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1 **Osmotic stress induces JNK-dependent embryo invasion in a model of implantation**

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18 **Abstract**

19 *In vitro* culture during assisted reproduction technologies (ART) exposes pre-implantation  
20 embryos to environmental stressors, such as non-physiological nutritional, oxidative and  
21 osmotic conditions. The effects on subsequent implantation are not well understood but  
22 could contribute to poor ART efficiency and outcomes. We have used exposure to  
23 hyperosmolarity to investigate the effects of stress on the ability of embryos to interact with  
24 endometrial cells in an *in vitro* model. Culturing mouse blastocysts for 2h in medium with  
25 osmolarity raised by 400mOsm induced blastocoel collapse and re-expansion, but did not  
26 affect subsequent attachment to, or invasion of, the endometrial epithelial Ishikawa cell line.  
27 Inhibition of stress-responsive c-Jun N-terminal kinase (JNK) activity with SP600125 did not  
28 affect the intercellular interactions between these embryos and the epithelial cells. Four  
29 successive cycles of hyperosmotic stress at E5.5 had no effect on attachment, but promoted  
30 embryonic breaching of the epithelial cell layer by trophoblast giant cells in a JNK-dependent  
31 manner. These findings suggest that acute stress at the blastocyst stage may promote  
32 trophoblast breaching of the endometrial epithelium at implantation, and implicates stress  
33 signalling through JNK in the process of trophoctoderm differentiation into the invasive  
34 trophoblast necessary for the establishment of pregnancy. The data may lead to increased  
35 understanding of factors governing ART success rates and safety.

36 **Introduction**

37 *In vitro* fertilisation (IVF) is widely used to treat infertility, however establishment of  
38 pregnancy after transfer of embryos generated *in vitro* remains a significant hurdle (Calhaz-  
39 Jorge, *et al.* 2017). Pregnancies arising from assisted reproductive technologies (ART) are  
40 associated with altered fetal growth, which continues into early childhood and may impact  
41 adult health (Ceelen *et al.* 2008, Hart & Norman 2013). Altered placental structure and  
42 function may underlie some of these effects (Haavaldsen *et al.* 2012, Feuer *et al.* 2014,  
43 Nelissen *et al.* 2014, Chen *et al.* 2015, Song *et al.* 2015). *In vitro* culture exposes embryos to

44 environmental conditions, including non-physiological nutritive, oxidative, osmotic,  
45 temperature, pH and light/electromagnetic conditions, as well as toxic stress and shear  
46 forces involved in handling (Xie *et al.* 2007a). Altered nutrient availability in the  
47 preimplantation period can affect blastocyst growth in rodents, with knock-on effects on  
48 offspring weight and developmental progression (Kwong *et al.* 2000), and even a brief period  
49 *in vitro* at the blastocyst stage can affect placental development and function (de Waal *et al.*  
50 2015). Conversely, some evidence suggests that exposure to sublethal stressors can  
51 improve developmental competence of gametes and embryos (Pribenszky *et al.* 2010).  
52 Effects of environmental stresses on intercellular interactions at implantation have not been  
53 examined, but a better understanding of this critical process has the potential to improve  
54 ART efficiency and efficacy.

55 Implantation involves attachment of the blastocyst trophoctoderm (TE) to endometrial  
56 epithelial cells (EEC), followed by trophoblast invasion into the underlying endometrial  
57 stroma and eventual access to the maternal vasculature (Aplin & Ruane 2017). Dissecting  
58 implantation requires *in vitro* models, and primary EEC and cell lines have been used to  
59 investigate the mechanisms of human and mouse embryo attachment (Weimar *et al.* 2013).  
60 Recent studies have characterised the effects of pharmacologic agents on human embryo  
61 attachment *in vitro* (Petersen *et al.* 2005, Lalitkumar *et al.* 2007, Lalitkumar *et al.* 2013,  
62 Boggavarapu *et al.* 2016), however studies of environmental impact on attachment have  
63 used only trophoblast cell spheroids as model embryos (Tsang *et al.* 2012, Tsang *et al.*  
64 2013). We recently described an *in vitro* model that allows the kinetics of mouse embryo-  
65 EEC attachment to be monitored and trophoblast breaching of EEC to be assessed; key  
66 parameters of the early stages of implantation which are difficult to investigate *in vivo*  
67 (Ruane *et al.* 2017).

68 Cells respond to environmental stressors by activating conserved signalling modules,  
69 including the mitogen-activated protein kinase (MAPK) superfamily member c-Jun N-terminal  
70 kinase (JNK). Active JNK phosphorylates transcription factors to coordinate the

71 transcriptional stress response, leading to the regulation of cell growth, survival and  
72 differentiation (Weston & Davis 2007). In mouse embryos, there is some evidence of a role  
73 for JNK in blastocyst formation from the 8-cell stage (Maekawa *et al.* 2005), and additional  
74 work has identified a role specific to preimplantation development in sub-optimal medium  
75 (Xie *et al.* 2006). Ultimately, stress signalling pathways are thought to impinge on cell growth  
76 to divert energy to homeostatic processes that support short-term survival, with increased  
77 extent or duration of signalling leading to senescence and apoptosis (Puscheck *et al.* 2015).  
78 Hyperosmolarity is a clinically relevant and experimentally tractable environmental variable  
79 that can be used to evaluate stress responses in mouse embryos and stem cells (Xie *et al.*  
80 2007b). Here we have characterised the effects of hyperosmotic stress on attachment and  
81 invasion parameters of mouse embryo implantation in an *in vitro* model.

