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RESEARCH

TNFRp55 deficiency promotes the development of ectopic endometriotic-like lesions in mouse

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Abstract

Endometriosis is an inflammatory disease depending on estradiol, with TNF-α being one of the most representative cytokines involved in its pathogenesis. TNF-α acts through its bond to the TNFRp55 and TNFRp75 membrane receptors. The aim of the present study was to analyze the effect of the TNFRp55 deficiency on the development of ectopic endometriotic-like lesions. Endometriosis was induced surgically in mice of the C57BL/6 strain, wild type (WT) and TNFRp55-/- (KO). After four weeks, the peritoneal fluid was collected and the lesions were counted, measured with a caliper, removed, weighed, fixed or kept at -80°C. We evaluated the cell proliferation by proliferating cell nuclear antigen (PCNA) immunohistochemistry and apoptosis by TUNEL technique in the ectopic lesions. MMP-2 and MMP-9 activities (factors involved in invasiveness) were measured by zymography in the peritoneal fluid; estradiol and progesterone levels were measured by radioimmunoassay in the lesions and in the peritoneal fluid. We found that in KO animals the mean number of lesions established per mouse, the lesion volume, weight and cell proliferation increased and apoptosis decreased. In addition, the activity of MMP-2 and the estradiol level increased, whereas the progesterone level was not significantly modified. In conclusion, the deficiency of TNFRp55 promoted the establishment and development of endometriosis through an increase in the lesion size and high levels of estradiol which correlate with an increase in the MMP-2 activity. This is evidence of the possible association of the deregulation of the TNFRp55 expression and the survival of the endometriotic tissue in ectopic sites.

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3

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20

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25 **Abstract**

26 Endometriosis is an inflammatory disease depending on estradiol, with TNF-α being one of
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29 analyze the effect of the TNFRp55 deficiency on the development of ectopic endometriotic-
30 like lesions. Endometriosis was induced surgically in mice of the C57BL/6 strain, *wild type*
31 (WT) and TNFRp55-/- (KO). After four weeks, the peritoneal fluid was collected and the
32 lesions were counted, measured with a caliper, removed, weighed, fixed or kept at -80°C. We
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34 immunohistochemistry and apoptosis by TUNEL technique in the ectopic lesions. MMP-2
35 and MMP-9 activities (factors involved in invasiveness) were measured by zymography in the
36 peritoneal fluid; estradiol and progesterone levels were measured by radioimmunoassay in the
37 lesions and in the peritoneal fluid. We found that in KO animals the mean number of lesions
38 established per mouse, the lesion volume, weight and cell proliferation increased and
39 apoptosis decreased. In addition, the activity of MMP-2 and the estradiol level increased,
40 whereas the progesterone level was not significantly modified. In conclusion, the deficiency
41 of TNFRp55 promoted the establishment and development of endometriosis through an
42 increase in the lesion size and high levels of estradiol which correlate with an increase in the
43 MMP-2 activity. This is evidence of the possible association of the deregulation of the
44 TNFRp55 expression and the survival of the endometriotic tissue in ectopic sites.

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48

49 **Introduction**

50 Endometriosis is a chronic disease, depending on estrogens, that affects women at
51 reproductive age, causing inflammation, intense pelvic pain and reduced fertility. It is
52 characterized by the implantation and growth of endometrial tissue outside the uterine cavity
53 (Greene *et al*. 2016). Sampson´s theory, which states that epithelial and stromal cells reach the
54 peritoneal cavity through late menstruation through the fallopian tubes and are then spread
55 and implanted in the peritoneal cavity, is the mostly accepted mechanism to explain its
56 etiology (Sampson 1927). However, this theory does not explain why more than 90% of
57 women with late menstrual bleeding do not develop endometriosis, which suggests that there
58 are genetic, immunological and/or biochemical factors which contribute to the development
59 of the disease (Ahn *et al*. 2015). A significant component in the pathogenesis of
60 endometriosis is the loss of normal patterns of communication between the endocrine and
61 immune systems. It is known that the endometrial cells show reduced their capacity of death
62 by apoptosis and increased their invasive capacity associated to hormonal alterations. These
63 changes promote the resistance of the endometrial cells to the normal cleaning of the
64 peritoneum by the immune system, responsible for the regulation of tissue homeostasis
65 (Kitawaki *et al.* 2002, Antsiferova & Sotnikova 2012).

66 Tumour necrosis factor-alpha (TNF-α), has an important function in the physiology of the
67 proliferation and desquamation of the endometrium during the menstrual cycle. In addition, it
68 plays a crucial role in inflammation, angiogenesis, cell proliferation and cell death. TNF-α
69 acts on the target cells via two receptors: TNFRp55 (type 1) and TNFRp75 (type 2).
70 TNFRp55 is characterized by a death domain in its intracellular region, whereas type 2
71 receptor lacks such domain. Thus, the activation of TNFRp55 leads primarily to pro-
72 inflammatory and programmed cell death pathways (Rojas-Cartagena *et al*. 2005). In

73 endometriosis, there is evidence that indicate an aberrant function of the TNF system. For
74 example, the levels of TNFRp55 expression in the endometrium of women with
75 endometriosis during the late secretory phase, stage related to the apoptosis, were lower in
76 relation to the endometrium without endometriosis, in coincidence with the almost absent
77 apoptosis in the eutopic and ectopic endometrium (Boric *et al.* 2013). In addition, in a recent
78 study that examined the serum levels of the soluble forms of both TNFRp55 and TNFRp75,
79 has been found that the first is significantly higher in serum of endometriosis patients than
80 controls and given that these soluble forms can modulate the effects of TNF by acting as
81 antagonists (Othman *et al.* 2016), this may also be involved in the altered cell death observed
82 in this pathology.

83 On the other hand, the metalloproteinases (MMPs) are essential factors in the processes of
84 invasiveness and tissue remodeling, which are secreted as latent pro-enzymes and activated by
85 proteolytic cleavage (Di Carlo *et al.* 2009). Gelatin is an important protein of the extracellular
86 matrix and it is sensitive to a high range of tissue proteinases, including MMP-2 and MMP-9
87 gelatinases (Jana *et al*. 2016, Pan *et al.* 2016). These enzymes participate in the physiological
88 cyclic changes of the endometrium, and several studies have shown that their steroid
89 regulation is critical for the formation of the fragments of the endometrial tissue in ectopic
90 sites (Bruner *et al.* 1997, Pitsos *et al.* 2009). In relation to this, there exists a correlation
91 between the levels of MMP-2 and estradiol in the peritoneal fluid of patients with
92 endometriosis (Huang *et al*. 2004).

