1 **Successful in vitro maturation of oocytes: a matter of follicular differentiation**

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14 Summary sentence: Successful IVM in cattle and humans starts with an optimally

15 differentiated ovarian follicle.

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21 **Abstract**

22   Folliculogenesis is remarkably similar in cattle and humans. In this review, we

23 consider the known differences and provide a possible explanation for the greater

24 success of oocyte in vitro maturation in cattle. Two different parallel processes that are

25 critical for oocyte competence acquisition are examined. The first occurs in the follicle

26 and in turn influences the oocyte, the second occurs within the oocyte itself and involves

27 the gradual cessation of the transcription machinery with additional changes observable

28 in the chromatin structure. We expect this insight to contribute to the improvement of

29 human fertility programs based on in vitro fertilization, and particularly to the

30 development of controlled ovarian stimulation protocols that yield more high-quality

31 oocytes and thereby improve the clinical performance of treatments for infertility.

32

33 **Premise**

34   The physiology of folliculogenesis in cattle and humans differs from that in mice

35 in several ways [1, 2], the most obvious being the number of follicles ovulated per cycle,

36 followed by the timing of the processes by which oocytes acquire all of the capabilities

37 necessary to yield healthy offspring. These include resumption of meiosis, proper

38 interaction with the sperm cell to produce a functional zygote and timely activation of the

39 embryonic genome, leading to successful implantation in the uterus and maintenance of

40 gestation to term. These capabilities collectively are called oocyte quality or

41 developmental competence. Though relying heavily on the competence of a single cell,

42 folliculogenesis is the basis of reproduction throughout the animal kingdom. The focus of

43 this review is the timing of follicular differentiation associated with the dominance period,

44 which appears to be the principal determinant of the relationship between follicular

45 events and oocyte quality [3].

46   Although the mouse is the most commonly used model of mammalian physiology

47   and has been used widely in the field of reproductive biology, several authors suggest

48   that mouse studies are not clinically relevant to human reproduction [2, 4-6]. In livestock

49   as in humans, an oocyte has a very limited capacity to form an embryo until several

50   events have occurred, none of which are fully understood and will be the main subject of

51   this paper. Two different but parallel processes will be examined. The first of these

52   occurs inside the follicle and influences the oocyte. The second occurs within the oocyte

53   and involves the gradual shutdown of the transcription machinery and additional

54   changes observable in the chromatin. These changes can be observed under the

55   microscope, but also through molecular analysis of stored and translated RNA.

56

57   **Acquisition of oocyte developmental competence**

58   Oocyte maturation is a complex process involving much more than the nuclear

59   maturation observed after the LH surge or after the release of the full-sized gamete from

60   its follicle. The nuclear changes that occur between the germinal vesicle (GV) stage and

61   metaphase II represent only the final steps and the easiest to observe by microscopy.

62   The complete process that leads to a competent oocyte begins a few days before

63   ovulation and involves progressive changes in the follicular environment, including the

64   antral stage, at the beginning of which the growing follicle reaches a diameter of about 2

65   mm [7, 8] and the oocyte acquires the ability to resume meiosis and become fertilizable.

66   In this review, the competence of an oocyte means its ability to reach the blastocyst

67   stage once fertilized. This is the most common definition used both in cattle breeding

68   and in the study of human fertility.

69   The first point to emphasize is that although in vitro maturation can take place

70   spontaneously in fully-grown oocytes released from antral follicles of any size, meiotic

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71   maturation occurs naturally only in large antral follicles (8.5 mm in cattle [9] and 11 mm

72   in humans [7]) with functional LH receptors (on granulosa cells) following a LH surge.

73   This difference is important, since completion of the oocyte differentiation program

74   depends very much on the LH surge [3, 9]. Moreover, even if an oocyte matures and is

75   fertilized in vivo, it still may fail to develop, especially following ovarian stimulation, as

76   shown in cows in which the number of viable embryos recovered from the uterus is

77   smaller than the number of ovulations, in spite of optimal insemination protocols [10].

78   It has been suggested that small follicles are lagging in development while larger

79   ones are too old at ovulation, both yielding lower quality oocytes. This hypothesis has

80   gained support recently, since prolonging FSH treatment has been shown to increase

81   cattle embryo quality, while stopping it 84 hours before ovulation leads to decreased

82   quality [11]. In our research, we have determined an optimal FSH withdrawal period of

83   about 48 h and have shown that shorter or longer durations both lead to decreased

84   oocyte quality [12]. This variability is likely present in humans, in which oocytes aspirated

85   34 h after an induced LH surge or hCG injection are not all competent as measured in

86   terms of embryo formation and pregnancy following regular in vitro fertilization [13].

87   In the bovine, initially oocytes were collected after in vivo maturation using

88   laparoscopy [14] but with the development of ultrasound recovery, all laboratories and

89   commercial units have moved to aspiration of immature oocytes followed by in vitro

90   maturation (IVM) and in vitro fertilization (IVF) [14]. After years of improvement, IVM now

91   leads to impressive success rates exceeding 80% (based on blastocyst yield). However,

92   these rates are achieved only if follicular differentiation has reached the optimal stage

93   before harvesting the oocytes [12, 15], again suggesting that competence is achieved

94   before the LH surge. With the new procedure, FSH is withdrawn (coasting) after several

95   days of stimulation in a permissive basal LH environment with progesterone levels that

96   prevent ovulation [12, 15]. If oocytes are aspired without this rise and fall in FSH level,

97   blastocyst yield falls to about 30 %, which can be obtained using slaughterhouse ovaries

98   [16]. Similar treatment has proven successful in humans when FSH is replaced

99   immediately by exogenous hCG injection [17]. In addition, in cases of ovarian hyper-

100   stimulation, complete cessation of FSH or any gonadotropin support for a few days often

101   allows recovery of fully competent oocytes [18].

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103   **Chromatin configuration and oocyte developmental competence**

104   The observations mentioned above indicate clearly that the oocyte acquires

105   developmental competence just prior to ovulation. However, identifying oocytes that

106   have achieved competence is extremely arduous. One indicator is large-scale chromatin

107   remodeling, which occurs before meiotic resumption (while the oocyte is in meiotic

108   arrest) and can be observed by microscopy in whole mount oocytes after DNA staining

109   with fluorescent dyes [19, 20]. This has led to the identification of distinct stages in which

110   the chromatin becomes more and more compacted and occupies a smaller area of the

111   oocyte nucleus or germinal vesicle (GV). These configurations reflect oocyte

112   competence status in all mammals studied so far [21].

