MANCHESTER 1824
The University of Manchester

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

DOI:
10.1530/REP-18-0154

Document Version
Accepted author manuscript

Link to publication record in Manchester Research Explorer

Citation for published version (APA):
Ruane, P. T., Koeck, R., Berneau, S. C., Kimber, S. J., Westwood, M., Brison, D. R., & Aplin, J. D. (2018). Osmotic stress induces JNK-dependent embryo invasion in a model of implantation. *Reproduction*, *156*(5), 421-428. https://doi.org/10.1530/REP-18-0154

Published in:
Reproduction

Citing this paper
Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

General rights
Copyright and moral rights for the publications made accessible in the Research Explorer are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Takedown policy
If you believe that this document breaches copyright please refer to the University of Manchester's Takedown Procedures [http://man.ac.uk/04Y6Bo] or contact uml.scholarlycommunications@manchester.ac.uk providing relevant details, so we can investigate your claim.

Download date:14. May. 2020

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

Peter T Ruane [1, 2 #], Rebekka Koeck [1, 2], Stéphane C Berneau[1, 2], Susan J Kimber [3], Melissa Westwood[1, 2], Daniel R Brison[1, 2, 4] and John D Aplin[1, 2]

[1]Maternal and Fetal Health Research Centre, Division of Developmental Biology and Medicine, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Sciences Centre, St. Mary's Hospital, Manchester, M13 9WL.

[2]Maternal and Fetal Health Research Centre, St. Mary's Hospital, Manchester University NHS Foundation Trust, Manchester Academic Health Sciences Centre, Manchester, M13 9WL.

[3]Division of Cell Matrix Biology and Regenerative Medicine, School of Biological Sciences, Faculty of Biology Medicine and Health, University of Manchester, Michael Smith Building, Manchester, M13 9PT.

[4]Department of Reproductive Medicine, Old St Mary's Hospital, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Oxford Road, Manchester M13 9WL.

[#] Corresponding author peter.ruane@manchester.ac.uk

Abstract

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

Introduction

In vitro fertilisation (IVF) is widely used to treat infertility, however establishment of pregnancy after transfer of embryos generated *in vitro* remains a significant hurdle (Calhaz-Jorge, *et al.* 2017). Pregnancies arising from assisted reproductive technologies (ART) are associated with altered fetal growth, which continues into early childhood and may impact adult health (Ceelen *et al.* 2008, Hart & Norman 2013). Altered placental structure and function may underlie some of these effects (Haavaldsen *et al.* 2012, Feuer *et al.* 2014, 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 trophectoderm (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 37ºC, 95% air and 5% CO_2 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 ≤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 140μm (E1.5 embryos) and 300μm (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 37ºC, 95% air and 5% CO_2. 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+/-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 μg/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 μg/ml streptomycin, 100 IU/ml penicillin and 0.4% w/v BSA, containing
113 400mM sorbitol (382+/-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 37ºC, 95% air and 5% CO_2. In some experiments, SP600125 (20μM; 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 37ºC, 95% air and 5% CO_2. 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 74.7μm). 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 μm). 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*
225 *al.* 2015), though signalling through the nutrient-responsive mTOR complex partially
226 mediates enhanced trophoblast formation from affected blastocysts (Eckert *et al.* 2012). To
227 investigate the impact of stresses relevant to embryo culture and blastocyst transfer in ART
228 on implantation, we used a defined stressor and a characterised *in vitro* model based on
229 mouse embryos and the Ishikawa EEC line. This enabled the analysis of blastocyst
230 attachment to EEC and trophoblast penetration of the EEC layer as key early steps in
231 implantation (Ruane *et al.* 2017). To assess stress effects on human implantation in this
232 model, careful powering would be required to account for variability of human embryo
233 quality. Further work is also merited with primary human endometrial epithelial and stromal
234 cells, especially in light of evidence that decidualised stromal cells respond differentially to
235 embryos of high and low quality (Brosens *et al.* 2014). Though it is difficult to generate
236 polarised primary human EEC layers *in vitro* (Campbell *et al.* 2000), recent developments in
237 epithelial organoid culture promise sophisticated models of human implantation (Boretto *et*
238 *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

295 **Funding**

296 This study was supported by funds from the charities Wellbeing of Women (RG1442) and
297 Diabetes UK (15/0005207), and studentship support for SCB from the Anatomical Society.