93 In turn, steroid hormones play an important role in the development of this pathology.
94 Endometriosis seems to be associated to a decrease in the response capacity of the
95 endometrial stromal cells to progesterone, which may be due to a decrease in the expression
96 of the progesterone receptors (PR). A recent study demonstrates that inflammatory cytokines

97 like TNFα reduce the expression of PR. Thus, this effect may contribute to the progesterone
98 resistance of women with endometriosis, which in turn is associated with hyperactive action
99 of estradiol (Bulun *et al.* 2006, Li *et al.* 2016, Grandi *et al.* 2016). The proinflammatory and
100 antiapoptotic effects of estradiol in endometrial cells appear to be exacerbated in women with
101 endometriosis (Reis *et al.* 2013).
102 In view of all the above, the aim of the present study was to analyze the effect of the
103 TNFRp55 deficiency on the establishment and growth of endometriotic-like lesions in an
104 induced endometriosis model in mouse. In addition, we evaluated cell proliferation and
105 apoptosis of endometriotic cells as well as factors related to invasiveness and endocrine
106 status.
107
108 **Materials and methods**
109 **Animals**
110 Female mice of the C57BL/6 strain, *wild type* (WT) and TNFRp55-/- (KO) of two months,
111 weighing 19-21 g were used. The TNFRp55-/- mice were obtained from the Max von
112 Pettenkofer Institute, Munich, Germany. Breeding colonies were established at the Animal
113 Facility of the National University of San Luis (San Luis, Argentina) under rigorous light
114 conditions (12 h light, 07:00-19:00, and 12 h darkness), controlled temperature (22±2ºC), with
115 *ad libitum* water and sterile food. The experiments were carried out according to the rules for
116 the care and use of laboratory animals of the National Institutes of Health (NIH, USA) and the
117 *Comité Institucional de Cuidado y Uso de Animales de Experimentación* (CICUA) of the
118 National University of San Luis, Argentina.
119
120

Surgical induction of endometriosis

The endometriotic-like lesions were induced experimentally, as reported previously (Bilotas *et al.* 2010). The animals were anaesthetized via intraperitoneal with 100mg/kg of ketamine (Holliday Scott, Buenos Aires, Argentina) and 10 mg/kg of xylazine (Richmond, Buenos Aires, Argentina), and a mid-ventral incision was made to expose the bowels. The right uterine horn is removed from the animal, placed in DMEM-F12 (Gibco, USA) and divided longitudinally. It is later cut in three square pieces of approximately $4mm^2$ which are sutured to the intestine mesentery with only one stitch (supralong 6-0, Ethicon, NJ, USA). The area is hydrated with sterile physiological solution supplemented with antibiotic-antimycotic before closing the abdominal wall with the same suture material, with continuous stitches. The mice are monitored daily in relation to body weight, food consumption, preening behavior and activity. The mice were sacrificed by cervix dislocation after four weeks. Then, a small medioventral hole was opened through which 1.5 ml of PBS was injected in the peritoneal cavity of each animal, and the peritoneal fluid was collected and centrifuged at 10.000 g for 1 minute. The supernatant was separated from the precipitate and kept at -80°C until the corresponding determinations. After that, the abdomen was completely opened to have access to the endometriotic-like lesions.

Evaluation of the ectopic uterine tissue

The lesions were identified, counted and measured with caliper in two perpendicular diameters. The volume of the developed lesions was calculated with the following equation: $V=(4/3)\ \pi\ r_1^2\ r_2$ (r_1 and r_2 are the radiuses and $r_1<r_2$). The lesions were removed, weighed and kept in as follows: one lesion per animal was fixed in buffer formaldehyde at 4% for 24h at 4°C. The fixed specimens are embedded in paraffin, cut in 5µm sections and stained with

145 hematoxylin-eosin in order to examine microscopically the presence of histological identity
146 signals of endometriosis (glands and stroma), or prepared for immunohistochemical technique
147 and/or TUNEL. The remaining lesions were kept at -80°C for the rest of the determinations.
148
149 **Immunohistochemistry**
150 Proliferating cell nuclear antigen (PCNA), also called cyclin, is a 36-KD auxiliary protein of
151 DNA polymerase-delta that has been found to be a useful marker in immunocytochemical
152 studies of cell proliferation because its expression correlates with the proliferative state of the
153 cell (Bravo *et al*. 1987). All sections were deparaffinized in xylene and rehydrated through
154 graded alcohols. Endogenous peroxidase was blocked by treatment with 3% H_2O_2 for 30 min,
155 followed by microwaving in 0.01M sodium citrate buffer for antigen retrieval. All the sections
156 were then blocked with 4% BSA in PBS for 2h at room temperature and incubated overnight
157 at 4°C with the anti-mouse PCNA rabbit polyclonal antibody (1:200, FL-261, Santa Cruz
158 Biotechnology Inc., Santa Cruz, CA, USA) in PBS with 1% BSA at room temperature. After
159 that, sections were incubated for 1h at room temperature with 1:200 goat biotinylated anti-
160 rabbit IgG antibody (Sigma-Aldrich, St Louis, MO, USA) followed by incubation with a
161 streptavidin-peroxidise conjugate (VectorLabs, Burlingame, CA, USA) for 30 min at room
162 temperature. The signal was developed with diaminobenzidine (DAB) as substrate (Cell
163 Marque, CA, USA), and finally, the sections were counterstaining with Gill´s hematoxylin,
164 dehydrated through grades alcohols, clarified in xylene, and properly mounted. As a negative
165 control, one section of each slide was assayed without the primary antibody. PCNA positive
166 cells were identified by the presence of brown nuclear reactivity. The percentage of PCNA
167 positive cells was established using a standard light microscope at 400X. Quantification of
168 cell proliferation was performed by counting a minimum of 100-150 randomly selected

169 epithelial and stromal cells per field and independently of the number of cells, 4-6 random
170 fields per section were counted. The percentage of proliferating cells was calculated on the
171 total and these percentages were then used to obtain the mean of each experimental group.
172
173 **TUNEL assay**
174 Fragmented DNA of apoptotic cells was detected using In Situ Cell Death Detection Kit POD
175 TUNEL assay (Cat N° 11684817910 Roche, Basel, Switzerland), according to the
176 manufacturer's instructions. The detection was achieved using the peroxidase substrate,
177 hydrogen peroxide, and the stable chromogen DAB. Using this procedure, apoptotic nuclei
178 are stained dark brown. Finally, sections were counterstained with hematoxylin, mounted and
179 analyzed in a light microscope. The number of apoptotic nuclei relative to total cells was
180 determined by counting 150 randomly selected epithelial and stromal cells per field and 5
181 random fields per section were counted. The percentage of apoptotic cells was calculated on
182 the total and these percentages were then used to obtain the mean of each experimental group.
183
184 **Gelatin Zymography**
185 MMP-2 and MMP-9 enzymatic activity was determined by SDS-PAGE gelatin zymography.
186 The samples, normalized in the same quantity of proteins, were mixed with the same volume
187 of the sample buffer (0.3 M Tris-HCl pH 6.8, 2% SDS, 40% glycerol and 0.1% Bromophenol
188 blue) and incubated for approximately 30 minutes at 37°C. Then they were subjected to
189 electrophoresis in polyacrylamide gels at 10% (SDS-PAGE) containing 0.2% of gelatin
190 (Merck, USA), under non reducing denaturalizing conditions at 4°C. Once electrophoresis
191 was finished, the gels were washed with 2.5% of TritonX-100 (v/v) in buffer TNC (50 mM
192 Tris-HCl pH 7.5, 0.5 M NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3), twice for 15 minutes to