## 82 **Materials and methods**

### 83 **Cell culture**

84 Ishikawa cells (ECACC 99040201) were cultured at 37°C, 95% air and 5% CO<sub>2</sub> in growth  
85 medium (1:1 Dulbecco's modified Eagle's medium:Ham's-F12 (Sigma, D5796 and N6658,  
86 respectively) containing 10% fetal bovine serum (Sigma) supplemented with 2mM L-  
87 glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin (Sigma)). Ishikawa cells between  
88 passage 6 and 25 were used for experiments. Cells were grown to confluency in 24-well  
89 plates (Greiner) on 13 mm glass coverslips coated with 2% Matrigel (Sigma).

### 90 **Mouse embryos**

91 Experiments were licensed under the authority of a UK Home Office project license (PPL  
92 70/07838), and were authorized by the Animal Welfare and Ethical Review Board of the  
93 University of Manchester, according to the Animal Act, 1986. CD1 mice were housed in the  
94 Biological Services Unit at the University of Manchester under standard environmental  
95 conditions of 12 h light and 12 h dark at 20–22°C and 40–60% humidity, with food and water

96 provided *ad libitum*. Eight-10-week old female CD1 mice (Charles River) were superovulated  
97 by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (Intervet), followed  
98 by 5 IU human chorionic gonadotrophin (Intervet) 46h later, then kept overnight with  $\leq 9$ -  
99 month-old CD1 stud males for mating. Midday the following day was designated E0.5.  
100 Embryos were gathered at E1.5 by flushing dissected oviducts with M2 medium (Millipore)  
101 containing 0.4% w/v BSA (Sigma). Embryo manipulation was performed using a Flexipet  
102 with 140 $\mu$ m (E1.5 embryos) and 300 $\mu$ m (E4.5-5.5 embryos) pipettes (Cook). E1.5 embryos  
103 were incubated for 72h in drops of KSOM medium (Millipore) containing 0.4% BSA under oil  
104 (Ovoil, Vitrolife) at 37°C, 95% air and 5% CO<sub>2</sub>. E4.5 blastocysts were chemically hatched in  
105 acid Tyrode's (pH 2.5) (Sigma) and washed in KSOM 0.4% w/v BSA.

#### 106 **Hyperosmolar treatment of blastocysts**

107 E4.5 blastocysts were treated with KSOM containing 0.4% w/v BSA and 400mM sorbitol  
108 (389 $\pm$ 2mOsm increase in osmolarity). E5.5 blastocysts were treated without prior co-  
109 culture, and were cultured in 1:1 DMEM:F12 supplemented with 2mM L-glutamine, 100  
110  $\mu$ g/ml streptomycin, 100 IU/ml penicillin and 0.4% w/v BSA to allow comparison with  
111 blastocysts co-cultured from E4.5. E5.5 blastocysts were treated with 1:1 DMEM:F12, 2mM  
112 L-glutamine, 100  $\mu$ g/ml streptomycin, 100 IU/ml penicillin and 0.4% w/v BSA, containing  
113 400mM sorbitol (382 $\pm$ 4mOsm increase in osmolarity). Repeated osmotic stress of  
114 blastocysts entailed four cycles of treatment, each of which consisted of a 30 minute  
115 incubation in hyperosmotic medium containing 400mM sorbitol followed by incubation in  
116 normosmotic medium for 30 minutes. Blastocysts were passed through 3 drops of treatment  
117 medium to prevent carry-over affecting osmolarity. Control blastocysts were treated as  
118 above but only normosmotic medium was used. All treatments were performed in drops  
119 under oil at 37°C, 95% air and 5% CO<sub>2</sub>. In some experiments, SP600125 (20 $\mu$ M; Sigma)  
120 was added to medium to inhibit JNK during treatments. Embryos were imaged throughout  
121 treatment using an inverted phase contrast microscope (Evos XL Core) and embryo  
122 diameter was measured using ImageJ software.

### 123 ***In vitro* implantation model**

124 The *in vitro* implantation model was employed as described previously (Ruane *et al.* 2017).  
125 Briefly, confluent Ishikawa cells in 24-well plates were incubated with co-culture medium (1:1  
126 DMEM:F12 containing 2 mM L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin)  
127 24h prior to co-culture with three hatched mouse blastocysts per well and incubation at  
128 37°C, 95% air and 5% CO<sub>2</sub>. Blastocyst attachment stability was recorded at 4h intervals from  
129 E5.5-6 and at E6.5, using an inverted phase contrast microscope. Attachment stability was  
130 graded upon gentle and continuous agitation of the plate, and observation of blastocyst  
131 movements (not attached) or oscillations (weakly, intermediately or stably attached). After  
132 co-culture, samples were washed in phosphate-buffered saline (PBS) and fixed with 4%  
133 paraformaldehyde in PBS for 20 minutes.