298 **Acknowledgments**

299 We acknowledge staff at the Biological Services Unit, University of Manchester, for their
300 support with animal care.

301 **References**

302 **Aplin JD & Ruane PT** 2017. Embryo-epithelium interactions during implantation at a glance.
303 J Cell Sci **130** 15-22.

304 **Awonuga AO, Zhong W, Abdallah ME, Slater JA, Zhou SC, Xie YF, Puscheck EE &**
305 **Rappolee DA** 2011. Eomesodermin, HAND1, and CSH1 proteins are induced by cellular
306 stress in a stress-activated protein kinase-dependent manner. *Mol Reprod Dev* **78** 519-528.

307 **Bell CE, Lariviere NM, Watson PH & Watson AJ** 2009. Mitogen-activated protein kinase
308 (MAPK) pathways mediate embryonic responses to culture medium osmolarity by regulating
309 Aquaporin 3 and 9 expression and localization, as well as embryonic apoptosis. *Hum*
310 *Reprod* **24** 1373-1386.

311 **Biggers JD, Bell JE & Benos DJ** 1988. Mammalian blastocyst: transport functions in a
312 developing epithelium. *Am J Physiol* **255** C419-432.

313 **Boggavarapu NR, Berger C, von Grothusen C, Menezes J, Gemzell-Danielsson K &**
314 **Lalitkumar PG** 2016. Effects of low doses of mifepristone on human embryo implantation
315 process in a three-dimensional human endometrial *in vitro* co-culture system. *Contraception*
316 **94** 143-51

317 **Boretto M, Cox B, Noben M, Hendriks N, Fassbender A, Roose H, Amant F,**
318 **Timmerman D, Tomassetti C, Vanhie A, *et al.*** 2017. Development of organoids from
319 mouse and human endometrium showing endometrial epithelium physiology and long-term
320 expandability. *Development* **144** 1775-1786.

321 **Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, Steel JH,**
322 **Christian M, Chan YW, Boomsma CM, *et al.*** 2014. Uterine selection of human embryos at
323 implantation. *Sci Rep* **4** 3894.

324 **Burton GJ, Jauniaux E & Charnock-Jones DS** 2009. The influence of the intrauterine
325 environment on human placental development. *Int J Dev Biol* **54** 303-312.

326 **Calhaz-Jorge C, De Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, Motrenko T,**
327 **Scaravelli G, Wyns C & Goossens V** 2017. Assisted reproductive technology in Europe,
328 2013: results generated from European registers by ESHRE. *Hum Reprod* **32** 1957-1973.

329 **Campbell S, Larsen J, Seif MW, Allen TD, Knox F, Jones CJ & Aplin JD** 2000. Mosaic
330 characteristics of human endometrial epithelium *in vitro*: analysis of secretory markers and
331 cell surface ultrastructure. *Mol Hum Reprod* **6** 41-49.

332 **Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE & Delemarre-van de**
333 **Waal HA** 2008. Cardiometabolic differences in children born after *in vitro* fertilization: follow-
334 up study. *J Clin Endocrinol Metab* **93** 1682-1688.

335 **Chen S, Sun FZ, Huang X, Wang X, Tang N, Zhu B & Li B** 2015. Assisted reproduction
336 causes placental maldevelopment and dysfunction linked to reduced fetal weight in mice. *Sci*
337 *Rep* **5** 10596.

338 **Choux C, Carmignac V, Bruno C, Sagot P, Vaiman D & Fauque P** 2015. The placenta:
339 phenotypic and epigenetic modifications induced by Assisted Reproductive Technologies
340 throughout pregnancy. *Clin Epigenetics* **7** 87.

341 **Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T & Gutkind JS** 1995.
342 The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK
343 signaling pathway. *Cell* **81** 1137-1146.

