TITLE: Three-dimensional culture in a bioengineered matrix and somatic cell complementation to improve growth and survival of bovine preantral follicles

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STRUCTURED ABSTRACT

Purpose: Here we explored poly(ethylene glycol) (PEG) bioengineered hydrogels for bovine preantral follicle culture with or without ovarian cell co-culture and examined the potential for differentiation of bovine embryonic stem cells (bESCs) towards gonadal somatic cells to develop a system more similar to the ovarian microenvironment.

Methods: Bovine preantral follicles were first cultured in two-dimensional (2D) control or within PEG hydrogels (3D) and then co-cultured within PEG hydrogels with bovine ovarian cells (BOCs) to determine growth and viability. Finally, we tested conditions to drive differentiation of bESCs towards the intermediate mesoderm and bipotential gonad fate.

Results: Primary follicles grew over the 10-day culture period in PEG hydrogels compared to 2D control. Early secondary follicles maintained a similar diameter within the PEG while control follicles decreased in size. Follicles lost viability after co-encapsulation with BOCs; BOCs lost stromal cell signature over the culture period within hydrogels. Induction of bESCs towards gonadal somatic fate under WNT signaling was sufficient to upregulate intermediate mesoderm (*LHX1*) and early coelomic epithelium/bipotential gonad markers (*OSR1*, *GATA4*, *WT1*). Higher BMP4 concentrations upregulated the lateral plate mesoderm marker *FOXF1*. *PAX3* expression was not induced, indicating absence of the paraxial mesoderm lineage.

Conclusions: Culture of primary stage preantral follicles in PEG hydrogels promoted growth compared to controls; BOCs did not maintain identity in the PEG hydrogels. Collectively, we demonstrate that PEG hydrogels can be a potential culture system for early preantral follicles pending refinements, which could include addition of ESC-derived ovarian somatic cells using the protocol described here.

KEYWORDS

Preantral follicle, three-dimensional culture, embryonic stem cells, ovary, bovine, biomimetic

CAPSULE SUMMARY

We demonstrate that three-dimensional bioengineered hydrogels could aid in the survival and growth of small bovine preantral follicles. Moreover, bovine embryonic stem cells have the potential to differentiate towards precursors of somatic gonadal cell types, presenting an alternative cell source for preantral follicle co-culture.

## INTRODUCTION

Folliculogenesis is a complex and selective process resulting in production of a limited quantity of meiotically and developmentally competent oocytes over a female's lifespan. The molecular processes that coordinate the activation, growth and maturation of ovarian follicles have yet to be fully elucidated, especially during the early preantral stages of development. Progress in understanding preantral folliculogenesis in non-rodent species has been hindered by the lack of appropriate in vitro culture systems and an insufficient knowledge base of ovarian development and function. This is particularly true in large mammal (i.e. cows and humans) preantral folliculogenesis which have prolonged duration and sizeable volumetric changes between the primordial and pre-ovulatory follicle stages. A great advantage of performing in vitro folliculogenesis beginning at the preantral stage includes establishing an alternative fertility preservation and restoration method by utilizing the vast preantral follicle pool for oocyte production [1]. More specifically, this approach would be beneficial for prepubertal girls suffering from blood-borne malignancies as isolated preantral follicles could be matured in vitro without the risk of reintroducing malignant cells or surgical intervention, as could be the case with the culture and reintroduction of ovarian tissue fragments.

Recapitulation of bovine preantral folliculogenesis has been attempted by several groups, but there has been little success in efficiently growing follicles to the preovulatory stage and yielding metaphase II oocytes. Although 2-dimensional culture systems have shown some promise in supporting bovine preantral follicle growth [2–4], mounting evidence suggests that 3-dimensional (3D) culture systems better promote development due to their ability to maintain a follicle's spherical configuration and preserve cell contact

74 both within the follicular unit (i.e. granulosa cell and oocyte interface) and outside the
75 basement membrane where theca cells are located [5–8]. Matrigel and alginate are
76 commonly used biomaterials to create hydrogels for in vitro follicle culture, however
77 pitfalls include uncontrollable degradation and biological inertness, respectively [9].
78 Therefore, a highlighted alternative is the use of bioengineered 3D hydrogel culture
79 systems that permit timely degradation by the follicle yet retaining the mechanical support
80 necessary to keep its spherical structure [10,11]. Poly(ethylene glycol) (PEG) hydrogels
81 constructed with proteolytically-degradable peptide crosslinkers have been employed for
82 mouse in vitro folliculogenesis and demonstrated success in growing preantral follicles to
83 the antral stage and producing metaphase II oocytes [12]. Likewise, the addition of
84 adipose-derived stem cells or conditioned media to PEG hydrogels containing mouse
85 preantral follicles further aided development and survival [13]. Despite the promise
86 demonstrated in mice, the use of PEG hydrogels for large mammal in vitro preantral
87 follicle culture has not been reported. Moreover, investigating cell supplementation in the
88 hydrogel system to determine their propensity to become theca cells and/or
89 advantageous effect on follicle growth has not been directly assessed. Indeed, early
90 preantral follicles do not contain theca cells and must recruit precursor cells from the
91 stromal environment to build the essential theca layer, hence addition of cells to the
92 system would be essential when starting with primordial- or primary-stage follicles. More
93 specifically, the hedgehog signaling pathway is key to driving theca cell differentiation and
94 recruitment [14–16], therefore cells added to the culture system should harbor the cellular
95 machinery to respond to hedgehog ligands secreted by the follicular granulosa cells.

Although native ovarian stromal cells have been used as feeder cells for 2D and 3D in vitro follicle culture [17–19], surgical intervention is required to obtain ovarian tissue and harvest cells. Moreover, their abilities to phenotypically-mirror theca cells upon co-culture with follicles has not been shown to date. Alternative cell sources, particularly those of renewable capacity (i.e. stem cells) present a unique advantage to generating cells that could give rise to a theca cell-like phenotype. To that end, stepwise in vitro differentiation of pluripotent stem cells into gonadal-like cells has been demonstrated in humans and mice [20–22] thus highlighting the potential to create a substitute cell source with prospective theca cell characteristics. Cell fate decisions that result in formation of the bipotential gonad in the embryo are the culmination of precisely timed cell signaling via gradients of morphogens such as FGF, BMP, and WNT proteins. Hence, protocols have been devised employing a stepwise induction of pluripotent stem cells using FGF, BMP, and WNT signaling such that cells progress through a mesoderm-like state, and finally a bipotential gonad-like state [23–25].

In this study, we describe the use of a PEG hydrogel culture system with or without native ovarian cells for the in vitro development of bovine preantral follicles and explore the potential of bovine embryonic stem cells to be induced towards gonadal-like cells in vitro. We first hypothesized that culture in PEG hydrogels would improve the growth and viability of bovine preantral follicles. By examining the gene expression of ECM-degrading enzymes of primary- and early secondary-stage follicles, we tailored the hydrogel design to respond to bovine follicle enzyme secretion. Based on the evidence that cell supplementation improves mouse follicle development, we also examined the effect of dissociated bovine ovarian cells (BOCs) on bovine preantral follicle growth and if BOCs

exhibit a pre-theca cell phenotype when cultured in vitro using PEG hydrogels. Finally, we hypothesized that bovine embryonic stem cells (bESCs) can be differentiated into the intermediate mesoderm and early coelomic epithelium/bipotential gonad.

## MATERIAL AND METHODS

All materials were purchased from Thermo Fisher Scientific unless otherwise specified.

