action of the mast
379 cell product tryptase on HFFF2 was blocked, implying that PGs derived from COX2
380 activity are crucially involved in this action. On the other hand, the nuclear PPARγ

381 receptor is expressed in the seminiferous tubule wall of infertile patients as well as
382 in HFFF2 cells, and its natural ligand 15d-PGJ2 directly increases fibroblast
383 proliferation (Frungieri *et al.* 2002a). Thus, there is a signalling pathway linked to
384 fibroblast proliferation that involves the mast cell product tryptase, its receptor
385 PAR2, induction of COX2, synthesis of 15d-PGJ2 and its action through PPARγ.
386 The initial events of the tryptase/PAR2 signalling pathway leading to COX2
387 induction and fibroblast proliferation involve up-regulation of the immediate-early
388 genes c-jun and c-fos, and phosphorylation of ERK1/2 (Frungieri *et al.* 2005).
389 It is important to bear in mind that PAR2 receptors are expressed in interstitial cells
390 while PPARγ receptors are found in the peritubular cells of the human testis.
391 Furthermore, mast cells containing tryptase accumulate in testes showing
392 abnormal spermatogenesis, and COX2 is mostly detected in biopsies of patients
393 with idiopathic infertility (Frungieri *et al.* 2002a). Thus, the fact that all components
394 involved in the tryptase/COX2/15d-PGJ2/PPARγ-induced proliferation of HFFF2
395 cells are also present in the testes of infertile patients showing fibrotic thickening in
396 the wall of the seminiferous tubules implies that COX2 and some PGs could be of
397 relevance for human diseases linked to fibrotic disorders.
398 To further explore the wall of the seminiferous tubules in health and disease, a new
399 and more reliable experimental model has recently been developed. Human
400 testicular peritubular cells were isolated from very small testicular tissue samples
401 from adult patients with obstructive azoospermia but normal spermatogenesis
402 (HTPCs), as well as from biopsies of men with non-obstructive azoospermia,
403 impaired spermatogenesis, and testicular fibrosis (HTPCFs) (Albrecht *et al.* 2006,
404 Schell *et al.* 2008, 2010, Spinnler *et al.* 2010, Mayerhofer 2013).

405 TNFα, a cytokine with pleiotrophic actions, which is known to be released from
406 human testicular macrophages (Frungieri *et al.* 2002b), induces inflammatory
407 markers in HTPCs such as COX2 and PGD2 (Schell *et al.* 2008). Previously, a
408 PGD2 system had been identified in the human testis (Schell *et al.* 2007). This
409 system includes the expression of PGD2 synthases and the existence of the
410 prostanoid receptor DP in the testes of men suffering from spermatogenic damage
411 and infertility (Schell *et al.* 2007).
412 On the other hand, 15d-PGJ2, via the generation of reactive oxygen species
413 (ROS), strongly influences cultured HTPCs and HTPCFs (Kampfer *et al.* 2012).
414 Upon 15d-PGJ2 treatment, cells become hypertrophic, and show a diminished
415 expression of smooth muscle cell markers (e.g. smooth muscle actin, MYH11,
416 calponin) as well as a reduced ability to contract. Interestingly, upon removal of
417 15d-PGJ2, cells spontaneously revert to the normal phenotype, an indication of a
418 high intrinsic degree of cellular plasticity (Schell *et al.* 2010, Welter *et al.* 2013,
419 Mayerhofer 2013). HTPCFs express higher levels of the H_2O_2-metabolizing
420 enzyme catalase than HTPCs, circumstantial evidence for increased ROS levels in
421 the tubular wall of infertility patients (Kampfer *et al.* 2012). Thus, it is possible to
422 speculate that up-regulation of COX2/15d-PGJ2 and generation of ROS are
423 interconnected events, forcing smooth muscle-like peritubular cells to adapt and
424 change their phenotype, and finally, to lose contractility (Mayerhofer 2013). Since
425 contractility of the tubular wall is crucial for sperm transport and fertility, COX2/15d-
426 PGJ2 could be, to date, an overlooked factor that contributes to male infertility.
427 Hence, results obtained from cellular studies and parallel examinations of human
428 testicular biopsies provide insights into the roles played by PGs on tubular fibrosis