### 134 **Fluorescence staining and microscopy**

135 Fixed attached embryo samples were washed with PBS, quenched with 50mM ammonium  
136 chloride solution and permeabilised with 0.5% Triton-X100 PBS. Alexa568-phalloidin (Life  
137 Technologies) and 4',6-diamidino-2-phenylindole (DAPI) (Sigma), in PBS was added for 1h  
138 before mounting in a chamber of 3% 1,4-diazabicyclo[2.2.2]octane (Sigma) in PBS.  
139 Fluorescence microscopy was performed with a Zeiss Axiophot microscope equipped with  
140 an Apotome module for optical sectioning. Images were analysed and processed using  
141 Zeiss Zen software.

### 142 **Statistical Analysis**

143 2-way ANOVA analysis with Dunnetts post hoc test was performed using SPSS and used to  
144 demonstrate significant differences from  $p < 0.05$ .

145 **Results**

146 **Single-episode hyperosmotic treatment of mouse blastocysts does not affect**  
147 **implantation in an *in vitro* model.**

148 We challenged mouse embryos with hyperosmolar conditions for 2h prior to implantation in  
149 our *in vitro* model system. Full activation of JNK in E3.5 early blastocysts has been shown to  
150 occur between 30min and 2h exposure to hyperosmotic conditions (Xie *et al.* 2007b).  
151 Placing E4+10h blastocysts in medium containing 400mM sorbitol caused immediate  
152 blastocoel collapse (Figure 1A, B, diameter reduced by 17% to 74.7 $\mu$ m). Recovery to  
153 expanded blastocyst morphology was apparent at the end of the 2h treatment (E4.5) and  
154 subsequent replacement in normosmotic medium led to enhanced expansion (Figure 1B,  
155 diameter increased by 7% to 99.8  $\mu$ m). Blastocysts were then co-cultured with Ishikawa cells  
156 and their attachment was monitored from E5.5, at 24, 28, 32 and 48h co-culture, as stable  
157 attachment occurs over this period (Ruane *et al.* 2017). The kinetics of stable attachment  
158 were not changed by hyperosmotic treatment (Figure 2A).

159 We previously showed that blastocyst apposition to Ishikawa cells from E4.5-5.5 activates  
160 them to breach Ishikawa cell layers during the following 24h, which resembles progression  
161 from attachment to invasion at implantation (Ruane *et al.* 2017). This process was not  
162 affected by hyperosmotic treatment (Figure 2B, C).

163 Attachment kinetics do not differ between embryos beginning co-culture with Ishikawa cells  
164 at E4.5 and those beginning co-culture at E5.5 (Ruane *et al.* 2017). E5.5 blastocysts, not  
165 previously co-cultured, were exposed to hyperosmolar conditions to establish whether  
166 concurrence of such stressors with the onset of attachment to Ishikawa cells affects  
167 attachment and breaching. The profile of stable attachment over time was not affected by  
168 hyperosmotic treatment from E5+10h to E5.5 (Figure 2D). Additionally, the limited embryonic  
169 breaching seen when co-culture was initiated at E5.5 was not affected by hyperosmotic  
170 treatment (Figure 2E).



171 **Inhibition of JNK signalling during hyperosmotic treatment does not affect embryo**  
172 **attachment or breaching**

173 To establish whether stress-activated JNK signalling is required for *in vitro* implantation after  
174 exposure to stress, we exposed blastocysts to hyperosmotic conditions for 2h at E4.5 and  
175 E5.5 in the presence of JNK inhibitor SP600125 prior to co-culture with Ishikawa cells.  
176 SP600125 did not affect E4.5 blastocyst collapse and re-expansion in hyperosmotic medium  
177 (data not shown). Moreover, SP600125 treatment during normosmotic or hyperosmotic  
178 treatment did not affect blastocyst attachment or breaching (Figure 3A-D).

179 **Repeated osmotic stress at E5.5 promotes breaching of Ishikawa cells through JNK**  
180 **signalling**

181 The re-expansion of mouse blastocysts within 2h after initial collapse in hyperosmotic  
182 medium revealed a remarkable ability to acclimatise to hyperosmolarity (Figure 1B). We  
183 hypothesised that repeated changes in osmolarity may result in stress responses which  
184 impact upon attachment and breaching. We therefore moved blastocysts between  
185 normosmotic and hyperosmotic media at 30 minute periods over 4h in the absence or  
186 presence of SP600125. Alongside this treatment blastocysts were moved between  
187 normosmotic media to control for repeated manipulation. Blastocysts were therefore  
188 exposed to a total of 2h hyperosmolarity, mirroring single episode exposures, and 30min  
189 exposure has been shown to activate JNK (Xie *et al.* 2007b).