*Follicle Isolation and bovine ovary single-cell dissociation*

Fresh bovine preantral follicles were isolated as previously described [26]. Follicles were maintained in warmed follicle wash media until subsequent experiments. For bovine ovary single-cell dissociation (n = 3 replicates), cortical tissue (250-300 mg) was fragmented into 500 $\mu m^2$ pieces incubated with Hank's balanced salt solution ($Ca^{2+}/Mg^{2+}$) containing 1 mg/mL collagenase IV, 1 U/mL DNase I, and 50 µg/mL Liberase (5401119001, Sigma Aldrich). The solution with tissue was shaken at 38.5 °C for 20 min, pipetted 15 times using a serological pipette, and shaken again for 20 min to mechanically aid in the dissociation of cells. Enzymatic activity was stopped by adding 20% v/v fetal bovine serum to the solution. The tissue-dissociated solution was filtered through a 100 µm then through a 35 µm cell strainer. Cells collected after final filtration were centrifuged (300 x*g* for 5 min) and the resulting pellet was resuspended in 1 mL Hank's balanced salt solution without $Ca^{2+}/Mg^{+}$ until encapsulation.

*PEG hydrogel materials, preparation, and follicle encapsulation*

PEG hydrogel experiments were conducted using 8-arm PEG vinyl sulfone (PEG-VS) (40 kDa, >99% purity, JenKem Technology) and crosslinked with MMP- and Plasmin-

143 sensitive peptide sequences (Ac-GCRD**VPMS**MRGGDRCG**YKNS**CG, i.e. YKNS/VPMS)
144 (2391.8 g/mol, >90%, Celtek Peptides). PEG and crosslinker peptide were dissolved in
145 isotonic 50 mM HEPES buffer and mixed to a final composition containing 5% PEG-VS
146 with 1:1 stoichiometric ratio of VS to thiol (side chain present on cysteines in peptide
147 sequence). Sixteen-µl hydrogels containing preantral follicles were formed between
148 parafilm-lined glass slides and allowed to gelatinize for up to 10 min. Then, hydrogels
149 were transferred to warmed follicle maintenance media to swell and placed in a humidified
150 incubator (37°C). Freshly isolated follicles were maintained in warmed follicle wash media
151 until encapsulation. In experiment 1, follicles (n = 1-4) were first washed in 10% PEG-VS
152 and either encapsulated in PEG hydrogels or placed into 2D culture. Hydrogel-
153 encapsulated and 2D culture follicles were placed into individual wells of a 96-well plate
154 containing follicle culture media (1:1 ratio of α-MEM and F12 with glutamine
155 supplemented with 3 mg/mL bovine serum albumin, 100 ng/mL penicillin/streptomycin,
156 1 mM sodium pyruvate, 1 % (v/v) non-essential amino acids, 10 mg/l insulin, 5.5 mg/l
157 transferrin and 6.7 µg/L selenium (ITS), 100 ng/mL human recombinant follicle stimulating
158 hormone (228-12609-2, RayBiotech), and 50 µg/mL ascorbic acid. Half of the spent media
159 was changed every 2 days and follicles were cultured for 10 days. To assess follicle
160 growth over the culture period, the average of 2 perpendicular diameter measurements
161 were taken at day 0, day 5, and day 10 using an inverted microscope (Revolve, Echo
162 Labs). The percentage of growing and non-growing follicles (growth defined as having at
163 least 5% increase in diameter by day 10 compared to day 0) and percent increase in size
164 in growing-only follicles was calculated. In total, 15 replicates were conducted with a total
165 of 216 follicles (n = 153 in PEG and n = 63 in 2D control)

166 *Cell viability staining and quantification*

167 Bovine ovarian cells and MeLCs were resuspended in PEG-VS + crosslinker
168 peptide VPMS/YKNS at a concentration of 1, 2, 10, and 20 x $10^6$ cells/mL to test cell
169 viability immediately after encapsulation, 5 and 10 days after in vitro culture. Hydrogels
170 were cultured individually in 96-well plates with 150 µl of follicle culture media with half
171 media changes every 2 days. After culture, hydrogels were incubated for 10 min with pre-
172 equilibrated alpha-MEM containing 2 µM propidium iodide (PI) and 1:1000 Hoechst (1
173 µg/mL final concentration). Negative control hydrogels were void of propidium iodide and
174 used for setting exposure limit during imaging. Hydrogels were imaged using an
175 epifluorescent microscope (Revolve) and the total number of cells and number of viable
176 cells were quantified using ImageJ after converting to binary images where PI/Hoechst-
177 positive cells were white, and background was black. Total number of cells positive for PI
178 were divided by total cells quantified by Hoechst staining to determine percentage of
179 unviable cells. Percentage of viable cells was determined by subtracting percentage of
180 unviable cells from 100. The experiment was repeated 4 times using 3 hydrogels per cell
181 density for each replicate.

182

183 *Bovine ovarian cells with follicle encapsulation and culture in PEG*

184 To test the impact of BOC and co-encapsulation on follicle viability and growth and
185 theca cell differentiation during in vitro culture, hydrogels were formed with either 1) only
186 follicles, 2) 5 x $10^6$ BOCs/mL only, or 3) follicles and 5 x $10^6$ BOCs/mL BOCs. Each
187 hydrogel contained 1-5 follicles (n = 65 follicles); 5 replicates). Hydrogel formation and
188 follicle encapsulation was performed as previously described with the addition of staining

189 follicles with the cell membrane marker PKH26 (2 μM final concentration; Sigma-Aldrich)
190 prior to encapsulation to improve visualization. Hydrogels were cultured for 10 days with
191 half of the medium changed every other day. Follicles were considered viable if the
192 basement membrane was attached, the follicles were void of vacuoles, and granulosa
193 cells were not darkened or mishappened. To recover cells for RT-qPCR, the hydrogels
194 were incubated in phenol red-free alpha-MEM with Liberase (1.3 wünsch units/mL) and
195 incubated at 37 °C and 5% $CO_2$ for 30 min with repeated pipetting to degrade the hydrogel
196 and extract the cells. After centrifugation, the cell pellet was used for RNA extraction,
197 cDNA synthesis and RT-qPCR as described below.
198
199 *Culture of bovine embryonic stem cells (bESCs)*
200 Bovine embryonic stem cells (n = 2 female lines) were routinely cultured feeder-
201 free in NBFR medium (N2B27 medium [1:1 DMEM/F12 and Neurobasal media, 0.5% v/v
202 N-2 supplement, 1% v/v B-27 supplement, 2 mM MEM Non-Essential Amino Acid
203 solution, 1% v/v GlutaMAX supplement, 0.1 mM 2-mercaptoethanol, 100 U/mL Penicillin,
204 and 100 μg/mL Streptomycin] supplemented with 1% bovine serum albumin (BSA), 20
205 ng/mL bFGF (100-18B, PeproTech), 2.5 μM IWR-1 (I0161, Sigma), and 20 ng/mL Activin
206 A (338-AC, R&D Systems)]. Cells were passaged onto vitronectin-coated plates every 2-
207 4 days using TrypleExpress at 1:4-1:6 split ratio and cultured in humidified incubators at
208 37 ºC and 5% $CO_2$. When cells were passaged, media was supplemented with 10 μm
209 ROCK inhibitor Y-27632. Media was changed daily and bESCs used for mesoderm
210 induction were between passage 18-26.

211 For mesoderm induction, bESCs were passaged onto 12-well vitronectin-coated
212 plates at 40,000 cells per well in mesoderm-induction media [GMEM medium, 15%
213 knockout serum replacement, 0.1 mM NEAA, 2mM GlutaMAX, 1mM sodium pyruvate,
214 0.1mM β-mercaptoethanol, 100 U/mL Penicillin, and 100 µg/mL streptomycin and
215 supplemented with 3 µm CHIR99021 and 70 ng/mL Activin A]. Cells were cultured in
216 mesoderm induction medium for 48 hours with daily medium change and thereafter the
217 cells were considered "mesoderm-like cells" (MeLCs) and used for subsequent
218 experiments.