429 and contractility. Consequently, PGs may be crucial factors for the active
430 transportation of immotile sperm that takes place in the seminiferous tubules.
431 Furthermore, these bioactive lipid substances might be key players in the paracrine
432 interactions taking place between peritubular cells and other testicular somatic
433 cells such as Leydig and Sertoli cells.
434

435 **Cyclooxygenase and prostaglandins in testicular immune cells**

436 The testis is one of a small number of so-called immunologically privileged tissues
437 of the body. In fact, the production, differentiation, and presence of germ cells
438 represent inimitable challenges to the immune system, because these cells appear
439 long after the maturation of the immune system and formation of systemic self-
440 tolerance (Fijak & Meinhardt 2006). The blood-testis barrier represents an essential
441 element for local immunosuppression. However, the existence of the blood-testis
442 barrier does not mean that the lymphatic drainage of the testis is deficient or that
443 immune cells are unable to access germ cells (Hedger 2002). Actually, immune
444 cells are seen in the capsule, interstitium and seminiferous tubules of the testis. In
445 particular, large numbers of macrophages are found in the testis. Significant
446 amounts of testicular mast cells, dendritic cells, as well as effector, regulatory and
447 natural killer T lymphocytes have also been reported (Itoh *et al.* 1995, Tompkins *et*
448 *al.* 1998, Meineke *et al.* 2000, Frungieri *et al.* 2002b, Hedger 2002, Jacobo *et al.*
449 2009).

450 Testicular immunoregulation depends on a delicate equilibrium between
451 immunoprivilege and inflammation in which immune cells play a dual role. Under
452 physiological conditions, antigen-specific auto immune responses are prevented by

453 systemic and local tolerance mechanisms involving the actions of dendritic cells
454 and regulatory T lymphocytes, as well as immunosuppressor cytokines mainly
455 secreted by resident macrophages. Breakdown of immune homeostasis in the
456 testis leads to inflammation (Pérez *et al.* 2013). It is known that male genital tract
457 inflammations are relevant co-factors in infertility. Human testicular macrophages
458 from infertile patients secrete pro-inflammatory cytokines such as IL1ß and TNFα
459 (Frungieri *et al.* 2002b). The number of macrophages and mast cells is markedly
460 increased in testes of patients showing impaired spermatogenesis (Meineke *et al.*
461 2000, Frungieri *et al.* 2002b). Furthermore, the distribution pattern and morphology
462 of these immune cells is altered in pathological states. For instance, there is a
463 significant shift in the location of macrophages and mast cells from the interstitium
464 to the tubular compartment in the testes of infertile men (Meineke *et al.* 2000,
465 Frungieri *et al.* 2002b). In samples with normal spermatogenesis, these immune
466 cells are round and located mainly in the interstitial spaces close to Leydig cells. In
467 pathological conditions, mast cells and macrophages are heterogeneous, with
468 rounded but also elongated shapes and signs of degranulation (Meineke *et al.*
469 2000, Frungieri *et al.* 2002b). In contrast to men, it has been described that mast
470 cells are located almost exclusively in the capsule adjacent to testicular blood
471 vessels in the testes of rodents, including hamsters (Frungieri *et al.* 1999, Rossi *et*
472 *al.* 2014).
473 COX2 is expressed in both testicular mast cells and macrophages of patients
474 suffering from hypospermatogenesis, germ cell arrest, mixed atrophy or SCO
475 syndrome (Matzkin *et al.* 2010, Welter *et al.* 2011, Rossi *et al.* 2014). Interestingly,
476 few mast cells which do not express COX2 are observed in testes with normal