190 E4+8h and E5+8h blastocysts subjected to repeated osmotic stress exhibited initial collapse  
191 in hyperosmotic medium that did not recover within 30 minutes or during the subsequent 30  
192 minutes in normosmotic medium. This decrease in size was exceeded upon the second  
193 exposure to hyperosmolarity, however the embryos then recovered, in both hyperosmotic  
194 and normomostic media, to the size seen after the initial hyperosmotic treatment. This latter  
195 collapse and re-expansion was observed in the following two cycles of hyperosmotic and  
196 normosmotic treatment and was not affected by SP600125 (Figure 4A, data shown for E5.5

197 embryos only). The manipulation control blastocysts were significantly decreased in size  
198 after two cycles through normosmotic medium and maintained this size, which was similar to  
199 that of blastocysts after two cycles of hyperosmotic shock, during subsequent manipulations  
200 (Figure 4A).

201 E4+8h blastocysts cycled through hyperosmotic and normosmotic medium until E4.5  
202 collapsed and expanded similarly to E5+8h blastocysts, however there was no effect on  
203 subsequent embryo attachment and breaching on Ishikawa cells (data not shown). E5.5  
204 blastocysts exposed to repeated osmotic stress in the presence or absence of SP600125  
205 also exhibited undisturbed attachment to Ishikawa cells (Figure 4B), however embryos  
206 subjected to repeated osmotic stress went on to breach the Ishikawa cell layer at a  
207 significantly higher rate than the manipulation control embryos (Figure 4C). Notably, this  
208 effect was dependent on JNK signalling since the presence of SP600125 during repeated  
209 osmotic stress abolished the increase in breaching.

## 210 **Discussion**

211 Despite concerted efforts to optimise the *in vitro* culture environment, exposure to stressors  
212 is inevitable during IVF and embryo culture. Using hyperosmolarity as a well-defined  
213 experimental stressor with some clinical relevance, we show that single hyperosmotic events  
214 do not compromise mouse embryo attachment and invasion of Ishikawa cells *in vitro*.  
215 Repeated changes from normosmotic to hyperosmotic conditions also do not affect TE  
216 function at attachment, however embryonic breaching of Ishikawa cells is increased in a JNK  
217 signalling-dependent manner.

218 Trophoblast responses to stress are coupled to development and invasion in interstitially  
219 implanting species, with hypoxic and nutritive stress promoting proliferation and migration  
220 and thus driving implantation and embryonic survival through access to maternal nutrients  
221 (Rosario *et al.* 2008, Watkins *et al.* 2015). Nutritive stress restricted to the preimplantation  
222 stage *in vivo* leads to more invasive trophoblast, suggesting that stress during cleavage and

223 first lineage allocation stages can affect subsequent trophoblast development at  
224 implantation. Effects on epigenetic reprogramming may underlie these outcomes (Choux *et al.* 2015), though signalling through the nutrient-responsive mTOR complex partially  
225 mediates enhanced trophoblast formation from affected blastocysts (Eckert *et al.* 2012). To  
226 investigate the impact of stresses relevant to embryo culture and blastocyst transfer in ART  
227 on implantation, we used a defined stressor and a characterised *in vitro* model based on  
228 mouse embryos and the Ishikawa EEC line. This enabled the analysis of blastocyst  
229 attachment to EEC and trophoblast penetration of the EEC layer as key early steps in  
230 implantation (Ruane *et al.* 2017). To assess stress effects on human implantation in this  
231 model, careful powering would be required to account for variability of human embryo  
232 quality. Further work is also merited with primary human endometrial epithelial and stromal  
233 cells, especially in light of evidence that decidualised stromal cells respond differentially to  
234 embryos of high and low quality (Brosens *et al.* 2014). Though it is difficult to generate  
235 polarised primary human EEC layers *in vitro* (Campbell *et al.* 2000), recent developments in  
236 epithelial organoid culture promise sophisticated models of human implantation (Boretto *et al.* 2017, Turco *et al.* 2017).

239 *In vitro* studies using rodent embryos and TE-derived trophoblast stem cells (TSC) have  
240 investigated acute and chronic stress effects on differentiation to early extraembryonic  
241 lineages, especially trophoblast giant cells (TGC) (Puscheck *et al.* 2015). The activity of the  
242 stress-responsive protein kinases, JNK, p38 MAPK and adenosine monophosphate-  
243 activated protein kinase (AMPK), increases in embryos and TSC subjected to sub-optimal  
244 culture medium, shear stress, hyperosmolarity, hypoxia and microgravity (Wang *et al.* 2005,  
245 Xie *et al.* 2007a, Xie *et al.* 2007b, Wang *et al.* 2009, Xie *et al.* 2013). Moreover,  
246 hyperosmolarity leads to downregulation of the TE and TSC progenitor marker *Cdx2* and  
247 upregulation of trophoblast differentiation markers *Eomes*, *Hand1*, *Stra13* and *Prl3d1* in a  
248 JNK- and AMPK-dependent manner (Awonuga *et al.* 2011). These studies strongly evidence  
249 stress signalling as a mechanism for promoting trophoblast differentiation, and this has been