113   In cows, four chromatin configurations corresponding to different stages of

114   developmental competence have been described, namely GV0 to GV3. In the GV0

115   configuration the chromatin appears mostly uncondensed and dispersed throughout the

116   nucleoplasm. The appearance of few foci of condensation marks the transition to the

117   GV1 configuration and further compaction into distinct aggregates characterizes the GV2

118   configuration. The highest level of compaction occurs in GV3, in which the chromatin

119   appears as a single clump occupying a restricted area of the nucleus [22]. These stages

120   accompany follicle development. Almost 90 % of the oocytes isolated from early antral

121 follicles (0.5 to 2 mm in diameter) have a GV0 configuration, while medium antral follicles

122 (2–8 mm) contain virtually no GV0-stage oocytes but GV1, GV2 and GV3 stages in

123 equal proportions. These are the follicles most commonly used for in vitro embryo

124 production following IVM.

125   Various patterns of chromatin organization and compaction in several other

126 mammals have been described. However, the nomenclature used to describe the

127 changes in configuration is not uniform, due in part to species specificity. In mice,

128 chromatin reconfigures from the non-surrounded nucleolus (NSN) configuration to the

129 surrounded nucleolus (SN) configuration, in which chromatin forms a rim around the

130 nucleolus, which itself is inactive [23]. Configurations intermediate between these

131 extremes include aggregates of condensed chromatin apposed to the nucleolus (partly

132 NSN) and a partial perinucleolar chromatin ring (partly SN) [23, 24]. Human oocyte

133 chromatin configuration also changes from dispersed to a rim condensed around the

134 nucleolus [25], and one or two intermediate stages of aggregation around the nucleolar

135 structure have been described [26, 27]. Murine, human and bovine configurations are

136 nevertheless similar, since the nucleolus of GV2 and GV3 oocytes is seen generally as

137 an inactive remnant encapsulated by condensed chromatin [28]. The changes that are

138 common to several species can be described as uncondensed (GV0), loosely

139 condensed (GV1), intermediate (GV2) and condensed (GV3).

140   Chromatin configuration is not merely transient ultrastructure but a marker of

141 gamete differentiation associated with various functional characteristics. In bovine

142 oocytes, the transition from GV0 to GV3 corresponds to progressive transcription

143 silencing [28], changes in epigenetic signatures such as overall methylation [29] and

144 histone modification [30, 31] and changes in nuclear architecture and cytoplasmic

145 organelle redistribution [28]. More importantly, the transition from dispersed to

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146   compacted chromatin is accompanied by progressive acquisition of meiotic and

147   developmental competence [22, 28, 32]. Similar correlations have been described in

148   mice and humans [24, 25, 27, 33, 34].

149   It is noteworthy that the changes in chromatin configuration also accompany

150   significant changes in the transcriptome signature in the oocyte and in the surrounding

151   cumulus cells [30, 35]. For example, the abundance of transcripts from the H2A, H2B,

152   H3, H4, and linker H1 family of histones in bovine GV oocytes clearly increases as

153   chromatin compaction advances through the four stages [30]. This begins with an initial

154   major drop in transcription during the GV0 to GV1 transition [28, 32]. In silico analysis

155   has predicted interactions between specific histone transcripts and bovine stem-loop

156   binding protein 2 (SLBP2), which regulates mRNA translation throughout oogenesis,

157   indicating active storage of selected histone-encoding transcripts, possibly to meet the

158   requirements of the first three cell cycles following fertilization until activation of genes

159   with embryological roles [30]. Meanwhile, the corresponding cumulus cell transcriptome

160   also varies significantly [35], suggesting that chromatin configuration also reflects phases

161   of follicle development (see Figure 1 and below). For example, ingenuity pathway

162   analysis (in silico) has revealed a major change in transcripts involved in lipid

163   metabolism during the GV0 to GV1 transition [35]. This is in agreement with recent

164   findings that cumulus cell lipid content reflects oocyte quality [36, 37] both in cattle [38]

165   and in humans [39]. It appears that oocyte survival is ensured by storing elevated levels

166   of free fatty acids obtained from follicular fluid [40] to maintain the cumulus metabolic

167   activities during meiotic progression [41]. Genes affecting cumulus "cell death and

168   survival" functions also appear to be involved. Transcriptomic data analysis predicts

169   inhibition of apoptosis in GV1/GV2-stage cumulus-oocyte complexes (COCs) and

170   stimulation in GV3-stage COCs [35]. Caspase cascade analysis has shown that cumulus

171   cells associated with GV0-stage oocytes are unlikely to undergo apoptosis, while those

172   associated with oocytes at stages GV1, GV2 and GV3 are progressively more likely to

173   do so [35].

174   Changes in oocyte chromatin configuration are apparently carried out through

175   remodeling mechanisms, which regulate chromatin structure and accessibility. How

176   these changes ultimately affect the above-mentioned cellular functions in the oocytes

177   remains the subject of intensive study. We believe that optimizing techniques applicable

178   to small samples such as oocytes and embryos will bring answers to this question in the

179   near future.

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181   **Contribution of follicular cells to the process of chromatin remodeling**

182   During folliculogenesis, oocyte and cumulus cells are involved in intense

183   metabolic crosstalk arbitrated by paracrine factors and gap-junction-mediated

184   communications. Recent bovine studies demonstrate that large-scale changes in

185   chromatin configuration are related to gap-junction functional status through cAMP

186   dependent mechanisms [32, 42, 43]. In cumulus-oocyte complexes isolated from early

187   antral follicles, the maintenance of functional gap-junction communications promotes

188   oocyte growth, gradual transcriptional silencing, large-scale chromatin remodeling and

189   competence acquisition, all of which are controlled via oocyte cAMP [32].

190   Several studies have been focused on mimicking the contribution of follicular

191   cAMP and the synchrony between nuclear and cytoplasmic maturation in attempts to

192   perfect in vitro oocyte culture. This derives from the concept of "prematuration" that is the

193   process occurring from the time a follicle is selected to become dominant and are

194   completed shortly before the LH surge initiates final maturation [44]. In the prematuration

195   design, oocytes are held in prophase I (GV stage) using different molecules that act on

196   the intracellular level of cyclic nucleotides associated with meiosis resumption in order to

197   promote the acquisition of complete oocyte developmental competence prior to IVM [45].