477 spermatogenesis (Welter *et al.* 2011). Human testicular macrophages secrete
478 IL1ß, and a positive correlation between IL1ß levels and COX2 expression has
479 been described in the testes of infertile patients (Matzkin *et al.* 2010).
480 Thus, mast cells and macrophages increased population number, secretion of pro-
481 inflammatory cytokines and the acquisition of the capability to produce
482 prostaglandin inflammatory mediators seem to play a decisive role in the
483 autoimmune basis of testicular inflammation associated with subfertility and
484 infertility.
485

486 **Concluding remarks and future perspectives**

487 In contraposition to initial data showing that fertility is not affected in *Cox*-deficient
488 male mice (Langenbach *et al.* 1999), and therefore that PGs might not be
489 significant to testicular function, research carried out in recent years describes a
490 plethora of PG functions in the male gonad.
491 A COX2/PG system has been described in the two key somatic cell types of the
492 testis: Leydig and Sertoli cells. Furthermore, studies have provided new insights
493 into how several hormones and cytokines (i.e. FSH, PRL, testosterone, IL1ß)
494 modulate COX2 expression and PG production in Leydig and Sertoli cells. Studies
495 performed mainly in rodents indicate that some PGs (i.e. PGD2 and PGF2α)
496 modulate androgen production in Leydig cells, while 15d-PGJ2 regulates glucose
497 transport in Sertoli cells and, consequently spermatogenic efficiency. Recently, an
498 additional physiological role of COX2 as protector of germ cells against
499 spermatogenic disturbance has been reported in an experimental cryptorchidism
500 mouse model (Kubota *et al.* 2011).

501 Most importantly, besides their action on testicular physiology, PGs seem to be
502 associated to pathogenesis or maintenance of infertility states in men.
503 For instance, 15d-PGJ2 has been associated to the fibrosis and loss of contractility
504 often seen in the wall of the seminiferous tubules in patients suffering from
505 idiopathic infertility. Furthermore, the existence of a COX2/PG system in testicular
506 immune cells (mast cells and macrophages) showing a significant increase in
507 number in some pathologies, strongly suggests the importance of PGs in the
508 development of local inflammation that might further compromise testicular function
509 in patients with hypospermatogenesis, germ cell arrest or SCO syndrome.
510 Currently, the majority of infertile men present disorders either untreatable or
511 treatable with drugs of questionable effectiveness. In this context, drugs targeting
512 COX, PGs and prostanoid receptors are being developed or are already in clinical
513 use for a variety of conditions. For example, there are widely marketed and
514 relatively safe drugs such as celecoxib, valdecoxib and rofecoxib, developed for
515 specific COX2 inhibition, that possess all of the analgesic, antipyretic, and anti-
516 inflammatory activities of the older nonselective NSAIDs (Simmons *et al.* 2004).
517 Therefore, the study of COX and PG actions appears a promising field of research
518 with potential impact on male fertility. Further advances in the knowledge of the
519 role played by COX, PGs and their receptors in the human testis, as well as future
520 investigations concerning the impact of drugs targeting COX/PGs at the testicular
521 level could lead to new therapeutic approaches in idiopathic male infertility. In this
522 context, non-selective inhibitors of COX usually used as mild analgesics such as
523 indomethacin, paracetamol and aspirin have been shown to display endocrine
524 disrupting properties in the adult human testis *in vitro* (Albert *et al.* 2013).

525 Nevertheless, the beneficial or disadvantageous effects of specific COX2 inhibitors
526 in the infertile human testis have not, to date, been fully explored.
527

528 **Declaration of interest**

529 The authors declare that there is no conflict of interest that could be perceived as
530 prejudicing the impartiality of the review.
531

532 **Fundings**

533 Studies mentioned in this review were supported by grants from Consejo Nacional
534 de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de
535 Promoción Científica y Técnica (ANPCyT), Ministerio de Ciencia, Tecnología e
536 Innovación Productiva (MINCYT), Facultad de Medicina-Universidad de Buenos
537 Aires, Fundación Antorchas, and Fundación Alberto J. Roemmers of Argentina,
538 and Deutscher Akademischer Austausch Dienst (DAAD) and Deutscher
539 Akademischer Austauschdienst and Deutsche Forschungsgemeinschaft (DFG) of
540 Germany, especially MA1080/21-1 and MA1080/25-1.
541

