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1 **Matrix metalloproteinase-dependent regulation of extracellular matrix**
2 **shapes the structure of sexually differentiating mouse gonads**

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Abstract

The extracellular matrix (ECM) proteins play an important role in the establishment of the sex-dependent structure of developing gonads. The matrix metalloproteinases (MMPs) are the major players in the regulation of ECM. Our hypothesis was that the MMPs-dependent regulation of EMC is crucial for the establishment of the correct, either testis or ovary, structure of developing gonad. We cultured developing mouse gonads *in vitro* in the presence of the MMPs inhibitors (α -2-macroglobulin, leupeptin, phosphoramidon) or the MMPs activator, APMA (4-aminophenylmercuric acetate). These inhibitors and activator inhibit/activate, to a different degree, matrix metalloproteinases, but the exact mechanism of inhibition/activation remains unknown. We found that the MMP inhibitors increased accumulation of ECM in the developing gonads. The α -2-macroglobulin had the weakest, and the phosphoramidon the strongest effect on the ECM and the structure of the gonads. The α -2-macroglobulin caused a slight increase of ECM and did not disrupt the gonad structure. Leupeptin led to the strong accumulation of ECM, resulted in the formation of the structures resembling testis cords in both testes and ovaries, and caused increase of apoptosis and complete loss of germ cells. Phosphoramidon caused the strongest accumulation of ECM, which separated individual cells and completely prevented intercellular adhesion both in the testes and in the ovaries. As a result of aberrant morphology, the sex of the phosphoramidon-treated gonads was morphologically unrecognizable. The APMA - the activator of MMP caused ECM loss, which led to the loss of cell adhesion, cell dispersion and an aberrant morphology of the gonads. These results indicate that the ECM accumulation is MMPs-dependent and that the correct amount and distribution of ECM during gonad development plays a key role in the formation of the gonad structure.

Highlights:

- Inhibition of MMPs in developing gonads leads to accumulation of ECM
- Inhibition of MMPs causes decrease of testis-markers expression
- Accumulation of ECM in developing gonads disrupts cell adhesion
- Accumulation of ECM disrupts gonad structure formation
- Activation of MMPs leads to dispersion of gonad cells

Key words: extracellular matrix; gonad differentiation; ovary; testis; metalloproteinases; α -2-macroglobulin; leupeptin; phosphoramidon; APMA

69 1. Introduction

70 During sexual differentiation of the gonads, the bipotential gonads differentiate into the
71 testis or ovary. Although this processes has been well studied in mice, the molecular and
72 cellular machinery governing the development of testes and ovaries is very complex and still
73 requires further studies. Gonad primordia, termed genital ridges, appear in mice soon before
74 10.5th day of embryonic life (E10.5) (Hu et al., 2013; reviewed by Pipek et al., 2016).
75 Between stage E10.5 and E12.5, the still undifferentiated gonads initiate the expression of
76 sex-determining genes (Bullejos and Koopman, 2001; Kobayashi et al., 2005). Depending on
77 the genetic sex, the male or female sex-determining pathway prevails and determines the
78 structure and fate of the gonad (Kim et al., 2006; Chassot et al., 2008; reviewed by Pipek,
79 2009a,b). The first differences in the structure between male and female gonads appear
80 around stage E12.5 (Schmahl et al., 2000; Nel-Themaat et al., 2009; reviewed by Pipek,
81 2010). A day later, i.e. at E13.5, the gonads are already sexually differentiated, and their sex
82 can be easily distinguished histologically (Nel-Themaat et al., 2009). In the differentiating
83 testes, the somatic cells derived from the coelomic epithelium proliferate leading to the
84 extensive growth of the male gonad (Schmahl et al., 2000). The presumptive Sertoli cells
85 enclose germ cells forming elongated testis cords surrounded by the basement membrane
86 (Svingen and Koopman, 2013). The cells migrating from the adjacent mesonephros give rise
87 to mainly the endothelial cells of the gonad vasculature (Brennan et al., 2002). The
88 subpopulation of the mesonephros-derived cells, and the cells derived from the coelomic
89 epithelium form the interstitium, which separate the testis cords, and thus, shape the testis
90 structure (Tilmann and Capel, 1999; DeFalco et al., 2011). The interstitium contains
91 steroidogenic fetal Leydig cells (FLCs) and abundant extracellular matrix (ECM). The
92 development of the ovary takes a different path. Although the germ cells in developing ovary
93 also become surrounded by the somatic cells (pre-follicular cells) (Albrecht and Eicher,
94 2001), the elongated cords do not develop. The ovigerous cords are built of many small and
95 irregularly shaped clusters of the somatic and germ cells, known as the germ cell nests,
96 embedded in the ovarian stroma (Lei and Spradling, 2013). Later in development, the
97 ovigerous cords split into ovarian follicles (Pepling and Spradling, 2001; Pepling et al., 2010).

98 It has been shown that in mouse, rat, cattle, chicken, slider (*Trachemys scripta*) and the
99 African clawed frog (*Xenopus laevis*) (Paranko et al., 1983; Yao et al., 2004; Hummitzsch et
100 al., 2013; Pipek et al., 2017a,2018) the ECM plays important role in gonad development. The
101 ECM contains many different proteins including collagens, laminins, fibronectin, and
102 proteoglycans (reviewed by Yue, 2014). The amount and distribution of ECM depends on two

103 processes: i.) synthesis of the ECM components and their deposition between cells, ii.)
104 degradation of the ECM components by the extracellular matrix enzymes (ECM enzymes).
105 Two main groups of ECM enzymes involved in the ECM formation/degradation are matrix
106 metalloproteinases (MMPs: MMP1 to MMP28) that digest ECM components, and inhibitors
107 of MMPs (TIMPs), which inhibit MMPs (Birkedal-Hansen, 1993; Stamenkovic, 2003; Arpino
108 et al., 2015). We hypothesize that a balance between the formation and degradation of ECM
109 components plays an important role in the regulation of the amount and distribution of ECM.

110 The knowledge on the role of the ECM in gonad development, especially during the
111 sexual differentiation, is very limited. We showed recently that in the mouse, between E11.5
112 and E13.5 (i.e. during the period of sexual differentiation) many genes encoding ECM
113 components and MMPs are expressed differentially in the male and female gonads (Piprek et
114 al. 2018). Considering the high number of ECM enzymes, the machinery of ECM remodeling
115 in developing gonads is probably very complex. Because the structure of the gonads is
116 different between sexes, the ECM has different distribution in the testes and ovaries;
117 presumably the sex-determining pathways (responsible for the gonad fate) also regulate the
118 sex-specific distribution of ECM. Indeed it has been shown that in the mouse, the TIMP3, an
119 enzyme inhibiting MMPs, is upregulated by male sex-determining pathway (Nishino et al.,
120 2002). Moreover, gonads develop in the close proximity of the mesonephros. Between these
121 two organs there is the vascular plexus. The vascular plexus disintegrates, and mesonephric
122 cells derived from the disintegrating vascular plexus contribute to the endothelium and
123 interstitium of the gonad, which is crucial for the patterning of testis cords (Coveney et al.,
124 2008). The ECM enzymes are probably involved in the disintegration of vascular plexus and
125 thus they facilitate the migration of the mesonephros-derived cells to the gonads. Several
126 studies showed the role of ECM enzymes in kidney development (Ota et al., 1998; Tanney et
127 al., 1998; Lelongt et al., 2005), however, a role of mesonephros in sexual differentiation of
128 gonads remains unknown. It is known that tubular system of mesonephros joins rete testis
129 later in development, however, molecular mechanisms driving this process are obscure
130 (Joseph et al., 2009; Davidson et al., 2018).

