

1 **Matrigel inhibits elongation and drives endoderm differentiation in**  
2 **aggregates of mouse embryonic stem cells**

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4 Atoosa Amel<sup>1</sup>, and Mubeen Goolam<sup>1,2</sup>

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6 1. Department of Human Biology, University of Cape Town, Cape Town, 7925, South Africa

7 2. UCT Neuroscience Institute, Cape Town, South Africa

8 \*Author for correspondence: [mubeen.goolam@uct.ac.za](mailto:mubeen.goolam@uct.ac.za)

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20 **Abstract**

21 Modelling peri-implantation mammalian development using the self-organising properties of stem cells is a  
22 rapidly growing field that has advanced our understanding of cell fate decisions occurring in the early embryo.  
23 Matrigel, a basement membrane matrix, is a critical substrate used in various protocols for its efficacy in  
24 promoting stem cell growth and self-organization. However, its role in driving stem cell lineage commitment,  
25 and whether this effect is driven by biochemical or physical cues is not being clearly defined. Here, we grow  
26 embryoid bodies in suspension, Matrigel, and agarose, an inert polysaccharide, to attempt to decouple the  
27 physical and biochemical roles of Matrigel and better understand how it drives stem cell differentiation. We  
28 show that stem cell aggregates in Matrigel are hindered in their ability to elongate compared to those grown  
29 in agarose or in suspension indicating that prohibitive role in self-organisation. Aggregates in Matrigel are  
30 also driven to differentiate into endoderm with ectoderm differentiation inhibited. Furthermore, these  
31 effects are not due to the physical presence of Matrigel as the same effects are not witnessed in aggregates  
32 grown in agarose. Our results thus indicate that Matrigel has a significant and complex effect on the  
33 differentiation and morphology of embryoid bodies.

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35 **Keywords**

36 embryonic morphogenesis, embryonic stem cells, stembyro, basement membrane, extracellular matrix  
37 Matrigel

38

## 39 Introduction

40 In mammals the establishment of the body plan begins with gastrulation, a process initiated in the posterior  
41 region of the embryo through the appearance of the primitive streak (PS) that establishes the formation of  
42 the endoderm, mesoderm, and ectoderm [1-4]. Studying these early, key developmental milestones is made  
43 challenging in mammals as implantation into the uterine lining renders the embryo inaccessible for direct  
44 experimentation and manipulation. However, understanding these early self-organising events remains a  
45 fundamental field of study in developmental biology.

46 Recent advances in stem cell culture techniques have revolutionised the study of early embryonic  
47 development as we are now able to study early embryonic milestones using *in vitro* stem cell-based embryo  
48 models, so-called 'stembryos' [5-13]. A variety of 'stembryo' techniques have been developed in a short  
49 space of time and have been shown to be able to resemble the blastocyst [14-17], undergo gastrulation [18,  
50 19], develop an anterior-posterior axis [20-22], develop a primitive neural tube [23-25], or even undergo the  
51 early stages of somitogenesis [23, 26] and even cardiogenesis [27, 28].

52 During the development of these protocols one of the critical factors found to drive stem cell self-  
53 organisation has been the addition of Matrigel. Matrigel is an extracellular matrix (ECM) derived from  
54 Engelbreth-Holm-Swarm mouse sarcoma [29, 30] that contains glycoproteins, proteoglycans, and growth  
55 factors and has been found to support embryo culture [31]. Used at different concentration the addition of  
56 Matrigel can drive 'stembryo' elongation, somitogenesis and neural tube development [23, 26]. These  
57 findings suggest that Matrigel plays a key role in driving stem cell differentiation in 'stembryos'. However,  
58 Matrigel has a complex and an unstandardised composition, and its composition can vary from batch to batch  
59 [32]. This variability may affect the reproducibility of experiments using Matrigel. Furthermore, Matrigel  
60 provides both structural support as well as a host of growth factors and signalling molecules making it unclear  
61 whether the advanced morphologies noted in 'stembryo' cultures with Matrigel are due to the presence of  
62 mechanical or biochemical cues. To uncouple these components, an inert material is needed to evaluate the  
63 influence of just mechanical cues on stem cell morphogenesis. Here, we investigate the morphological and  
64 gene expression changes induced in embryoid bodies (EBs), the simplest three-dimensional aggregates of

65 stem cells used to study their differentiation, when grown in suspension, Matrigel, and agarose, an inert  
66 polysaccharide. In doing so we help to dissect the role of structural support on the fate of mouse embryonic  
67 stem (mES) cells.

## 68 **Materials and Methods**

### 69 **mES cell culture**

70 In order to culture 129/Ola mouse embryonic stem (mES) cells, a feeder layer of inactive murine embryonic  
71 fibroblast cells was cultured in 6-well plates coated with 0.1% gelatin (Sigma-Aldrich G7041-100G) at a  
72 density of  $1.6 \times 10^4$  cells/cm<sup>2</sup> in a base medium containing DMEM (1x) +GlutaMAX™ (Gibco 10566016), 15%  
73 FBS (Gibco 10493106), 0.05 mM β-mercaptoethanol (Gibco 21985023), and 1% PenStrep (100 U penicillin/0.1  
74 mg/ml streptomycin, Gibco 15140122). Two h before seeding the mES cells, the medium was changed to 2i  
75 + LIF medium consisting of base medium supplemented with CHIR99021 (Chiron, 3 μM, Sigma-Aldrich  
76 SML1046-5MG), PD0325901 (1 μM, Sigma-Aldrich PZ0162-5MG), and Leukaemia inhibitory factor (10 ng/ml,  
77 LIF, Thermo Fisher Scientific A35934). The mES cells were then seeded onto the feeder layer at a density of  
78  $7 \times 10^3$  cells/cm<sup>2</sup>, and the medium was changed daily, with cells being passaged every other day.