198   A similar perspective characterizes studies conducted on human oocytes [27, 46, 47]. A

199   series of bovine studies demonstrates that prematuration in the presence of cilostamide,

200   3-isobutyl-1-methyl-xanthine (phosphodiesterase inhibitors) or natriuretic peptide

201   precursor C (which stimulates cGMP synthesis by binding its cognate receptor NPR2)

202   plus FSH at physiological concentrations stimulates the opening of gap junctions and

203   gradual chromatin transition. This transition ultimately increases oocyte embryonic

204   developmental competence after standard IVM and IVF, as evaluated in terms of

205   blastocyst quality parameters such as cell number and hatching rate [35, 42, 43, 48, 49].

206   However, as several groups have demonstrated over the past decade, the resulting

207   increase in embryo quality, though statistically significant, is modest. This has led some

208   to suggest that the heterogeneity of the ovarian follicle population in naturally cycling

209   animals affects the success obtained with the different bovine embryo production

210   systems [50]. A study conducted using an oocyte selection enriched on the basis of

211   chromatin configuration has shown that prematuration may be beneficial for the

212   developmental competence of GV1 oocytes but detrimental for that of GV3 oocytes [35].

213   The involvement of the follicle in the acquisition of developmental competence is

214   illustrated dramatically by studies using bovine oocytes obtained from slaughterhouses.

215   These oocytes are recovered from ovaries of un-stimulated animals at random stages of

216   the estrous cycle. The bovine estrous cycle consists of a 15-day luteal phase and a 6-7-

217   day follicular phase. Throughout the luteal phase, two or three follicular waves are

218   generated, each characterized by the appearance of a dominant follicle and the

219   regression of subordinate follicles, since cattle are mono-ovulatory, as are humans [51].

220   Ovaries obtained from slaughtered cows thus contain all types and sizes of antral

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221   follicles representing all stages of oocyte development. For example, oocytes from 2-8

222   mm follicles are heterogeneous in GV chromatin distribution as well as competence [22,

223   35]. It is interesting that oocytes in the GV3 configuration show initial degradation of the

224   gap junction coupling between the follicle and oocyte compartments as well as early

225   signs of atresia (based on ultrastructure), although they still have a relatively high

226   developmental capability [22, 28]. This is in agreement with previous studies in which a

227   correlation was identified between COC morphology and developmental competence

228   [52, 53]. In particular, oocytes within COCs with compact cumulus and homogeneous

229   ooplasm are less competent than those in which the ooplasm appears granulated and

230   the outer layers of cumulus cells exhibit the slight expansion often seen in early atretic

231   follicles. This suggests that oocytes inside follicles in the early stages of atresia are

232   more competent than those inside growing follicles [52]. The possible association of

233   developmental competence with follicle differentiation has been explored in conjunction

234   with FSH withdrawal [12, 15]. In compact COCs, the oocyte tends to be in the GV1

235   chromatin configuration (loosely condensed) while in COCs with slight expansion and/or

236   granulated cytoplasm the oocyte chromatin is in either the GV2 or the GV3 configuration

237   only [35]. It is interesting that in the case of GV1-stage oocytes, standard IVM without 6

238   h of prematuration leads to poor pre-implantation development and yet the same pre-

239   treatment decreases the competence of oocytes that have reached the GV2 or GV3

240   stage [35].

241   These findings suggest strongly that in naturally cycling animals, follicle size is

242   not a reliable criterion for selecting a population of oocytes that is homogeneous in terms

243   of chromatin configuration. The GV1 to GV2 transition, which marks the acquisition of

244   high embryonic developmental potential, occurs in oocytes almost regardless of the

245   antral size reached before FSH levels decline and the dominant follicle emerges [35].

246 Once the FSH level peaks within a follicular wave and then begins to decline, the atretic

247 events start and the GV2 to GV3 transition eventually occurs. The timing of such a

248 sequence would result in oocyte chromatin compaction regardless of whether the follicle

249 ovulates or undergoes atresia. This supports a previously proposed hypothesis that

250 chromatin condensation is complete in oocytes that became fully competent during

251 follicular growth and were collected from follicles undergoing the early events of atresia

252 [22, 28], the latter being corroborated by gene expression in cumulus cells [35]. This is

253 summarized in Figure 1 and is consistent with the finding that 33 % of oocytes obtained

254 from follicles in the early stages of atresia have a relatively high developmental potential

255 [12, 15, 52]. If such oocytes were at the GV2 stage and therefore maximally competent,

256 this would explain the extraordinarily stable blastocyst yield obtained by IVM since 1995.

257 Oocytes in growing follicles would be mainly at the GV1 stage, while those in plateau

258 phase follicles (low FSH) would be at GV2 and those in early atretic follicles at GV3, with

259 respectively low, high and medium developmental competence.

260 　　　　The interesting paradox described above is that neither the size nor the

261 healthiness of the follicle is the sole determinant of oocyte quality or competence.

262 Indeed, both small follicles (2-3 mm) and early atretic follicles may yield embryos and

263 newborns [52, 54, 55]. Furthermore, embryos with increased competence can be

264 obtained from oocytes harvested from larger follicles (> 9 mm in cattle), especially those

265 expressing LH receptors [56], the latter indicating that the gradual progression toward

266 developmental competence is complete. Analysis of follicle size, stimulation conditions

267 and time spent in the dominant phase (LH receptor) indicates that several factors

268 contribute to the progression towards a flawless oocyte with full developmental

269 competence. However, the complexity of the process is greater than meets the eye,

270 since oocyte competence can be reduced or lost suddenly if the follicle does not proceed

271   to timely ovulation [11, 12, 56-60]. It is now recognized that at any follicle size above 3

272   mm, three phases of follicular differentiation occur, namely growth, plateau and atresia,

273   and these phases correspond to the rise and decline of oocyte quality [61, 62]. A

274   speculative association between chromatin configuration and follicle size is presented

275   with estimated blastocyst yield in Table 1.

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277   **Bovine to human comparisons**

278   In the field of human fertility, IVM needs semantic clarification [63]. Maturation of

279   human oocytes aspirated without prior ovarian treatment was attempted a few times [64,

280   65], while most investigators worked with GV-stage oocytes collected following regular

281   controlled ovarian stimulation (COS) cycles [66, 67]. Meanwhile, a McGill University

282   group working with non-stimulated patients examined the effect of 34 h of IVM following

283   injection of hCG and aspirating from the first follicle to reach 11-12 mm [68]. These three

284   types of IVM protocols produce oocytes with quite different characteristics. The first of

285   these is analogous to the ovum pickup (OPU) procedure developed for slaughterhouse

286   material or non-stimulated, non-synchronized animals. The follicles aspirated are non-

287   dominant, early or late atretic, and the granulosa cells are not yet expressing LH

288   receptors. Each year, hundreds of thousands of oocytes are recovered from cows

289   without ovarian stimulation or synchronization, notably in Brazil where this is the main

290   system of reproduction on many large commercial farms [69]. Such oocytes have been

291   used in research for at least three decades [55] and their quality is well known, the yield

292   of transferrable embryos being 20–40 % and 40–50 % of transfers leading to pregnancy.

