

Title: A 3D approach to reproduction

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27 **Abstract**

28 For over a century, 2D cell culture has been extensively used for all the different research fields.
29 However, this in vitro system does not allow to reproduce the natural structures of the original tissue,
30 causing several changes and, in most cases, the loss of cell-to-cell communications and cell-to-
31 extracellular matrix interactions. Based on this, during the last years, novel 3D platforms, able to
32 mimic the in vivo milieu, are being developed. The advantages of the use of 3D models are: the
33 reduction of the gap between cell culture and physiological environment; imitation of the specific
34 architecture; partially maintenance of the mechanical and biochemical cues of the original tissue.
35 Currently, 3D systems are used in a broad range of studies, including the field of reproduction, where
36 they have been applied to promote maturation of follicles and oocytes and embryo culture.
37 Here, we review 2D and 3D cell culture methods, discussing advantages and limitations of these
38 techniques. We report the fundamental mechanisms involved in cell ability to perceive and respond
39 to mechanical cues and their role in transmitting signals to and between cells and in regulating
40 intracellular signaling pathways. In particular, we focus on the main effectors of the Hippo pathway,
41 Yes-associated protein (YAP) and WW domain-containing transcription regulator protein 1 (TAZ),
42 describing their behavior and function in oocytes and embryos. Lastly, we provide an overall
43 perspective of the most recent 3D technologies developed in the field of reproduction, describing how
44 their use may revolutionize the understanding of cellular behavior and provide novel tools, useful in
45 reproductive technologies and livestock production.

46

47 **Keywords:** 3D culture system; Hippo pathway; in vitro reproductive technologies; mechano-sensing

48

49 **1. Introduction**

50 Over the last decades, great effort was dedicated to identify the best chemical composition of the
51 culture media to improve in vitro oocyte maturation and embryo development. Established
52 approaches had proven extremely useful and had largely contributed to increase success rates in
53 reproductive technologies. Nevertheless, a large body of literature, has highlighted that, not only the
54 chemical composition of culture media needs to be considered, but also the physical requirements
55 play a fundamental key role and are necessary in order to improve in vitro culture systems. Indeed,
56 several recent studies have demonstrated that all cells respond to the mechanical stimuli of their
57 environment, such as the elasticity and stiffness of the extracellular matrix (ECM) and traction or
58 compression forces exerted by neighboring cells [1–4]. Similarly, oocyte maturation, as well as
59 oocyte and embryo progression through the female reproductive tract, are influenced by gentle
60 mechanical stimulations, which significantly affect gamete and embryo development, beside their
61 exposure to a changing fluid chemical compositions [5–7]. Physical and mechanical characteristics
62 experienced within the genital tract stimulate cell mechano-sensing responses, inducing modification
63 in their morphology, polarity, growth, gene expression and functions. Recent advances in new three-
64 dimensional (3D) culture systems, has allowed for the development of complex matrix that closely
65 mimic the in vivo milieu, and its mechanical cues, greatly improving standard in vitro models within
66 reproductive technologies.

67 Aim of this review is to provide the reader with an overall perspective of the most recent 3D
68 approaches applied to the field of assisted reproduction. The pros and cons of their use and how they
69 may revolutionize the understanding of cellular behavior will be described, providing novel tools
70 useful in reproductive technologies in human and animal species.

71

72 **2. From 2D to 3D**

73 The first two-dimensional (2D) in vitro cell culture was carried out in 1907 by Harrison, during
74 research into the origin of nerve fibers [8]. Since then, this method was widely applied to study many

cell types, ranging from fibroblasts to gametes and embryos. During several years, 2D approaches were improved and used to characterize the mechanisms underlying cell biology and to better understand diseases and drug action outside the body [9]. Advantages of 2D cultures are associated with simple and low-cost maintenance as well as with ease of functional test performance [10]. On the other hand, adherent cultures have severe disadvantages (Fig. 1). They poorly mimic the natural structures of the original tissue representing only in a limited way the cellular environment found in organisms. 2D monolayers induce changes in cell-to-cell and cell-to-extracellular environment communications that play a key role in several cellular processes, regulating morphology, polarity, differentiation, proliferation, gene expression, responsiveness to stimuli, and drug metabolism [11–15]. As a consequence, cells isolated from tissues and transferred on flat and hard plastic substrates, tend to lose their standard morphology and functions.