344 **de Waal E, Vrooman LA, Fischer E, Ord T, Mainigi MA, Coutifaris C, Schultz RM &**
345 **Bartolomei MS** 2015. The cumulative effect of assisted reproduction procedures on
346 placental development and epigenetic perturbations in a mouse model. *Hum Mol Genet* **24**
347 6975-6985.

348 **Eckert JJ, Porter R, Watkins AJ, Burt E, Brooks S, Leese HJ, Humpherson PG,**
349 **Cameron IT & Fleming TP** 2012. Metabolic induction and early responses of mouse
350 blastocyst developmental programming following maternal low protein diet affecting life-long
351 health. *PLoS One* **7** e52791.

352 **Feuer SK, Liu X, Donjacour A, Lin W, Simbulan RK, Giritharan G, Piane LD, Kolahi K,**
353 **Ameri K, Maltepe E *et al.*** 2014. Use of a mouse *in vitro* fertilization model to understand the
354 developmental origins of health and disease hypothesis. *Endocrinology* **155** 1956-1969.

355 **Furst J, Gschwentner M, Ritter M, Botta G, Jakab M, Mayer M, Garavaglia L, Bazzini C,**
356 **Rodighiero S, Meyer G, *et al.*** 2002. Molecular and functional aspects of anionic channels
357 activated during regulatory volume decrease in mammalian cells. *Pflugers Arch* **444** 1-25.

358 **Haavaldsen C, Tanbo T & Eskild A** 2012. Placental weight in singleton pregnancies with
359 and without assisted reproductive technology: a population study of 536,567 pregnancies.
360 *Hum Reprod* **27** 576-582.

361 **Hart R & Norman RJ** 2013. The longer-term health outcomes for children born as a result of
362 IVF treatment: Part I--General health outcomes. *Hum Reprod Update* **19** 232-243.

363 **Kwong WY, Wild AE, Roberts P, Willis AC & Fleming TP** 2000. Maternal undernutrition
364 during the preimplantation period of rat development causes blastocyst abnormalities and
365 programming of postnatal hypertension. *Development* **127** 4195-4202.

366 **Lalitkumar PG, Lalitkumar S, Meng CX, Stavreus-Evers A, Hambiliki F, Bentin-Ley U &**
367 **Gemzell-Danielsson K** 2007. Mifepristone, but not levonorgestrel, inhibits human blastocyst
368 attachment to an *in vitro* endometrial three-dimensional cell culture model. *Hum Reprod* **22**
369 3031-3037.

370 **Lalitkumar S, Boggavarapu NR, Menezes J, Dimitriadis E, Zhang JG, Nicola NA,**
371 **Gemzell-Danielsson K & Lalitkumar LP** 2013. Polyethylene glycated leukemia inhibitory

372 factor antagonist inhibits human blastocyst implantation and triggers apoptosis by down-
373 regulating embryonic AKT. *Fertil Steril* **100** 1160-1169.

374 **Maekawa M, Yamamoto T, Tanoue T, Yuasa Y, Chisaka O & Nishida E** 2005.
375 Requirement of the MAP kinase signaling pathways for mouse preimplantation development.
376 *Development* **132** 1773-1783.

377 **Nelissen EC, Dumoulin JC, Busato F, Ponger L, Eijssen LM, Evers JL, Tost J & van**
378 **Montfoort AP** 2014. Altered gene expression in human placentas after IVF/ICSI. *Hum*
379 *Reprod* **29** 2821-2831.

380 **Petersen A, Bentin-Ley U, Ravn V, Qvortrup K, Sorensen S, Islin H, Sjogren A,**
381 **Mosselmann S & Hamberger L** 2005. The antiprogesterone Org 31710 inhibits human
382 blastocyst-endometrial interactions *in vitro*. *Fertil Steril* **83** Suppl 1 1255-1263.

383 **Pribenszky C, Vajta G, Molnar M, Du Y, Lin L, Bolund L & Yovich J** 2010. Stress for
384 stress tolerance? A fundamentally new approach in mammalian embryology. *Biol Reprod* **83**
385 690-697.

386 **Puscheck EE, Awonuga AO, Yang Y, Jiang Z & Rappolee DA** 2015. Molecular biology of
387 the stress response in the early embryo and its stem cells. *Adv Exp Med Biol* **843** 77-128.