542 **Acknowledgments**

543 We are grateful to our colleagues, Drs. Cigorraga SB, Gonzalez B, Gonzalez CR,
544 Gonzalez-Calvar SI, Levalle O, Lustig L, Parborell F, Pellizarri EH, Pomata P,
545 Ponzio R, Puigdomenech E, Rossi SP, and Terradas C (Buenos Aires, Argentina),
546 and Drs. Albrecht M, Jessberger B, Kampfer C, Köhn FM, Mayer C, Meineke V,
547 Raemsch R, Schell C, Schwarzer JU, Spillner S, Spinnler K, Vogt H-J, Weidinger
548 S, and Windschuettl S (Munich, Germany).

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846

847 **Figure legends**

848 Figure 1: Schematic representation of prostaglandin (PG) biosynthetic pathway.
849 The process is initiated by the action of the cyclooxygenase (COX) enzyme, which
850 catalyzes both the conversion of arachidonic acid into PGG2, and the subsequent
851 reduction of PGG2 to PGH2. Afterward, PGH2 is the common precursor for the
852 synthesis of the remaining major PGs.

853

854 Figure 2: Immunohistochemical images of consecutive testicular sections of a
855 patient with hypospermatogenesis immunostained for 3β-hydroxysteroid
856 dehydrogenase (3β-HSD) and cyclooxygenase 2 (COX2). Most, but not all, 3β-
857 HSD-immunoreactive Leydig cells found in the human testis are also positively
858 stained for COX2.

859 A polyclonal rabbit anti-COX2 serum (Oxford Biomedical Research, Oxford, UK,
860 1:200) and a polyclonal rabbit anti-3β-HSD serum (kindly provided by Prof. Dr. JI
861 Mason, University of Edinburgh Centre of Reproductive Biology, Scotland, 1:2000),
862 were used. Bar: 100 μm.

863

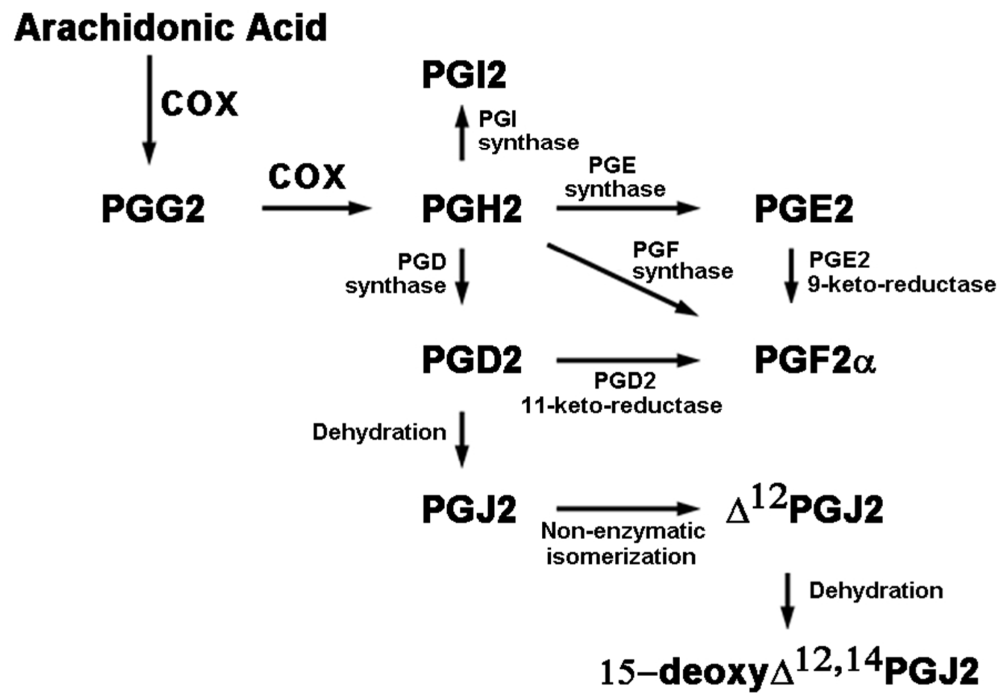
864 Figure 3: Using laser capture microdissection, androgen receptor (AR)-
865 immunoreactive peritubular (A) and Sertoli (B) cells were isolated from a testicular
866 biopsy of a patient suffering from germ cell arrest, and then subjected to RT-PCR
867 studies.