293   It is also known that using ultrasound transvaginal aspiration, mostly early and late

294   atretic oocytes are recovered, the former yielding more embryos than the latter, in which

295   the cumulus cell investment is often partially lost [52]. There is reason to believe that the

296   situation would be similar in humans, although the number of studies is limited [70, 71].

297   In the second type of protocol, IVM is performed on oocytes collected in the context of a

298   regular COS. Isolated typically from follicles that did not respond to the hCG injection,

299   these oocytes tend to be surrounded by non-expanded cumulus cells and/or are still at

300   the GV stage. Several groups have tried to rescue them with a further 24, 36 or 44 h of

301   IVM in a culture system similar to that used for cattle oocytes. Their success has been

302   very limited [66] and currently very few clinics if any still try to produce embryos in this

303   manner. Follicles containing such oocytes are most likely in the rapid growth phase,

304   stimulated by several days of FSH treatment but not differentiated enough to express

305   functional LH receptors in numbers above the threshold of response to the ovulation

306   trigger. Bovine oocytes aspirated from follicles in this phase are less competent than

307   those harvested later on, even from early atretic follicles of the same size [52, 57], and

308   oocytes in growing follicles may be still at the GV1 stage (Table 2). It should be noted

309   that oocytes with the GV still intact after exposure to hCG are often used in research

310   protocols designed to optimize IVM. This has contributed to confounding the effects

311   seen in comparative studies conducted on IVM/IVP with oocytes isolated from normal

312   cycling animal models and has been discussed in recent debate on defining clearly what

313   should not be considered as IVM [63, 72]. A third type of protocol although not sustained

314   by statistical analysis further confuses the issue. In this procedure, hCG is injected with

315   minimal or no FSH pre-treatment and aspiration is performed 30–34 h later [68]. The

316   resulting dominant follicle is often large enough (11-12 mm) to have sufficient LH

317   receptors to start the process of ovulation and accordingly a mature oocyte with an

318   expanded cumulus is recovered. Subsequent IVF is performed twice: immediately with

319   the mature oocyte and two days later with immature oocytes kept in culture. Based on

320   our experience with livestock, the mature oocyte is more likely to become an embryo and

321 lead to pregnancy, especially when embryos of different stages are transferred to the

322 same uterus [71].

323 Subordinate follicles that were still growing at the time of the hCG trigger cannot

324 respond to LH since they received less FSH and have begun the atretic process, which

325 is not immediately detrimental if our experience with cattle is any indication. In fact, it is

326 likely that the LH (hCG) trigger itself promotes atresia by stimulating androgen

327 production in theca cells of follicles in which the granulosa cells bear no LH receptors [3].

328 If the dominant follicle is already functional (12 mm or larger) the subordinates have

329 already started the atresia process and will be on hold for the 30–34 h period prior to

330 aspiration. These oocytes are associated with cumulus cells exhibiting various degrees

331 of dispersion or apoptosis, suggesting that hCG triggering did not have the same effect

332 as it did on the dominant follicle [71]. The procedure has been tried in many laboratories

333 around the world but is now seldom used for regular patients since the results are not

334 superior to regular IVF and smaller numbers of eggs are produced [70].

335 Recent work [73] has demonstrated convincingly a crucial upper limit on

336 dominant follicle diameter. The observations of Son's group [73] make it clear that

337 human COCs collected from 6–12 mm follicles when the dominant follicle was larger

338 than 12 mm had very little developmental competence.

339 A modified IVM procedure has been developed to include a few days of FSH

340 support to increase follicle recruitment before aspiration [74]. When OPU is performed

341 before FSH coasting (withdrawal), the recovery rate is poor because COCs remain

342 attached solidly to their follicles (De Vos, personal communication). The oocytes that are

343 recovered under these conditions may have a less compacted cumulus but are

344 comparable in developmental capacity to what is observed in cattle treated similarly [57].

345   Again, these oocytes are harvested while the follicular process is incomplete and are

346   therefore less competent.

347   The quality of oocytes obtained using the three IVM protocols described above

348   cannot be stated with certainty, due to the limited reproductive data available. Table 2

349   shows blastocyst yields ranging from 5 % to 30 % based on the number of fertilized or

350   cleaved embryos, which is somewhat lower than for mice or livestock. The best results

351   for true IVM come surprisingly from ovariectomy patients who received no treatment

352   before oocyte collection, but no embryo was transferred in these cases [75]. The poorest

353   results come from GV oocytes recovered during a stimulated cycle 34–36 h after hCG,

354   probably indicating that such follicles were recruited later, were still growing or bore

355   insufficient LH receptor (Table 2). We speculate that ovaries selected at random would

356   have a balanced distribution of growing (mainly GV1), plateau (mainly GV2) and early

357   atretic (mainly GV3) follicles (Figure 1). With such a distribution, we would obtain the

358   same blastocyst yield (30%) as from cows selected randomly or from slaughterhouse

359   ovaries. If oocytes were collected from patients while a follicle was establishing

360   dominance (> 9 mm), this follicle would be at the GV1 or GV2 stage, depending on how

361   many days into the process before ovulation. Oocytes from subordinate follicles would

362   be at the GV2 stage for 1-2 days and then would enter final atresia and the GV3 stage.

363   Immature oocytes obtained 34–36 h post hCG in the context of in a stimulated cycle

364   would most likely be in the GV1 stage, due to the follicle not responding adequately to

365   the ovulation trigger. This is the worst-case scenario if these follicles are otherwise totally

366   healthy and responsive to stimulation by FSH. Under such conditions in the cow, the

367   larger growing follicles contain oocytes of the lowest quality [57]. What has not been

368   done is the collection of immature oocytes from large (with LH receptors) growing

369   follicles before the LH surge. There is no reason to expect these follicles to respond to

370 hCG and generate mature eggs in vivo within 34 h. Based on animal models, we would

371 even predict that without proper coasting (FSH withdrawal), these oocytes would be less

372 competent after IVM than those matured in vivo.