131 Because the ECM is differentially patterned in developing testes and ovaries, and the
132 genes encoding ECM components and enzymes responsible for ECM remodeling are
133 differentially expressed, we hypothesized that the ECM and its enzymes are important factors
134 controlling sexual differentiation of the gonads. The aim of this study was to explore how the
135 structure of differentiating mouse testes and ovaries changes upon inhibition or activation of
136 ECM regulating enzymes. Fetal gonads isolated at E11.5, i.e. just before the onset of sexual

137 differentiation, were cultured in a medium supplemented with the inhibitors of MMPs (α -2-
138 macroglobulin, leupeptin, or phosphoramidon) or with the activator (APMA, 4-
139 aminophenylmercuric acetate) (Table 1). The gonads were analyzed after 3 days in culture
140 using histological techniques, immunohistochemistry and gene expression analysis.

141

142 **2. Material and methods**

143 *2.1. Animals and genotyping*

144 The gonads were isolated from the C57bl/6 mouse strain. The study was approved by
145 the I Local Commission for Ethics in Experiments on Animals. The animals were bred and
146 housed in the Animal Facility at the Jagiellonian University (Krakow, Poland). The number of
147 studied animals is presented in Table 1. Timed matings were performed by placing a male
148 with 2 females overnight. The following morning, females were checked for the presence of
149 the vaginal plug, and the pregnancies were estimated as E0.5 (embryonic day). Females were
150 euthanized by spinal dislocation at 11.5. The sex of all studied animals was confirmed by
151 genotyping using primers for *Sly* (Y chromosome) and *Xlr* (X chromosome) (McFarlane et al.,
152 2013). Primers used for genotyping are listed in Suppl. Table 1. PCR reactions were
153 performed in a final volume of 10 μ l with primers (0.5 μ L each), extracted DNA (1 μ L), water
154 (3.5 μ L), and 2X PPP Master Mix (Top-Bio) (5 μ L) and the following PCR parameters: initial
155 denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s,
156 followed by final elongation at 72°C for 5 min. PCR products were electrophoresed on 2%
157 agarose gels containing GelRed (Biotium) and visualized under UV-illumination.

158

159 *2.2. In vitro culture*

160 Gonads were dissected from embryos at E11.5 along with mesonephroi and cultured
161 on agar in DMEM high glucose GlutaMAX (ThermoFisher, 10566) medium supplemented
162 with 10% fetal bovine serum (FBS, Biomedical Industries, 04-001) and a mixture of
163 antibiotics (penicillin 100 U/ml, streptomycin 100 μ g/ml, amphotericin B 0.25 μ g/ml,
164 Biomedical Industries, 03-033). For MMP inhibition, α -2-macroglobulin, leupeptin,
165 phosphoramidon were added to the medium at concentration listed in Table 2. To activate
166 MMPs, an activator (APMA, 4-aminophenylmercuric acetate) was added to the medium
167 (Table 2). Inhibitors and the activator were dissolved in DMSO (Sigma, D2650) and added in
168 amount of 1 μ l/ml of the medium. Only DMSO (1 μ l/ml) was added to the medium in the
169 control. Organs were cultured at the air/medium interface for 3 days at 37°C in 5% CO₂.

170

171 2.3. MMPs modulators

172 α -2-macroglobulin is an endogenous large plasma protein, synthesized mainly in the
173 liver (Rehman et al., 2013). It has a broad spectrum of action in an organism, including
174 modulation of growth factors activities, regulation of blood coagulation, and functions in
175 developmental process, such as the development of liver in zebrafish (Westwood et al., 2001;
176 Hong and Dawid, 2008). Leupeptin (N-acetyl-L-leucyl-L-leucyl-L-argininal) is a protease
177 inhibitor produced by actinomycetes. It also has a broad spectrum of action, and inhibits
178 numerous enzymes, such as MMPs, serine and threonine proteases, calpain, cathepsin,
179 trypsin, plasmin, papain (Kuramochi et al., 1979). Phosphoramidon derives from the
180 bacterium *Streptomyces tanashiensis* isolated from the soil in Japan (Kitagishi and Hiromi
181 1984). It inhibits MMPs and bacterial thermolysin, and regulates endothelins that have a key
182 role in vascular homeostasis (Plumpton et al., 1994; McMahon et al., 1991; Keller et al.,
183 1996). APMA (4-aminophenylmercuric acetate) is an organomercurial compound and thiol-
184 blocking reagent, which activates MMPs and collagenase proteolytic enzymes (Rosenfeldt et
185 al., 2005).

186

187 2.4. Gelatin zymography

188 Activity of MMPs was studied as previously described (Hibbs et al., 1985). After 3
189 days of *in vitro* culture, gonads were lysed for 30 min. at 4°C with 20 μ l of 1% NP-40 and
190 5mM EDTA. Lysates were mixed with Zymogram sample buffer (ZymoResearch) with 2%
191 SDS and 10% glycerol and subjected to electrophoresis on a 10% SDS-polyacrylamid gel
192 containing 1mg/mL gelatin (Sigma) in the absence of any reducing agent, at room
193 temperature. The gel was washed 4x in the washing buffer (50 mM Tris-HCl, 5 mM CaCl₂, 5
194 μ M ZnCl₂, 0.02% NaN₃ and 2.5% Triton X-100). The gel was stained with Coomassie
195 Brilliant blue R-250, washed in Coomassie washing solution, dried, and documented.

196

197 2.5. RNA isolation and Real-Time Quantitative PCR (qPCR)

198 After 3 days of culture the gonads were pooled accordingly to the genetic sex. Total
199 RNA was isolated using Trizol and further purified with RNeasy Mini kit per manufacturer's
200 instructions (Qiagen, Valencia, CA). Total RNA in RNase-free water was frozen at -80°C and
201 then used for multigene qPCR analysis. 50 ng RNA of each sample was reverse-transcribed
202 into cDNA using random primers and SuperScript III Reverse Transcriptase (Invitrogen,
203 18080044) following manufacturer's instructions. A list of primers is presented in Suppl.
204 Table 1. The RT-qPCR procedure was performed in 5 μ l reactions using SYBR Green Master

205 Mix (Life Technologies, 4312704) on a 7500 Fast Real-Time PCR System (Applied
206 Biosystems) with universal cycling parameters and analyzed as previously described (Svingen
207 et al., 2009). Data were collected as raw C_T values and analyzed using the $2^{-\Delta\Delta C_T}$ method.
208 Beta-actin (*Actb*) was used as a reference gene. Gene expression was normalized on an
209 arbitrary scale with *Actb* as 1.0. Statistical analysis was performed using the nonparametric
210 ANOVA Kruskal-Wallis test followed by the Tukey's test. Statistica 7.0 software was used
211 for the analyses.