250 suggested as a response to the developmental hurdle of implantation when the embryo  
251 signals to establish maternal recognition of pregnancy and strives to secure resources for  
252 growth. Conversely, excessive stress-driven differentiation at the expense of TSC renewal  
253 has been proposed to negatively affect placental homeostasis and adaptation due to a  
254 reduction in the TSC pool (Puscheck *et al.* 2015). The data presented here provide the first  
255 evidence of preimplantation embryo exposure to an environmental stressor promoting  
256 subsequent invasion into maternal cells. In addition, we evidence JNK signalling as a  
257 mediator of stress-induced embryonic invasion at implantation because pharmacological  
258 inhibition of JNK signalling during embryo exposure to stress blocked the stimulation of  
259 trophoblast breaching of Ishikawa cell layers. We previously showed that blastocyst  
260 apposition to Ishikawa cells during E4.5-5.5 induced gene expression changes characteristic  
261 of TGC differentiation, and in turn TGC were seen to mediate breaching of the Ishikawa cell  
262 layer at E6.5 (Ruane *et al.* 2017). We therefore speculate that stress signalling through JNK  
263 in the TE at E5.5 induces the expression of genes underpinning TGC differentiation, such as  
264 *Hand1*.

265 The observation that breaching was promoted only by repeated osmotic stress may reflect a  
266 stress signalling threshold that must be reached to advance trophoblast differentiation faster  
267 than in unstressed conditions (Puscheck *et al.* 2015). The observation that blastocyst  
268 expansion was fully rescued 2h after initial collapse in hyperosmotic medium highlights the  
269 homeostatic resilience of pre-implantation mouse embryos and suggests that stress signals  
270 may have rapidly abated after initial exposure to hyperosmolarity. Our data suggest that a  
271 single episode of collapse and re-expansion may cause stress equivalent to simple embryo  
272 manipulation by pipetting (Xie *et al.* 2007a). Blastocysts undergo repeated cycles of partial  
273 collapse and re-expansion during normal development, likely due to transient losses of  
274 epithelial integrity during cytokinesis in the TE layer. Once the blastocoel fluid has  
275 equilibrated with the external environment and epithelial integrity has been restored,  
276 directional ion pumping rapidly restores osmotic pressure allowing the blastocyst to re-

277 expand (Biggers *et al.* 1988). Repeated osmotic stress caused repeated blastocyst collapse-  
278 expansion cycles, perhaps indicating prolonged or higher magnitude stress signalling which  
279 reached a threshold that led to altered gene expression. One possibility is that reduced  
280 actomyosin tension upon repeated blastocoel collapse acts upstream of JNK through Rho  
281 GTPases (Coso *et al.* 1995). Other mechanisms of osmotic sensing which persist through  
282 repeated osmotic stress, such as ion channel activity, may also act upon JNK (Furst *et al.*  
283 2002).

284 There is a lack of understanding regarding the role of stress signalling in human embryos,  
285 especially at the implantation stage, despite the exposure to sub-optimal environmental  
286 conditions that is inherent in *in vitro* culture. Environmental stressors have been shown to  
287 impact on human trophoblast development and function (Burton *et al.* 2009), and if TE  
288 differentiation to trophoblast at implantation is positively regulated by stress signalling in  
289 human as in mouse, it follows that stress invoked during ART procedures may not impede  
290 implantation. Whether this response is capable of rescuing a failing conceptus, with possible  
291 implications for fetal development and long-term health, or whether stress-affected embryos  
292 may implant only to fail at a later stage of pregnancy, will require investigation *in vivo*.

### 293 **Declaration of interest**

294 The authors declare no conflicts of interest

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#### 436 **Figure legends**

437 **Figure 1 A** Brightfield images of E4.5 blastocysts in normosmotic medium, and immediately  
438 after placement in hyperosmotic medium. Scale bars 50µm. **B** E4.5 blastocyst diameter was  
439 measured from brightfield images taken before incubation in treatment medium  
440 (normosmotic for control treatment and hyperosmotic for experimental treatment),  
441 immediately after the start of treatment, 2h after incubation in treatment medium, and  
442 immediately after transfer from treatment medium to normosmotic medium. 36 embryos  
443 were measured per treatment group over 3 independent experiments, data expressed as  
444 mean diameter +/- SEM. \* p<0.05, \*\* p<0.01 ANOVA compared to pre-treatment group.

445 **Figure 2 A** E4.5 blastocysts were incubated in normosmotic or hyperosmotic medium for 2h  
446 before co-culture with Ishikawa cells. Stability of embryo attachment to Ishikawa cells was  
447 monitored from E5.5 every 4h to E6, and at E6.5. A total of 64 blastocysts were used across  
448 3 independent experiments. Mean stable attachment was plotted +/-SEM. No significant  
449 differences in stable attachment were seen. **B** Attached embryos were fixed and labelled  
450 with actin (red) and nuclear (blue) stains (phalloidin and DAPI, respectively) before optical  
451 sectioning by fluorescence microscopy to reveal the embryo-Ishikawa interface. The top  
452 image shows an upper section of the embryo above the Ishikawa cell plane, while the  
453 bottom image shows breaching of the Ishikawa cell layer by embryonic cells (area within  
454 dotted line) in a lower section. Scale bars 20µm. **C** E4.5 normosmotic and hyperosmotic-  
455 exposed embryos that had attached to Ishikawa cells by E6.5 were analysed by  
456 fluorescence microscopy. Embryo breaching of the Ishikawa cell layer was scored for 52  
457 embryos from 3 independent experiments and the mean +/- SEM percentage of imaged  
458 embryos to have breached the Ishikawa cells was plotted. No significant difference in  
459 breaching was seen (NS). **D** E5.5 blastocysts were cultured in normosmotic or hyperosmotic  
460 medium for 2h before incubation with Ishikawa cells. Stability of attachment to Ishikawa cells  
461 was monitored every 4h to E6, and at E6.5. A total of 60 blastocysts were used in 3  
462 independent experiments. Mean +/- SEM stable attachment was plotted. No significant  
463 differences in stable attachment were observed. **E** E5.5 normosmotic and hyperosmotic-  
464 exposed embryos attached to Ishikawa cells at E7.5 were subjected to fluorescence  
465 microscopic analysis. Mean +/- SEM percentage of 30 imaged embryos from 3 independent  
466 experiments to have breached the Ishikawa cells was plotted. No significant difference in  
467 breaching was seen (NS).