All these aspects have suggested the need of the development of new three-dimensional (3D) culture systems that more closely mimic the *in vivo* milieu. One of the first 3D model was created in soft agar solution by Hamburg and Salmon in the 1970s [16]. Since then, several studies have demonstrated striking similarities between the morphology and behavior of cells growing *in vivo* and those cultured under 3D conditions [11,17]. The use of 3D scaffolds, in fact, reduces the gap between cell cultures and physiological tissues, imitating the architecture of the original organ more accurately than in 2D models [18]. Tissue engineering was able to create *in vitro* 3D models that facilitate cell growth, organization and differentiation, re-establishing the proper physiological cell-to-cell and cell-to-environment interactions. Currently, 3D cultures are used in a broad range of *in vitro* studies, including cancer and stem cells biology, drug testing and discovery, cell adhesion and migration and. In addition, they have been proposed as promising tools in the field of reproduction to provide optimal microenvironment for follicle, oocyte and embryo culture.

98

99 **3. Mechanical signaling**

100 Mechanical cues result from both intracellular-generated and externally-applied forces and, similarly
101 to intrinsic and extrinsic biochemical factors, they have broad impact on cell behavior. At present, it
102 is largely recognized that external mechanical forces are able to regulate cell growth, differentiation,
103 and functions. Indeed, cell-to-cell interactions produce intrinsic stimuli through various activities,
104 such as cytoskeletal assembly. Similarly, adhesion to extracellular matrix (ECM) generates extrinsic
105 forces that are constantly applied on cells [19,20]. All these mechanisms are crucial in transmitting
106 signals to and between cells and regulate intracellular signaling pathways [21].

107 The term "mechano-transduction" is currently used to describe the cell ability to perceive
108 microenvironment and appropriately respond to physical stimuli. These processes have been shown
109 to be regulated by the highly conserved Hippo pathway and, in particular, by the two main
110 downstream effectors of the cascade, namely the Yes-associated protein (YAP) and the WW domain-
111 containing transcription regulator protein 1 (WWTR1 or TAZ) [22]. Several studies have
112 demonstrated that the Hippo pathway responds to various stimuli from the cellular microenvironment,
113 including mechanical signals, cellular stress, extracellular stimuli, polarity, and adhesion cues [23].

114 This is made possible thanks to the presence of specific upstream regulators, such as the mammalian
115 STE20-like protein kinase 1/2 (MST1/2), the Salvador family WW domain containing protein 1
116 (SAV1), the MOB kinase activator 1A/B (MOB1A/B), and the large tumor suppressor 1/2
117 (LATS1/2). All these factors promote the activation of the Hippo kinase cascade [24] and induce the
118 phosphorylation/dephosphorylation and subsequent cytoplasmic/nuclear retention of YAP and TAZ,
119 controlling cell fate. These two proteins are transcriptional coactivators, unable to directly interact
120 with DNA, but rather binding other transcription factors (TFs) that elicit their functions [25]. In
121 particular, accumulating evidences show that the TEAD protein family is the major mediator of YAP
122 and TAZ activity, modulating the expression of different target genes involved in cell growth and
123 proliferation as well as organ development [26].