193 remove the SDS and thus allow the proteinases to recover their activity. After washing, the
194 gels were incubated in TNC buffer with 1% TritónX-100 for 24 h at 37°C. Finally, the
195 coloration with a 0.5% of Coomassie brilliant blue R-250 solution was carried out with the
196 subsequent decoloration. The MMPs activity was evaluated observing the lysis zones. The
197 bands intensity was determined by densitometry using ImageJ software and was expressed in
198 arbitrary units.
199
200 **Radioimmunoassay (RIA)**
201 Samples of peritoneal fluid supernatant and homogenates of endometriotic lesions were used
202 to measure progesterone and estradiol levels using a RIA kit (Beckman Coulter and
203 DIAsource, respectively, DiagnosMed SRL, Buenos Aires, Argentina) following the
204 manufacturer's instructions. The assays sensitivity was 0.05ng progesterone/ml and < 2.7pg
205 estradiol/ml. The inter- and intra-assay coefficients of variation in all the assays were <10.0%.
206
207 **Western blot**
208 Protein extracts were obtained using TRIzol reagent and following the manufacturer's
209 indications (Invitrogen Life Technologies, Buenos Aires, Argentina). Protein concentration
210 was determined by Lowry method. Aliquots containing 40μg of total protein were subjected
211 to electrophoresis in 10% SDS-PAGE gels and then electrotransferred to PVDF membrane
212 (Millipore Corporation, Bedford, MA, USA) at 100V for 1h in a transfer buffer (25mM Tris,
213 192mM glycine and 20% v/v methanol, pH 8.3). The membrane was immersed in 5% non-fat
214 milk in a PBST solution [KH_2PO_4 0.015M, NaH_2PO_4 0.017M, KCl 0.076M, NaCl 0.14M (pH
215 7.4), 0.5% Tween-20] for 1h at room temperature, followed by an overnight incubation at 4°C
216 with either rabbit anti-P450aromatase (SC-30086) or goat anti β-actin (SC-1615, Santa Cruz

217 Biotechnology Inc, CA, USA), 1:1000 dilution in 1% solution of non-fat powdered milk in
218 PBST. After incubation with primary antibody, membranes were washed in PBST and
219 incubated with donkey anti-goat IgG peroxidase-linked antibody (Cat sc-2020, Santa Cruz
220 Biotechnology Inc, CA, USA) 1:5000 dilution in 1% milk for 1h at room temperature and
221 goat anti-rabbit IgG peroxidase-linked antibody (Cat: sc-2004, Santa Cruz Biotechnology Inc,
222 CA, USA) 1:5000 dilution in 1% milk for 3h at room temperature, respectively. Following
223 washing in PBST, blots were developed using an enhanced chemiluminescence Western
224 blotting detection system Thermo Scientific Super signal West Pico chemiluminescence
225 (Pierce Biotechnology, Rockford, IL, USA) and exposed to X-ray films Thermo Scientific
226 CL-XPosureTM Film (Pierce Biotechnology, Rockford, IL, USA). The mean of intensity of
227 each band was measured using the NIH ImageJ software (Image Processing and Analysis in
228 Java fromhttp://rsb.info.nih.gov/ij/). P450aromatase (P450arom) protein levels were
229 normalized against β-actin (endogenous control).
230
231 **RNA isolation and RT-PCR analysis**
232 Total RNA was isolated from endometriotic lesions using TRIzol Reagent (Invitrogen Life
233 Technologies, Buenos Aires, Argentina), according to the manufacturer's instructions.
234 Purified total RNAs were then quantified and assessed for purity by measurement of the
235 260/280 ratio using an UV spectrophotometer Beckman DU-640 B (CA, USA). Only samples
236 with 260/280 ratio of 1.8 to 2.0 were used. The integrity was confirmed by running 2μg RNA
237 on a 0.8 % agarose gel. After GelRed™ (Biotium, Hayward, CA, USA) staining RNA bands
238 were visualized with a UV transilluminator and 28S and 18S rRNA band patterns were
239 analyzed. Two micrograms of total RNA were reverse transcribed at 37°C using random
240 primers and M-MLV Reverse Transcriptase (Promega Inc., Buenos Aires, Argentina) in a

241 26μl reaction mixture. For amplification of the reverse transcription (RT) products, the
242 reaction mixture consisted of 1× Green Go Taq reaction buffer, 0.2mM deoxynucleoside
243 triphosphates, 0.5μM specific oligonucleotide primers and 1.25U Go Taq DNA polymerase
244 (Promega Inc. Buenos Aires, Argentina) in a final volume of 50μl. The PCR primers were
245 designed using Primer Express 3.0 software (Applied Biosystems, USA). The primers
246 information is shown in Table 1. The amplification of the cDNA was performed using a
247 thermalcycler (My Cycler, BioRad, Buenos Aires, Argentina). Reaction products were
248 electrophoresed on 2% agarose gels, visualized with GelRed, and examined by ultraviolet
249 transillumination. Band intensities of RT-PCR products were quantified using ImageJ (Image
250 Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). Relative levels of mRNA
251 were expressed as the ratio of signal intensity for the target genes relative to that for the
252 housekeeping gene β-actin.
253
254 **Statistical analysis**
255 Statistical analysis was performed using GraphPad Prism (Version 5, GraphPad Software Inc.
256 San Diego SA).Values are presented as the mean ± SEM. Differences between the means of
257 each group were analyzed using the Student´s *t*-test. Pearson's correlation coefficient was
258 used to evaluate the relationship between estradiol levels and MMP-2 activity in peritoneal
259 fluid. Differences were considered to be statistically significant when $P<0.05$.
260
261
262
263
264

265 **Results**

266 **Effect of the TNFRp55 deficiency on the endometriotic-like lesions establishment and**
267 **growth**

268 The macroscopic evaluation of the ectopic uterine tissue revealed that the number of
269 established lesions increased in animals with receptor deficiency (P<0.05) (Fig. 1A). In
270 addition, we observed that the lesions volume and weight were higher in KO animals than in
271 WT animals (P<0.05 and P<0.001, respectively) (Fig. 1B and C). These results show the
272 relevance of TNFRp55 in the development of the ectopic endometrial tissue.

273

274 **Effect of the TNFRp55 deficiency on the cell proliferation and apoptosis in**
275 **endometriotic-like lesions**

276 The TNFRp55 deficiency caused an increase in the percentage of cell proliferation (P<0.01)
277 (Fig. 2A) and a decrease in the percentage of apoptosis cells (P<0.001) (Fig. 2B). There were
278 no significant differences between epithelial and stromal cell. These results suggest that the
279 increase in the volume and weight of the lesions in the KO animals might be associated to the
280 low elimination rate and high proliferation rate of the endometriotic cells.