373

374 **Conclusions**

375 Bovine oocytes perform better in IVM than human oocytes do, mainly because the

376 oocyte harvesting is made from follicles that have acquired LH receptors (dominant),

377 which can optimize differentiation in absence of FSH. In human fertility treatment, such

378 follicles mature in vivo without FSH arrest. When FSH is replaced by low doses of LH

379 (hCG), human oocytes in IVM appear to be as competent as bovine oocytes [76]. We

380 believe that if the concepts outlined in this review were applied to human IVF programs,

381 COS protocols could be adjusted to make it easier to obtain high-quality oocytes,

382 especially for patients who do not respond well to standard COS protocols or present a

383 risk of developing the hyper-stimulation syndrome.

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392 **Competing interest**

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398 **References**

399 1.   Adams GP, Pierson RA. Bovine model for study of ovarian follicular dynamics in

400 humans. Theriogenology 1995; 43:113-120.

401 2.   Sirard MA. The ovarian follicle of cows as a model for human. In: Schatten H,

402 Constantinescu GM (eds.), Animal Models and Human Reproduction: Cell and Molecular

403 Approaches with Reference to Human Reproduction: Wiley-Blackwell; 2017: 127-144.

404 3.   Sirard MA. Somatic environment and germinal differentiation in antral follicle: The

405 effect of FSH withdrawal and basal LH on oocyte competence acquisition in cattle.

406 Theriogenology 2016; 86:54-61.

407 4.   Quinn P, Horstman FC. Is the mouse a good model for the human with respect to

408 the development of the preimplantation embryo in vitro? Hum Reprod 1998; 13 Suppl

409 4:173-183.

410 5.   Neuber E, Powers RD. Is the mouse a clinically relevant model for human

411 fertilization failures? Hum Reprod 2000; 15:171-174.

412 6.   Menezo YJ, Herubel F. Mouse and bovine models for human IVF. Reprod

413 Biomed Online 2002; 4:170-175.

414 7.   Gougeon A. Human ovarian follicular development: from activation of resting

415 follicles to preovulatory maturation. Ann Endocrinol (Paris) 2010; 71:132-143.

416 8.   Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational

417 competence and transcriptional activity. Mol Reprod Dev 1995; 42:437-442.

418 9.   Beg MA, Ginther OJ. Follicle selection in cattle and horses: role of intrafollicular

419 factors. Reproduction 2006; 132:365-377.

420   10.   Hasler JF. Forty years of embryo transfer in cattle: a review focusing on the

421   journal Theriogenology, the growth of the industry in North America, and personal

422   reminisces. Theriogenology 2014; 81:152-169.

423   11.   Dias FC, Costa E, Adams GP, Mapletoft RJ, Kastelic J, Dochi O, Singh J. Effect

424   of duration of the growing phase of ovulatory follicles on oocyte competence in

425   superstimulated cattle. Reprod Fertil Dev 2013; 25:523-530.

426   12.   Nivet AL, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P, Sirard MA.

427   FSH withdrawal improves developmental competence of oocytes in the bovine model.

428   Reproduction 2012; 143:165-171.

429   13.   Verberg MF, Eijkemans MJ, Macklon NS, Heijnen EM, Baart EB, Hohmann FP,

430   Fauser BC, Broekmans FJ. The clinical significance of the retrieval of a low number of

431   oocytes following mild ovarian stimulation for IVF: a meta-analysis. Hum Reprod Update

432   2009; 15:5-12.

433   14.   Sirard MA, Lambert RD. Birth of calves after in vitro fertilisation using

434   laparoscopy and rabbit oviduct incubation of zygotes. Vet Rec 1986; 119:167-169.

435   15.   Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. Manipulation of

436   follicular development to produce developmentally competent bovine oocytes. Biol

437   Reprod 2002; 66:38-43.

438   16.   Hendriksen PJ, Vos PL, Steenweg WN, Bevers MM, Dieleman SJ. Bovine

439   follicular development and its effect on the in vitro competence of oocytes.

440   Theriogenology 2000; 53:11-20.

441   17.   Filicori M, Cognigni GE, Gamberini E, Parmegiani L, Troilo E, Roset B. Efficacy of

442   low-dose human chorionic gonadotropin alone to complete controlled ovarian

443   stimulation. Fertil Steril 2005; 84:394-401.

444   18.   Kovacs P, Matyas S, Kaali SG. Effect of coasting on cycle outcome during in vitro

445   fertilization/intracytoplasmic sperm injection cycles in hyper-responders. Fertil Steril

446   2006; 85:913-917.

447   19.   De La Fuente R. Chromatin modifications in the germinal vesicle (GV) of

448   mammalian oocytes. Dev Biol 2006; 292:1-12.

449   20.   Luciano AM, Lodde V. Changes of Large-Scale Chromatin Configuration During

450   Mammalian Oocyte Differentiation. In: Coticchio G, Albertini DF, De Santis L (eds.),

451   Oogenesis: Springer London; 2013: 93-108.

452   21.   Luciano AM, Franciosi F, Dieci C, Lodde V. Changes in large-scale chromatin

453   structure and function during oogenesis: a journey in company with follicular cells. Anim

454   Reprod Sci 2014; 149:3-10.

455   22.   Lodde V, Modina S, Galbusera C, Franciosi F, Luciano AM. Large-scale

456   chromatin remodeling in germinal vesicle bovine oocytes: interplay with gap junction

457   functionality and developmental competence. Mol Reprod Dev 2007; 74:740-749.

458   23.   Mattson BA, Albertini DF. Oogenesis: chromatin and microtubule dynamics

459   during meiotic prophase. Mol Reprod Dev 1990; 25:374-383.

460   24.   Bouniol-Baly C, Hamraoui L, Guibert J, Beaujean N, Szollosi MS, Debey P.

461   Differential transcriptional activity associated with chromatin configuration in fully grown

462   mouse germinal vesicle oocytes. Biol Reprod 1999; 60:580-587.

463   25.   Combelles CM, Albertini DF, Racowsky C. Distinct microtubule and chromatin

464   characteristics of human oocytes after failed in-vivo and in-vitro meiotic maturation. Hum

465   Reprod 2003; 18:2124-2130.

466   26.   Combelles CM, Cekleniak NA, Racowsky C, Albertini DF. Assessment of nuclear

467   and cytoplasmic maturation in in-vitro matured human oocytes. Hum Reprod 2002;

468   17:1006-1016.

469   27.   Sanchez F, Romero S, De Vos M, Verheyen G, Smitz J. Human cumulus-

470   enclosed germinal vesicle oocytes from early antral follicles reveal heterogeneous

471   cellular and molecular features associated with in vitro maturation capacity. Hum Reprod

472   2015; 30:1396-1409.