212

213 2.6. *Histology and Immunohistochemistry*

214 Freshly isolated gonads and gonad after 3 days of *in vitro* culture, were rinsed in PBS
215 and fixed in Bouin's solution, dehydrated and embedded in paraffin (Paraplast, Sigma,
216 P3683). Histological staining was performed according to Debreuil's trichromatic method as
217 previously described (Kiernan, 1990; Piprek et al., 2017b). For immunohistochemistry, heat-
218 induced epitope retrieval was conducted in sodium citrate buffer (10 mM sodium citrate,
219 0.05% Tween-20, pH 6) at 95°C for 20 minutes. Subsequently, the sections were blocked with
220 3% H₂O₂ and 10% goat serum (Sigma, G9023). Sections were incubated with primary
221 antibodies (all rabbit polyclonal: anti-AMH, Santa Cruz Biotechnology, sc-166752; anti-
222 collagen I, Abcam, ab34710; anti-laminin, Abcam, ab11575; anti-cleaved caspase 3, Assay
223 BioTech, L0104) at 4°C overnight, and with UltraVision Quanto Detection System (TL-125-
224 QHD). Mayer's hematoxylin was used as a counterstain. Sections were examined under
225 Nikon Eclipse E600 microscope. The germ cells and somatic cells were identified by the size
226 and morphological features. The germ cells were larger than the somatic cells, and had a
227 large, round and pale nuclei. The somatic cells were smaller than the germ cells and had small
228 and dark nuclei. The apoptotic cells were identified by the presence of small, dark, pyknotic
229 nuclei with a highly condensed chromatin, and by caspase 3 immunostaining.

230

231 2.7. *Quantification of apoptotic cells*

232 The number of (caspase 3-positive) apoptotic cells was calculated within the 10,000
233 μm^2 area in 5 cross sections from each gonad using ImageJ software. The number of
234 apoptotic cells in gonads cultured in medium supplemented with MMP inhibitors and
235 activator was compared to the control using χ^2 test. Statistical data were analyzed using
236 Statistica 6 PL Software (Krakow, Poland).

237

238 3. Results and discussion

239 3.1. Activity of MMPs in the gonads after incubation in the presence of inhibitors and 240 activator

241 Zymography analyses showed that α -2-macroglobulin, leupeptin and phosphoramidon
242 inhibited MMP2, MMP3 and MMP9 in the gonads after 3 days of *in vitro* culture (Fig. 1).
243 Phosphoramidon inhibited MMP2, MMP3 and MMP9 to the higher degree than a α -2-
244 macroglobulin and leupeptin did. As expected, APMA activated MMP2, MMP3 and MMP9
245 (Fig. 1). The results of these experiments are summarized in Table 3.

246

247 3.2. Development of gonads under control *in vitro* conditions

248 The histology of the freshly isolated XY and XX gonads, before the start of the *in*
249 *vitro* culture, was identical (Fig. 2A,C).

250 3.2.1. XY gonads

251 After three days of *in vitro* culture the XY gonads contained cell clusters (Fig. 3A).
252 These clusters, which are the early testis cords, contained the germ cells with large, round
253 nuclei, surrounded by AMH (anti-müllerian hormone) positive pre-Sertoli cells with the small
254 nuclei (Fig. 3C). The clusters of pre-Sertoli/germ cells were enclosed by the basement
255 membrane. The space (interstitium) between the cords was filled with the thin layers of ECM
256 (Fig. 3E). Immunostaining showed that this interstitial ECM contained collagen I and laminin
257 (Fig. 3E,G). Only singular apoptotic cells were present (Fig. 3I). The overall structure of the
258 testis was similar to the structure of physiologically developing testes of the same age (Fig.
259 2B).

260 3.2.2. XX gonads

261 The XX gonad after 3 days of *in vitro* culture had poorly defined clusters of somatic
262 and germ cells (Fig. 5A), which were separated by a small amount of ECM containing
263 collagen I and laminin (Fig. 5C,E). Only singular apoptotic cells were present (Fig. 5G). The
264 overall structure of the ovary was similar to the physiologically developing ovaries of the
265 same age (Fig. 2D).

266

267 3.3. The effect of α -2-macroglobulin inhibitor on ECM and gonad structure

268 3.3.1. XY gonads

269 The structure of XY gonads cultured for 3 days in the medium supplemented with α -2-
270 macroglobulin, which inhibits MMPs, was similar to the control gonad cultured in the absence
271 of inhibitor. Both contained well defined testis cords containing germ and somatic cells (Fig.

272 3B). The only noticeable difference was the higher amount of ECM around the cords and the
273 stronger collagen I and laminin immunostaining in the gonad cultured in the presence of
274 inhibitor (Fig. 3F,H). This indicates that α -2-macroglobulin, which inhibits MMPs, inhibited
275 disintegration of ECM. The AMH immunostaining showed the presence of solid, well
276 differentiated clusters of pre-Sertoli cells in the testis cords both in control and in cultured
277 gonads (Fig. 3D). Singular apoptotic (caspase 3-positive) cells were observed, indicating cell
278 death (Fig. 3J, Table 4).

279 3.3.2. XX gonads

280 The overall structure of the XX gonads after three days of culture in the medium
281 supplemented with α -2-macroglobulin was similar to the control. However, in the inhibitor
282 treated gonads, the amount of ECM was higher (Fig. 5B). The streams of ECM separated
283 clusters of somatic/germ cells (Fig. 5D,F).

284 These results indicate that the *in vitro* exposure of gonads to the α -2-macroglobulin
285 inhibitor causes only moderate increase of ECM and does not affect the structure of
286 developing XX or XY gonads.

287

288 3.4. Leupeptin increases ECM content and changes gonad structure

289 3.4.1. XY gonads

290 Our zymography analysis showed that leupeptin had a stronger inhibitory effect on
291 MMPs than α -2-macroglobulin (Fig. 1). Accordingly, we found that the gonad cultured in the
292 medium supplemented with leupeptin had higher content of ECM than the gonads cultured
293 with α -2-macroglobulin (Fig. 4A). After three days of culture in the medium supplemented
294 with leupeptin, XY gonads contained strong accumulations of ECM components, i.e. collagen
295 I and laminin (Fig. 4E,G). AMH immunostaining showed that the integrity of testis cords was
296 compromised; the clusters of AMH-positive cells were much looser than in control (Fig. 4C).
297 Such testis cords were surrounded by a high accumulation of ECM. The germ cells were
298 absent, indicating that leupeptin led to the loss of germ cells. Occasionally apoptotic cells
299 were observed, indicating enhanced cell death (Fig. 4I, Table 4).

300 3.4.2. XX gonads

301 The XX gonads cultured in the medium with leupeptin, similar to the XY gonads, had
302 higher content of ECM (Fig. 6A,C,E) and lacked the germ cells.

303

304 3.5. The phosphoramidon causes very high accumulation of ECM and changes the structure
305 of XY and XX gonads

306 The structure of the gonads cultured for three days in the medium supplemented with
307 phosphoramidon had a very high accumulation of ECM components collagen I and laminin
308 (Fig. 4B,D,F,H, 6B,D,F). The ECM was so abundant that it separated all cells preventing
309 intercellular adhesion. As a result, the clusters of cells, such as testis cords, were absent, and
310 AMH-positive cells were dispersed (Fig. 4D). The germ cells were absent and the apoptotic
311 cells were present and in XX and XY gonads (Fig. 4B,I, 6B,H). Among all used MMPs
312 inhibitors, the number of apoptotic cells in the phosphoramidon supplemented medium was
313 the highest (Table 4). Because of the profound changes in the gonad structure, the gonad sex
314 was morphologically unrecognizable. The XY and XX had the same structure with the
315 dispersed cells embedded in ECM. This indicated that phosphoramidon had very strong
316 inhibitory effect on MMPs, which resulted in excessive accumulation of ECM. This in turn
317 caused complete disruption of the gonad structure and the loss of the germ cells.