868 (A) Each panel depicts the same specimen before laser microdissection (left), after
869 UV-laser delimitation of AR-immunoreactive peritubular cells (middle), and after IR-

870 laser microdissection (right) of target cells. A polyclonal rabbit anti-AR serum
871 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:200) was used. Bar, 50
872 μm.
873 (B) Each panel depicts the same specimen before laser microdissection (left), after
874 UV-laser delimitation of AR-immunoreactive Sertoli cells (middle), and after IR-
875 laser microdissection (right) of target cells. A polyclonal rabbit anti-AR serum
876 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:200) was used. Bar, 50
877 μm.
878 (C) COX2 mRNA expression was detected in human peritubular and Sertoli cells
879 by RT-PCR assays performed using oligonucleotide primers from the reference
880 Matzkin *et al.* (2010). PCR products were separated on 2% agarose gels and
881 visualized with ethidium bromide. The identity of the cDNA products was confirmed
882 by sequencing, using a fluorescence-based dideoxysequencing reaction and an
883 automated sequence analysis on an ABI 373A DNA sequencer.
884

885 Figure 4: Testicular morphology in Bouin's fluid fixed and haematoxylin stained
886 cross sections of a patient suffering from hypospermatogenesis (A) and a
887 reproductively regressed adult hamster (B).
888 Sertoli cells (black arrows), spermatogonia (white arrows), spermatocytes (black
889 arrowheads) and prematurely detached spermatocytes (white arrowheads) are
890 shown. Bar, 50 μm.
891

892 Figure 5: Summary view of COX2 expression/PGs synthesis regulation, and the
893 modulatory effect of some PGs on steroidogenesis in hamster Leydig cells.
894 Based on experimental results, PRL induces COX2 expression as well as PGD2
895 and PGF2α production in Leydig cells through activation of p38-MAPK and
896 JAK2/STAT5. In addition, testosterone (T) via androgen receptors and a non-
897 classical mechanism that involves phosphorylation of ERK1/2 also increases
898 COX2 expression and PGs production.
899 While PGD2 through DP receptors stimulates testosterone (T) production under
900 basal conditions, PGF2α via FP receptors inhibits StAR and 17β-hydroxysteroid
901 dehydrogenase (17β-HSD) expression and consequently testosterone production in
902 the presence of LH/hCG, thus setting a brake on testicular steroidogenesis.
903
904 Figure 6: Summary view of COX2 expression/PGs production regulation, and the
905 signalling pathway involved in the PG modulation of glucose uptake in Sertoli cells.
906 Based on experimental results, testosterone (T) exerts a stimulatory effect on
907 COX2 expression and 15d-PGJ2 production in Sertoli cells through a non-classical
908 mechanism that involves the presence of androgen receptors (AR) and ERK1/2
909 activation. FSH also stimulated COX2/PGs via ERK1/2 phosphorylation.
910 FSH and testosterone (T) stimulate glucose uptake in Sertoli cells. Nevertheless,
911 these hormones also exert an indirect negative regulation on glucose uptake which
912 involves the COX2/15d-PGJ2/PPARγ system.
913