388 **Rosario GX, Konno T & Soares MJ** 2008. Maternal hypoxia activates endovascular
389 trophoblast cell invasion. *Dev Biol* **314** 362-375.

390 **Ruane PT, Berneau SC, Koeck R, Watts J, Kimber SJ, Brison DR, Westwood M & Aplin**
391 **JD** 2017. Apposition to endometrial epithelial cells activates mouse blastocysts for
392 implantation. *Mol Hum Reprod* **23** 617-627.

393 **Song S, Ghosh J, Mainigi M, Turan N, Weinerman R, Truongcao M, Coutifaris C &**
394 **Sapienza C** 2015. DNA methylation differences between *in vitro*- and *in vivo*-conceived
395 children are associated with ART procedures rather than infertility. *Clin Epigenetics* **7** 41.

396 **Tsang H, Cheung TY, Kodithuwakku SP, Chai J, Yeung WS, Wong CK & Lee KF** 2012.
397 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) suppresses spheroids attachment on
398 endometrial epithelial cells through the down-regulation of the Wnt-signaling pathway.
399 *Reprod Toxicol* **33** 60-66.

400 **Tsang H, Cheung TY, Kodithuwakku SP, Chai J, Yeung WS, Wong CK & Lee KF** 2013.
401 Perfluorooctanoate suppresses spheroid attachment on endometrial epithelial cells through
402 peroxisome proliferator-activated receptor alpha and down-regulation of Wnt signaling.
403 *Reprod Toxicol* **42** 164-171.

404 **Turco MY, Gardner L, Hughes J, Cindrova-Davies T, Gomez MJ, Farrell L, Hollinshead**
405 **M, Marsh SGE, Brosens JJ, Critchley HO, *et al.*** 2017. Long-term, hormone-responsive
406 organoid cultures of human endometrium in a chemically defined medium. *Nat Cell Biol* **19**
407 568-577.

408 **Wang Y, Puscheck EE, Lewis JJ, Trostinskaia AB, Wang F & Rappolee DA** 2005.
409 Increases in phosphorylation of SAPK/JNK and p38MAPK correlate negatively with mouse
410 embryo development after culture in different media. *Fertil Steril* **83** Suppl 1 1144-1154.

411 **Wang Y, Xie Y, Wygle D, Shen HH, Puscheck EE & Rappolee DA** 2009. A major effect of
412 simulated microgravity on several stages of preimplantation mouse development is lethality
413 associated with elevated phosphorylated SAPK/JNK. *Reprod Sci* **16** 947-959.

414 **Watkins AJ, Lucas ES, Marfy-Smith S, Bates N, Kimber SJ & Fleming TP** 2015.
415 Maternal nutrition modifies trophoblast giant cell phenotype and fetal growth in mice.
416 *Reproduction* **149** 563-575.

417 **Weimar CH, Post Uiterweer ED, Teklenburg G, Heijnen CJ & Macklon NS** 2013. In-vitro
418 model systems for the study of human embryo-endometrium interactions. *Reprod Biomed*
419 *Online* **27** 461-476.

420 **Weston CR & Davis RJ** 2007. The JNK signal transduction pathway. *Curr Opin Cell Biol* **19**
421 142-149.

422 **Xie Y, Awonuga A, Liu J, Rings E, Puscheck EE & Rappolee DA** 2013. Stress induces
423 AMPK-dependent loss of potency factors Id2 and Cdx2 in early embryos and stem cells
424 [corrected]. *Stem Cells Dev* **22** 1564-1575.

425 **Xie Y, Awonuga AO, Zhou S, Puscheck EE & Rappolee DA** 2011. Interpreting the stress
426 response of early mammalian embryos and their stem cells. *Int Rev Cell Mol Biol* **287** 43-95.

427 **Xie Y, Puscheck EE & Rappolee DA** 2006. Effects of SAPK/JNK inhibitors on
428 preimplantation mouse embryo development are influenced greatly by the amount of stress
429 induced by the media. *Mol Hum Reprod* **12** 217-224.