318 It has been shown that the proper cell adhesion is important for the germ cell survival
319 in mouse gonads (Nagano et al., 2000; Luaces et al., 2014). This explains why the excessive
320 accumulation of ECM around the cells, which prevents cell adhesion caused germ cell loss in
321 the gonads cultured in the presence of MMPs inhibitors.

322 Although, the phosphoramidon was described previously as a weak MMP inhibitor
323 (Kitagishi and Hiromi, 1984; Matsumura et al., 1990), in our experimental system
324 phosphoramidon had the strongest inhibitory effect on MMPs in developing gonads. The
325 strong correlation between the zymography-measured anti-MMP activity of used inhibitors,
326 ECM content, and the changes in the gonads structure, described here, argues for the
327 important role of MMPs and ECM in the process of gonad development and germ cell
328 survival, and thus for the future fecundity of the individual.

329 Mazaud and coauthors (2005) showed that the rat ovaries cultured *in vitro* in the
330 presence of α -2-macroglobulin or phosphoramidon had only sporadic ovarian follicles, which
331 indicated a partial inhibition of folliculogenesis. However, a culture of ovaries with leupeptin
332 led to a complete absence of ovarian follicles (ibid). In our experimental system the leupeptin
333 had much lower impact on gonad development than the phosphoramidon. This points to the
334 profound differences between the processes of sexual differentiation of gonads and the
335 folliculogenesis.

336

337 *3.6. MMPs activator APMA decreases ECM content and disrupts structure of XY and XX*
338 *gonads*

339 The structure of the gonads after three days of culture in the medium supplemented
340 with APMA was drastically altered (Fig. 7A-C). XY and XX gonads were morphologically
341 undistinguishable. All somatic cells were widely dispersed within the gonad, did not adhere
342 one to another, and only very low amount of ECM was present, and the germ cells were either
343 absent or morphologically unrecognizable.

344

345 3.7. Modifications of gene expression pattern by MMPs modulators

346 To identify molecular effects of the MMPs modulators, we studied the expression of
347 marker gene for the female germ cells (*Oct4*), testis specific Sertoli cells (*Sox9*, *Amh*), ovary
348 specific follicular cells (*Fst*) The actin β encoding gene (*Actb*), was used as a control (Fig. 8).
349 Inhibitors of MMPs caused a significant decrease of *Oct4* expression in both XY and XX
350 gonads. The APMA activator of MMPs, caused a slight increase in *Oct4* expression (Fig. 8).
351 A decrease of *Oct4* expression likely reflected the observed germ cell loss.

352 The Sertoli cell marker *Sox9* had decreased expression in XY gonads cultured with
353 MMP inhibitors. *Sox9* expression was almost completely lost in the gonads treated with MMP
354 activator APMA (Fig. 8). The expression of *Amh*, another Sertoli cells marker, was slightly
355 lower in XY gonads cultured with MMPs inhibitors, and was almost completely lost in
356 gonads treated with MMP activator. These results indicate that dysregulation (increase or
357 decrease) of the amount) of ECM content disrupts differentiation of Sertoli cells. This, in turn,
358 implies that a proper content and distribution of ECM may be critical for the expression of
359 genes directing sex determination and differentiation of Sertoli cells.

360 *Fst*, a marker of the developing ovary, remained unchanged (low in XY gonads and
361 elevated in XX gonads) in the presence of MMP inhibitors and slightly downregulated in the
362 presence of MMP activator (Fig. 8). This indicates that the expression of ovarian markers is
363 not affected by the changes in ECM. It is possible that the changes in ECM and cell adhesion
364 are less important for ovarian than testis development.

365 The gene expression analysis showed that MMP inhibition or activation did not
366 impair sex determination in the gonads; the ovarian marker (*Fst*) was not upregulated in the
367 XY gonads, and Sertoli cells markers (*Sox9*, *Amh*) were not upregulated in the XX gonads
368 (Fig. 8). Thus, no sex reversal was detected. Importantly, the *Actb* gene (used as a control)
369 was expressed at the constant level in all analyzed gonads, which indicated that the observed
370 changes in the expression of markers were not caused by the *in vitro* culturing conditions.

371

372 4. Conclusion

373 We showed that the modulators, both the inhibitors and the activator, of MMPs trigger
374 important changes in the structure of sexually differentiating developing mouse gonads (Table
375 3). 1. MMPs inhibitors causes accumulation of ECM, which drives cells dispersion and
376 disappearance of testis cords. 2. MMPs activator APMA causes ECM loss and a complete
377 disruption of the gonad structure. Thus, both the excessive accumulation of ECM and its
378 decrease or loss leads to a dramatic impairment of the tissue architecture in developing
379 gonads. In addition, leupeptin and phosphoramidon led to the enhanced apoptosis and the loss
380 of germ cells, and thus lowered expression of germ cell marker *Oct4*. APMA decreased the
381 expression level of Sertoli cell markers *Sox9*, *Amh*, which indicated disruption of Sertoli cell
382 integrity. Thus, we postulate that the ECM amount, which depends on a balanced synthesis
383 and degradation of its components, is critical for the establishment of the proper structure of
384 the gonads, and that MMPs play a crucial role in this process.

385

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389

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600 **Figure legends**

601 **Fig. 1. Gelatin zymography of developing XY and XX mouse gonads cultured *in vitro* for**
 602 **3 days.** Zymography shows that phosphoramidon inhibits matrix-metalloproteinase 2, 3 and
 603 9, (MMP2, MMP3, and MMP9) to a higher degree than α -2-macroglobulin and leupeptin, and
 604 that APMA (4-aminophenylmercuric acetate) activates these three MMPs.

605
 606 **Fig. 2. Development of mouse XY and XX gonads *in vivo* at E11.5 and E13.5. A. XY**
 607 undifferentiated gonad structure at E11.5. The somatic (arrows) and germ cells (arrowheads)
 608 are evenly distributed within the gonad. **B.** Testis at E13.5 developed *in vivo*. The testis cords
 609 (encircled) are present, the interstitium (i) is located between the testis cords, and the germ
 610 cells (arrowhead) are located within the cords. **C.** XX undifferentiated gonad at E11.5. The
 611 somatic (arrows) and germ cells (arrowheads) are evenly distributed within the gonad. **D.**
 612 Ovary at E13.5 developed *in vivo*. The ovigerous cords are small, irregular and not well
 613 differentiated. Scale bar is equal to 25 μ m.

614
 615 **Fig. 3. Effect of 3-day *in vitro* culture of XY gonads in the control and in the presence of**
 616 **α -2-macroglobulin. A.** Control XY gonad after 3-day *in vitro* culture in the absence of MMP
 617 inhibitors. The basement membranes (arrow) and the blue-stained ECM surrounding the
 618 testis cords (encircled) are visible. The germ cells (arrowhead) are present in the testis cords.
 619 **B.** XY gonad after 3-day *in vitro* culture in the presence of α -2-macroglobulin. The amount
 620 of blue-stained ECM is slightly higher than in the control; the testis cords (encircled) and the
 621 germ cells (arrowheads) are present. The apoptotic cells are marked by the asterisks. **C,D.**
 622 Immunostaining of AMH (anti-müllerian hormone – a marker of Sertoli cells). The strongest
 623 signal is visible in the control gonads. **E,F.** Immunostaining of collagen I. There is an increase
 624 in collagen I accumulation between cells in gonads treated with α -2-macroglobulin. **G,H.**
 625 Immunostaining of laminin. There is an increase of signal in gonads treated with α -2-
 626 macroglobulin. **I,J.** Immunostaining of caspase 3 (apoptosis marker). Only singular apoptotic
 627 cells are present. Scale bar is equal to 25 μ m.