281

282 **Effect of TNFRp55 deficiency on the factors regulating invasiveness**

283 We analyzed the enzymatic activity of MMP-2 and MMP-9 in homogenates of endometriotic
284 lesions and peritoneal fluid, and although no significant changes were observed in the lesion
285 in both experimental groups (Fig. 3A), in peritoneal fluid, the activity of MMP-2 (P<0.001)
286 and its zymogene increased significantly in the KO animals (P<0.01) (Fig. 3B). These results
287 suggest that the TNFRp55 deficiency modifies the peritoneal environment, which contributes
288 to the remodeling and establishment of lesions.

289 **Effect of the TNFRp55 deficiency on the steroid hormones levels**

290 In the KO animals, the levels of estradiol increased in the peritoneal fluid and in lesion
291 (*P*<0.05) (Fig. 4A and B) whereas the expression, analyzed in ectopic uterine tissue, (mRNA
292 and protein) of P450arom, synthesis enzyme, was not significantly modified (Fig. 4C and D).
293 These results indicate the close relationship between estradiol and TNFRp55. Pearson's test
294 was applied to determine the possible correlation between estradiol levels and MMP-2
295 activity in peritoneal fluid samples. For the entire group of mice (n=16), positive correlation
296 was found (coefficient (r) Pearson: 0.67; *P*<0.01). While, there was no correlation in the wild
297 type mice (n=8) and deficient mice (n=8) (Fig. 5).

298 We also analyzed the effect of TNFRp55 deficiency on the progesterone levels in peritoneal
299 fluid and homogenates of endometriotic lesions, the expression of the metabolic enzymes and
300 progesterone receptors (PR). In KO animals, the levels of progesterone were not significantly
301 modified (Fig. 6A and B), whereas the expression (mRNA) of 3β-HSD, synthesis enzyme,
302 and of 20α-HSD, degradation enzyme, decreased in endometriotic tissue (*P*<0.01 and
303 *P*<0.005, respectively) (Fig. 6C); therefore, the hormone levels were not modified. To
304 determine if the TNFRp55 deficiency modifies the action sites of progesterone, we analyzed
305 the expression (mRNA) of the progesterone receptors PR A and PR B, but no changes were
306 observed (Fig. 6D).

307

308 **Discussion**

309 Extensive cell proliferation, tissue remodelling and aberrant apoptosis occur at the ectopic
310 sites where endometrial tissue deposits develop into endometriotic lesions. In addition, there
311 is evidence of an aberrant function of the TNF system in this pathology. In the endometrium
312 of control women, the highest levels of TNF and TNFRp55 are produced during the late

313 secretory phase. This phase is related to the apoptosis, which is the physiological process
314 involved in the menstrual shedding in a non-conceptional cycle. On the contrary, the levels of
315 TNFRp55 expression in the endometrium of women with endometriosis during the late
316 secretory phase are lower in relation to the endometrium without endometriosis. This is in
317 coincidence with the almost absent apoptosis in the eutopic and ectopic endometrium, which
318 might favor the survival and growth of the menstrual debris outside the uterus (Rojas-
319 Cartagena *et al*. 2005, Boric et al. 2013). Considering that the abnormal survival of
320 endometrial cells may result in their continuing growth into ectopic locations and that
321 TNFRp55 plays a rol in triggering apoptosis, in the present study we selected TNFRp55 as
322 molecular target and we investigate the effect of TNFRp55 deficiency on the development of
323 endometriotic-like lesion in a murine model. One of the major limitations of mouse model is
324 the lack of menstruation and subsequent spontaneous endometriosis. However, this model has
325 many similarities to the disease in humans, such as the growth of ectopic endometrial tissue
326 estrogen-dependent. In addition, it is a versatile model that has been used to investigate the
327 mechanisms involved in the peritoneal attachment of endometrial cells, how the immune
328 system and hormones affect endometriosis as well as the effects of drugs and therapeutic
329 products (Grümmer 2006).
330 The obtained results show that the TNFRp55 deficiency promoted the establishment and the
331 growth of endometriotic lesions associated to a high rate of cell proliferation and a low rate of
332 apoptosis. Altogether, these data constitute strong evidence of the association of the
333 deregulation of the TNFRp55 expression and the survival of the endometriotic tissue in
334 ectopic sites.
335 The MMPs have a key role in the pathogenesis of endometriosis. Thus, in a model of induced
336 endometriosis in mouse, it has been demonstrated that the activity suppression of these

337 enzymes leads to inhibition in the endometriosis progression with the subsequent decrease in
338 the weight of the endometriotic-like lesions (Bruner *et al.* 1997). In addition, increased levels
339 of MMP-2 and MMP-9 have been detected in peritoneal fluid of patients with endometriosis
340 (Amălinei *et al.* 2010). In our study, we detected an increase of the enzymatic activity of
341 MMP-2 in the peritoneal fluid of the KO mice, which indicates that the TNFRp55 deficiency
342 modifies the peritoneal environment contributing to the lesions remodeling and establishment.
343 In turn, several studies have shown that the steroid regulation of MMP is critical for the
344 formation of the fragments of the endometrial tissue in ectopic sites. In relation to this, there
345 exists a correlation between the levels of MMP-2 and estradiol in the peritoneal fluid of
346 patients with endometriosis (Huang *et al.* 2004). In agreement with this, we also observed the
347 presence of high levels of estradiol that correlate with an increase in MMP-2 activity, which
348 might favor the establishment and development of endometriotic lesions. It is important to
349 emphasize that the TNFRp55-/- mice lack expression of TNFRp55 but display normal
350 numbers of high affinity TNFRp75 molecules (Pfeffer *et al.* 1993). In surgically induced
351 endometriosis in rat and mouse, the treatment with etanercept, a fusion protein consisting of
352 human recombinant soluble TNFRp75 conjugated to a human Fc antibody subunit, was
353 associated with negative immunohistochemical staining for TNFRp75 and with reduced
354 endometriosis development (Islimye *et al.* 2011, Liu *et al*. 2016). These data indicate that the
355 lack of activity of the TNFRp75, a receptor associated mainly with proliferation, cell survival
356 and angiogenesis (Haider *et al*. 2009, Cabal-Hierro & Lazo 2012) suppresses the development
357 of endometriosis and that the effects observed in TNFRp55 deficient animals of the present
358 study may be due to activation of TNFRp75 dependent pathways.
359 Specifically in relation to the steroid hormones and endometriosis, estradiol is a factor
360 favoring its development while the role of progesterone seems to be attenuating. The

361 TNFRp55 deficiency did not modify either the levels of progesterone or its action sites. On
362 the other hand, we observed that the TNFRp55 deficiency caused a strong effect on the
363 estradiol levels, which suggests the close relationship between estradiol and TNFRp55. It is
364 well known that endometriotic lesions have the capacity to produce estradiol since they
365 express the complete set of steroidogenic enzymes (Kianpour *et al*. 2015). Among these
366 enzymes, P450arom plays an important role in endometriosis (Bulun *et al*. 2000, Bilotas *et al*.
367 2010). However, in our experimental model, this enzyme was not modified by the TNFRp55
368 deficiency. It is also important to take into account that the beginning and development of this
369 disorder might be promoted not only by the estrogen synthesized at local level but also by the
370 systemic estrogen (Rizner 2009). Thus, the estradiol from the ovary and from the conversion
371 of androstenedione circulating in the adipose tissue and skin reaches the lesion via circulation.
372 Therefore, the previous mechanism might explain the increase observed in the estradiol level
373 in peritoneal fluid and endometriotic lesion.