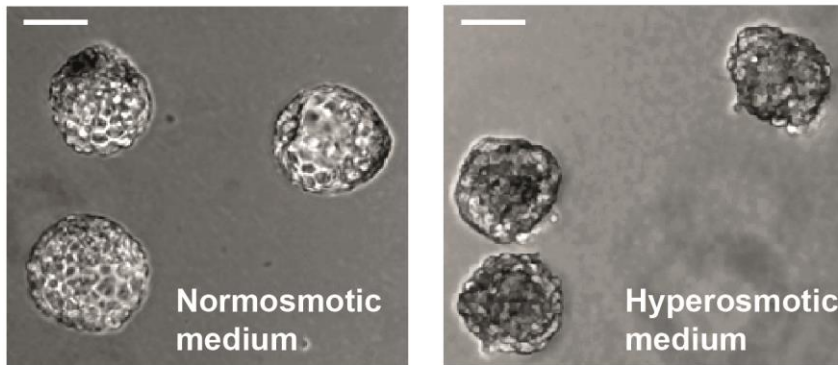
468 **Figure 3 A** E4.5 blastocysts were incubated in normosmotic or hyperosmotic medium for 2h  
469 in the presence or absence of 20µM JNK inhibitor SP600125 before co-culture with Ishikawa  
470 cells. Stability of embryo attachment was monitored from E5.5 to E6.5. A total of 134  
471 blastocysts were used in 3 separate experiments. Mean +/- SEM stable attachment was

472 plotted. No significant differences in stable attachment were detected. **B** Embryos from A  
473 that had attached to Ishikawa cells by E6.5 were stained with phalloidin and DAPI to detect  
474 embryos breaching the Ishikawa cell layer. Mean +/- SEM percentage of 134 imaged  
475 embryos from 3 independent experiments to have breached was plotted. No significant  
476 differences in breaching were observed (NS). **C** E5.5 blastocysts were cultured in  
477 normosmotic or hyperosmotic medium for 2h in the presence or absence of 20 $\mu$ M JNK  
478 inhibitor SP600125 before incubation with Ishikawa cells. Stability of attachment was  
479 monitored to E6.5. A total of 147 blastocysts were used in 3 independent experiments. Mean  
480 +/- SEM stable attachment was plotted and no significant differences were found. **D** E7.5  
481 attached embryos from C were labelled with phalloidin and DAPI and imaged to analyse  
482 embryo breaching. Mean +/- SEM percentage of 96 imaged embryos from 3 independent  
483 experiments to have breached the Ishikawa cells was plotted. No significant differences in  
484 breaching were seen (NS).

485 **Figure 4 A** E5+8h blastocysts were incubated in hyperosmotic medium for 30min followed  
486 immediately by incubation in normosmotic medium for 30min. This regimen was repeated  
487 four times in the presence or absence of SP600125. Manipulation control blastocysts were  
488 passed through normosmotic medium only. Brightfield images of embryos were taken after  
489 1min and 30min in each culture condition and embryo diameter measured. Mean +/-SEM  
490 diameter of 106 embryos over 3 independent experiments was plotted. **B** 104 E5.5  
491 blastocysts exposed to repeated osmotic stress in the presence or absence of SP600125, or  
492 control conditions, were co-cultured with Ishikawa cells from E5.5 and the stability of  
493 attachment monitored to E6.5 in 3 independent experiments. Mean +/- SEM stable  
494 attachment was plotted. No significant differences in stable attachment were observed (NS).  
495 **C** Embryos attached to Ishikawa cells by E7.5 from B were stained with phalloidin and DAPI  
496 to image the embryo-Ishikawa interface. Mean +/- SEM percentage of 62 imaged embryos  
497 from 3 independent experiments to have breached the Ishikawa cells was plotted. \*  $p < 0.05$   
498 ANOVA.