628
 629 **Fig. 4. Effect of 3-day *in vitro* culture of XY gonads in the presence of leupeptin and**
 630 **phosphoramidon. A.** XY gonad after 3-day *in vitro* culture in the presence of leupeptin.
 631 There is high amount of ECM, the germ cells are absent. The testis cords (encircled) are small
 632 and sterile. Occasionally, the apoptotic cells (asterisk) are visible. **B.** XY gonad after 3-day *in*
 633 *vitro* culture with phosphoramidon. There is very high amount of ECM and the germ cells are

634 absent. Because of the high amount of ECM all cells are dispersed and the testis cords do not
 635 form. The apoptotic cells (asterisk) are present. **C,D.** Immunostaining of AMH. In the gonads
 636 treated with MMP inhibitors, the stronger the inhibitor the weaker the AMH signal. **E,F.**
 637 Immunostaining of collagen I. The gonads cultured in the presence of phosphoramidon had
 638 the strongest collagen I signal. **G,H.** Immunostaining of laminin. The strongest signal is in the
 639 gonads cultured in the presence of phosphoramidon. **I,J.** Immunostaining of caspase 3
 640 (apoptosis marker). The most numerous apoptotic cells are present in the gonads cultured in
 641 the presence of phosphoramidon. Scale bar is equal to 25 μm .

642

643 **Fig. 5. Effect of 3-day *in vitro* culture of XX gonads in the control and in the presence of**
 644 **α -2-macroglobulin. A.** Control XX gonad after-3 day *in vitro* culture in the absence of MMP
 645 inhibitors. The somatic and germ cells (arrowhead) are dispersed; blue-stained ECM is
 646 present between groups of cells. **B.** XX gonad after 3-day *in vitro* culture in the presence of α -
 647 2-macroglobulin. The amount of blue-stained ECM is slightly higher than in the control.
 648 Occasionally, the apoptotic cells (asterisk) are present. **C,D.** Immunostaining of **collagen I**.
 649 There is increase in collagen I content between the cells in gonads treated with α -2-
 650 macroglobulin. **E,F.** Immunostaining of **laminin**. There is increase in laminin content in the
 651 gonads treated with α -2-macroglobulin. **G,H.** Immunostaining of caspase 3 (apoptosis
 652 marker). Only singular apoptotic cells are present. Scale bar is equal to 25 μm .

653

654 **Fig. 6. Effect of 3-day *in vitro* culture of XX gonads in the presence of leupeptin and**
 655 **phosphoramidon. A.** XX gonad after 3-day *in vitro* culture in the presence of leupeptin. The
 656 amount of ECM is higher; the germ cells are absent; occasionally, the apoptotic cells
 657 (asterisk) are visible. **B.** XX gonad after 3day *in vitro* culture in the presence of
 658 phosphoramidon. There is very high amount of ECM. The germ cells are absent. Because of
 659 the high amount of ECM all cells are dispersed. The apoptotic cells (asterisk) are present.
 660 **C,D.** Immunostaining of **collagen I**. The gonads cultured in the presence of phosphoramidon
 661 have the strongest collagen I signal. **E,F.** Immunostaining of **laminin**. The gonads cultured in
 662 the presence of phosphoramidon have the strongest laminin signal. **G,H.** Immunostaining of
 663 caspase 3 (apoptosis marker). The most numerous apoptotic cells are present in the gonads
 664 cultured in the presence of phosphoramidon. Scale bar is equal to 25 μm .

665

666 **Fig. 7. Effect of 3-day *in vitro* culture of XY and XX gonads in the presence of APMA.**

667 In both XY (A) and XX (B) gonads, the cells are completely dispersed and only miniscule
668 amount of ECM is present between the cells. (C) There is no positive signal in gonads
669 immunostained for AMH. Scale bar is equal to 25 μ m.

670

671 **Fig. 8. Gene expression analysis after 3-day *in vitro* culture of XY and XX gonad in the**
672 **absence or presence of MMP inhibitors (α -2-macroglobulin, leupeptin,**
673 **phosphoramidon) and MMP activator (APMA). The expression of *Oct4* (marker of germ**
674 **cells), was lower in the gonads cultured with MMP inhibitors in comparison to the to the**
675 **control gonads and gonads cultured with APMA. The expression of testis-specific markers**
676 **(*Sox9* and *Amh*) was slightly decreased in gonads cultured with MMP inhibitors, and**
677 **significantly decreased in gonads cultured with APMA. The expression of ovary-specific**
678 **follistatin (*Fst*) and control gene (*Actb*) show no significant changes in the gonads cultured**
679 **with MMP inhibitors or APMA. Relative quantitation (Y-axis) determines the changes in**
680 **steady-state mRNA level, data are normalized to the level of *Actb* expression (value = 1.0).**

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 701 **Table 1.** Number of XY and XX mouse fetuses used for the *in vitro* culture in the medium
 702 supplemented with inhibitors/activator of metalloproteinases.

Chemical reagent	Number of XY fetuses	Number of XX fetuses
α -2-macroglobulin	14	16
Leupeptin	20	15
Phosphoramidon	17	21
APMA, 4-aminophenylmercuric acetate	13	12
DMSO - control	17	15

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 706 **Table 2.** Inhibitors and activator of metalloproteinases used in the experiment.

Chemical reagent	Action	Concentration	Product number
α-2-macroglobulin	an inhibitor of endoproteases, including metalloproteinases (Cawston and Mercer, 1986)	100 μ g/ml	Sigma, M3398
Leupeptin (N-acetyl-L-leucyl-L-leucyl-L-argininal)	an inhibitor of metalloproteinases, serine and threonine proteases, calpain, cathepsin, trypsin, plasmin, papain (Kuramochi et al., 1979)	100 μ M	Sigma, L5793
Phosphoramidon	a weak inhibitor of metalloproteinases (Kitagishi and Hiromi, 1984; Matsumura et al., 1990)	200 μ M	Sigma, R7385
APMA (4-aminophenylmercuric acetate)	an activator of metalloproteinases (Galazka et al., 1996)	1.5 mM	Sigma, A9563

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715 **Table 3. Summary of the experimental results.**
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Substance	MMPs activity in zymography	Histology and IHC	Gene expression
Control	Activity detected	- Gonadal sex recognizable by morphological features - Testis cords present - Germ cells present	<i>Oct4</i> , <i>Sox9</i> , <i>Amh</i> and <i>Fst</i> expressed
α-2-macroglobulin	Slightly decreased	Slightly increased ECM accumulation - Gonadal sex recognizable - Testis cords present - Germ cells present - Apoptosis	Lower <i>Oct4</i> expression
Leupeptin	Decreased	Increased ECM accumulation - Gonadal sex recognizable - Testis cords present - Germ cells absence - Apoptosis	
Phosphoramidon	The strongest decrease	High structure impairment by strong ECM accumulation - Gonadal sex unrecognizable by morphological features - Cells dispersed in ECM - No testis cords - Germ cells absence - Apoptosis	
APMA	High activity	High structure disturbance by strong ECM dispersion - Gonadal sex unrecognizable by morphological features - No testis cords - Cells dispersed	Lower <i>Sox9</i> and <i>Amh</i> expression

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Table 4. Mean number and standard deviation of apoptotic (caspase 3-positive) cells per 10,000 μm^2 in XY and XX gonads after 3 days of *in vitro* culture.