### 79 **Cell aggregates in suspension**

80 The aggregate formation method was adopted from Bailie Johnson *et al.* [33]. mES cells colonies were treated  
81 with Dispase II (5mg/ml, Sigma-Aldrich D4693-1G) at 37°C for 20 min, followed by inactivation of Dispase and  
82 transfer of the cell suspension to a 15 ml tube. The cells were spun down and resuspended in PBS to remove  
83 residual medium. Following the PBS washes, the cells were resuspended in N2B27 medium which consists of  
84 base medium supplemented with 1% N2 supplement (Gibco 17502048) and 1% B27 plus supplement. (Gibco  
85 A3582801). The solution was diluted in N2B27 to give  $5 \times 10^4$  cells per 5ml of medium for one 96-well plate.  
86 The suspension was then added in 40μl droplets/well to a non-adhesive 96-well U-bottom plate (Greiner Bio-  
87 One 650185). The plate was incubated at 37°C and 5% CO<sub>2</sub> for 48hrs after which a Chiron pulse (CHIR99021,  
88 3μM) was added to the aggregates and the plate was incubated for an additional 24hrs. The following day,



89 the Chiron pulse was removed and fresh N2B27 was added. The medium was changed every day until the  
90 required endpoint and not more than 168hrs.

#### 91 **Cell aggregates on agarose**

92 The 96-well flat bottom plates were coated with 30µl of 1.2% agarose solution (1.2% w/v of Lonza Bioscience  
93 50004 agarose added to deionised water and sent for autoclaving) and left to dry for 10min at RT. mES cell  
94 aggregates were made as above, however, instead of adding the cell suspension to 96-well U-bottom plates,  
95 40µl drops were placed in each well of the agarose-coated plate. The plate was incubated at 37°C and 5% CO<sub>2</sub>  
96 for 48hrs after which a Chiron pulse (3µM) was added to the aggregates, and they were incubated for an  
97 additional 24hrs. The following day, the Chiron pulse was removed and fresh N2B27 was added. The medium  
98 was changed every day until the required endpoint and no longer than 168hrs.

#### 99 **Cell aggregates embedded in Matrigel**

100 mES cells were lifted using Dispase II after a 20 min incubation period and the pellet was washed twice in PBS  
101 before counting. The cells were counted, and the appropriate volume was aliquoted and spun down again to  
102 give a concentration of 3x10<sup>4</sup> cells/ml. The pellet was resuspended in 1ml of Matrigel (Corning, 356234).  
103 Droplets of 20µl were evenly placed on a 10 cm culture plate and Matrigel was left to solidify for 5 min at  
104 37°C and subsequently covered with N2B27 medium. The Chiron pulse was added to the medium in the dish  
105 on day 2 of the culture and removed after 24hrs. The medium was changed every second day until the  
106 required endpoint, but not more than 168hrs.

#### 107 **RNA extraction and cDNA synthesis**

108 RNA was collected from aggregates grown in suspension, agarose or Matrigel 144 hrs post aggregation using  
109 the High Pure RNA Isolation Kit (Roche 11828665001) following the manufacturer's instructions. Due to the  
110 limited amount of material obtained from each experiment, two experiments were combined to form one  
111 biological replicate. This was done for a total of two biological replicates. The ImProm-II™ Reverse  
112 Transcription System (Promega) was utilised for cDNA synthesis. A minimum of 1 µg of RNA was combined

113 with 1  $\mu$ l of Oligo (dT)15 primer (Promega, C110B) and nuclease-free water to make up a total of 5  $\mu$ l and  
114 incubated at 70°C and 4°C for 5 min each. PCR master mix made up of 6.1  $\mu$ l nuclease-free water, 4  $\mu$ l 5x  
115 reaction buffer (Promega, M289A), 2.4  $\mu$ l MgCl<sub>2</sub> (Promega, A351H), 1 dNTP mix (Promega, C114B), 0.5  $\mu$ l  
116 Recombinant RNasin® Ribonuclease Inhibitor (Promega, N251A), 1  $\mu$ l Reverse Transcriptase (Promega,  
117 M314A), was added to each sample. Thermal cycling was as follows: 25°C for 5 min, 42°C for 60 min and 70°C  
118 for 15 min.

## 119 **qPCR**

120 The StepOnePlus™ Real-Time PCR System was used for quantitative PCR with SYBR green PCR Master-Mix  
121 (Thermo-Fisher Scientific 4368708). Primer sequences are available in supplementary material Table S1.  
122 Briefly, 2  $\mu$ l of cDNA (diluted 1:1 with nuclease-free water) was mixed with 8  $\mu$ l master which consisted of: 5  
123  $\mu$ l SYBR green Master-Mix, 0.4  $\mu$ l of 10  $\mu$ M forward and reverse primer mixture (5  $\mu$ l of 100  $\mu$ M stock with 40  
124  $\mu$ l nuclease-free water), and 2.6  $\mu$ l nuclease-free water. Each reaction had three technical replicates. The run  
125 parameters can be found in supplementary material Table S2. *Gapdh* was used as a housekeeping gene to  
126 calculate relative expression via the 2- $\Delta\Delta$ Ct method. Expression was normalised to cell aggregates cultured  
127 in base medium that did not receive a Chiron pulse. Data analysis was performed using MS Excel, while  
128 statistical analysis and graph generation were done using GraphPad Prism8.

## 129 **Immunofluorescence**

130 Cell aggregates were fixed at 4°C in 4% PFA for 1 h on a shaker. Subsequently, the aggregates were washed  
131 three times for 5-10 minutes with PBST (PBS, 0.05% Tween-20) on a rotating shaker and were permeabilised  
132 with 0.5% Triton-X-100 in PBS at room temperature for 1 h. After a brief washing step with PBST (Three times  
133 for 5-10 minutes), the aggregates were blocked with PBS, 10% FBS, and 0.2% Triton-X-100 at room  
134 temperature for 1 h. Primary antibody incubation was performed overnight at 4°C in blocking buffer. The  
135 following day, the aggregates were washed three times quickly with PBS, followed by three 20 min washes.  
136 Secondary antibody incubation was performed overnight at 4°C in blocking buffer, after which the washing  
137 procedure was repeated. Hoechst was added to the 20 min washes following secondary antibody incubation

138 and the samples were left to incubated overnight at 4°C. Prior to imaging, aggregates mounted onto Mowiol  
139 drops in glass-bottomed dishes. Antibody details and dilutions used are provided in Table S3.