219 Mesoderm-like cells were cultured in mesoderm induction medium without activin
220 A for an additional 72 hours and supplemented with 10 ng/mL bFGF beginning
221 immediately after MeLC induction, 24 hours after MeLC induction, 48 hours after MeLC
222 induction, or no supplementation (mesoderm medium only). Based on the finding of which
223 bFGF regimen best upregulated gene expression, in a second experiment, MeLCs were
224 cultured in mesoderm induction media without activin A, with 10 ng/mL bFGF and with
225 either 0, 1, 10, or 20 ng/mL of BMP4 for an additional 72 hours. All experiments contained
226 duplicate wells for each condition tested and had 3 biological replicates/independent
227 differentiations. Cells were imaged every day during each differentiation on the EVOS
228 (Thermo Fisher) inverted microscope.

229

230 *End-point (RT-PCR) and quantitative reverse transcription PCR (qRT-PCR)*

231 Freshly isolated follicles were pooled by stage and snap frozen in minimal PBS. To
232 examine the expression of transcripts for extracellular-matrix degrading enzymes,
233 primary follicles (n = 3 pools of 46-56 follicles per pool) and early secondary follicles (n =

234 3 pools of 34-40 follicles per pool) were subjected to RNA isolation using a protocol with
235 a combination of Trizol, Purelink DNase treatment, and Qiagen RNeasy Micro Kit. Final
236 total RNA was eluted into 14 µl of RNase-free water. Bovine ovarian cells (BOCs), bESCs,
237 MeLCs and differentiated cells were pelleted, snap frozen, and subjected to RNA isolation
238 following manual instructions using the Qiagen RNeasy Micro Kit. Maximum available
239 RNA mass was used for cDNA synthesis of primary and early secondary follicles and 250
240 ng - 1 µg of total RNA was used for cDNA synthesis of bESCs, BOCs, MeLCs, and
241 differentiated cells (RevertAid cDNA Reverse Transcription Kit). For RT-qPCR, 1-2 µl of
242 cDNA (50 ng total), 1x SsoAdvanced Universal SYBR Green Supermix (Bio-Rad,
243 Hercules, CA), and 250 nM of forward and reverse primers were mixed for 10 µl total
244 volume and pipetted into 96-well plates. The reaction was carried out in the CFX96 Touch
245 Deep Well Real-Time PCR Detection System (Bio-Rad). Cycling conditions were an initial
246 denaturation step at 95 ºC for 30 sec followed by 40 cycles of denaturation at 95 ºC for
247 10 sec, annealing at 60 ºC for 30 sec, and extension at 60 ºC for 5 sec. To confirm
248 specificity of PCR products for each gene, each assay included a melt curve analysis and
249 non-template control. Data was normalized to *ACTB* by the delta-Ct method and relative
250 expression data are represented as 1/ΔCT. For conventional PCR, PCR products from
251 quantitative PCR reactions were ran on a 2% agarose gel to visualize the PCR products.
252 For RT-qPCR experiments using *BAX* and *BCL2* primers, a standard curve of fresh
253 bovine ovarian cells was used to estimate absolute quantity of mRNA present in unknown
254 samples of BOCs. The ratio of *BAX*/*BCL2* was calculated from mRNA quantity of
255 respective genes and used to determine if cells were in a pro- or anti-apoptotic state. All
256 primers used in these experiments are listed in Table 1.

*Statistical analysis*

All data was first assessed for normality using a Shapiro-Wilk test. PEG growth data was subjected to a two-way ANOVA using RStudio with effect of replicate included in the model. Cell viability and RT-qPCR data were subjected to a one-way ANOVA or Kruskal-Wallis test if data was not normally distributed using Graph Prism. Post-hoc tests (Tukey's and Dunn's for ANOVA and Kruskal-Wallis, respectively) were conducted when appropriate. RT-qPCR data were analyzed based on delta-Ct values. Comparisons were considered significantly different when associated with $P < 0.05$.

## RESULTS

To design a proteolytically-degradable PEG hydrogel that would most likely be compatible with bovine preantral follicles, we first examined the gene expression of ECM-degrading enzymes in primary and early secondary follicles that express the key follicle marker *FSHR*. Matrix metalloproteinases (MMPs) and plasminogen activators are ECM-degrading enzymes and play a pivotal role in tissue remodeling. We found the bovine primary and early secondary follicles express *MMP2*, although in one pool we only found *MMP2* in early secondary follicles. However, neither follicle stage expressed *MMP9* (Figure 1A). Plasminogen-activator urokinase (*PLAU*) was expressed in primary and early secondary follicles from one pool, but not in the second or third pool. Similarly, Plasminogen-activator tissue (*PLAT*) was expressed in primary follicles of one pool and in early secondary follicles from two other pools (Figure 1A). Since we collectively found mRNA expression of *MMP2* and plasminogen enzymes, we proceeded with using a PEG

279 hydrogel that is functionalized with VPMS (MMP-sensitive) and YKNS (plasminogen-
280 sensitive) peptide sequences (Figure 1B).

281 Next, we tested the hypothesis that bovine preantral follicles are more likely to
282 grow and demonstrate greater growth rate when encapsulated in PEG hydrogels
283 compared to 2D traditional methods after 10 days of culture. We found no difference in
284 the percentage of growing follicles between PEG (35.8% ± 6.23%) and 2D control (30.3%
285 ± 7.88%) ($P$ = 0.56) (Figure 2A). We found that replicate (i.e. individual ovary) affected
286 the percentage of growing follicles ($P$ < 0.05) regardless of culture condition. It is worth
287 noting that in the control group, no follicle growth was observed in four out of the 15
288 replicates, whereas in the PEG group there was growth, although variable, in every
289 replicate (Figure 1A). Of the replicates where at least one follicle demonstrated growth in
290 either condition, we found no difference ($P$ = 0.50) in the growth rate from initial size (PEG:
291 18.8% ± 3.32%, control: 22.7% ± 3.49%) (Figure 2B). There was no effect of replicate on
292 growth rate ($P$ = 0.78).

293 We further examined the diameter changes between day 0, 5, and 10 in follicles
294 within a narrow range of starting diameters corresponding to their stage of development
295 between the primary to early secondary. When we examined follicles with starting
296 diameters corresponding to the early primary stage (40-60 μm), we found PEG hydrogels
297 outperformed the control conditions as day 5 follicles were larger in PEG compared to 2D
298 control ($P$ < 0.05). Moreover, by day 10, PEG hydrogel follicles had a significant increase
299 in diameter compared to day 0 ($P$ < 0.05), whereas control follicles showed no difference
300 ($P$ = 0.72). Follicles starting at 61-70 μm showed a decrease in diameter by day 10 of
301 culture in control conditions ($P$ < 0.05), whereas follicles cultured in PEG maintained a

302 similar diameter over time ($P$ = 0.68; Figure 2C). Therefore, day 5 and 10 follicles were
303 significantly smaller ($P$ < 0.05) in control follicles when compared to similar timepoints of
304 PEG follicles. A representative image of a follicle grown in a PEG hydrogel over the 10-
305 day period is depicted in Fig. 2D.

306 We evaluated cell viability of BOCs when encapsulated in PEG hydrogels and
307 cultured for 10 days (Figure 3A-C). When encapsulated at 1 or 2 x $10^6$ cells/mL, BOCs
308 showed low cell viability (data not shown), whereas concentrations of 10 or 20 x $10^6$
309 cells/mL showed high viability (70-90%) and cell aggregation (Figure 3B and C). In this
310 experiment, we found that neither control (no BOCs encapsulated) or hydrogels with
311 BOCs supported follicle growth over the 10 day-culture period (Figure 3D). Moreover,
312 gene expression analysis of pre-theca cell markers in the BOCs after 10 days of culture
313 revealed a decrease in expression of *PTCH1* ($P$ < 0.01) and *GLI1* ($P$ < 0.01) when
314 compared to day 0 fresh cells in both BOCs cultured alone and co-encapsulated with
315 follicles (Figure 3E). We found a decrease in both *BAX* and *BCL2* in BOCs that were
316 cultured with follicles and a decrease in *BCL2* in BOCs cultured in PEG hydrogels alone
317 ($P$ < 0.05; Figure 3F). However, there was no difference in *BAX*/*BCL2* ratio, thus indicating
318 no difference in the pro- versus anti-apoptotic balance between cultured and fresh BOCs.