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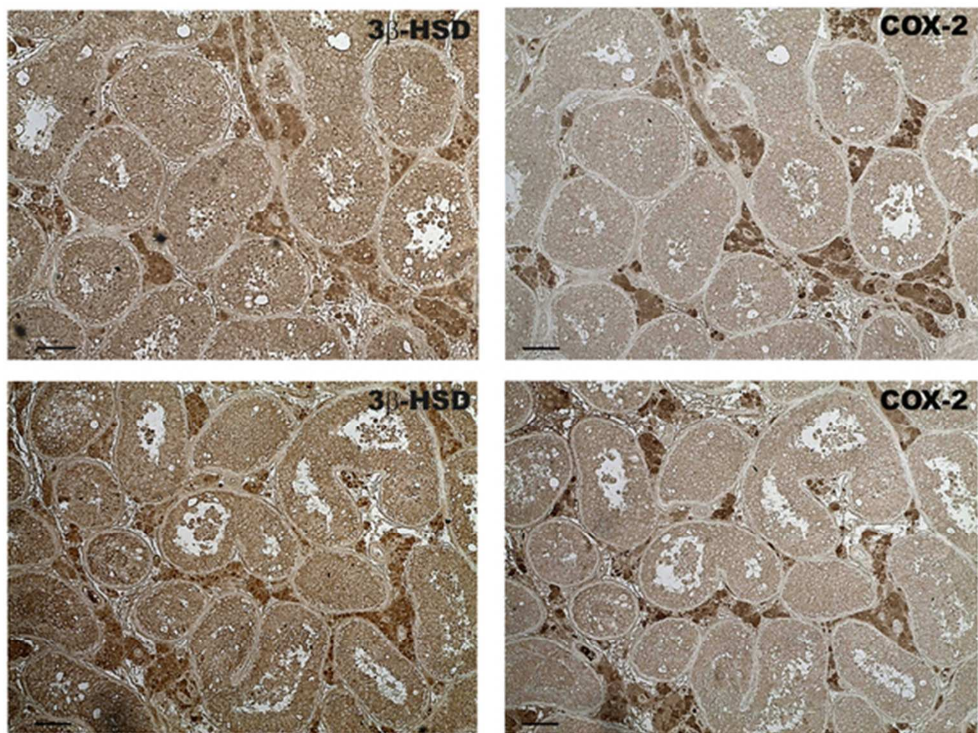

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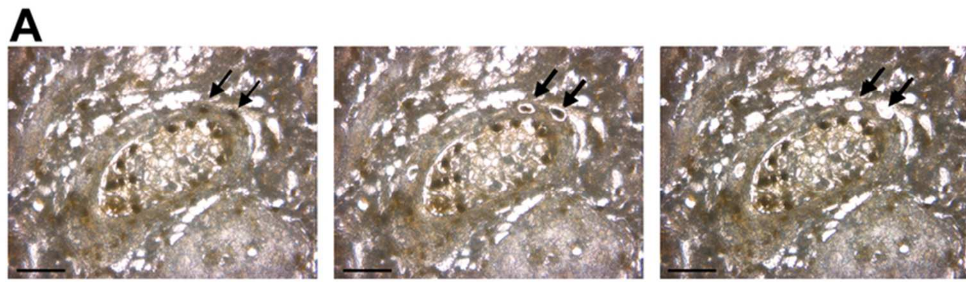