## 140 **Image analysis**

141 The EVOS™ M5000 Imaging System microscope (Thermo Fisher Scientific, USA) was used to capture images  
142 at a magnification of 10X. ImageJ was used to optimise the brightness and contrast of all images and for  
143 measurements. The outlines of the entire aggregate were traced manually to determine the perimeter, while  
144 the minor and major axial lengths were determined using the line tool. The elongation index was calculated  
145 by dividing the major axial length by the minor axial length. Multichannel images were taken using a Zeiss  
146 LSM 880 Airyscan confocal microscope (Zeiss, Germany) using a 20x or a 40x water-immersion objective. Z-  
147 stack slices were spaced at 0.4µm and the final image was deconvolved and displayed at maximum projection  
148 using the open-source ImageJ2 platform.

## 149 **Statistical analysis**

150 Statistical analyses were carried out using the functions provided by GraphPad Prism 8.4.2 (679). Because of  
151 the scarcity of biological material, two biological replicates (each consisting of two pooled experiments) were  
152 used for statistical analysis. When appropriate, an unpaired, nonparametric Mann-Whitney test was  
153 performed. Alternatively, a Two-way Anova was performed followed by a Fisher's Least Significant Difference  
154 test. In all cases, the error bars represent the mean ± standard error of the mean (mean ± s.e.m.). P values  
155 are represented as follows: ns =  $p \leq 0.1234$ , \* =  $p \leq 0.0332$ , \*\* =  $p \leq 0.0021$ , \*\*\* =  $p \leq 0.0002$ , \*\*\*\* =  $p < 0.0001$ .

## 156 **Results**

### 157 **The effect of Matrigel and agarose on the morphology of mES cell aggregates**

158 To examine the role of physical support in mES cell differentiation, we grew aggregates of ES cells in  
159 suspension, on agarose, and in Matrigel (Fig. 1A). Under all conditions embryoid bodies (EBs) were subjected  
160 to a pulse of Chiron at 48 h for 24 h and shape changes were quantified over time by measuring the aspect  
161 ratio of the aggregates (the longest axis of the aggregate, the major axis, divided by the distance

162 perpendicular to the midpoint of the longest axis, the minor axis) (Fig. 1A). Aggregates were also classified  
163 based on visual characteristics and categorised as spherical, ovoid, tear-shaped, budding, or elongating based  
164 on their appearance and aspect ratio (Fig. S1). While aggregates grown in suspension (Fig. 1B and E) or  
165 agarose (Fig. 1C and F) started to elongate at 72 h, following a pulse of Chiron, those grown in Matrigel  
166 primarily retained their spherical or ovoid structures at the same time (Fig. 1D and G). By 144 h, all aggregates  
167 in suspension (Fig. 1B and E) and 8/20 of those in agarose (Fig. 1C and F) were able to elongate and resemble  
168 the cup-shaped morphology of the embryo. However, aggregates in Matrigel retained a more oval shape by  
169 the end of the culture period and did not elongate as easily (Fig. 1D and G). It was also noted that agarose  
170 aggregates had a unique, temporary balloon-like morphology (Fig. S1) that appeared at 24 h (16/20) and  
171 were mostly no longer present by 96 h (1/20) (Fig. 1F).

172 Aspect ratio analyses revealed that suspension aggregates were the only group that underwent a rapid period  
173 of elongation at 72 h and an additional increase in axial length at 144 h (Fig. 1H). Interestingly, there was a  
174 dip in aspect ratio at 120 h under both suspension and agarose conditions, meaning that the aggregates grew  
175 in width rather than length. The suspension aggregates had the highest rate of growth in perimeter, followed  
176 by the agarose aggregates, and the Matrigel embedded aggregates showed the smallest increase in  
177 perimeter during the 144 h period (Fig. 1I).

### 178 **Matrigel drives endoderm differentiation in mES cell aggregates**

179 Physical support from the maternal environment plays an essential role in early embryonic development  
180 during induction of the PS, when cells first undergo an epithelial-to-mesenchymal transition (EMT) and form  
181 mesodermal and endodermal progenitors [34]. In this study, we sought to determine the effects of physical  
182 constraints on the development of the PS and in driving EMT in EBs. To investigate this, we measured the  
183 expression of EMT and PS markers using qPCR in EBs grown in suspension, agarose, and Matrigel with a  
184 Chiron pulse, and compared this to EBs grown in suspension without any exposure to a Wnt agonist.

185 The posterior epiblast marker *Wnt3* was significantly increased in suspension aggregates compared to  
186 agarose and Matrigel aggregates while there was little variation in the expression of  *$\beta$ -catenin* (Fig. 2).

187 *Brachyury*, a marker of the PS and mesoderm, was also upregulated at 144 h under suspension conditions,  
188 with no increase in expression noted in agarose or Matrigel conditions (Fig. 2). However, another PS and  
189 mesoderm marker, *Nodal*, was significantly up-regulated in both agarose and Matrigel conditions (Fig. 2).  
190 The mesendoderm marker *Eomes* was up-regulated in EBs in suspension, as well as in Matrigel (Fig. 2), while  
191 another mesendoderm marker *Mixl1* was significantly up-regulated significantly in suspension and agarose  
192 conditions but not in Matrigel (Fig. 2). Interestingly, the early endoderm marker, *Gata6*, had increased levels  
193 of expression in Matrigel aggregates compared to the control (although not significantly), while a marker of  
194 the definitive endoderm marker, *Sox17*, was significantly increased in Matrigel compared to the other  
195 conditions, suggesting that Matrigel may be directing differentiation towards the endoderm lineage. A key  
196 EMT marker, *Snai1* was down-regulated significantly under suspension and agarose conditions compared to  
197 the control (Fig. 2) and *E-cadherin*, an epithelial marker, was down-regulated in suspension compared to the  
198 other two conditions (Fig. 2). The anterior epiblast marker *Pou3f1* had higher levels of expression in all three  
199 conditions compared to controls, albeit with a significant increase only observed in suspension (Fig. 2).  
200 Similarly, *Slc7a3*, another anterior epiblast marker, had increased expression levels in all three conditions  
201 compared to controls with a significant increase in suspension and agarose conditions (Fig. 2). The  
202 anterior/neuroectoderm marker *Sox2* had an increased relative level of expression in the three conditions,  
203 while *Pax6*, a late neuroectoderm marker, was only up-regulated in suspension aggregates. This suggests that  
204 neuroectoderm formation may be at a more advanced stage in suspension aggregates exposed to a Chiron  
205 pulse.