319 Given that native BOCs did not show maintenance of pre-theca identity or
320 contribute to follicle growth, we then explored the differentiation of bESCs into progenitor
321 cells of the bipotential gonad by testing effect of WNT, bFGF and BMP4 signaling (Figure
322 4A). We first tested the hypothesis that MeLCs exposed to a pulse of bFGF during
323 intermediate mesoderm/bipotential gonad differentiation would upregulate gene
324 expression of key markers at the end of induction (Figure 4B and C). Compared to bESCs

325 and 0, 24, 48, and 72 hours of 10 ng/mL bFGF exposure, MeLCs had higher expression
326 of the early lateral plate/intermediate mesoderm marker *LHX1* (Figure 4B; $P < 0.001$). We
327 also found the intermediate mesoderm marker *OSR1* to be upregulated in all bFGF
328 regimens compared to bESCs ($P < 0.01$). When analyzing expression of bipotential gonad
329 markers, there was no difference in *GATA4* ($P = 0.15$) across all experimental groups. We
330 also found higher expression of *WT1* after 0, 24, 48, and 96 hours of bFGF (i.e., after 4
331 additional days of culture) compared to bESCs and MeLCs ($P < 0.001$). There was no
332 difference in expression of *WT1* between bESCs and MeLCs ($P = 0.15$). There was also
333 no difference in expression of the paraxial mesoderm marker *PAX3* across all
334 experimental groups ($P = 0.06$), indicating induction conditions did not show strong
335 propensity to drive cells to the paraxial mesoderm fate. However, the lateral plate
336 mesoderm marker *FOXF1* was significantly upregulated compared to bESCs in 0, 24, and
337 48 hours of bFGF exposure ($P < 0.05$).

338 Because all bFGF regimens tested caused elevation in *WT1* and *OSR1*
339 expression, we chose to use a 24-hour pulse of bFGF in the next experiments when
340 testing the synergistic effects of including various concentrations of BMP4 over the same
341 culture period time and in two bESC lines (Figure 5A and B). Similar to the bFGF
342 experiment, we found the highest level of *LHX1* expression in MeLCs (Figure 5A; $P <$
343 $0.0001$). However, 10 and 20 ng/mL of BMP4 led to lower expression when compared to
344 bESCs ($P < 0.05$). Also confirming the previous results, *OSR1* was elevated in all
345 experimental groups, including MeLCs, when compared to bESCs ($P < 0.01$). Unlike in
346 the bFGF experiment where there was no difference amongst exposure times to bFGF,
347 we found an increase in *GATA4* in MeLCs and when 1, 10 or 20 ng/mL of BMP4 were

348 present ($P < 0.05$). *WT1* was upregulated in 0, 1, 10, or 20 ng/mL BMP4, but not in MeLCs
349 ($P < 0.05$). Again, we found no difference in *PAX3* expression among all concentrations
350 of BMP4 when compared to bESCs. As expected, increasing the concentration of BMP4
351 led to a significant increase in *FOXF1* ($P < 0.05$), indicating a likely shift towards the lateral
352 plate mesoderm lineage with rising levels of BMP4 which is similar to in vivo development.
353 We further assessed WT1 and OCT4 expression in bESCs, MeLCs, and cells
354 cultured with 0 and 1 ng/mL BMP4 as these concentrations led to elevated *WT1* without
355 greatly upregulating *FOXF1* (Figure 5B). We found positive nuclear staining for OCT4 in
356 bESCs and MeLCs and no signal in cells exposed to 0 or 1 ng/mL BMP4, indicating likely
357 loss of pluripotency after mesoderm induction. We found nuclear localization of WT1 in
358 bESCs and nuclear and cytoplasmic localization in MeLCs (48 h culture) and 0 ng/mL
359 BMP4 (120 h culture). Interestingly, we did not observe WT1 expression at the protein
360 level when cells were exposed to 1 ng/mL BMP4.
361
362 DISCUSSION
363 To our knowledge, this study is the first to utilize a novel bioengineered PEG
364 hydrogel system for in vitro culture of bovine preantral follicles and assess whether
365 bESCs could be differentiated towards progenitor cells of the bipotential gonad using
366 stepwise in vitro culture protocols. The ultimate goal of this work is to develop novel
367 assisted reproductive technologies that enable the use of small preantral follicles in
368 conjunction with renewable theca-like cells for oocyte production. Since maintaining
369 structural integrity of the follicle is a main concern with 2D culture, hydrogels represent a
370 viable option as they provide framework to maintain the 3D configuration of follicles while

371 permitting growth. Indeed, the theory to build a culture system complete with extracellular
372 matrix components like the ovary has been suggested when early studies first explored
373 bovine preantral in vitro development [27]. Further adaptations to include sophisticated
374 ECM components in the hydrogel design have permitted mouse follicle growth in vitro
375 starting as early as the primordial stage [28]. Our finding that ECM-degrading enzymes
376 such as MMPs, PLAT and PLAU are expressed by bovine preantral follicles agrees with
377 previous investigations in follicles and spent medium [37,38]. However, the fact that these
378 transcripts were not uniformly expressed in all pools examined could indicate an intrinsic
379 variability in the ability of the follicles to remodel the ECM. This could explain at least in
380 part the variability observed in growth rate in the present study. Although we found that
381 PEG hydrogels outperformed 2D control over 10 days in primary follicles, there was a
382 notable difference in the percentage of follicles that grew between replicates (each
383 represented by one ovary), further underlining the biological variability between samples
384 and possibly an inherent propensity of some follicles for growth versus atresia. A limitation
385 of this study using slaughterhouse-derived oocytes is the inability to track factors known
386 to affect follicle viability such as animal age, nutritional and health status. Of note, early
387 secondary follicles showed a stagnant rate of growth compared to primary follicles
388 cultured in PEG hydrogels; this stagnation suggests that either 1) the 10 day-culture
389 conditions remain substandard for bovine preantral follicles or 2) follicles canonically slow
390 their growth at this stage of development which is a phenomenon seen in mice [29]. It is
391 worth noting that in vivo, 10 days represents a very narrow window within the estimated
392 180 days of folliculogenesis in the cow, and therefore relatively small changes in growth
393 could be expected in normally-developing follicles.

394 We tested the hypothesis of enhancing the bovine follicle culture system with
395 cellular supplementation as others have shown that the addition of ovarian stromal cells,
396 fibroblasts, and adipose-derived stem cells promote survival and growth of preantral
397 follicles in several species [13,17–19,30]. When BOCs were encapsulated in PEG
398 hydrogels and cultured, we found cell aggregates which may be due to absence of
399 basement membrane binding proteins in the PEG composition, rendering the cells unable
400 to adhere to the matrix and resulting in self-aggregation. Indeed, cell-extracellular matrix
401 interactions are important for mediating complex cellular responses such as migration,
402 proliferation, and differentiation [31], thus BOCs were likely incapable of integrating well
403 throughout the hydrogel. Furthermore, the finding of decreased expression of genes
404 known to be important for the pre-theca cell phenotype after culture shows that the BOCs
405 did not maintain their identity, which could have contributed to the lack of success of this
406 3D co-culture system. Others have reported that mouse ovarian stromal cells transition
407 from a predominately theca-like identity to almost entirely macrophage identity after 12
408 days of in vitro culture with follicles, partially corroborating our findings of theca cell loss
409 [32]. Since we found similarly high signs of atresia between follicles co-encapsulated and
410 follicles in PEG-only hydrogels, we pose the possibility that the prolonged protocol
411 required to dissociate ovarian tissue, isolate and label follicles, and process for
412 encapsulation may have contribute to decrease follicle viability beginning at day 0. In
413 future experiments, pre-theca cells could be sorted (pending availability of reliable
414 antibodies against specific theca cell surface markers) before incorporation into the PEG
415 hydrogel. Furthermore, culture conditions that specifically support theca cell phenotype
416 would likely need to be investigated.