Inhibitor	XY	XX
Control without inhibitor	0.3 +/- 0.48 ^a	0.4 +/- 0.7 ^a
α-2-macroglobulin	1.1 +/- 1.1 ^a	1.2 +/- 1.32 ^a
Leupeptin	2.1 +/- 1.73 ^a	2.4 +/- 1.71 ^a
Phosphoramidon	6.4 +/- 2.72 ^a	5.7 +/- 2.54 ^a

723 ^aSignificant difference between the experimental and control gonads (χ^2 test, $P < 0.05$).

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725 **Suppl. Table 1. Primers used for genotyping and RT-qPCR.**

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Gene	Primers
Primers used for genotyping	
<i>SX</i> (sex genotyping)	F: GATGATTTGAGTGGAAATGTGAGGTA R: CTTATGTTTATAGGCATGCACCATGTA
Primers used for RT-qPCR	
<i>Oct4</i>	F: GCATTCAAACCTGAGGCACCA R: AGCTTCTTTCCCATCCCA
<i>Sox9</i>	F: GTGCAAGCTGGCAAAGTTGA R: TGCTCAGTTCACCGATGTCC
<i>Amh</i>	F: TCAACCAAGCAGAGAAGGTG R: AGTCATCCGCGTGAAACAG
<i>Fst</i>	F: AAAACCTACCGCAACGAATG R: TTCAGAAGAGGAGGGCTCTG
<i>Actb</i>	F: CATGTACGTTGCTATCCAGGC R: CTCCTTAATGTCACGCACGAT

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Fig. 1.

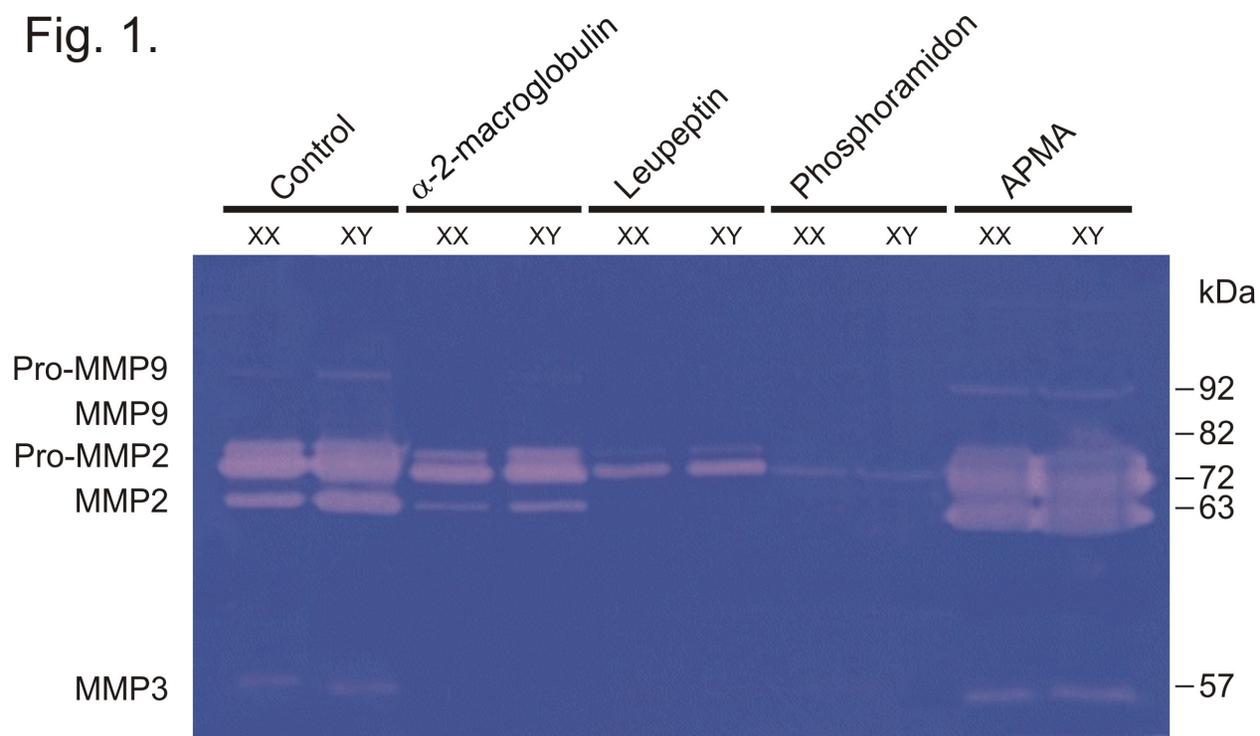


Fig. 2.

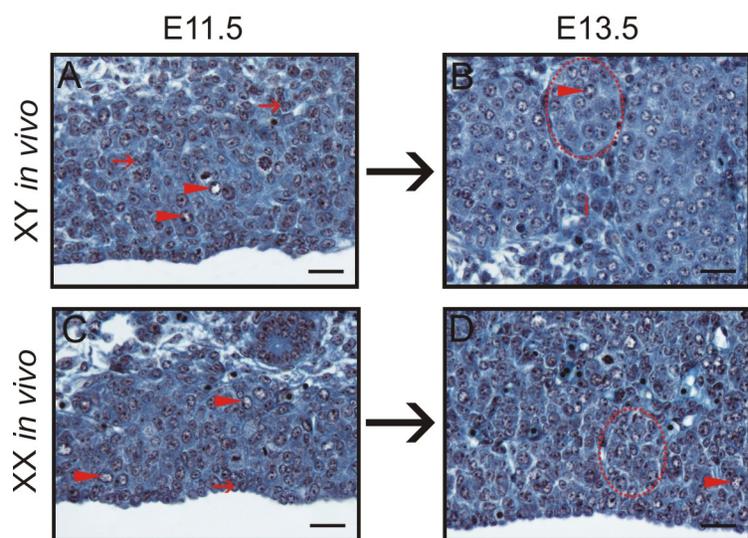


Fig. 3.