#### 206 **Aggregates in Matrigel form pro-amniotic-like cavities**

207 The effects that physical constraints had on mesoderm and endoderm gene expression next led us to  
208 investigate the spatial expression of the mesoderm marker *Brachyury* and the endoderm marker *Sox17* in  
209 our aggregates using immunofluorescence. When in suspension very few aggregates expressed *Brachyury*.  
210 When they did, this expression was either restricted to one pole of the aggregate (Fig. 3A, 1/50) or dispersed  
211 throughout the entire aggregate (Fig. 3B, 2/50). A highly similar pattern was observed in the agarose  
212 aggregates with only a few aggregates being positive for *Brachyury*, and when they were, this staining was

213 either localised (Fig. 3C, 2/46) or dispersed (Fig. 3D, 2/46). Aggregates in Matrigel, however, have significantly  
214 more Brachyury positive aggregates with 18/60 showing a dispersed pattern of expression (Fig. 3E).  
215 Interestingly, all of these also developed a Brachyury lined lumen in their centers (Fig. 3F, 18/60). All  
216 aggregates in Matrigel were found to have formed a similar cavity, even those without any Brachyury-positive  
217 staining. The cells surrounding these cavities appeared as epithelial-like and were brightly stained with E-  
218 cadherin (Fig. 3G). The suspension and agarose aggregates clearly lacked such cavities, but they had regions  
219 that were negative for E-cadherin (Fig. 3H and 3I) suggesting that EMT has occurred.

220 Endoderm staining was observed infrequently in all conditions investigated. In suspension aggregates, it was  
221 noted as either small pockets of Sox17-positive cells near the periphery (Fig. 3J, 7/46) or dispersed across the  
222 surface of the aggregate (Fig. 3K, 7/46). A similar expression pattern was observed in agarose aggregates  
223 with small pockets of Sox17-positive cells (Fig. 3L, 3/49) or a dispersed expression pattern (Fig. 3M, 5/49).  
224 Aggregates in Matrigel were noted to express Sox17 in dispersed groups across the surface of the aggregate  
225 (Fig. 3N, 5/60).

## 226 **Discussion**

227 The emergence and subsequent development of 'stembryo' culture systems has generated a non-invasive,  
228 scalable, and novel way to investigate early mammalian embryonic morphogenesis and cell fate decisions  
229 gastrulation and germ layer formation in mammals. One of the key aspects missing from 'stembryo'  
230 structures is the presence of physical support provided by the ECM normally generated by the basement  
231 membrane of the visceral endoderm. To address this issue, many of these systems incorporate the  
232 commercial basement membrane substitute Matrigel. Although Matrigel can promote stem cell self-  
233 organisation and provides some degree of mechanical constraint, it is ill-defined and suffers from lot-to-lot  
234 variability. In addition, its effect on organoid structure and differentiation is influenced by both mechanical  
235 and biochemical cues, and the specific component/s responsible for the observed morphologies remain  
236 unknown. Matrigel also does not support any controlled modifications of its stiffness or components, and  
237 thus there is a need to use an inert and manipulatable scaffold to separate the effects of physical and  
238 chemical cues on stem cell self-organisation in this burgeoning field. In this study, we used agarose, an inert

239 polysaccharide, to test the role of mechanical cues on the fate of embryoid bodies and to try to create similar  
240 physical constraints to those provided by the uterus.

241 We found that EB morphology was significantly influenced by the physical constraints present during culture,  
242 with suspended aggregates exhibiting more effective growth and elongation than counterparts grown in the  
243 presence of Matrigel or agarose. At 48 h, some suspension and agarose aggregates began to exhibit tear-  
244 shaped or budding structures, indicating an intrinsic breaking of symmetry. At 72 h, there was a sudden  
245 increase in the variety of morphologies, including balloon-like structures, which were observed as  
246 intermediates in the elongation process. Interestingly, we found that the presence of Matrigel inhibited  
247 elongation, causing aggregates to maintain their oval or spherical shape for longer. When we measured  
248 aspect ratios, we found that aggregates in suspension underwent a drop in aspect ratio at 120 h, followed by  
249 a sudden increase at 144 h. This suggests that cell proliferation occurs before elongation, along the major  
250 axis. Aggregates grown in agarose exhibited a similar dip and rise in aspect ratio, but the same pattern was  
251 not found in aggregates grown on Matrigel. Overall, we found that early self-patterning events in stem cell  
252 aggregates in culture are hindered by the presence of Matrigel. These aggregates neither grow nor elongate  
253 as effectively as those in suspension or in agarose, with Matrigel playing a prohibitive role in self-organisation.

254 Previous studies have shown that after 144 h in culture 'stembryo' should start to undergo a gastrulation-  
255 like process [20, 23, 35, 36]. Although we noted elongation in EBs in both suspension and agarose conditions  
256 by this time point, there were marked differences in the expression of key markers between the two  
257 conditions. Aggregates in suspension had increased levels of *Brachyury* suggesting that the PS had been  
258 initiated. In contrast, aggregates grown in agarose did not have this same up-regulation in *Brachyury*  
259 expression. They did, however, have up-regulated  $\beta$ -*catenin*, an upstream regulator of *Brachyury*. This could  
260 indicate a delay in the gastrulation process due to the differences in the physical properties of the  
261 environments as the more restrictive agarose may present a less conducive environment for elongation and  
262 subsequent gastrulation. Aggregates in Matrigel had similarly low levels of *Brachyury* and  $\beta$ -*catenin*, further  
263 supporting the theory that physical support hinders stem cell driven *in vitro* gastrulation-like events.  
264 Aggregates in suspension also had the lowest relative levels of *E-cadherin*, an epithelial marker, and the

265 highest levels of mesenchymal marker *Snai1*, suggesting that these aggregates are likely undergoing EMT, a  
266 critical process in gastrulation. This is further support for suspension aggregates being more developmentally  
267 advanced than aggregates in either agarose or Matrigel.