Figure 1

A



B

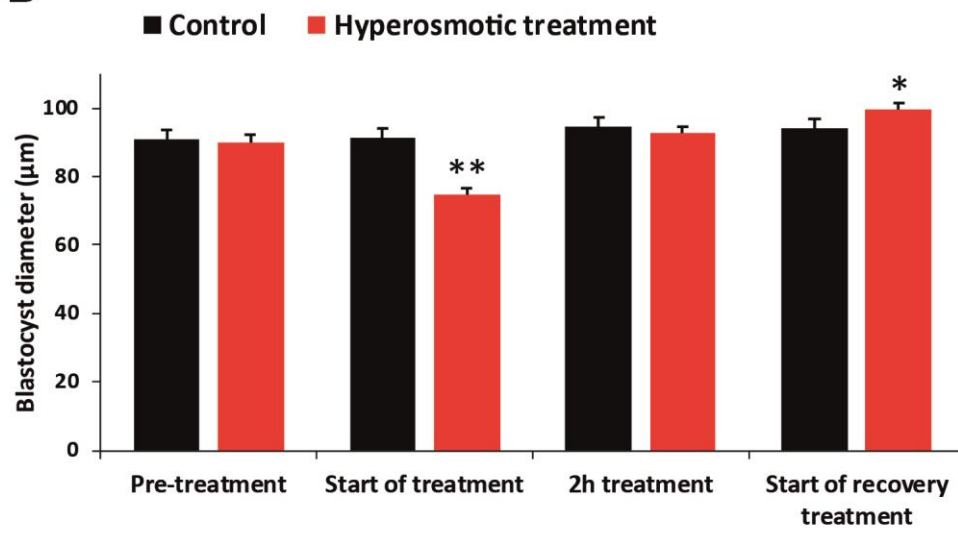
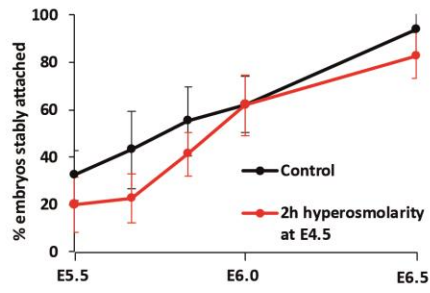
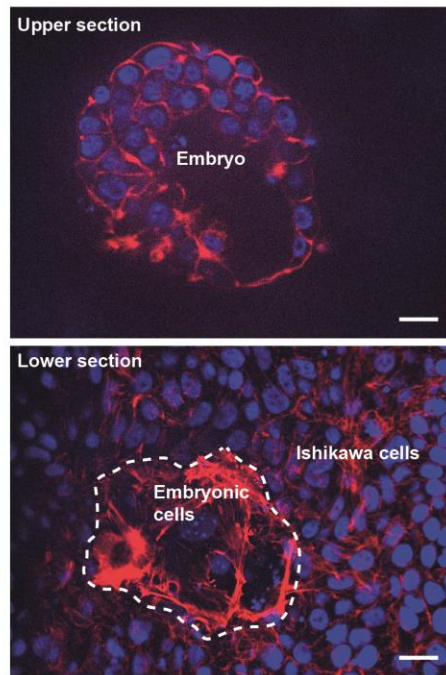


Figure 2

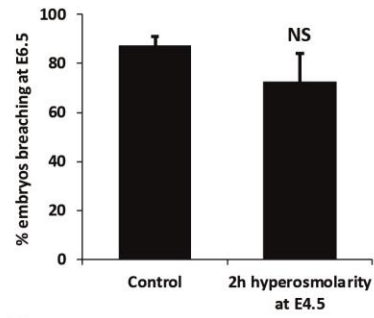
A



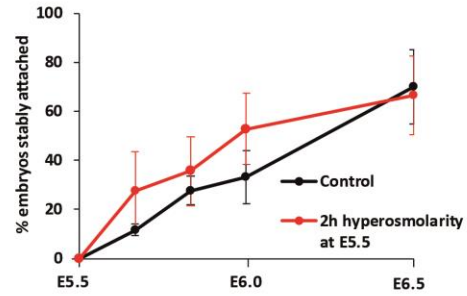
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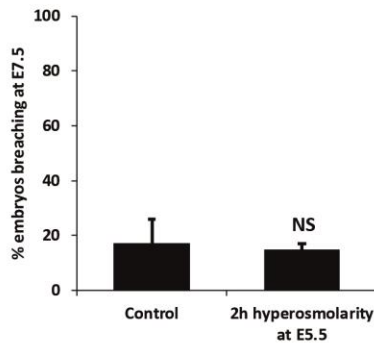
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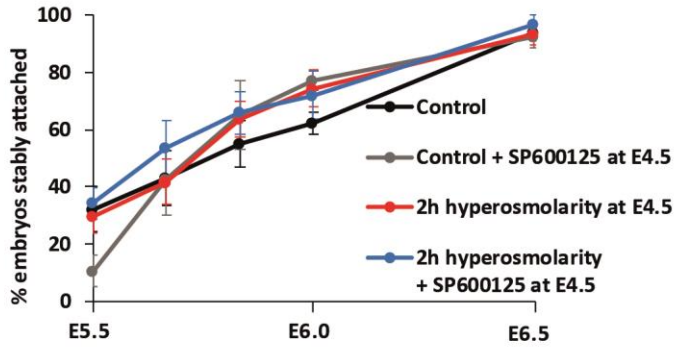