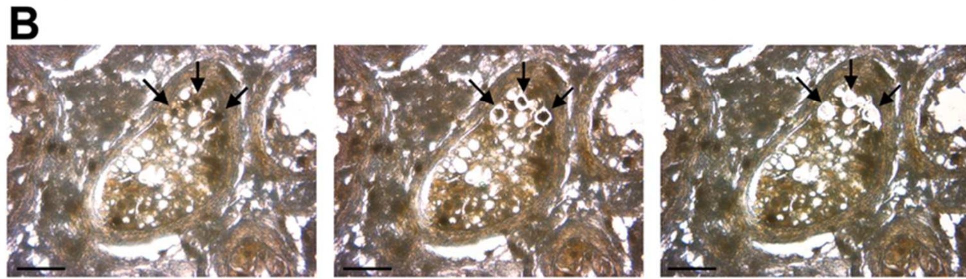

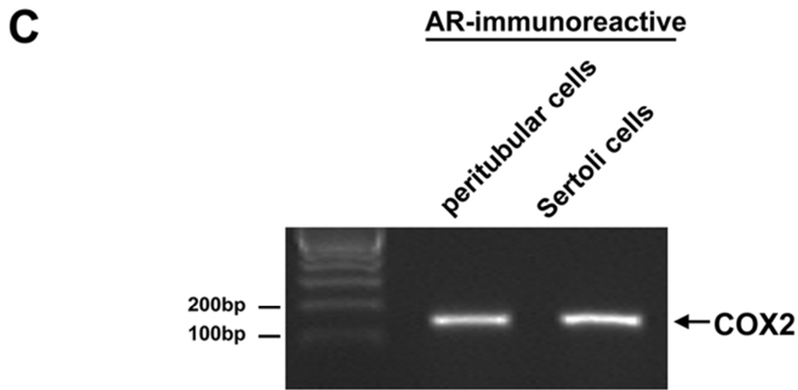

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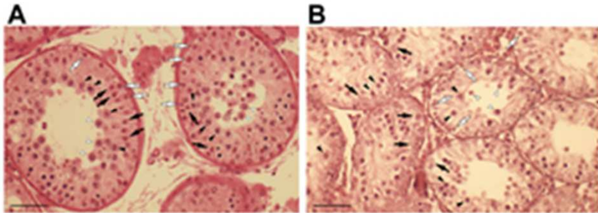

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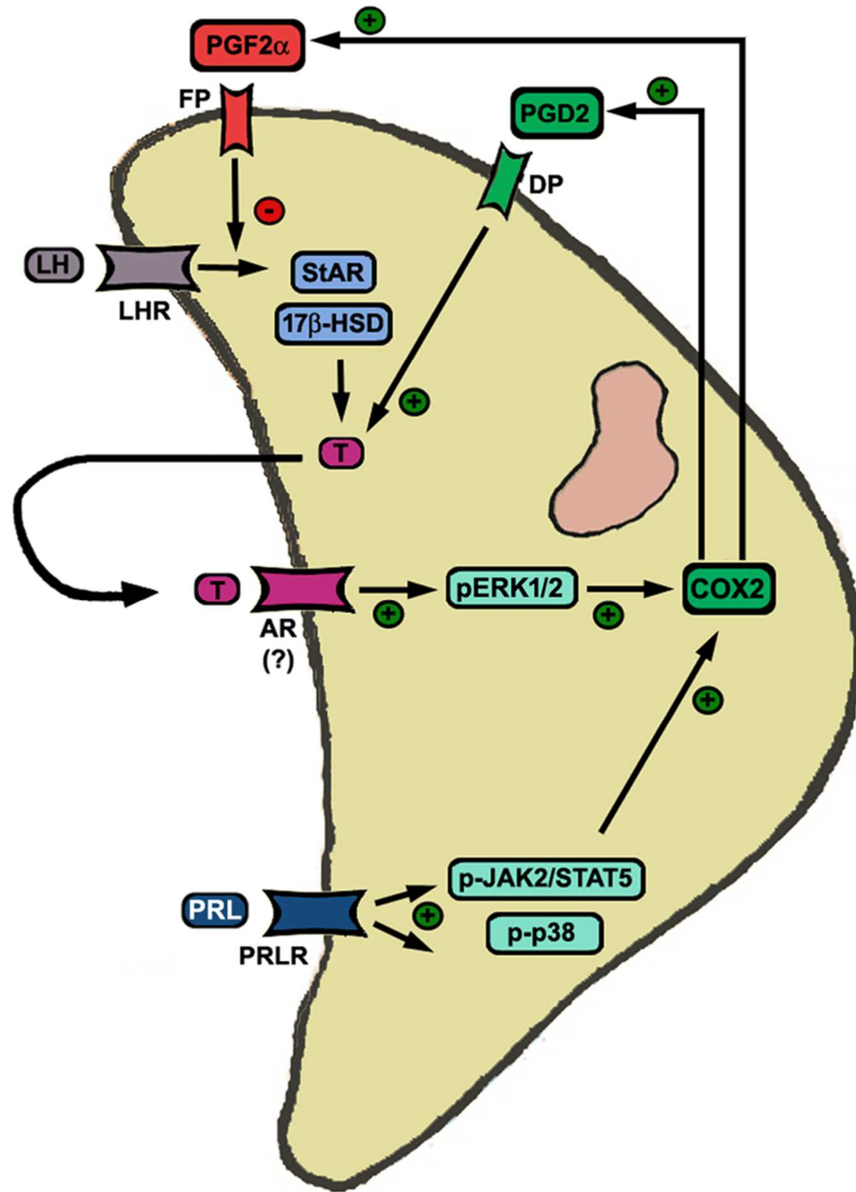