268 However, the expression patterns of *Nodal* and *Wnt3* in our aggregates indicate that the developmental  
269 landscape in our conditions is more complex than this. *Nodal* and *Wnt3* are known to work together to induce  
270 the expression of *Brachyury* and *Eomes*, another marker of gastrulation [1]. We observed that *Nodal* and  
271 *Wnt3* have opposing expression patterns in suspension aggregates, with elevated levels of *Wnt3* and low  
272 levels of *Nodal*. Interestingly, the opposite pattern, high levels of *Nodal* and relatively low levels of *Wnt3* was  
273 observed in aggregates in agarose and Matrigel. These results would indicate that the aggregates under these  
274 conditions are more advanced in terms of mesoderm differentiation. As *Eomes* has been shown to precede  
275 *Brachyury* expression [37], and based on the high levels of *Eomes* we can assume that *Brachyury* expression  
276 would increase in both suspension and Matrigel aggregates if cultured further. As both aggregates in Matrigel  
277 and agarose show a similar pattern of expression in this case, we hypothesise that it is principally mechanical  
278 cues driving this expression pattern and this warrants further investigation.

279 Analysing the expression of key markers of PS formation and EMT transition, it appears that our aggregates  
280 most closely resemble mouse embryos at E5.5-E6.5, between the emergence of the germ layers and the  
281 formation of the anterior-posterior axis (A-P axis), which occurs at approximately 120-144 h of  
282 development[6]. It is noted that this is significantly delayed compared to other 'stembyro' culture processes,  
283 where aggregates can undergo a gastrulation-like process after 96 h and exhibit similarities to the E8.5 mouse  
284 embryo [20, 23, 35, 38].

285 Despite this apparent developmental delay, suspension aggregates had high levels of  
286 anterior/neuroectoderm markers, *Pou3f1*, *Slc7a3*, *Sox2*, indicating the initiation of the anterior-posterior(A-  
287 P) axis. We observed a distinct increase in *Pax6*, a late neuroectoderm marker, in suspension aggregates  
288 similar to what is observed in E8.5 mouse embryos and gastruloids (one of the most robust 'stembyro'  
289 systems) at 120 h [38]. This is possibly due to the high levels of *Sox2* present in mES cells prior to



290 differentiation, allowing rapid neural differentiation [39], provided they were able to differentiate without  
291 any confounding effects, such as physical constraints.

292 Aggregates in Matrigel demonstrated the highest expression of endoderm markers compared to the other  
293 two conditions. *Gata6* is typically a marker for cardiac mesoderm and DE progenitors [40], while *Sox17* is a  
294 well-known DE marker [41]. *Gata6* mRNA was up-regulated in Matrigel aggregates, followed by up-regulation  
295 of *Sox17*, indicating that this condition likely favoured endoderm formation. It is unclear if this preference is  
296 due to physical restrictions imposed by Matrigel or the presence of certain signalling factors. However,  
297 evidence suggests that laminin, a major component of Matrigel, can direct human ES cells toward the  
298 endoderm lineage [42]. Furthermore, studies have demonstrated that culturing EPI stem cells in Matrigel  
299 results in up-regulated levels of endoderm-related transcription factors such as *Sox17* [43], further  
300 supporting the notion that Matrigel influences lineage commitment towards endoderm.

301 Immunofluorescent staining for Brachyury revealed that the positive cells were located exclusively at the  
302 elongated pole of the aggregates in both suspension and agarose, as observed in other studies [20, 35, 38,  
303 44], although the frequency of this pattern was significantly lower than reported by others. Aggregates in  
304 Matrigel, however, had many more aggregates that were Brachyury-positive, and these cells were principally  
305 in the centre of the aggregate surrounding the lumen. Studies have demonstrated that Matrigel can increase  
306 the expression of Wnt3 antagonists such as *Dkk1* and *Sfpr1* [45]. Therefore, it is plausible that cells located  
307 in the periphery of our Matrigel aggregates express Wnt3 agonists, resulting in the localisation of Wnt3  
308 activity in the centre of the aggregates, which leads to the up-regulation of Brachyury expression. By  
309 embedding aggregates in Matrigel, we were able to observe cavity formation that resembled the pro-  
310 amniotic cavity. This was likely due to the cells pulling apart, facilitated by E-cadherin localisation around the  
311 membrane. Matrigel is used as a substitute for ECM derived from extra-embryonic tissue [18] and the  
312 interaction between cells and Matrigel via integrin receptors facilitates cell polarisation and cavity formation  
313 [46]. In the embryo, cavity formation is mediated by the interaction between laminin and the  $\beta 1$ -integrin  
314 receptor between embryonic and extra-embryonic tissue [46]. The same mechanism may operate in Matrigel

315 aggregates, allowing them to form pro-amniotic cavity-like structures. To test the establishment of polarity  
316 in our aggregates, future work should involve immunofluorescence staining for aPKC and Par6 [47, 48].

317 Overall, our results suggest that Matrigel has a significant and complex effect on the differentiation and  
318 morphology of mES cells in culture. It can hinder stem cell self-organisation and neural differentiation, drive  
319 endoderm differentiation, and induce the formation of a pro-amniotic cavity. Its effects are not driven simply  
320 by the mechanical force it provides as its effects on stem cells are not mimicked by using agarose. Although  
321 Matrigel is a critical component of 'stembryo' cultures, caution should be taken when viewing it as a  
322 substitute for an ECM. Future research in the field should focus on finding a more defined and manipulatable  
323 replacement for Matrigel in order to advance the field and the accuracy of *in vitro* embryo models.

324

#### 325 **Author contributions**

326 M.G. conceived the project. A.A carried out the experiments. A.A. and M.G. wrote the paper.

327

#### 328 **Declaration of competing interest**

329 The authors have no financial or competing interests to declare.

330

#### 331 **Data availability**

332 Data will be made available upon request.

333

334

335

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356 **Figure captions**

357 **Fig. 1: Comparative analysis of the effect of various physical constraints on ES cell aggregate morphology.**