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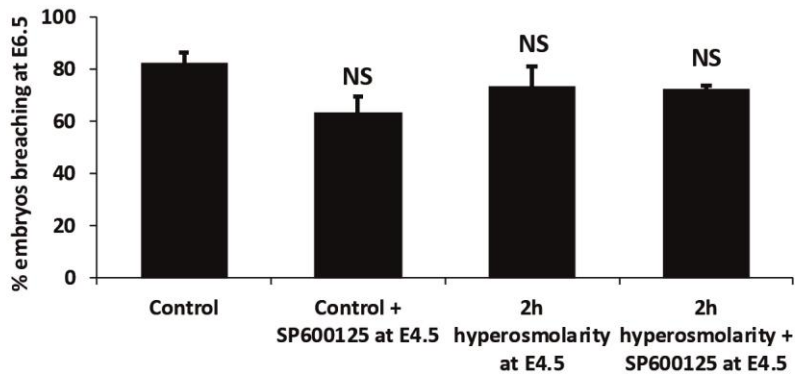


**Figure 3**

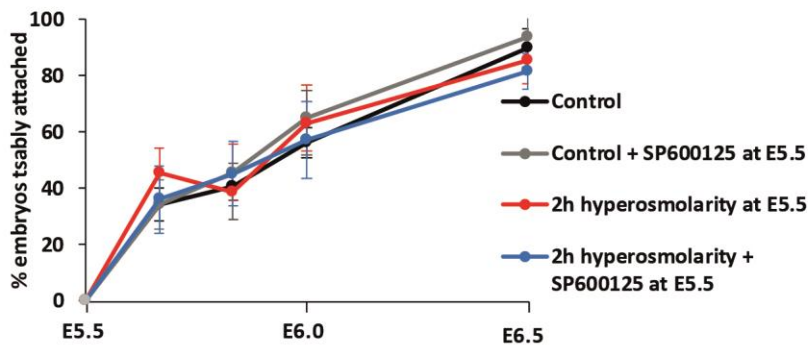
**A**



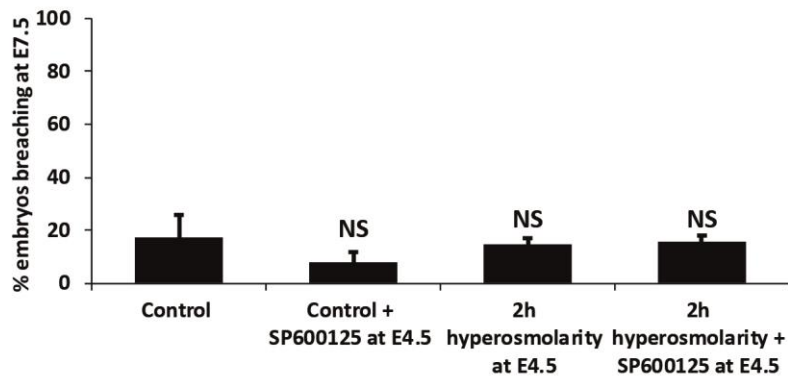
**B**



**C**



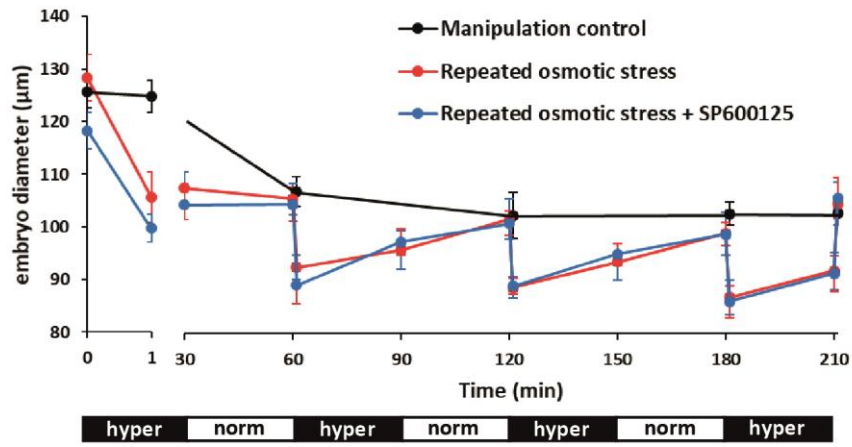
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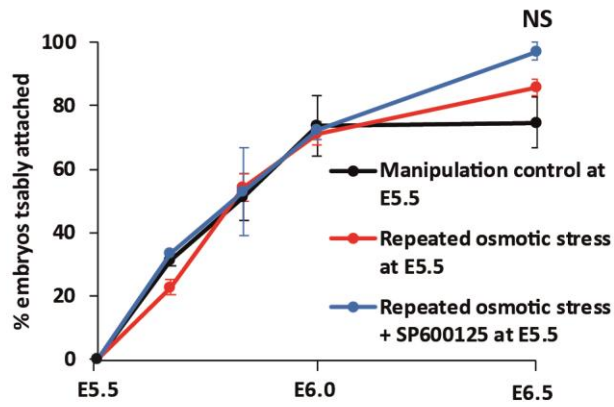


**Figure 4**

**A**



**B**



**C**