473   28.   Lodde V, Modina S, Maddox-Hyttel P, Franciosi F, Lauria A, Luciano AM. Oocyte

474   morphology and transcriptional silencing in relation to chromatin remodeling during the

475   final phases of bovine oocyte growth. Mol Reprod Dev 2008; 75:915-924.

476   29.   Lodde V, Modina SC, Franciosi F, Zuccari E, Tessaro I, Luciano AM. Localization

477   of DNA methyltransferase-1 during oocyte differentiation, in vitro maturation and early

478   embryonic development in cow. Eur J Histochem 2009; 53:e24.

479   30.   Labrecque R, Lodde V, Dieci C, Tessaro I, Luciano AM, Sirard MA. Chromatin

480   remodelling and histone mRNA accumulation in bovine germinal vesicle oocytes. Mol

481   Reprod Dev 2015; 82:450-462.

482   31.   Lodde V, Luciano AM, Franciosi F, Labrecque R, Sirard MA. Accumulation of

483   Chromatin Remodelling Enzyme and Histone Transcripts in Bovine Oocytes. Results

484   Probl Cell Differ 2017; 63:223-255.

485   32.   Luciano AM, Franciosi F, Modina SC, Lodde V. Gap junction-mediated

486   communications regulate chromatin remodeling during bovine oocyte growth and

487   differentiation through cAMP-dependent mechanism(s). Biol Reprod 2011; 85:1252-

488   1259.

489   33.   Zuccotti M, Piccinelli A, Giorgi Rossi P, Garagna S, Redi CA. Chromatin

490   organization during mouse oocyte growth. Mol Reprod Dev 1995; 41:479-485.

491   34.   Miyara F, Migne C, Dumont-Hassan M, Le Meur A, Cohen-Bacrie P, Aubriot FX,

492   Glissant A, Nathan C, Douard S, Stanovici A, Debey P. Chromatin configuration and

493   transcriptional control in human and mouse oocytes. Mol Reprod Dev 2003; 64:458-470.

494 35. Dieci C, Lodde V, Labreque R, Dufort I, Tessaro I, Sirard MA, Luciano AM.

495 Differences in cumulus cell gene expression indicate the benefit of a pre-maturation step

496 to improve in-vitro bovine embryo production. Mol Hum Reprod 2016; 22:882-897.

497 36. Auclair S, Uzbekov R, Elis S, Sanchez L, Kireev I, Lardic L, Dalbies-Tran R,

498 Uzbekova S. Absence of cumulus cells during in vitro maturation affects lipid metabolism

499 in bovine oocytes. Am J Physiol Endocrinol Metab 2013; 304:E599-613.

500 37. Montani DA, Cordeiro FB, Regiani T, Victorino AB, Pilau EJ, Gozzo FC, Ferreira

501 CR, Fraietta R, Lo Turco EG. The follicular microenviroment as a predictor of pregnancy:

502 MALDI-TOF MS lipid profile in cumulus cells. J Assist Reprod Genet 2012; 29:1289-

503 1297.

504 38. Kim JY, Kinoshita M, Ohnishi M, Fukui Y. Lipid and fatty acid analysis of fresh

505 and frozen-thawed immature and in vitro matured bovine oocytes. Reproduction 2001;

506 122:131-138.

507 39. Matorras R, Ruiz JI, Mendoza R, Ruiz N, Sanjurjo P, Rodriguez-Escudero FJ.

508 Fatty acid composition of fertilization-failed human oocytes. Hum Reprod 1998; 13:2227-

509 2230.

510 40. Aardema H, Lolicato F, van de Lest CH, Brouwers JF, Vaandrager AB, van Tol

511 HT, Roelen BA, Vos PL, Helms JB, Gadella BM. Bovine cumulus cells protect maturing

512 oocytes from increased fatty acid levels by massive intracellular lipid storage. Biol

513 Reprod 2013; 88:164.

514 41. Sanchez-Lazo L, Brisard D, Elis S, Maillard V, Uzbekov R, Labas V,

515 Desmarchais A, Papillier P, Monget P, Uzbekova S. Fatty acid synthesis and oxidation in

516 cumulus cells support oocyte maturation in bovine. Mol Endocrinol 2014:me20141049.

517 42. Lodde V, Franciosi F, Tessaro I, Modina SC, Luciano AM. Role of gap junction-

518 mediated communications in regulating large-scale chromatin configuration remodeling

519   and embryonic developmental competence acquisition in fully grown bovine oocyte. J

520   Assist Reprod Genet 2013; 30:1219-1226.

521   43.   Franciosi F, Coticchio G, Lodde V, Tessaro I, Modina SC, Fadini R, Dal Canto M,

522   Renzini MM, Albertini DF, Luciano AM. Natriuretic peptide precursor C delays meiotic

523   resumption and sustains gap junction-mediated communication in bovine cumulus-

524   enclosed oocytes. Biol Reprod 2014; 91:61.

525   44.   Dieleman SJ, Hendriksen PJ, Viuff D, Thomsen PD, Hyttel P, Knijn HM,

526   Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers MM, Vos PL. Effects of in vivo

527   prematuration and in vivo final maturation on developmental capacity and quality of pre-

528   implantation embryos. Theriogenology 2002; 57:5-20.

529   45.   Gilchrist RB, Luciano AM, Richani D, Zeng HT, Wang X, Vos MD, Sugimura S,

530   Smitz J, Richard FJ, Thompson JG. Oocyte maturation and quality: role of cyclic

531   nucleotides. Reproduction 2016; 152:R143-157.

532   46.   Nogueira D, Ron-El R, Friedler S, Schachter M, Raziel A, Cortvrindt R, Smitz J.

533   Meiotic arrest in vitro by phosphodiesterase 3-inhibitor enhances maturation capacity of

534   human oocytes and allows subsequent embryonic development. Biol Reprod 2006;

535   74:177-184.

536   47.   Sanchez F, Lolicato F, Romero S, De Vos M, Van Ranst H, Verheyen G,

537   Anckaert E, Smitz JEJ. An improved IVM method for cumulus-oocyte complexes from

538   small follicles in polycystic ovary syndrome patients enhances oocyte competence and

539   embryo yield. Hum Reprod 2017; 32:2056-2068.