358 (A) ES cell aggregates were cultured in suspension, on agarose or embedded in Matrigel. All aggregates  
359 received a 24 h Chiron pulse on the third day of culture. To calculate the elongation of aggregates, the major  
360 axis (the longest axis; blue line) was measured and divided by the minor axis- the line perpendicular to the  
361 major axis at its midpoint (red line). (B) Morphology of aggregates in suspension. Aggregates had elongated  
362 by 144 h. (C) Morphology of aggregates cultured on agarose. At 24 h the aggregates had already showed  
363 signs of asymmetry and by 144 h the aggregates had elongated. (D) Morphology of aggregates embedded in  
364 Matrigel. The aggregates were relatively smaller than the other two conditions and did not elongate  
365 significantly. (E) Characteristics of n = 20 aggregates grown in suspension for the period of 144 h. Major  
366 morphological changes occurred at 72 h and at 144 h all the aggregates had elongated. (F) Characteristics of  
367 n = 20 aggregates grown on agarose for the period of 144 h. The aggregates had a unique balloon-like  
368 morphology in the first 96 h of culture and by 144 h some aggregates were still in the budding stage and had  
369 not elongated fully. (G) Characteristics of n = 20 aggregates grown in Matrigel for the period of 144 h.  
370 Aggregates had a delayed growth and only showed signs of asymmetry at 96 h. (H) Aspect ratio of aggregates  
371 in suspension (black line, empty circle), on agarose (grey line, dotted circle) and in Matrigel (dotted line, solid  
372 circle) for n = 20 aggregates per condition. (I) Perimeter of aggregates in suspension (black line, empty circle),  
373 on agarose (grey line, dotted circle) and in Matrigel (dotted line, solid circle) for n = 20 aggregates per  
374 condition. Error bars indicate mean  $\pm$  SEM. The aspect ratio within the same condition between two  
375 consecutive time points was evaluated for significance. An unpaired, nonparametric Mann-Whitney test was  
376 performed and \* =  $p \leq 0.0332$ , \*\* =  $p \leq 0.0021$ , \*\*\* =  $p \leq 0.0002$ , \*\*\*\* =  $p < 0.0001$ . Scale bar: 100  $\mu$ m.

377 **Fig. 2: Effects of various physical constraints on gene expression of aggregates.** QPCR was performed on

378 reverse transcribed RNA extracted at 144 h. Primitive streak markers: *Wnt3*,  *$\beta$ -catenin*, *Brachyury* and *Nodal*.

379 Mesendoderm markers: *Eomes* and *Mixl1*. Endoderm markers: *Gata6* and *Sox17*. EMT markers: *Snai1* and *E-*

380 *cadherin*. Anterior/neurectoderm markers: *Pou3f1*, *Slc7a3*, *Sox2* and *Pax 6*. Relative fold expression to cell

381 aggregates without a Chiron pulse. Error bars indicate mean  $\pm$  SEM. An unpaired, nonparametric Mann-  
382 Whitney test was performed and \* =  $p \leq 0.0332$ , \*\* =  $p \leq 0.0021$ , \*\*\* =  $p \leq 0.0002$ , \*\*\*\* =  $p < 0.0001$ .

383 **Fig. 3: Immunofluorescence staining of Brachyury, Sox 17 and E-cadherin in aggregates cultured under**  
384 **various physical constraints.** (A) Localised Brachyury positive cells at the elongating tip of suspension  
385 aggregates. (B) Brachyury positive cells spread throughout suspension aggregates that remained spherical.  
386 (C) Localised Brachyury positive cells in agarose aggregates. (D) Dispersed Brachyury-positive cells in agarose  
387 aggregates. (E) Brachyury positive cells dispersed throughout Matrigel aggregates. (F) Brachyury positive cells  
388 lining the lumen of Matrigel aggregates. (G) Epithelial-like cells surrounding the cavity in Matrigel aggregates  
389 stained positive for epithelial marker E-cadherin. (H) Suspension aggregates stained for E-cadherin had  
390 regions with reduced expression that may represent EMT. (I) Agarose aggregates stained for E-cadherin had  
391 regions with reduced expression that may represent EMT. (J) Pockets of Sox17 positive cells in the periphery  
392 of suspension aggregates. (K) Sox17 positive cells dispersed across the surface of suspension aggregates. (L)  
393 Pockets of Sox17 positive cells in aggregates cultured on agarose. (M) Sox17 positive cells dispersed across  
394 the surface of aggregates grown on agarose. (N) Sox17 positive cells in dispersed groups across the surface  
395 of aggregates in Matrigel. Scale bar: 25 $\mu$ m.