374 There is strong evidence of the bidirectional communication between the endocrine and
375 immune system. For example, it has been described that estrogens may contribute to tumor
376 development, blocking the ability of immune cells to induce apoptosis of target cancer cells
377 (Jiang *et al.* 2008). In endometriosis, the high level of estradiol can also play an important
378 role in a local decrease of immunosurveillance (Vetvicka *et al.* 2016). In addition, *in vitro*
379 studies suggest that endometriotic cells respond to estrogen-induced antiapoptotic signaling
380 more intensely than normal cells (Reis *et al.* 2013). We postulate that hormones, especially
381 estradiol, may regulate the expression of the gene that codifies TNFRp55 and that this might
382 be a mechanism involved in the survival of the endometriotic cells in ectopic sites. In support
383 of this hypothesis, we have mapped 1800 bp upstream from the initial site of the TNFRp55
384 gene transcription using MatInspector software (Quandt *et al.* 1995), and we identified four

385 putative oestrogen response element and one putative progesterone response elements (results
386 not shown). In addition, a study through hybridization in situ demonstrated that the uterus of
387 adult mice expresses mRNA of TNFRp55 and after the ovariectomy and 72 h of estradiol
388 administration, the mRNA that codifies TNFRp55 in uterine cells decreased (Roby *et al.*
389 1996). These date indicate that estradiol may modulate the expression of the gene encoding
390 TNFRp55. Whether this mechanism is involved in the decreased susceptibility of
391 endometriotic cells to apoptosis associated to estradiol remains to be investigated.
392 In conclusion, the deficiency of TNFRp55 promoted the establishment and development of
393 endometriosis through an increase in the lesion size and high levels of estradiol which
394 correlate with an increase in the MMP-2 activity. Further studies on the deregulation of the
395 TNFRp55 expression and the survival of the endometrial cells in ectopic sites might
396 contribute to improve the knowledge about pathophysiology and to discover possible
397 therapies or biomarkers of endometriosis.
398
399 **Declaration of interest**
400 There is no conflict of interest that could be perceived as prejudicing the impartiality of the
401 research reported.
402
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406
407
408

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414
415

416 **References**

417 Ahn SH, Monsanto SP, Miller C, Singh SS, Thomas R & Tayade C 2015 Pathophysiology
418 and immune dysfunction in endometriosis. *Biomed Research International* 795976.

419 Amălinei C, Căruntu ID, Giuşcă SE & Bălan RA 2010 Matrix metalloproteinases
420 involvement in pathologic conditions. *Romanian Journal of Morphology and Embryology*
421 **51** 215–28.

422 Antsiferova Y & Sotnikova N 2012 The local immune mechanisms involved in the
423 formation of endometriotic lesions. In *Endometriosis: basic concepts and current*
424 *research trends*, pp 211–44. Eds K Chaudhury, BN Chakravarty. Croatia: Intech.

425 Bilotas M, Meresman G, Stella I, Sueldo C & Barañao RI 2010 Effect of aromatase
426 inhibitors on ectopic endometrial growth and peritoneal environment in a mouse model of
427 endometriosis. *Fertility and Sterility* **93** 2513–8.

428 Boric MA, Torres M, Pinto C, Pino M, Hidalgo P, Gabler F, Fuentes A & Johnson MC
429 2013 TNF system in eutopic endometrium from women with endometriosis. *Open*
430 *Journal of Obstetrics and Gynecology* **3** 271–278.

431 Bravo R & Macdonald-Bravo H 1987 Existence of two populations of cyclin/proliferating
432 cell nuclear antigen during the cell cycle: association with DNA replication sites. *Journal*
433 *Cell Biology* **105** 1549−1554.
434 Bruner KL, Matrisian LM, Rodgers WH, Gorstein F & Osteen KG 1997 Suppression of
435 matrix metalloproteinases inhibits establishment of ectopic lesions by human
436 endometrium in nude mice. *Journal of Clinical Investigation* **99** 2851−7.
437 Bulun SE, Cheng YH, Yin P, Imir G, Utsunomiya H, Attar E, Innes J & Julie Kim J 2006
438 Progesterone resistance in endometriosis: link to failure to metabolize estradiol.
439 *Molecular Cell Endocrinology* **248** 94−103.
440 Bulun SE, Zeitoun KM, Takayama K & Sasano H 2000 Molecular basis for treating
441 endometriosis with aromatase inhibitors. *Human Reproductive Update* **6** 413−8.
442 Cabal-Hierro L & Lazo PS 2012 Signal transduction by tumor necrosis factor receptors.
443 *Cell Signal* **24** 1297−305.
444 Di Carlo C, Bonifacio M, Tommaselli GA, Bifulco G, Guerra G & Nappi C 2009
445 Metalloproteinases, vascular endothelial growth factor, and angiopoietin 1 and 2 in
446 eutopic and ectopic endometrium. *Fertility and Sterility* **91** 2315−23.
447 Grandi G, Mueller MD, Papadia A, Kocbek V, Bersinger NA, Petraglia F, Cagnacci A &
448 McKinnon B 2016 Inflammation influences steroid hormone receptors targeted by
449 progestins in endometrial stromal cells from women with endometriosis. *Journal of*
450 *Reproductive Immunology* **117** 30−8.
451 Greene AD, Lang SA, Kendziorski JA, Sroga-Rios JM, Herzog TJ & Burns KA 2016
452 Endometriosis: where are we and where are we going? *Reproduction* **152** R63−78.

453 Grümmer R 2006 Animal models in endometriosis research. *Human Reproduction*
454 *Update* **12** 641–649.
455 Haider S & Knöfler M 2009 Human tumour necrosis factor: physiological and
456 pathological roles in placenta and endometrium. *Placenta* ***30*** 111–23.
457 Huang HF, Hong LH, Tan Y & Sheng JZ 2004 Matrix metalloproteinase 2 is associated
458 with changes in steroid hormones in the sera and peritoneal fluid of patients with
459 endometriosis. *Fertility and Sterility* **81** 1235–9.
460 Islimye M, Kilic S, Zulfikaroglu E, Topcu O, Zergeroglu S & Batioglu S 2011 Regression
461 of endometrial autografts in a rat model of endometriosis treated with etanercept.
462 *European Journal of Obstetric Gynecology Reproductive Biology* **159** 184–9.
463 Jana S, Chatterjee K, Ray AK, DasMahapatra P & Swarnakar S 2016 Regulation of
464 matrix metalloproteinase-2 activity by COX-2-PGE2-pAKT axis promotes angiogenesis
465 in endometriosis. *Plos One* **11** e0163540.
466 Jiang X, Patterson NM, Ling Y, Xie J, Helferich WG & Shapiro DJ 2008 Low
467 concentrations of the soy phytoestrogen genistein induce proteinase inhibitor 9 and block
468 killing of breast cancer cells by immune cells. *Endocrinology* **149** 5366–5373.
469 Kianpour M, Nematbakhsh M & Ahmadi SM 2015 Asymmetric dimethylarginine
470 (ADMA), nitric oxide metabolite, and estradiol levels in serum and peritoneal fluid in
471 women with endometriosis. *Iranian Journal of Nursing and Midwifery Research* **20** 48–9.
472 Kitawaki J, Kado N, Ishihara H, Koshiba H, Kitaoka Y & Honjo H 2002 Endometriosis:
473 the pathophysiology as an estrogen-dependent disease. *Jouranal Steroid Biochemistry*
474 *Molecular Biology* **83** 149–55.