124

125 **3.1 TAZ/YAP in oocytes and embryos**

126 Mammalian oocytes are highly specialized non-proliferative cells. Upon fertilization, they are
127 reprogrammed into embryos that gain the ability to proliferate, differentiate and develop into new
128 individuals [27,28]. The most critical step is represented by the maternal-zygotic transition (MZT),
129 which is controlled by maternally stored RNAs and proteins [29,30]. Indeed, the zygotic genome is
130 transcriptionally quiescent. However, after MZT, it becomes active and takes control of development
131 [31–33]. A recent study has demonstrated that maternally accumulated YAP protein is essential for
132 zygotic genome activation (ZGA) [34]. This protein is highly expressed in both human and mouse
133 gametes and in early embryos [35,36], although no specific role has been fully identified yet. Indeed,
134 while YAP has been shown to mediate mechano-sensing related controls of survival, proliferation
135 and differentiation in somatic cells, neither oogenesis nor spermatogenesis was affected by its
136 knockout in germ cells [34]. Interestingly, YAP was shown to be highly expressed in early zygotic
137 stage and, together with TAZ, required for the first phases of mammalian embryogenesis, when the
138 maternal RNAs and proteins are exhausted [34]. These results clearly indicate a developmental stage-
139 specific role of the proteins. In particular, distinct changes in TAZ/YAP localization are essential for
140 determining early differentiation events, where nuclear/cytoplasmic compartmentalization of TAZ
141 and YAP defines the first cell fate choice in the embryos. Subsequently, at the blastocyst stage,
142 YAP/TAZ distribution is strictly compartmentalized to the nucleus in the inner cell mass (ICM), from
143 which embryonic stem cells (ESCs) are derived, while appears more diffused in the outer cells. This
144 localization allows the two molecules to elicit their transcriptional co-activator functions. Indeed,
145 YAP/TAZ interaction with nuclear transcriptional factors, TEAD1/3/4 sustains self-renewal and
146 pluripotency in ICM [37,38]. More in detail, once sorted to the nucleus, YAP and TAZ are able to
147 directly interact with SMAD2/3, forming a YAP/TAZ-SMAD2/3 complex [39] (Fig. 2). This newly
148 formed complex binds to TEAD transcription factors as well as OCT4, induces pluripotency-related
149 gene transcription, buffering pluripotency and repressing differentiation processes [37,38]. In
150 contrast, nuclear exclusion of YAP/TAZ is directly related to the specification of trophectoderm (TE)
151 with the expression of CDX2 in TE [40,41].

152 A study recently performed in our laboratory further supports these observations, and demonstrates
153 that YAP/TAZ activity is significantly up-regulated in parthenogenetic ESCs and compartmentalized
154 to the nucleus [42]. This appears to be related to the exclusive maternal origin of these cells and their
155 inability to give rise to functional TE. As expected, a higher ability to form outgrowths [43–45],
156 generate 3D spheroid colonies and increased high plasticity [46–48] parallel YAP/TAZ localization.
157 All these data point to the key role played by adequate 3D supports that, by activating mechano-
158 sensing response and related effector molecules, may provide the optimal microenvironment for
159 follicle, oocyte and embryo culture.

160

161 **4. 3D approaches applied to reproduction**

162 During the last years, many efforts were addressed to improve in vitro culture systems in order to
163 generate reliable and predictive models in reproductive biology. Several studies are currently focusing
164 on the derivation of in vitro 3D culture approaches that better mimic the physical structures and the
165 biochemical context of the reproductive tissues.

166

167 ***4.1 3D models for gamete maturation and embryo culture***

168 The first engineered in vitro culture system designed to mature oocytes isolated from cryopreserved
169 ovarian tissues was described in 2003 by Pangas et al. These authors used alginate hydrogel beads to
170 encapsulate and grow immature granulosa cell–oocyte complexes (GOCs) in specific spatial
171 arrangement. Oocytes retrieved and matured were able to achieve the specific morphology of mature
172 oocytes (cortical granule formation, a well-developed zona pellucida with microvilli, normal
173 mitochondria) and resume meiosis [49]. Subsequently, Xu et al. used the same 3D hydrogel-based
174 culture system, obtaining mature oocytes with fertilization ability similar to that of in vivo oocytes.
175 Moreover, the embryos produced from these oocytes were able to generate male and female fertile
176 offspring [50]. Hydrogel systems were also adopted to create oviduct organoids [51,52] and
177 endometrium engineered models [53–55] to improve oocyte maturation, fertilization and early

178 embryo development as well as to study interactions between embryos and endometrium, or for drug
179 testing. Very promising results were also obtained with agarose gels, another polysaccharide-based
180 biomaterial that is widely used in tissue engineering. A 3D artificial ovary was created to home the
181 three follicular cell types, namely theca, granulosa cells, and oocytes, and allowed the maturation of
182 early antral follicle into metaphase II oocytes [56]. Although encouraging, these approaches, based
183 on the use of biomaterials alone, provided suboptimal environments. One plausible hypothesis is the
184 lack of ECM proteins that are essential for all mammalian cells. Based on this, other studies were
185 carried out adding different ECM components, such as collagen (type I and IV), fibrin and/or
186 fibronectin, to polysaccharide-based matrix. These experiments demonstrated improved growth,
187 differentiation, and meiotic competence of the oocytes [57–59]. It is however, important to keep in
188 mind that general problems related to the use of biomaterials, such as batch-to-batch variability and
189 less-defined molecular composition, must be addressed before these methods can be widely utilized
190 to obtain consistent and reliable results.