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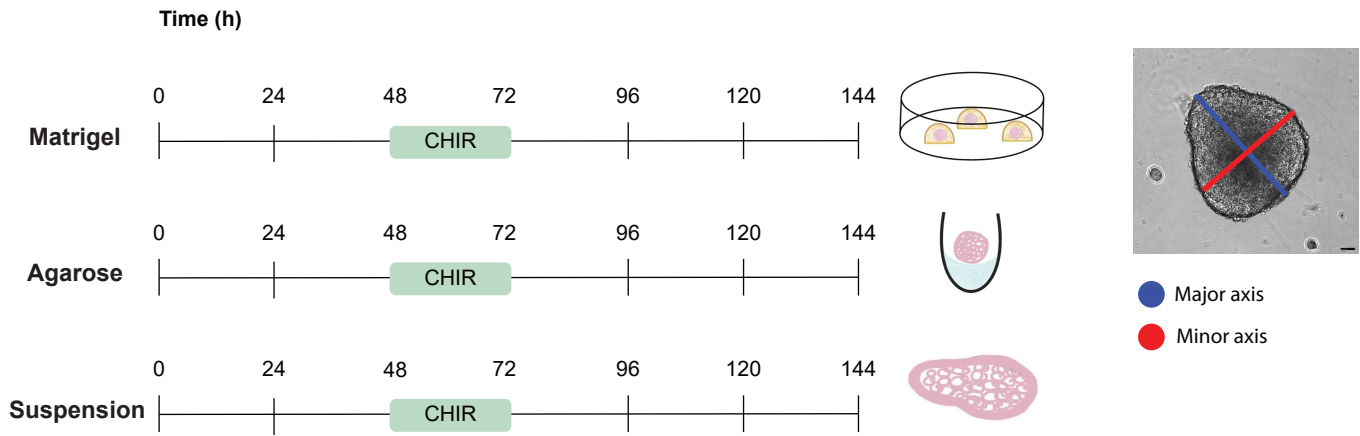
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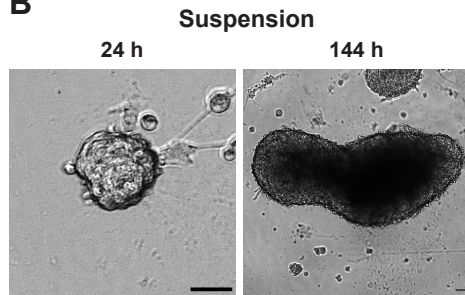
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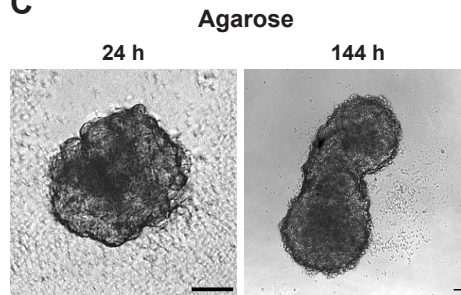
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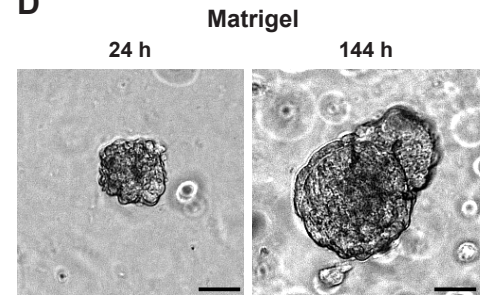
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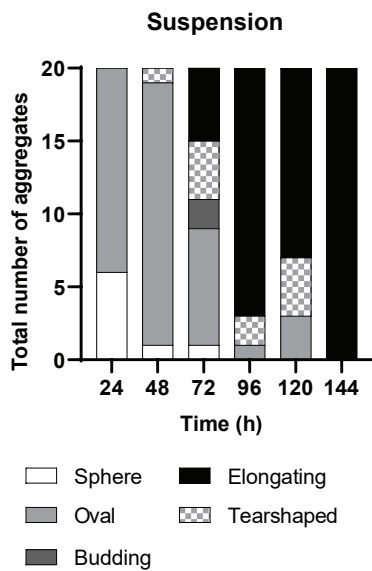
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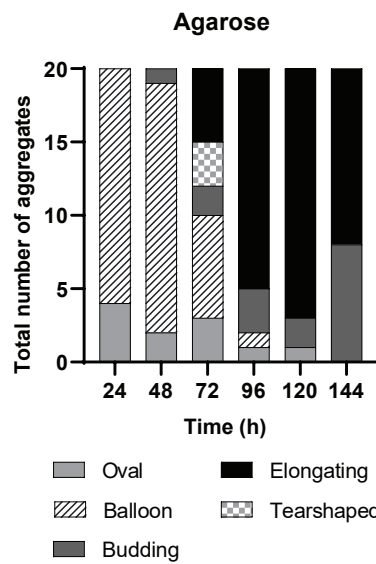
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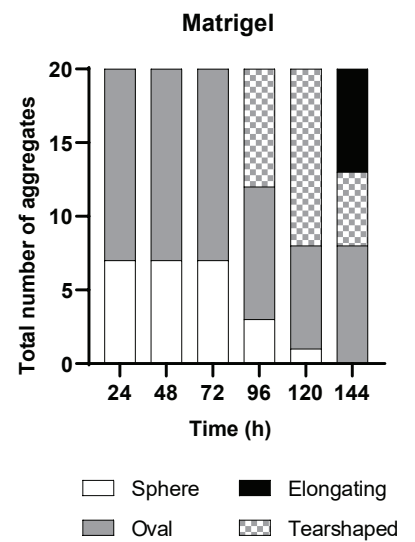
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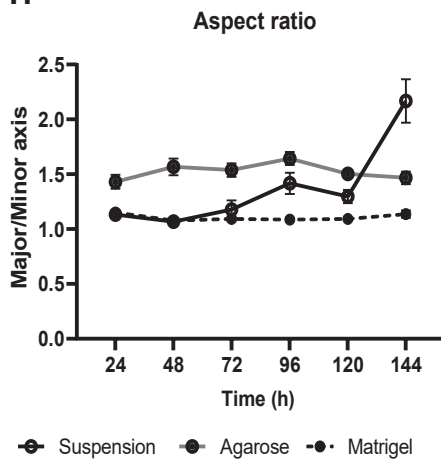
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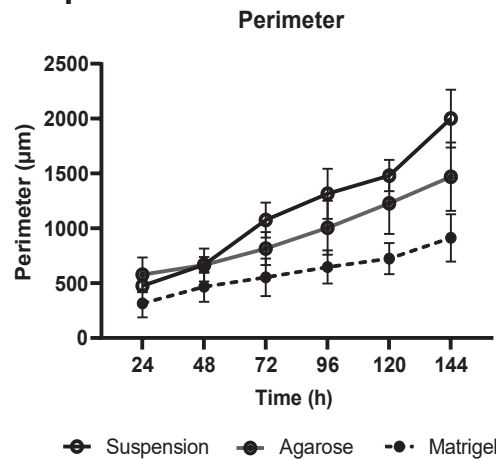
**G**