475 Li Y, Adur MK, Kannan A, Davila J, Zhao Y, Nowak RA, Bagchi MK, Bagchi IC & Li Q
476 2016 Progesterone alleviates endometriosis via inhibition of uterine cell proliferation,
477 inflammation and angiogenesis in an immunocompetent mouse model. *Plos One* **11**
478 e0165347.

479 Liu Y, Sun L, Hou Z, Mao Y, Cui Y & Liu J 2016 rhTNFR: Fc Suppresses the
480 development of endometriosis in a mouse model by downregulating cell proliferation and
481 invasiveness. *Reproductive Science* **23** 847–57.

482 Othman ER, Hornung D, Hussein M, Abdelaal II, Sayed AA, Fetih AN & Al-Hendy A
483 2016 Soluble tumor necrosis factor-alpha receptors in the serum of endometriosis
484 patients. *European Journal Of Obstetrics Gynecology And Reproductive Biology* **200**
485 1–5.

486 Pan H, Zhang P, Li JR, Wang H, Jin MF, Feng C & Huang HF 2016 c-Fos-regulated
487 matrix metalloproteinase-9 expression is involved in 17β-estradiol-promoted invasion of
488 human endometrial stromal cell. *Current Molecular Medicine* **16** 266–75.

489 Pfeffer K, Matsuyama T, Kündig TM, Wakeham A, Kishihara K, Shahinian A,
490 Wiegmann K, Ohashi PS, Krönke M & Mak TW 1993 Mice deficient for the 55 Kd tumor
491 necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L.*
492 *monocytogenes* infection. *Cell* **73** 457–67.

493 Pitsos M & Kanakas N 2009 The role of matrix metalloproteinases in the pathogenesis of
494 endometriosis. *Reproductive Science* **16** 717–26.

495 Quandt K, Frech K, Karas H, Wingender E & Werner T 1995 MatInd and MatInspector:
496 new fast and versatile tools for detection of consensus matches in nucleotide sequence
497 data. *Nucleic Acids Research* **23** 4878–84.

498 Reis FM, Petraglia F & Taylor RN 2013 Endometriosis: hormone regulation and clinical
499 consequences of chemotaxis and apoptosis. *Hum Reprod Update* **19** 406-18.
500 Reis FM, Petraglia F & Taylor RN 2013 Endometriosis: hormone regulation and clinical
501 consequences of chemotaxis and apoptosis. *Human Reproductive Update* **19** 406–418.
502 **Rizner TL** 2009 Estrogen metabolism and action in endometriosis. *Molecular Cell*
503 *Endocrinology* **307**:8−1.
504 Roby KF, Laham N & Hunt JS 1996 Cellular localization and steroid hormone regulation
505 of mRNA encoding tumour necrosis factor receptor I in mouse uterus. *Journal of*
506 *Reproductive Fertility* **106** 285-90.
507 Rojas-Cartagena C, Appleyard CB, Santiago OI & Flores I 2005 Experimental intestinal
508 endometriosis is characterized by increased levels of soluble TNFRSF1B and
509 downregulation of Tnfrsf1a and Tnfrsf1b gene expression. *Biology of Reproduction* **73**
510 1211−8.
511 Sampson JA 1927 Metastatic or Embolic Endometriosis, due to the Menstrual
512 Dissemination of Endometrial Tissue into the Venous Circulation. *American Journal*
513 *Pathology* **3** 93−110.43.
514 Vetvicka V, Laganà AS, Salmeri FM, Triolo O, Palmara VI, Vitale SG, Sofo V &
515 Králíčková M 2016 Regulation of apoptotic pathways during endometriosis: from the
516 molecular basis to the future perspectives. *Archives of Gynecology and bstetrics* **294**
517 897–904.

Figure legends

Figure 1. Establishment and growth of endometriotic lesions. The number of established endometriotic lesions (A), the volume (B), the weight (C) and were assessed in mice *wild type* (WT) n=12 y TNFRp55-/- (KO) n=11 after 4 week of induced endometriosis. Statistical comparisons were performed by Student "t" test. * $P<0.05$, ■ $P<0.001$.

Figure 2. Cell proliferation and apoptosis in endometriotic lesions. (A) The percentage of proliferating cells was assessed by immunohistochemistry for PCNA in endometriotic lesions. Micrographs show representative histological sections of endometriotic lesions of WT (n=11) (i) and KO (n=7) (ii). As a negative control, one section of each slide was assayed without the primary antibody (iii). Statistical comparisons were performed by Student "t" test. •$P<0.01$. (B) The percentage of apoptosis cells was assessed by TUNEL in endometriotic lesions. Micrographs show representative histological sections of endometriotic lesions of WT (n=8) (iv) and KO (n=8) (v). As a negative control, one section of each slide was assayed without the primary antibody (vi). Statistical comparisons were performed by Student "t" test. ■ $P<0.001$.

Figure 3. Enzyme activities of MMP-2 and MMP-9. MMP-2 and MMP-9 (proenzyme and active forms) enzymatic activity was determined by SDS-PAGE gelatin zymography in endometriotic lesions (A) and peritoneal fluid (B). The gel photographs were quantified using ImageJ and expressed as in relative units. Results are expressed as mean ± S.E.M of eight animals per experimental group. Student's t-test was used. • $P<0.01$, ■ $P<0.001$.

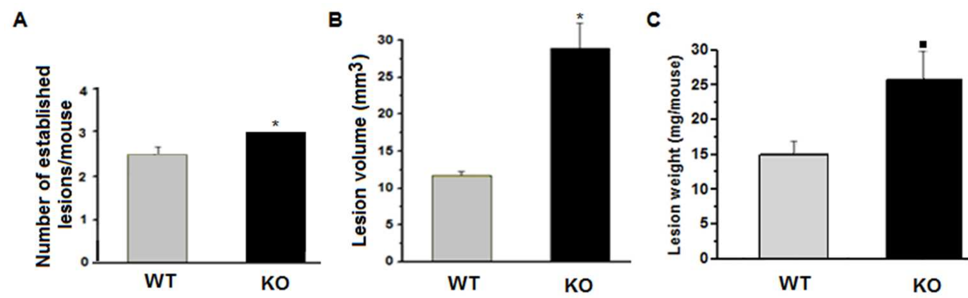
Figure 4. Estradiol levels and P450aromatase expression. Estradiol levels were measured by RIA in peritoneal fluid (A) and endometriotic lesions (B). Measurement by RT-PCR of expression (mRNA) of P450arom and β-actin as housekeeping gene (C). Measurement by Western Blot of expression (protein) of P450arom and β-actin as load control (D). The gel photographs were quantified using ImageJ and expressed as in relative units. Results are expressed as mean ± S.E.M of eight animals per experimental group. Student's t-test was used. *$P<0.05$.