In an attempt to produce gonadal-like cells for supplementation as pre-theca-like cells in follicle culture, we employed bovine embryonic stem cells to recapitulate mesoderm formation and early differentiation of the bipotential gonad with defined media conditions. We show that inclusion of exogenous signaling factors such as the GSK3β inhibitor CHIR 99021 for activation of WNT. WNT signaling can drive bESCs into intermediate mesoderm-like cells that show early signs of coelomic epithelial-like phenotype as indicated by gene and protein expression. During embryonic development, the sexually dimorphic gonad (ovary or testis) must develop beginning from the mesoderm germ layer, and then further differentiate into the intermediate mesoderm, then coelomic epithelium which ingresses and gives rise to the sexually amorphic “bipotential gonad” [33,34]. More specifically, upon Nodal, WNT and FGF signaling to induce mesoderm differentiation [35], a subset of cells in the caudal aspect of the early gastrulated embryo are specified to become precursors of the bipotential gonad. Signaling by WNT, Nodal, and FGF during anterior-posterior (A-P) body axis extension, which occurs at peri-gastrulation and is regulated by TBXT and other morphogens in the mesoderm, drives cell fate decisions that result in caudal body axis formation [36,37]. Concomitantly, mediolateral segmentation occurs when a low, medium, and high gradient of BMP signaling drives mesoderm cells into the paraxial, intermediate, and lateral plate mesoderm domains, respectively [38,39]. Following this knowledge of developmental biology, we chose to modulate WNT, Nodal, FGF and BMP signaling to mimic the molecular events that drive cell differentiation for pre-gonad development during early embryogenesis as shown in several mammalian species [34]. However, through these experiments, we found that under WNT activation, bFGF and BMP4 are not essential to

440 induce the intermediate mesoderm or early coelomic epithelium. We observed peak
441 expression of the intermediate mesoderm marker *LHX1* after 48 h of mesoderm induction
442 under WNT and activin A, the latter a known inducer of *Lim1* (i.e. *Lhx1*) that has an
443 important role in IM specification/kidney development [40]. We also found *OSR1*
444 upregulated in all conditions after MeLC induction. *OSR1* is an early marker of IM and
445 others have shown its necessity for the urogenital phenotype [41–43] and generating
446 somatic cells of the gonad from PSCs [44]. Thus, we propose these cells may be reaching
447 the IM state with possible capability of becoming progenitor bipotential gonad-like.
448 Nevertheless, *OSR1* is not exclusive of the IM or gonads; kidney progenitor and lateral
449 plate mesoderm cells express *OSR1* [43,45] and this marker is used to demarcate kidney
450 progenitor-like cells during in vitro differentiation of PSCs [46–48]. Therefore, our
451 intermediate mesoderm/early coelomic epithelium-like cells should be further evaluated
452 for markers of early nephrogenesis.

453 We additionally found a rise in *WT1,* which is also an early, and more restrictive,
454 marker of gonadal progenitor cells [49], in induced cells across all bFGF and BMP4
455 regimens compared to bESCs. This indicates that continuous WNT activation for 3
456 additional days after initial mesoderm induction can provoke *WT1* expression. WT1 is
457 found along the A-P axis of mice as early as E9.0, with overlapping expression of GATA4
458 and NR5A1 in the coelomic epithelium by E10.0 and onward [49]. Moreover, WT1
459 activates the NR5A1 promoter [50], thus driving early gonad development. Interestingly,
460 *WT1* upregulation was accompanied by *GATA4* upregulation when 10 or 20 ng/mL of
461 BMP4 was included in culture which aligns with other studies [51]. However, we found
462 variable expression of the protein WT1 in cells cultured beyond MeLC induction and after

3 additional days under 0 or 1 ng/mL of BMP4. Although typically found in the nucleus, WT1 isoforms with capability of shuttling between the nucleus and cytoplasm, therefore we postulate various WT1 isoforms with nuclear and/or cytoplasmic activity may be expressed in our cells [52]. We additionally highlight that there was no difference in *PAX3* in all experiments, demonstrating that our culture conditions did not favor differentiation of the paraxial mesoderm lineage. Interestingly, increasing BMP4 concentrations led to increased expression of *FOXF1* (lateral plate mesoderm marker), which aligns with in vivo studies showing a higher gradient of BMPs specifies the lateral plate of developing embryos [39].

Our findings provide seminal context to previous work looking at differentiation of ESCs from humans and mouse to gonad-like cells by using combinations of BMP4, FGF, CHIR, and activin A [53–55]. Alternative approaches to inducing gonad-like cell formation include overexpression of key transcription factors such as *SF1*, *WT1*, *RUNX1*, and *GATA4* [56,57]. The efficiency of induction can be variable when using small molecules or genetic engineering for differentiating stem cells, therefore heterogeneity in cell identity would be expected in the present study. Although not done in this study, cell-sorting based on expression of key markers could aid in purifying cell populations of interest and used for downstream in vitro culture to achieve bipotential and even ovary or testis- like cells. Yet, in vivo early somatic progenitor cells of the bipotential gonad are not specified homogeneously, therefore some heterogeneity could be warranted, especially when forming organoids as self-organizing structures. Finally, future studies should examine early bovine embryonic development in vivo as findings from these studies could shed

light on the species-specific molecular mechanisms that drive gonad formation and inform future in vitro culture conditions to recapitulate the process.

In conclusion, these findings provide a key step in expanding current understanding of in vitro preantral folliculogenesis using a non-rodent model as well as sheds light on methodologies in differentiating bESCs into precursor-like cells of the ovary. The convergence of these two technologies will be pivotal to broadening the knowledge base of follicle development and early gonadogenesis such that novel assisted reproductive technologies can be expanded.

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AUTHOR CONTRIBUTION

Conceptualization: Juliana Candelaria, Anna Denicol; Methodology: Juliana Candelaria, Carly Guiltinan, Ramon Botigelli, Ariella Shikanov, Anna Denicol; Formal analysis and investigation: Juliana Candelaria, Anna Denicol; Writing - original draft preparation: Juliana Candelaria; Writing - review and editing: Juliana Candelaria, Anna Denicol, Ariella Shikanov, Carly Guiltinan, Ramon Botigelli,; Funding acquisition: Juliana Candelaria, Anna Denicol; Resources: Anna Denicol, Ariella Shikanov; Supervision: Anna Denicol.

COMPETING INTERESTS

The authors declare no competing interests.