430 **Xie Y, Wang F, Puscheck EE & Rappolee DA** 2007a. Pipetting causes shear stress and
431 elevation of phosphorylated stress-activated protein kinase/jun kinase in preimplantation
432 embryos. *Mol Reprod Dev* **74** 1287-1294.

433 **Xie Y, Zhong W, Wang Y, Trostinskaia A, Wang F, Puscheck EE & Rappolee DA** 2007b.
434 Using hyperosmolar stress to measure biologic and stress-activated protein kinase
435 responses in preimplantation embryos. *Mol Hum Reprod* **13** 473-481.

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).

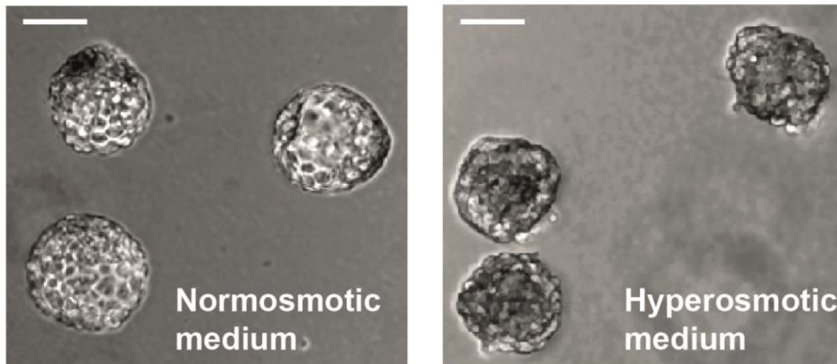
468 **Figure 3 A** E4.5 blastocysts were incubated in normosmotic or hyperosmotic medium for 2h
469 in the presence or absence of 20μM JNK inhibitor SP600125 before co-culture with Ishikawa
470 cells. Stability of embryo attachment was monitored from E5.5 to E6.5. A total of 134
471 blastocysts were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

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

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

Figure 1

A



B

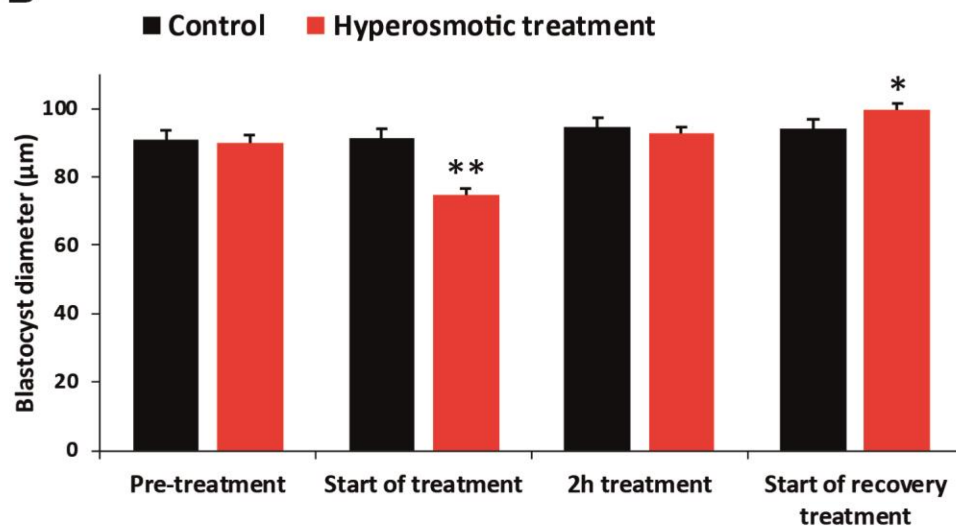
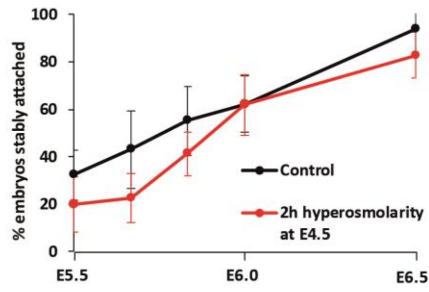
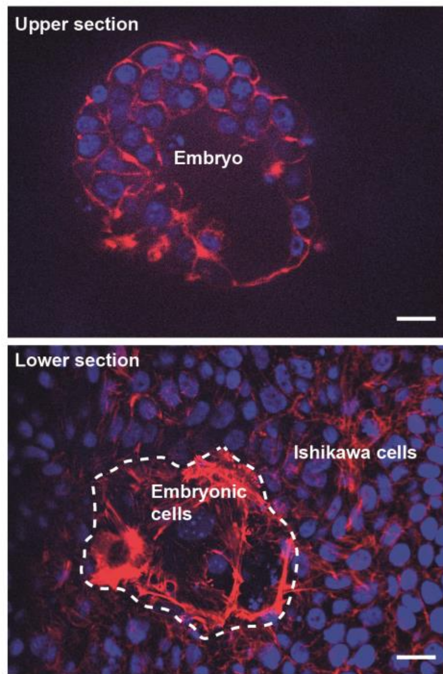