**H**



**I**

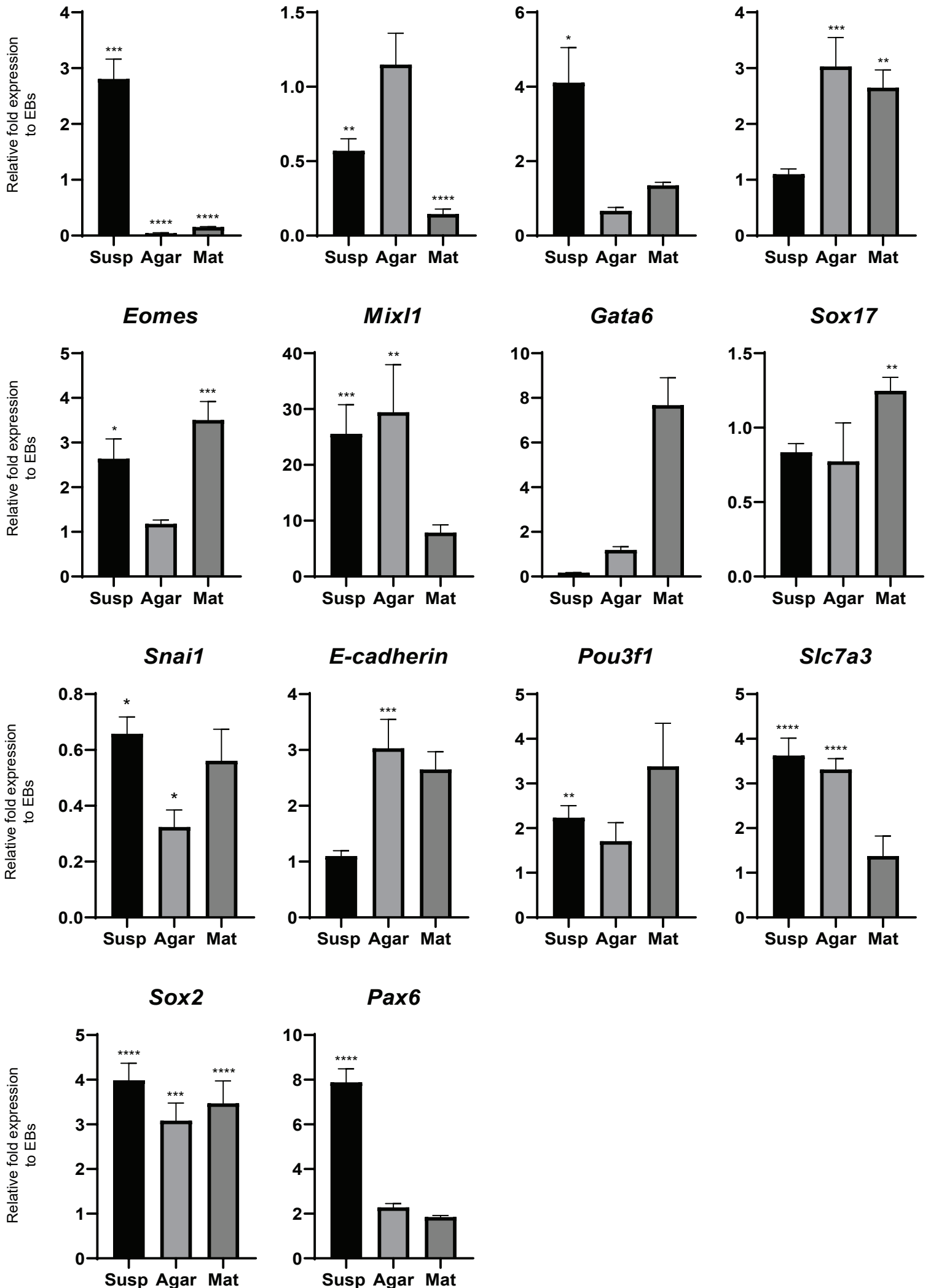


*Wnt3*

*β-catenin*

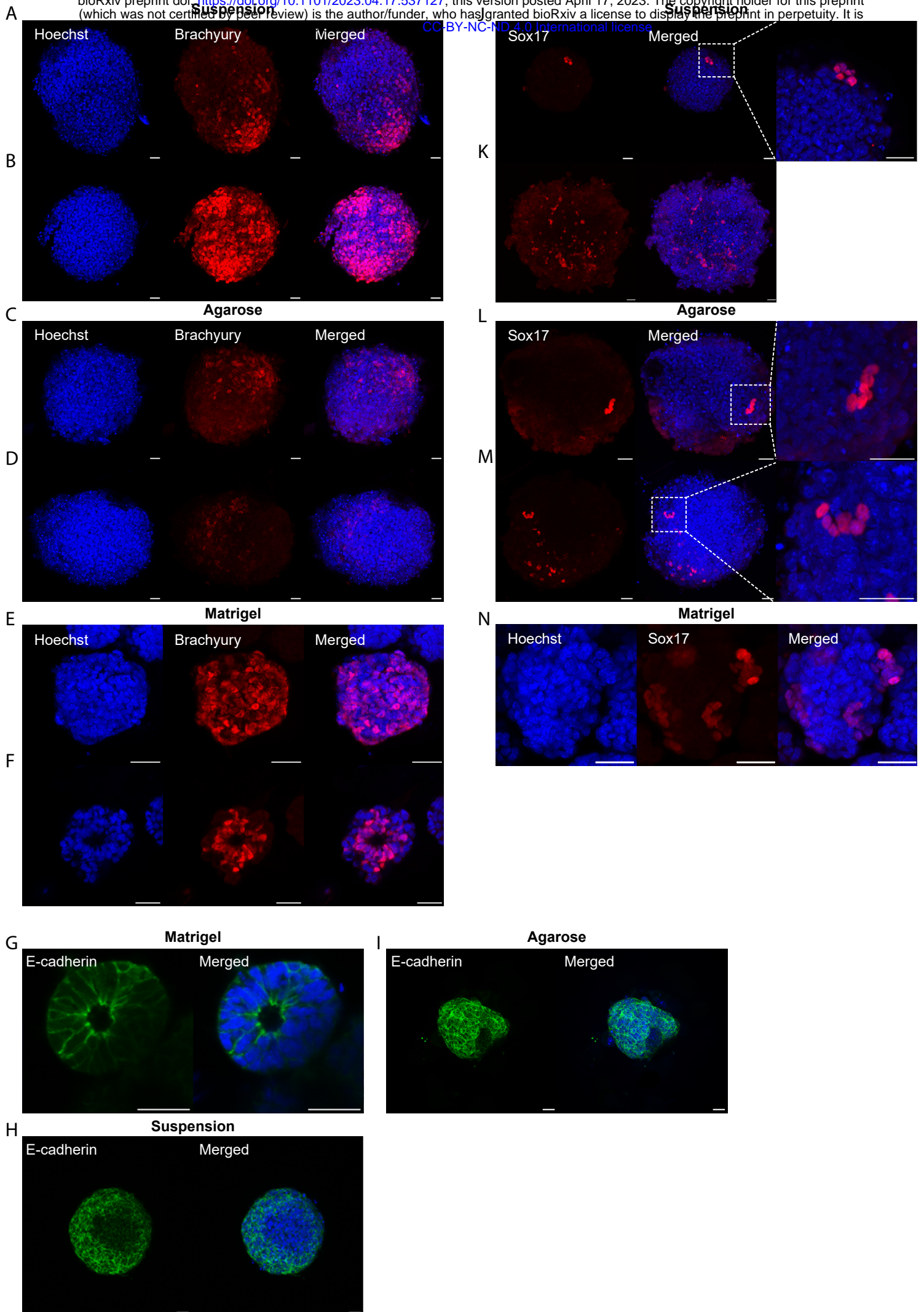
*Brachyury*

*Nodal*



**Fig. 2**





**Fig. 3**



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