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694 **Table 1** Primer list.

| Gene | Forward Sequence (5'-3') | Reverse Sequence (5'-3') | Product size (base pairs) |
|---|---|---|---|
| *BAX* | CATGAGGTCAACCCTCGGGA | TAG GGA AGC CCA TGCTGAGT | 117 |
| *BCL2* | GAGTTCGGAGGGGTCATGTG | GGG CCA TAC AGC TCCACAAA | 158 |
| *CSPG4* | CTGGAGGAACAA AGGTCTCTGG | ACTGCGTGATCTGGAAAAGCA | 140 |
| *DHH* | GCATGCATTGCAGGTCGTC | ACGTGGGCAGAATGCTAGTC | 145 |
| *FOXF1* | GCCTCCTACATCAAGCAGCA | GTTCTGGTGCAGATACGGCT | 108 |
| *GATA4* | TGCGCCCCATCAAAACAGAG | GGGCCATACAGCTCCACAAA | 77 |
| *GLI1* | CCTTCAAAGCCCAGTACATGC | GTGCGTCTTCAGATTTTCCAG | 122 |
| *IHH* | CGGCTTCGACTGGGTGTATT | GGCTGAGTGCTCGGACTTG | 70 |
| *LHX1* | TGGACCGCTTCCTCTTGAAC | CTTGGTACCGAAACACCGGA | 152 |
| *MMP2* | ATCGTCTTCGACGGC ATCTC | TTCGCCAGATGAATCGGTCC | 70 |
| *MMP9* | CTTCACCTTTGAGGGTCGCT | TGGGTGTAGAGTCTCTCGCT | 138 |
| *OSR1* | CCTGTGGAAATCCAGCGCC | CAGTCTGTTCTGACTTCCTGCTTA | 111 |
| *PAX3* | CCTTCAAAGCCCAGTACATGC | GTGCGTCTTCAGATTTTCCAG | 122 |
| *PLAT* | TCGGCAGGCCCTCTATTCTT | ACGTGGCGGTAGCATCTATTT | 89 |
| *PLAU* | TGGGTCAGTCACGGCTTAAC | ACGGATCTTCAGCAAGGCAAT | 131 |
| *WT1* | CCTTCACCGTCCACTTCTCT | CCGTGCTGTATCCCTGGTTG | 183 |
| *H2A.2* | GAGGAGCTGAACAAGCTGTTG | TTGTGGTGGCTCTCAGTCTTC | 104 |
| *ACTB* | CTCTTCCAGCCTTCCTTCCT | GGGCAGTGATCTCTTTCTGC | 178 |

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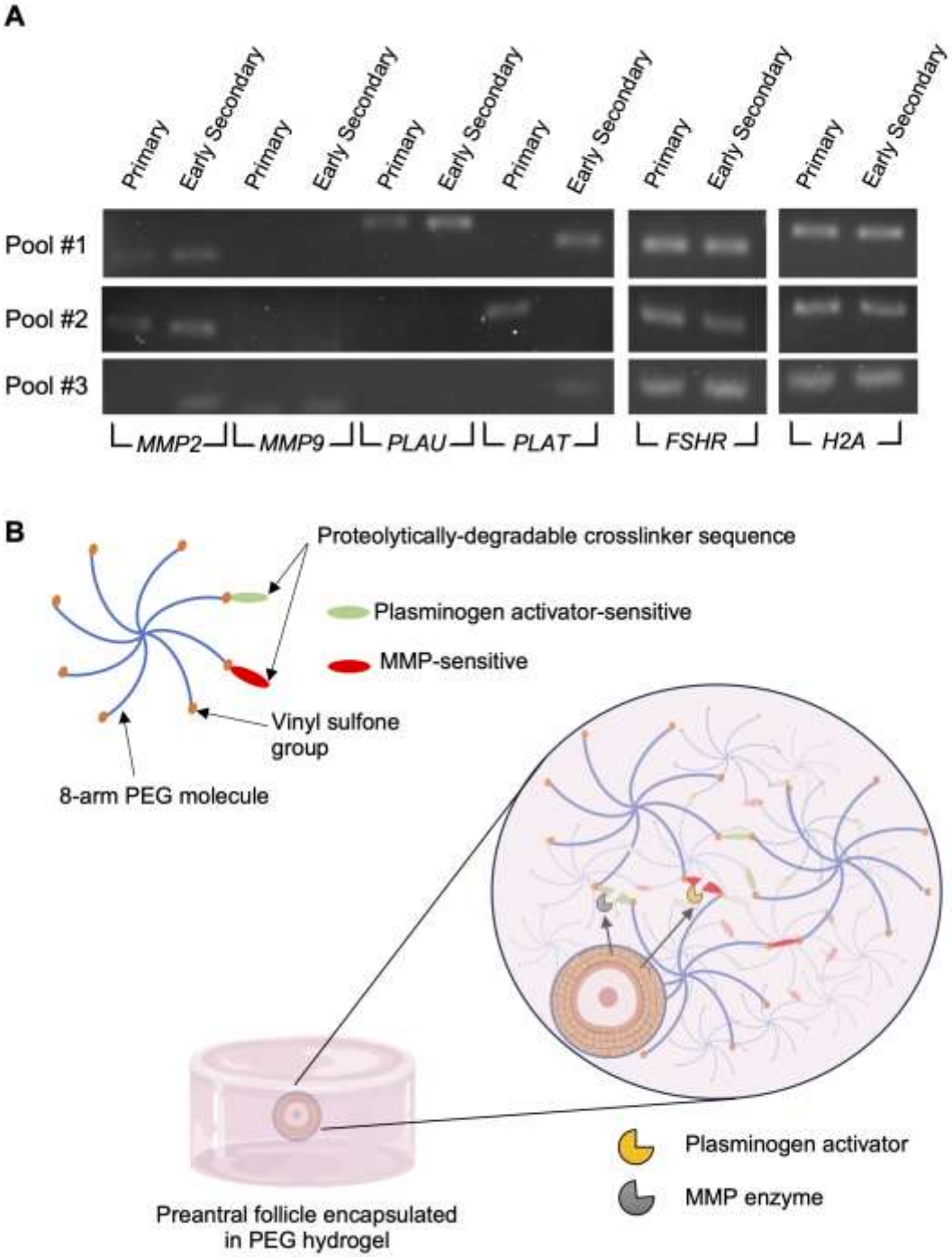


**Fig 1** Bovine preantral follicles express extracellular matrix-degrading enzymes, allowing the design of a PEG hydrogel containing target peptides for controlled degradation. (**A**) Agarose gel with products of RT-PCR of primary and early secondary follicles expressing mRNA for *MMP2, MMP9, PLAU, PLAT, FSHR,* and *H2A.* Each pool of primary and early secondary follicles (n = 3 pools per stage) contained 46-56 and 34-40 follicles,

702 respectively. (**B**) Schematic of PEG hydrogel for follicle encapsulation. PEG =
703 poly(ethylene glycol). MMP = matrix metalloproteinase
704