Figure 5. Pearson's correlation between estradiol levels and MMP-2 activity in peritoneal fluid samples. For the entire group of mice (n=16), coefficient (r) Pearson: 0.67.

Figure 6. Progesterone levels, expression of metabolic enzymes and progesterone receptors. Progesterone levels were measured by RIA in peritoneal fluid (A) and endometriotic lesions (B) Measurement by RT-PCR of expression (mRNA) of 3β-HSD, 20α-HSD (C), PR (D). β-actin was used as housekeeping gene. The gel photographs were quantified using ImageJ and expressed as in relative units. Results are expressed as mean ± S.E.M of eight animals per experimental group. Student's t-test was used. • $P<0.01$, ♦$P<0.005$.

Table 1. Primer used for semi-quantitative RT-PCR amplification.

Gen	Sequences (sense above, antisense below; 5'-3')	GenBank accession #	Amplicon length (pb)	N° of cycles
3β-HSD	GTCTTCAGACCAGAAACCAAG CCTTAAGGCACAAGTATGCAG	M58567	213	35
20α-HSD	TTCGAGCAGAACTCATGGCTA CAACCAGGTAGAATGCCATCT	NM_134066	141	35
PR-AB	CTGTGCCTTACCATGTGGCA TTCACCATGCCCGCCAGGAT	NM_008829.2	389	35
PR-B	GGTCCCCCTTGCTTGCA CAGGACCGAGGAAAAAGCAG	NM_008829	121	35
P450arom	CCCGAGCCTTTGGAGAACAA TGAGGGTCAACACATCCACG	NM_007810.3	161	40
β-actin	CGGAACCGCTCATTGCC ACCCACACTGTGCCCATCTA	NM_007393.5	289	35



Establishment and growth of endometriotic lesions. The number of established endometriotic lesions (A), the volume (B), the weight (C) and were assessed in mice wild type (WT) n=12 y TNFRp55-/- (KO) n=11 after 4 week of induced endometriosis. Statistical comparisons were performed by Student "t" test. * P<0.05, ■ P<0.001.

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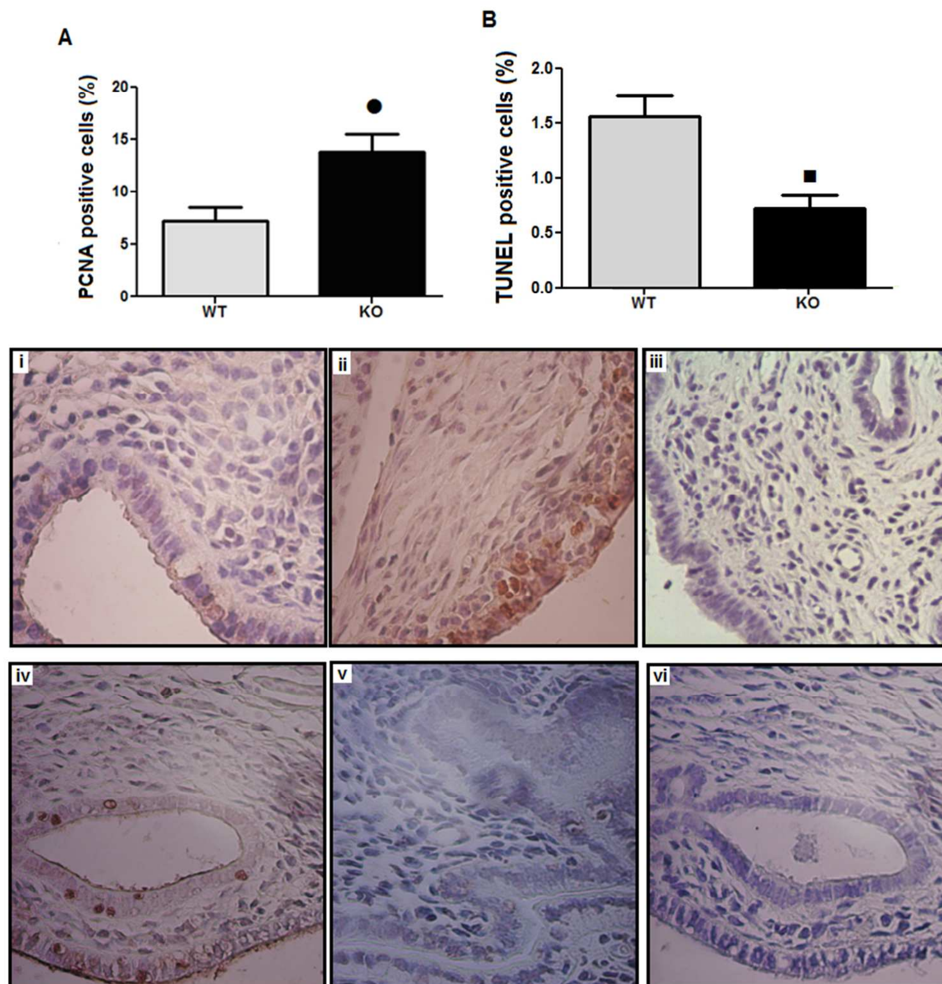

Cell proliferation and apoptosis in endometriotic lesions. (A) The percentage of proliferating cells was assessed by immunohistochemistry for PCNA in endometriotic lesions. Micrographs show representative histological sections of endometriotic lesions of WT (n=11) (i) and KO (n=7) (ii). As a negative control, one section of each slide was assayed without the primary antibody (iii). Statistical comparisons were performed by Student "t" test. •P<0.01. (B) The percentage of apoptosis cells was assessed by TUNEL in endometriotic lesions. Micrographs show representative histological sections of endometriotic lesions of WT (n=8) (iv) and KO (n=8) (v). As a negative control, one section of each slide was assayed without the primary antibody (vi). Statistical comparisons were performed by Student "t" test. ▪ P<0.001.