191 A new 3D strategy is based on the production of polytetrafluoroethylene (PTFE) micro-bioreactors,
192 also known as “Liquid Marbles”. This method was described for the first time by Aussillous and
193 Quere [60] and consists of a drop of liquid encapsulated by hydrophobic PTFE. The powder particles
194 are able to adhere to the surface of the drop and to act as coating material, confining cells in a small
195 space and boosting them to freely interact with each other while, at the same time, allowing gas
196 exchange between the interior liquid and the surrounding environment. The results reported in the
197 literature demonstrated that PTFE microbioreactors may represent a promising tool for reproductive
198 studies and showed that liquid marbles provide an optimal microenvironment for oocyte in vitro
199 maturation [61] as well as for long-term culture of high plasticity cells [62].

200

201 ***4.2 3D models for reproductive organ reconstruction***

202 Very interesting results were recently obtained with the production of 3D-printed supports based on
203 the use of compatible bio-materials, that can be repopulated with specific cell subpopulations, or

204 directly printing cells in the matrix, recreating layer-by-layer an artificial environment that closely
205 mimics the architecture of the original tissue. This approach has recently allowed the assembly of
206 ovarian prostheses for supporting in vitro follicle and/or oocyte maturation and embryo development
207 [63]. Similarly, poly (DL-lactide-co-glycolide) degradable scaffolds were successfully used for ex
208 vivo creation of neovaginal constructs for patients suffering from vaginal aplasia [64]. On the other
209 hand the use of ECM derived from decellularized tissue has become increasingly frequent in
210 regenerative medicine and tissue engineering strategies, with recent applications including the use of
211 three-dimensional ECM scaffolds prepared by whole organ decellularization [65]. These bio-
212 scaffolds are obtained through the removal of cells from the original explant, while maintaining intact
213 all the ECM structures. In the field of reproduction, recent studies have described a successful
214 production of these type of biological supports, starting from ovarian cortical slides and fragments
215 [66–69]. The decellularization process has also been successfully used to obtain whole-uterus bio-
216 scaffolds from rat [70,71] and porcine [72,73] species. In our laboratory, we are currently developing
217 a specific protocol for whole-ovary decellularization. This strategy is a key step in order to supply
218 physiological-size-matched scaffolds for organ engineering. The cornerstone of this advance is the
219 reliance on the native organ vascular system as a cue for perfusion decellularization, recellularization,
220 and nutrients/oxygen delivery after in vivo transplantation [74]. The promising results obtained so far
221 with this approach pave the way for a possible in vitro re-construction of ovarian tissue that may
222 result advantageous for a general improvement of reproductive technologies and, possibly, future
223 application to organ transplantation.

224

225 **5. Conclusions**

226 New culture approaches are continuously appearing and constantly evolving to address the new
227 challenges and improve standard in vitro models that may ensure reliability and high predictivity. In
228 the field of reproduction, this trend acquires a fundamental importance, considering the huge practical
229 and clinical implications. On the other hand, one major problem of tissue engineering is the lack of

230 standardized approaches in 3D cultures and further studies are mandatory for their identification. As
231 a result, during the last years, an exponential increase in the number of in vitro models, proposed in
232 the literature tried to answer this requirement. The complexity of the developed supports is increasing
233 day-by-day to obtain more reproducible, reliable and predictive systems. Overall, this will hopefully
234 lead, in the near future, to a significant improvement of the existing ones, or to the creation of radical
235 innovative approaches, with significant benefits for reproductive technologies in livestock as well as
236 in human reproduction.

237

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241

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441

442 **Figure legend**

443 **Figure 1. Schematic representation of the Hippo signaling pathways.**

444 **Figure 2. Pros and cons of 2D vs. 3D culture systems.**