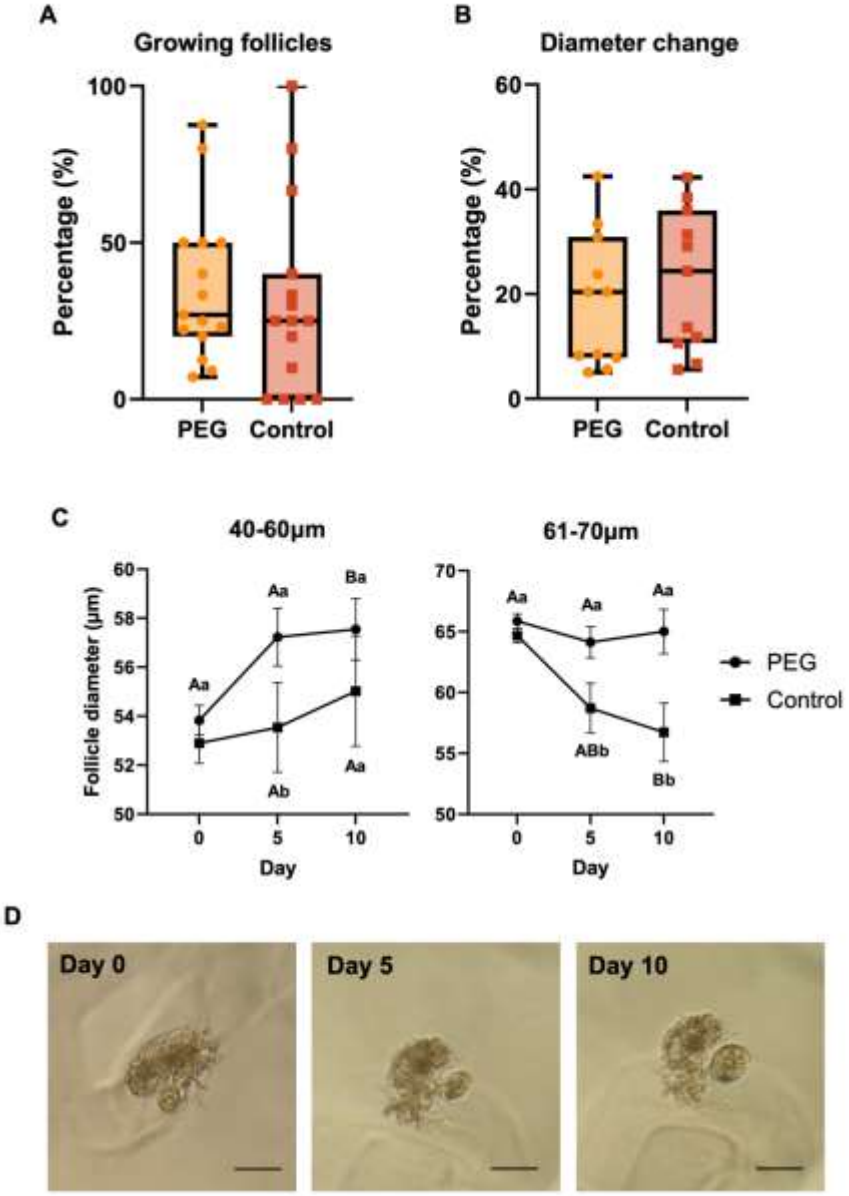


**Fig 2** Bovine preantral follicle in vitro growth using PEG or 2D control culture systems. Percentage of preantral follicles that grew (**A**) and percent change in diameter from follicles that grew (**B**). (**C**) Bovine preantral follicle diameter growth during in vitro culture at indicated starting diameters/stages. Lowercases letters that differ = significant difference ($P < 0.05$) between treatment groups in the same day. Uppercase letters that differ = significant difference ($P < 0.05$) within the same treatment group but compared to day 0. Data are presented as mean ± SEM. (**D**) Representative images of follicle grown in PEG over 10-day culture period. Scale bar = 100 µm.

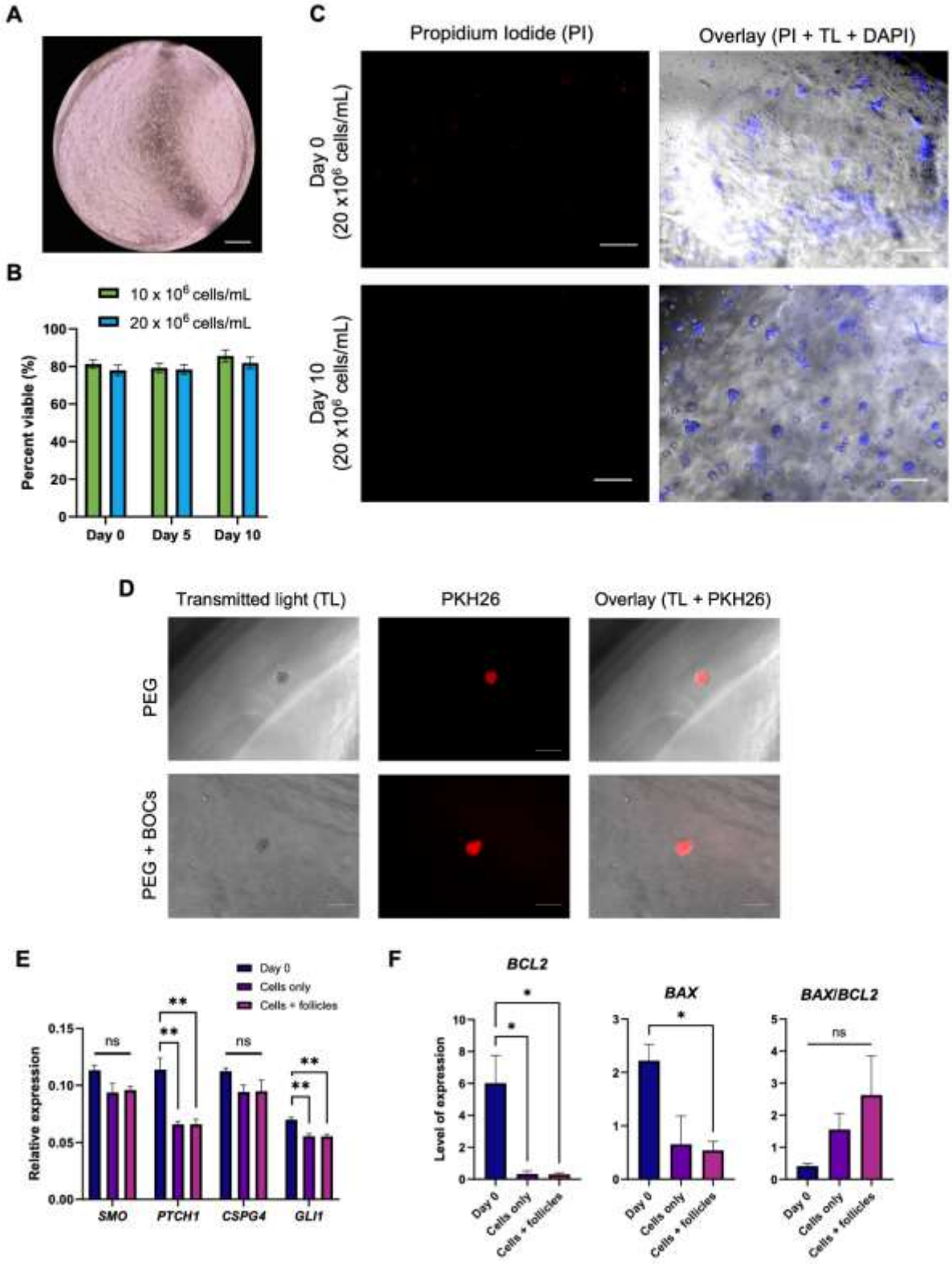


**Fig 3** Co-culture of bovine preantral follicles with bovine ovarian cells (BOCs) in PEG hydrogels. (**A**) Representative image of BOCs in PEG hydrogel (scale bar = 500 µm). (**B**) Viability of BOCs encapsulated in PEG hydrogels during in vitro culture. (**C**) Propidium