Figure 2

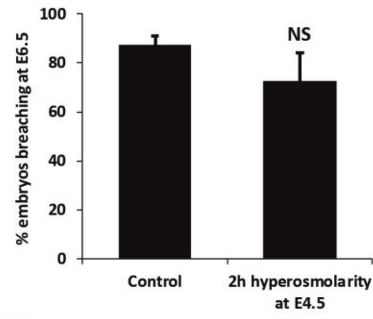
A



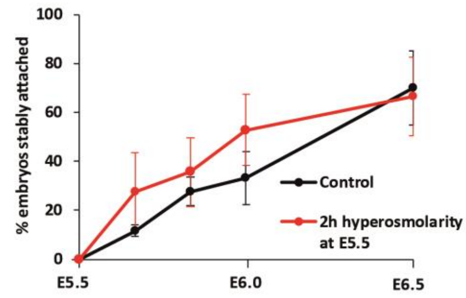
B



C



D



E

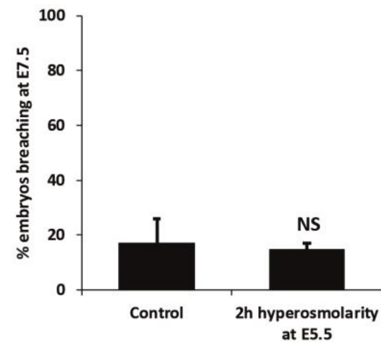
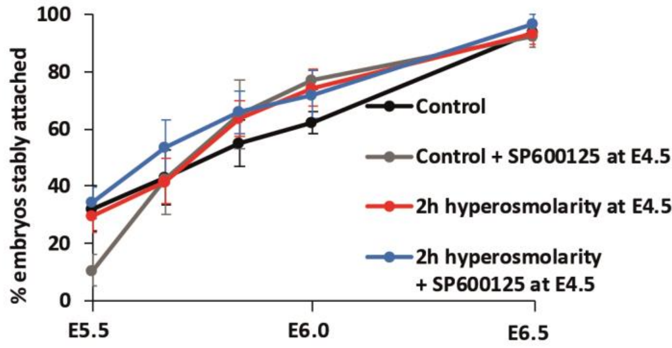
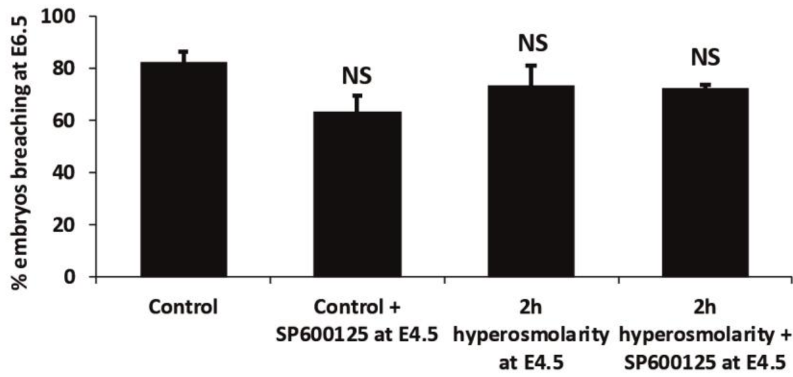