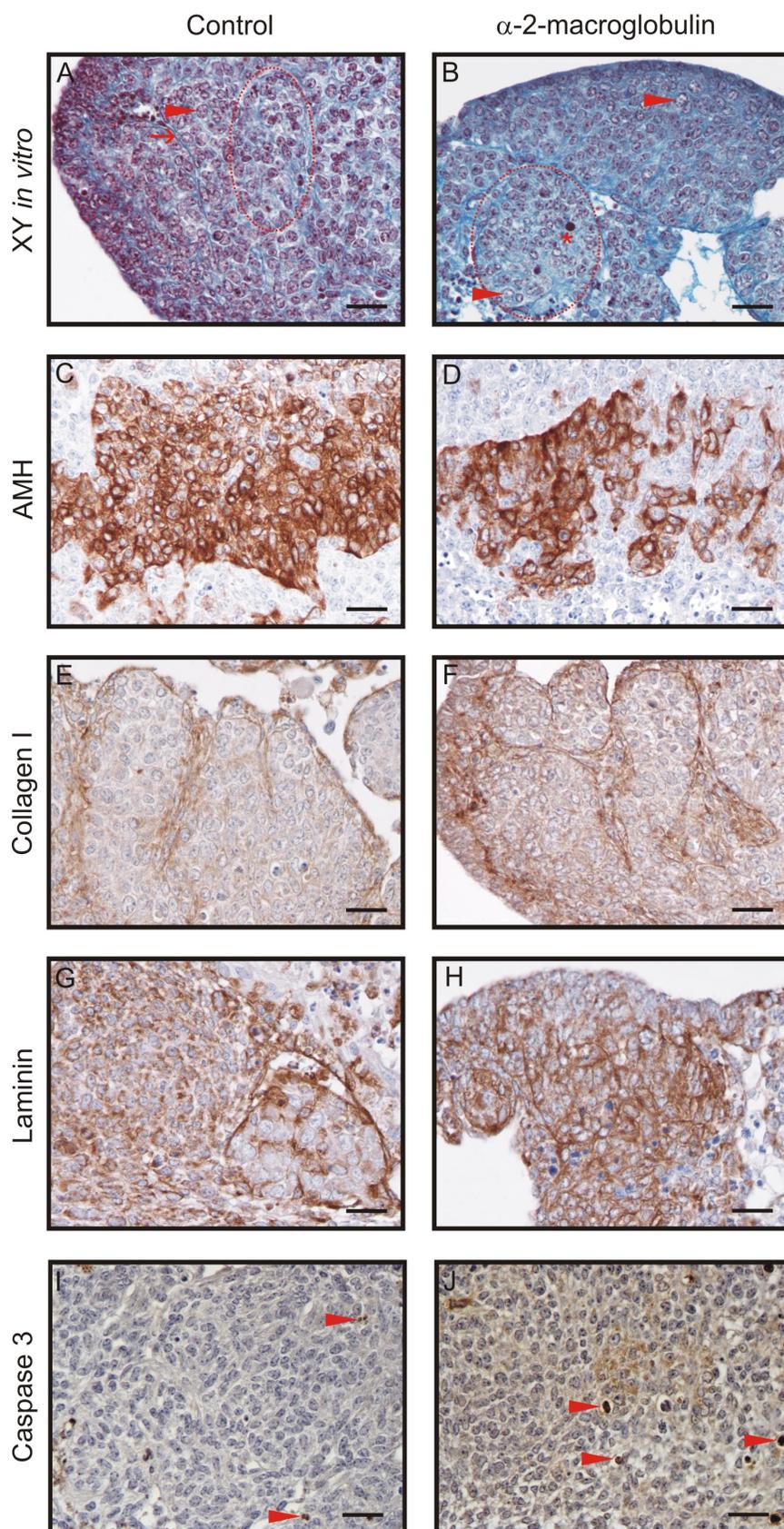


Fig. 4.

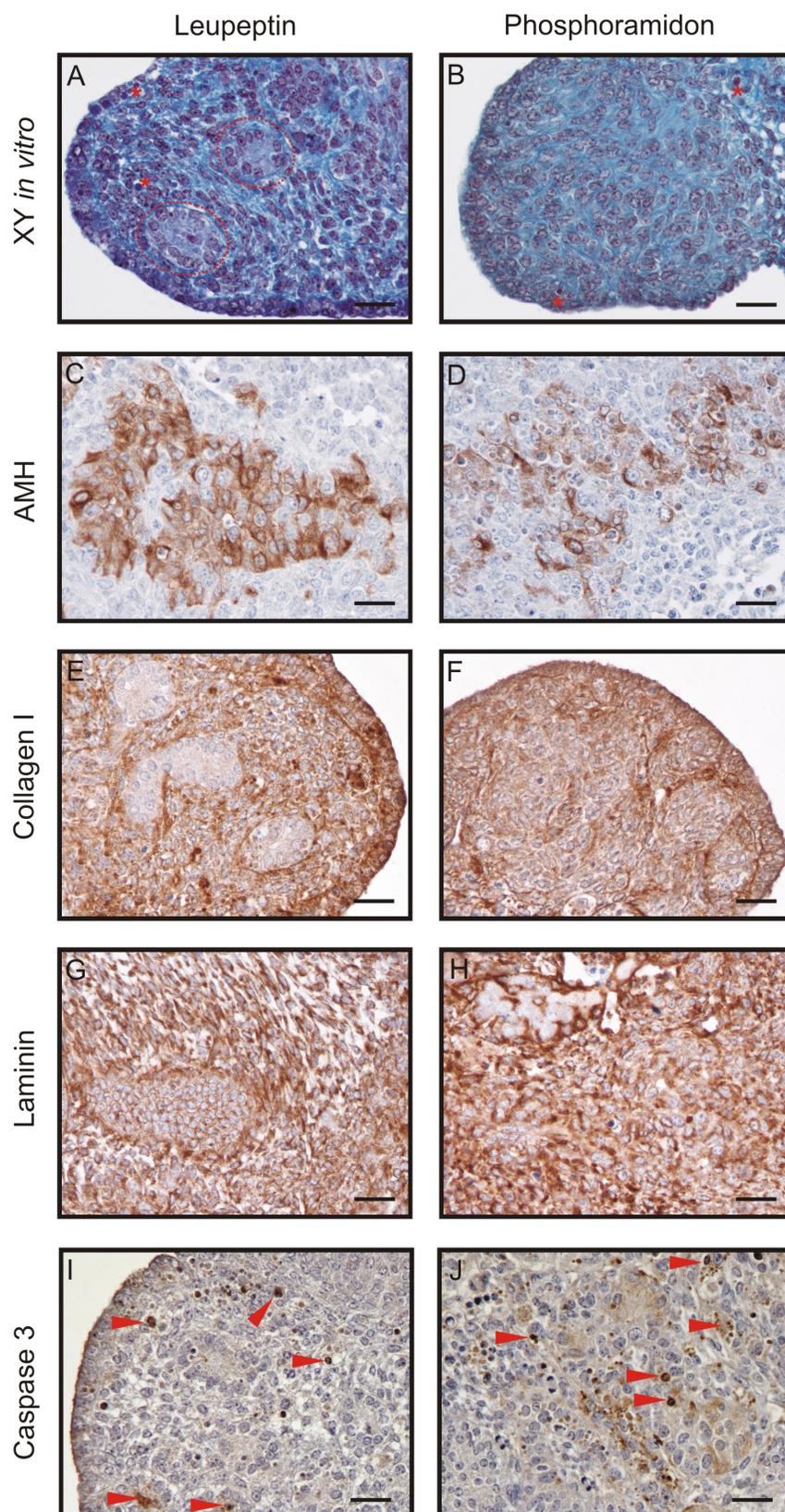


Fig. 5.