719 iodine (PI) staining in BOCs encapsulated in PEG hydrogels at day 0 and day 10 of
720 culture. Counterstained with DAPI (TL = transluminescent; scale bar = 100 μm). (**D**)
721 Unviable preantral follicle encapsulated in PEG hydrogel with or without BOCs using
722 PKH26 to label follicles at day 10 of culture (scale bar = 100 μm). (**E**) Gene expression of
723 pre-theca cell markers in BOCs (relative expression = 1/ΔCT). (**F**) Anti- and pro-apoptotic
724 gene marker expression in BOCs (level of expression = absolute quantification from
725 standard curve). Data are presented as mean ± SEM.
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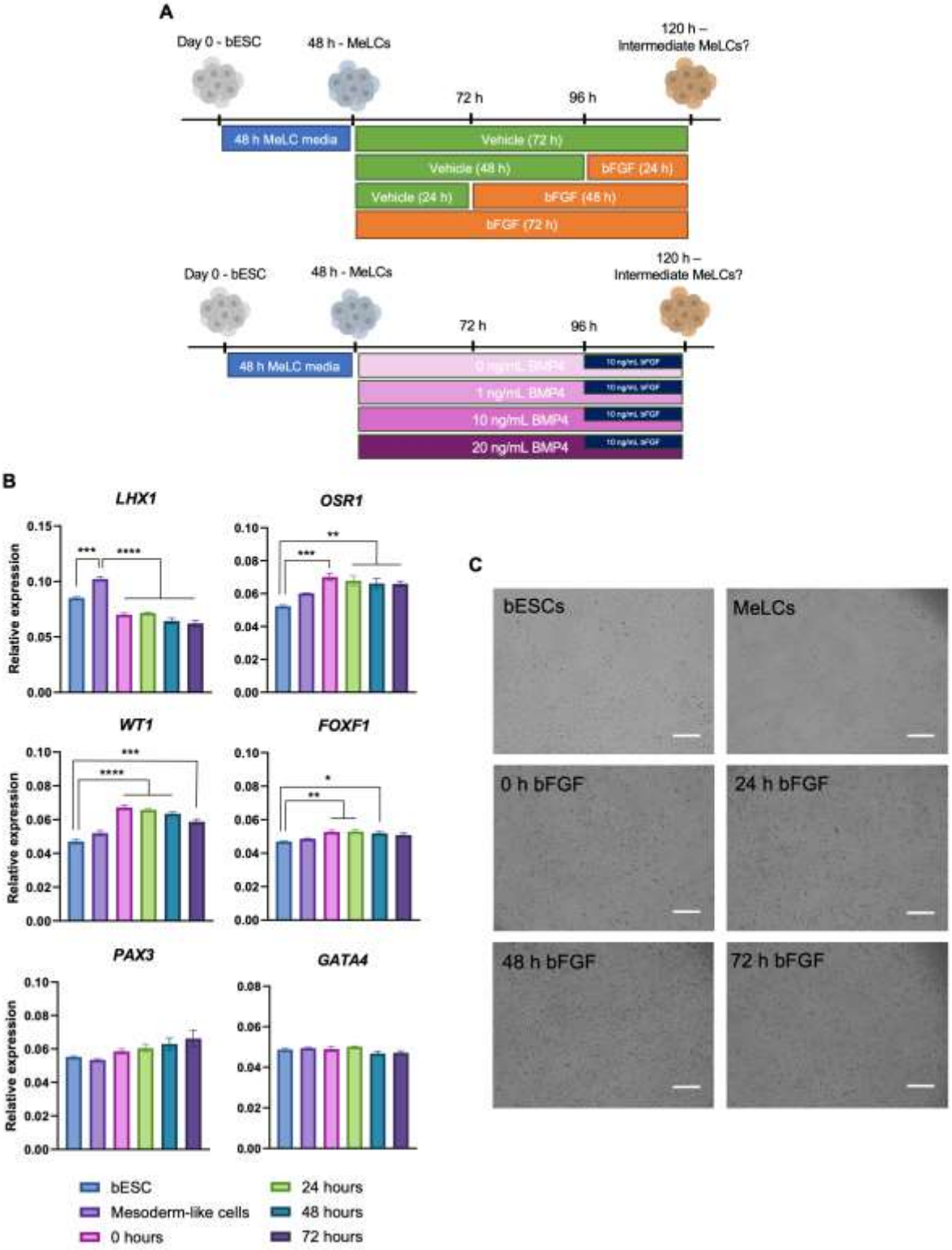


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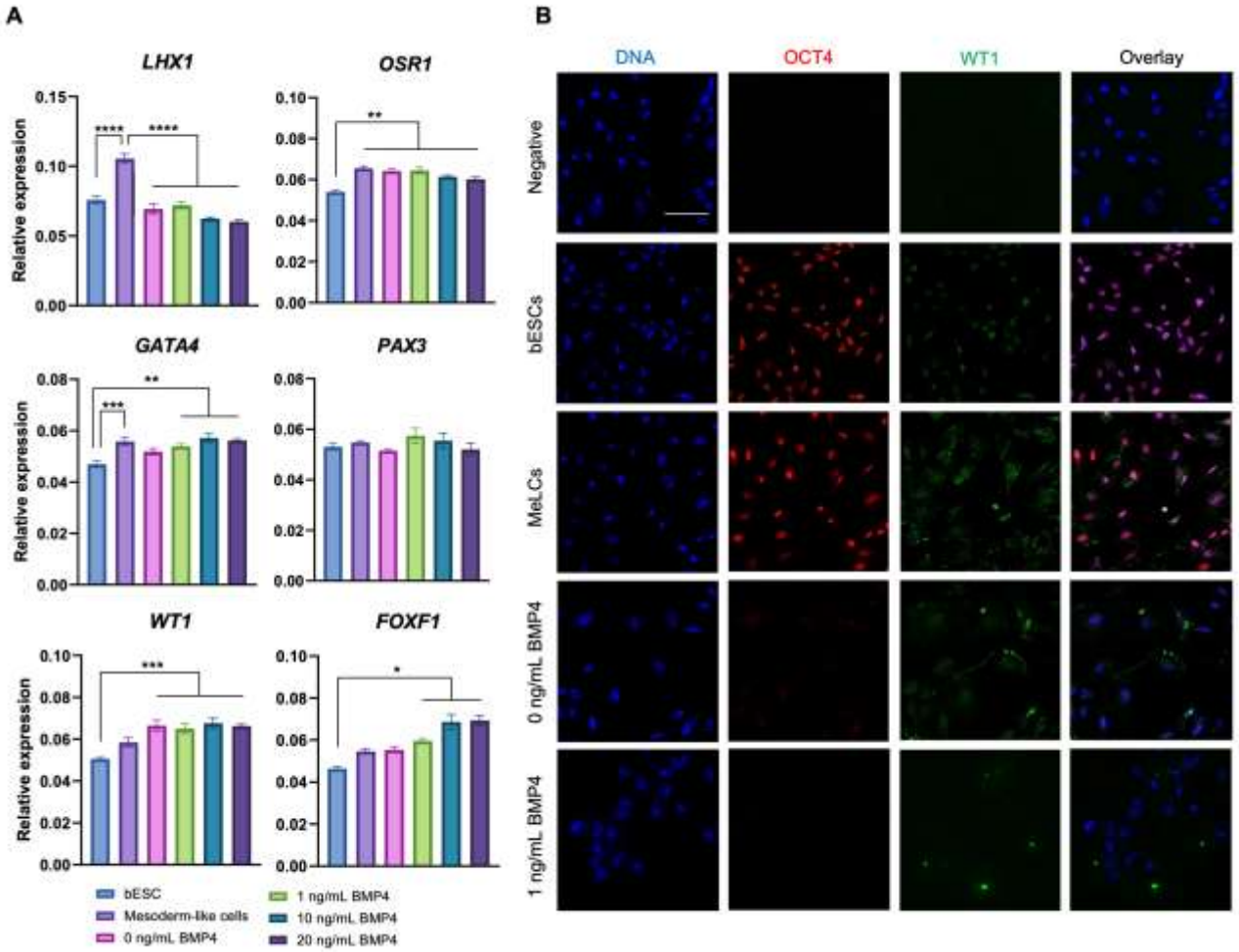
731 **Fig 4** In vitro differentiation of bovine embryonic stem cells (bESCs) towards progenitors
732 of somatic bipotential gonad-like cells. (**A**) Schematic diagram of experimental design for
733 bFGF and BMP4 experiments. (**B**) Gene expression of intermediate mesoderm,
734 bipotential gonad, paraxial and lateral plate mesoderm markers before and after bESC
735 differentiation using bFGF at various days of exposure. (**C**) Representative images of
736 cells from each treatment group. Data are presented as mean ± SEM. Significant
737 differences are noted by * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.
738

739

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741
742 **Fig 5** In vitro differentiation of bovine embryonic stem cells (bESCs) towards progenitors
743 of somatic bipotential gonad-like cells testing BMP4 concentrations. (**A**) Gene expression
744 of intermediate mesoderm, bipotential gonad, paraxial and lateral plate mesoderm
745 markers before and after bESC differentiation (**B**) Representative images of cells from
746 each treatment group. Data are presented as mean ± SEM. Significant differences are
747 noted by * = $P$ <0.05, ** = $P$ <0.01, *** = $P$ <0.001, **** = $P$ <0.0001.