Figure 3

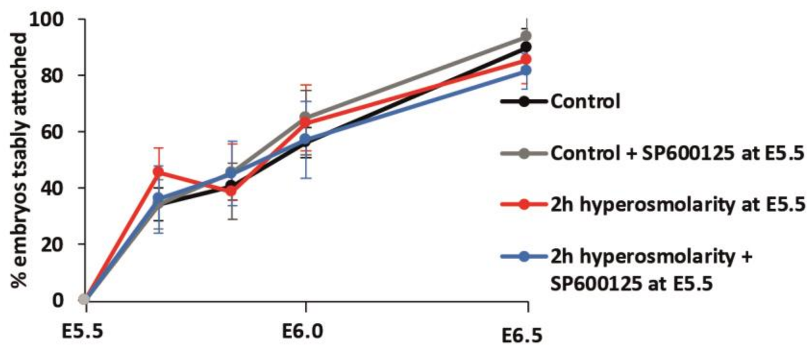
A



B



C



D

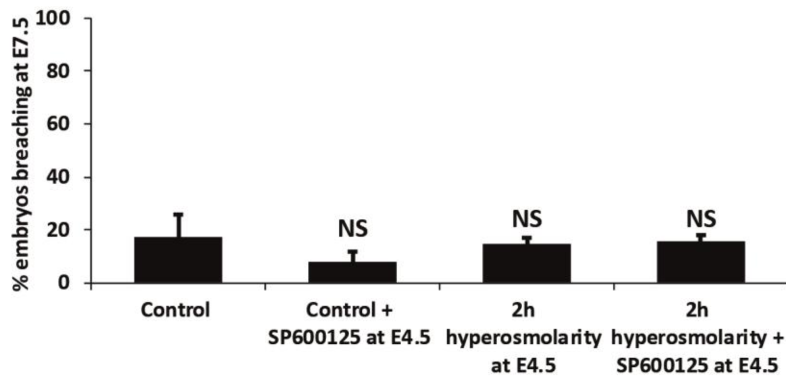
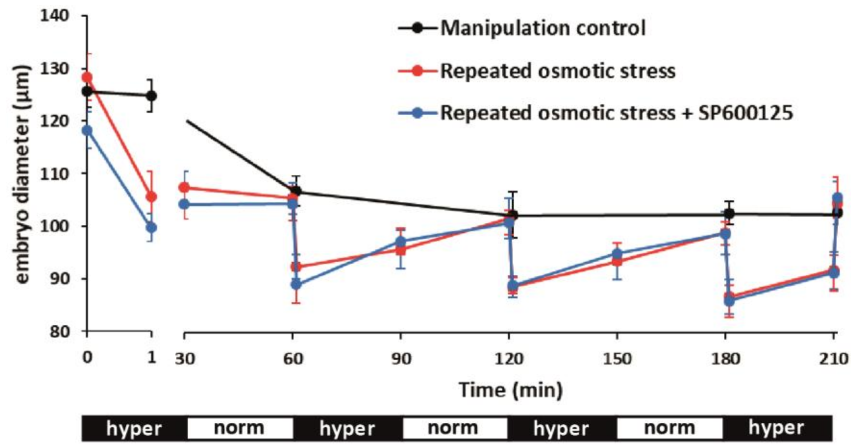
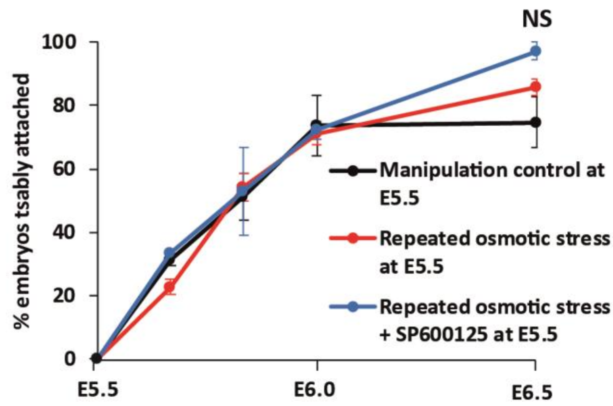


Figure 4

A



B



C