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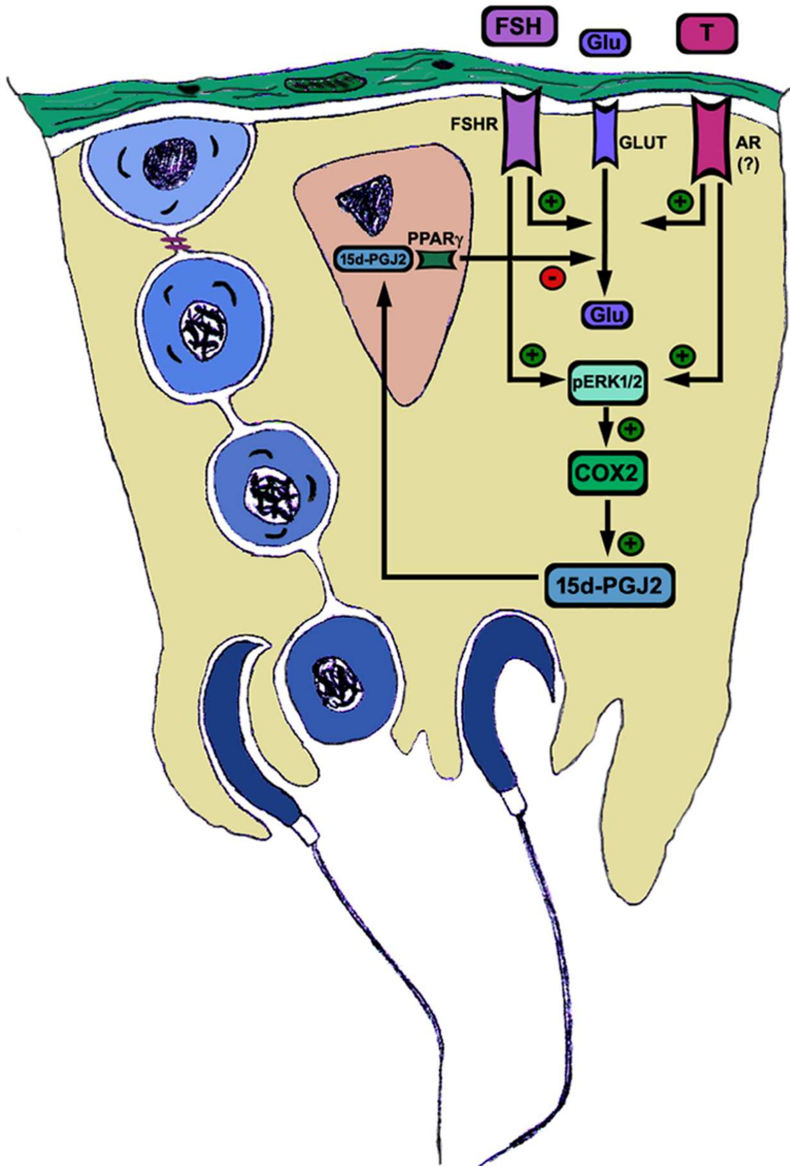

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