540   48.   Dieci C, Lodde V, Franciosi F, Lagutina I, Tessaro I, Modina SC, Albertini DF,

541   Lazzari G, Galli C, Luciano AM. The effect of cilostamide on gap junction communication

542   dynamics, chromatin remodeling, and competence acquisition in pig oocytes following

543   parthenogenetic activation and nuclear transfer. Biol Reprod 2013; 89:68.

544 49.   Soares AC, Lodde V, Barros RG, Price CA, Luciano AM, Buratini J. Steroid

545 hormones interact with natriuretic peptide C to delay nuclear maturation, to maintain

546 oocyte?cumulus communication and to improve the quality of in vitro-produced embryos

547 in cattle. Reprod Fertil Dev 2017; 29:2217-2224.

548 50.   Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, Dieleman SJ.

549 Factors affecting oocyte quality and quantity in commercial application of embryo

550 technologies in the cattle breeding industry. Theriogenology 2003; 59:651-674.

551 51.   Ginther OJ, Beg MA, Donadeu FX, Bergfelt DR. Mechanism of follicle deviation in

552 monovular farm species. Anim Reprod Sci 2003; 78:239-257.

553 52.   Blondin P, Sirard MA. Oocyte and follicular morphology as determining

554 characteristics for developmental competence in bovine oocytes. Mol Reprod Dev 1995;

555 41:54-62.

556 53.   Hazeleger NL, Hill DJ, Stubbing RB, Walton JS. Relationship of morphology and

557 follicular fluid environment of bovine oocytes to their developmental potential in vitro.

558 Theriogenology 1995; 43:509-522.

559 54.   Blondin P, Dufour M, Sirard MA. Analysis of atresia in bovine follicles using

560 different methods: flow cytometry, enzyme-linked immunosorbent assay, and classic

561 histology. Biol Reprod 1996; 54:631-637.

562 55.   Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo

563 quality. Theriogenology 2006; 65:126-136.

564 56.   Hagemann LJ, Beaumont SE, Berg M, Donnison MJ, Ledgard A, Peterson AJ,

565 Schurmann A, Tervit HR. Development during single IVP of bovine oocytes from

566 dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. Mol

567 Reprod Dev 1999; 53:451-458.

568   57.   Blondin P, Coenen K, Guilbault LA, Sirard MA. Superovulation can reduce the

569   developmental competence of bovine embryos. Theriogenology 1996; 46:1191-1203.

570   58.   Blondin P, Coenen K, Guilbault LA, Sirard MA. In vitro production of bovine

571   embryos: developmental competence is acquired before maturation. Theriogenology

572   1997; 47:1061-1075.

573   59.   Blondin P, Coenen K, Sirard MA. The impact of reactive oxygen species on

574   bovine sperm fertilizing ability and oocyte maturation. J Androl 1997; 18:454-460.

575   60.   Sirard MA, Picard L, Dery M, Coenen K, Blondin P. The time interval between

576   FSH administration and ovarian aspiration influences the development of cattle oocytes.

577   Theriogenology 1999; 51:699-708.

578   61.   Douville G, Sirard MA. Changes in granulosa cells gene expression associated

579   with growth, plateau and atretic phases in medium bovine follicles. J Ovarian Res 2014;

580   7:50.

581   62.   Girard A, Dufort I, Douville G, Sirard MA. Global gene expression in granulosa

582   cells of growing, plateau and atretic dominant follicles in cattle. Reprod Biol Endocrinol

583   2015; 13:17.

584   63.   De Vos M, Smitz J, Thompson JG, Gilchrist RB. The definition of IVM is clear-

585   variations need defining. Hum Reprod 2016; 31:2411-2415.

586   64.   Trounson A, Anderiesz C, Jones GM, Kausche A, Lolatgis N, Wood C. Oocyte

587   maturation. Hum Reprod 1998; 13 Suppl 3:52-62; discussion 71-55.

588   65.   Guzman L, Adriaenssens T, Ortega-Hrepich C, Albuz FK, Mateizel I, Devroey P,

589   De Vos M, Smitz J. Human antral follicles <6 mm: a comparison between in vivo

590   maturation and in vitro maturation in non-hCG primed cycles using cumulus cell gene

591   expression. Mol Hum Reprod 2013; 19:7-16.

592   66.   Chian RC, Tan SL. Maturational and developmental competence of cumulus-free

593   immature human oocytes derived from stimulated and intracytoplasmic sperm injection

594   cycles. Reprod Biomed Online 2002; 5:125-132.

595   67.   Goud PT, Goud AP, Qian C, Laverge H, Van der Elst J, De Sutter P, Dhont M. In-

596   vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and

597   epidermal growth factor in the culture medium. Hum Reprod 1998; 13:1638-1644.

598   68.   Chian RC, Buckett WM, Abdul Jalil AK, Son WY, Sylvestre C, Rao D, Tan SL.

599   Natural-cycle in vitro fertilization combined with in vitro maturation of immature oocytes is

600   a potential approach in infertility treatment. Fertil Steril 2004; 82:1675-1678.

601   69.   Vieira LM, Rodrigues CA, Netto AC, Guerreiro BM, Silveira CR, Freitas BG,

602   Braganca LG, Marques KN, Sa Filho MF, Bo GA, Mapletoft RJ, Baruselli PS. Efficacy of

603   a single intramuscular injection of porcine FSH in hyaluronan prior to ovum pick-up in

604   Holstein cattle. Theriogenology 2016; 85:877-886.

605   70.   Fadini R, Coticchio G, Brambillasca F, Mignini Renzini M, Novara PV, Brigante C,

606   De Ponti E, Dal Canto M. Clinical outcomes from mature oocytes derived from

607   preovulatory and antral follicles: reflections on follicle physiology and oocyte

608   competence. J Assist Reprod Genet 2015; 32:255-261.

609   71.   Dal Canto M, Brambillasca F, Mignini Renzini M, Coticchio G, Merola M, Lain M,

610   De Ponti E, Fadini R. Cumulus cell-oocyte complexes retrieved from antral follicles in

611   IVM cycles: relationship between COCs morphology, gonadotropin priming and clinical

612   outcome. J Assist Reprod Genet 2012; 29:513-519.

613   72.   Coticchio G. IVM in need of clear definitions. Hum Reprod 2016; 31:1387-1389.

614   73.   Son WY, Chung JT, Herrero B, Dean N, Demirtas E, Holzer H, Elizur S, Chian

615   RC, Tan SL. Selection of the optimal day for oocyte retrieval based on the diameter of

616 the dominant follicle in hCG-primed in vitro maturation cycles. Hum Reprod 2008;

617 23:2680-2685.