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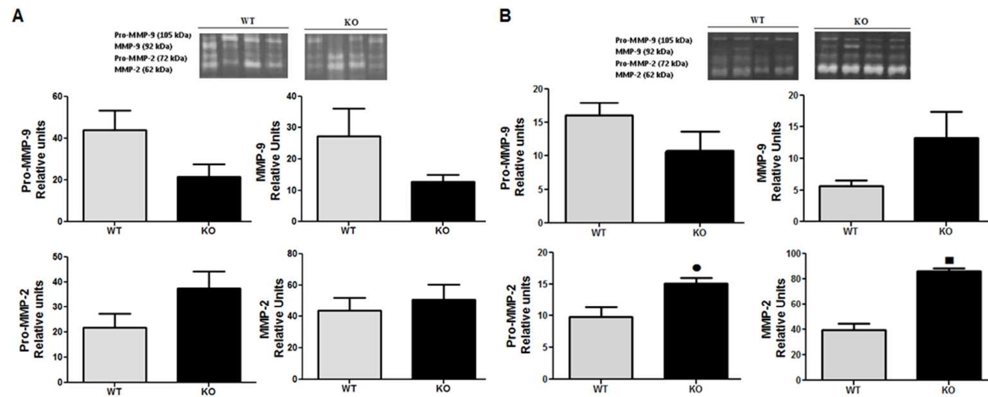

Enzyme activities of MMP-2 and MMP-9. MMP-2 and MMP-9 (proenzyme and active forms) enzymatic activity was determined by SDS-PAGE gelatin zymography in endometriotic lesions (A) and peritoneal fluid (B). The gel photographs were quantified using ImageJ and expressed as in relative units. Results are expressed as mean ± S.E.M of eight animals per experimental group. Student's t-test was used. • $P<0.01$, ▪ $P<0.001$.

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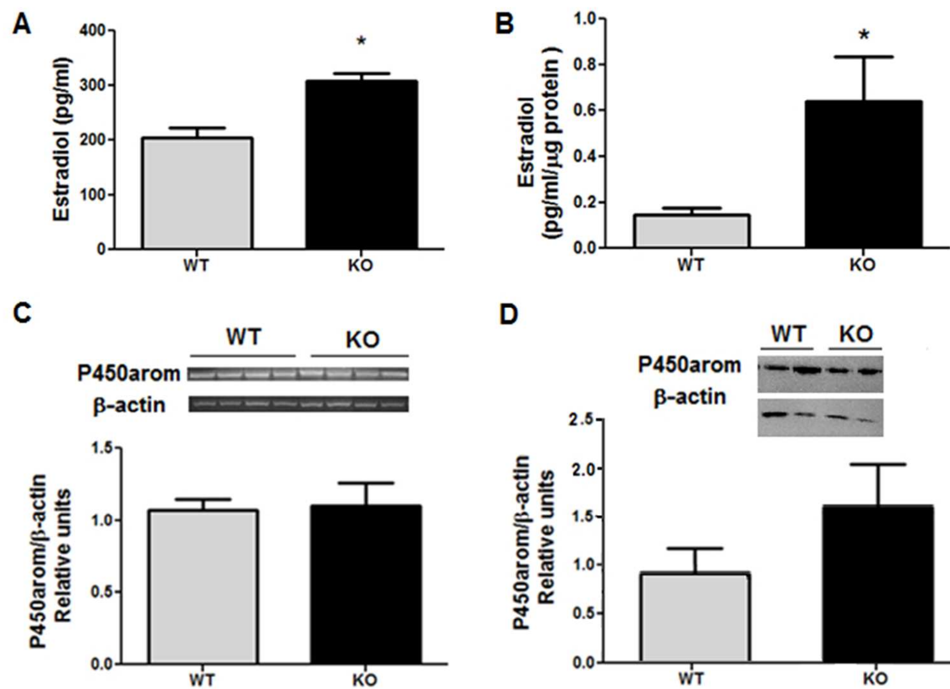

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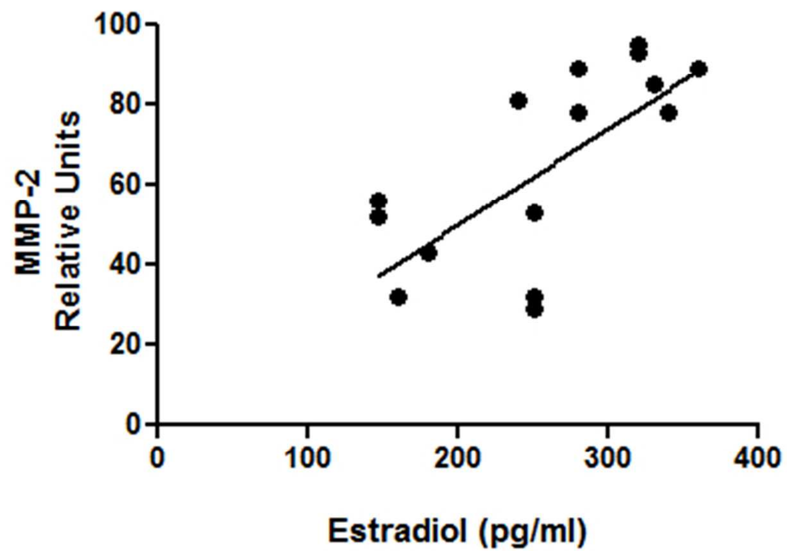
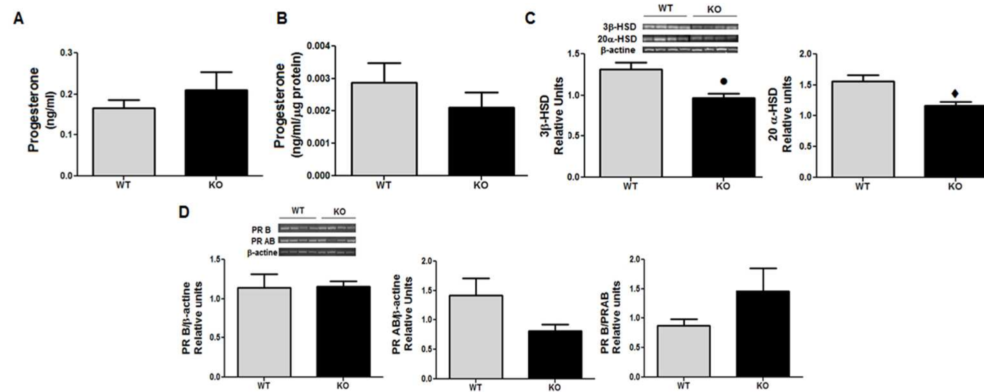
59x43mm (300 x 300 DPI)



Pearson's correlation between estradiol levels and MMP-2 activity in peritoneal fluid samples. For the entire group of mice (n=16), coefficient (r) Pearson: 0.67.

88x66mm (120 x 120 DPI)



Progesterone levels, expression of metabolic enzymes and progesterone receptors. Progesterone levels were measured by RIA in peritoneal fluid (A) and endometriotic lesions (B) Measurement by RT-PCR of expression (mRNA) of 3β-HSD, 20α-HSD (C), PR (D). β-actin was used as housekeeping gene. The gel photographs were quantified using ImageJ and expressed as in relative units. Results are expressed as mean ± S.E.M of eight animals per experimental group. Student's t-test was used. • $P<0.01$, ♦$P<0.005$.

104x41mm (300 x 300 DPI)