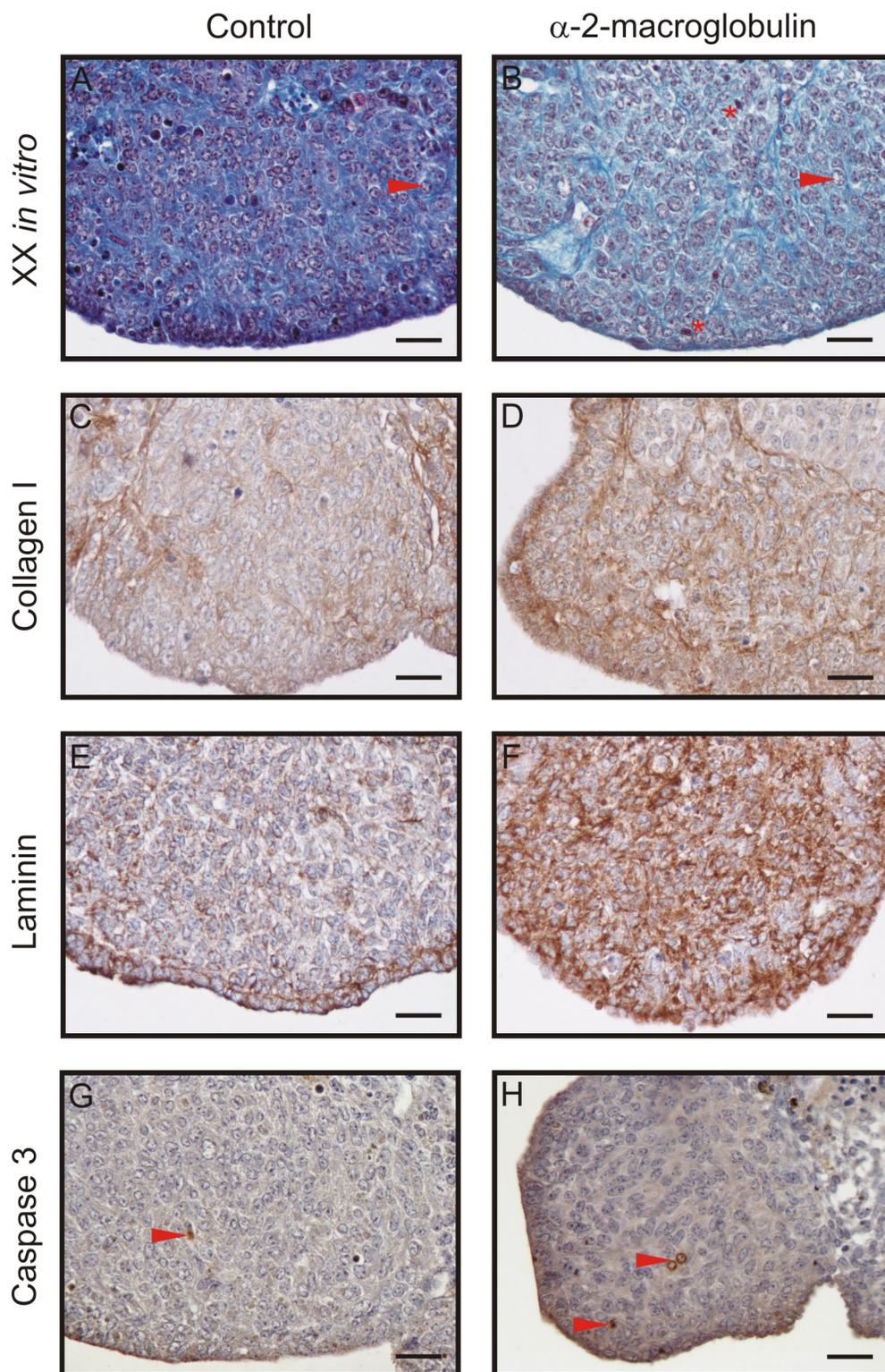


Fig. 6.

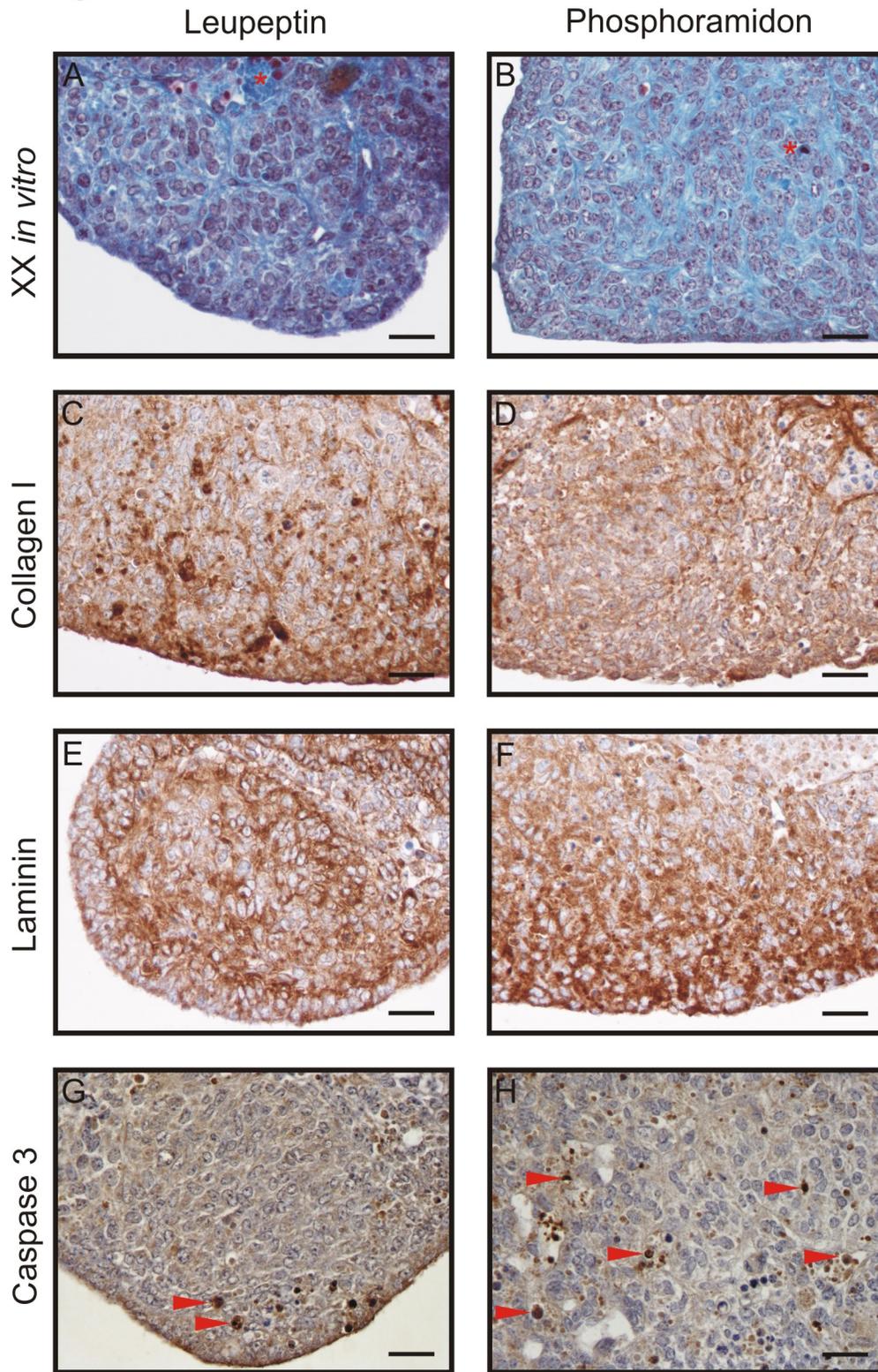


Fig. 7.