618 74.   Roesner S, Von Wolff M, Eberhardt I, Beuter-Winkler P, Toth B, Strowitzki T. In

619 vitro maturation: a five-year experience. Acta Obstet Gynecol Scand 2012; 91:22-27.

620 75.   Hwu YM, Lee RK, Chen CP, Su JT, Chen YW, Lin SP. Development of hatching

621 blastocysts from immature human oocytes following in-vitro maturation and fertilization

622 using a co-culture system. Hum Reprod 1998; 13:1916-1921.

623 76.   Filicori M, Cognigni GE, Taraborrelli S, Spettoli D, Ciampaglia W, de Fatis CT.

624 Low-dose human chorionic gonadotropin therapy can improve sensitivity to exogenous

625 follicle-stimulating hormone in patients with secondary amenorrhea. Fertil Steril 1999;

626 72:1118-1120.

627 77.   Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. Effect of follicle size on

628 bovine oocyte quality and developmental competence following maturation, fertilization,

629 and culture in vitro. Mol Reprod Dev 1994; 37:48-53.

630 78.   Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro

631 fertilization of human follicular oocytes collected from nonstimulated cycles, their culture

632 in vitro and their transfer in a donor oocyte program. Fertil Steril 1991; 55:109-113.

633 79.   Son WY, Chung JT, Demirtas E, Holzer H, Sylvestre C, Buckett W, Chian RC,

634 Tan SL. Comparison of in-vitro maturation cycles with and without in-vivo matured

635 oocytes retrieved. Reprod Biomed Online 2008; 17:59-67.

636 80.   Fadini R, Dal Canto MB, Mignini Renzini M, Brambillasca F, Comi R, Fumagalli

637 D, Lain M, Merola M, Milani R, De Ponti E. Effect of different gonadotrophin priming on

638 IVM of oocytes from women with normal ovaries: a prospective randomized study.

639 Reprod Biomed Online 2009; 19:343-351.

640

641   **Table 1**. Apparent association of bovine follicle size category and growth status at OPU

642   with oocyte developmental potential[†]

Size cat.	Follicle status (%)				Blastocyst yield (estimated %)			
	Growing[1]	Plateau[2]	Atresia[3]	Ref.	Growing	Plateau	Atresia	Ref.
< 3 mm	85	10	5	[22, 35]	Nil	< 25	Low[4]	[77]
3-5 mm	30	40	30	[22, 35]	Nil	Low	Low	[77]
5-9 mm	10	30	60	[22, 35]	Nil	25–40	< 25	[56]
9 mm to ovulation	?	?	?	NA	Low	25–40	< 25	[56]
FSH withdrawn after 3 days	10	80	10	(*)	Low	> 80	Low	[12, 15]

643   [†]Based on published data [22, 35] and our unpublished results (*)
644   [1]GV chromatin was at stage 1 in all categories
645   [2]GV chromatin was at stage 2 in all categories
646   [3]GV chromatin was at stage 3 in all categories
647   [4]Low means close to nil
648

649 **Table 2.** Apparent association of human follicle size category and surmised germinal

650 vesicle stage with oocyte developmental potential

Follicle size (mm)	Matured (% of total)	Blastocyst yield (%)	Reference	Surmised stage
2–5	56	35	[78]	GV-1-2-3
2–6		30	[75]	GV-1-2-3
6–9 (subordinate)	75	12	[66] [73]	GV-2-3
> 9–12		23	[79]	GV-1-2-3
9–12 + hCG		40	[79]	M-II
> 9 (GV, COS)	52	5	[67]	GV1
2–10	48	27	[80]	GV-1-2-3

651

652   **Figure 1.** Schematic depiction of germinal vesicle chromatin remodeling during bovine

653   ovarian follicle development: Small spheroids colored white, pink or purple represent

654   oocytes inside non-atretic, early atretic or atretic follicles respectively. Most **growing**

655   **phase** follicles contain a GV1-stage oocyte, most **plateau phase** follicles contain a GV2-

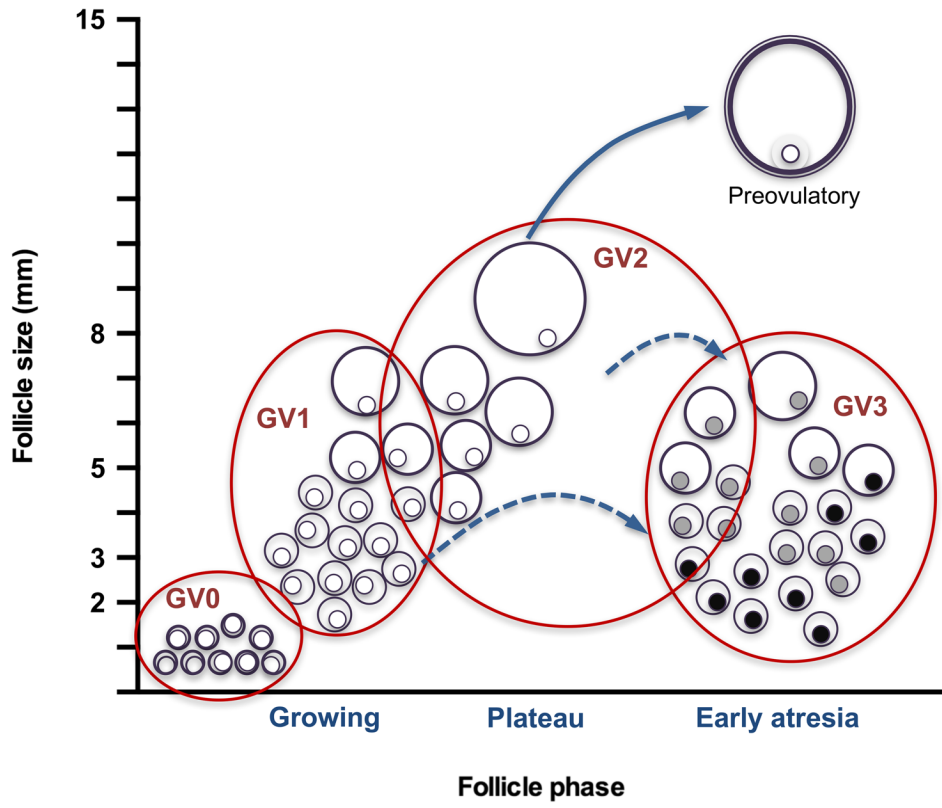656   stage oocyte and most **early atretic phase** follicles contain a GV3-stage oocyte.

657   Modified from Dieci et al., 2016 [35].

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