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

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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 trophoctoderm 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

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

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

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

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

Materials and methods

Cell culture

Ishikawa cells (ECACC 99040201) were cultured at 37°C, 95% air and 5% CO₂ in growth medium (1:1 Dulbecco's modified Eagle's medium:Ham's-F12 (Sigma, D5796 and N6658, respectively) containing 10% fetal bovine serum (Sigma) supplemented with 2mM L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin (Sigma)). Ishikawa cells between passage 6 and 25 were used for experiments. Cells were grown to confluency in 24-well plates (Greiner) on 13 mm glass coverslips coated with 2% Matrigel (Sigma).

Mouse embryos

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

provided *ad libitum*. Eight-10-week old female CD1 mice (Charles River) were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (Intervet), followed by 5 IU human chorionic gonadotrophin (Intervet) 46h later, then kept overnight with ≤ 9 -month-old CD1 stud males for mating. Midday the following day was designated E0.5. Embryos were gathered at E1.5 by flushing dissected oviducts with M2 medium (Millipore) containing 0.4% w/v BSA (Sigma). Embryo manipulation was performed using a Flexipet with 140 μ m (E1.5 embryos) and 300 μ m (E4.5-5.5 embryos) pipettes (Cook). E1.5 embryos were incubated for 72h in drops of KSOM medium (Millipore) containing 0.4% BSA under oil (Ovoil, Vitrolife) at 37°C, 95% air and 5% CO₂. E4.5 blastocysts were chemically hatched in acid Tyrode's (pH 2.5) (Sigma) and washed in KSOM 0.4% w/v BSA.

Hyperosmolar treatment of blastocysts

E4.5 blastocysts were treated with KSOM containing 0.4% w/v BSA and 400mM sorbitol (389 \pm 2mOsm increase in osmolarity). E5.5 blastocysts were treated without prior co-culture, and were cultured in 1:1 DMEM:F12 supplemented with 2mM L-glutamine, 100 μ g/ml streptomycin, 100 IU/ml penicillin and 0.4% w/v BSA to allow comparison with blastocysts co-cultured from E4.5. E5.5 blastocysts were treated with 1:1 DMEM:F12, 2mM L-glutamine, 100 μ g/ml streptomycin, 100 IU/ml penicillin and 0.4% w/v BSA, containing 400mM sorbitol (382 \pm 4mOsm increase in osmolarity). Repeated osmotic stress of blastocysts entailed four cycles of treatment, each of which consisted of a 30 minute incubation in hyperosmotic medium containing 400mM sorbitol followed by incubation in normosmotic medium for 30 minutes. Blastocysts were passed through 3 drops of treatment medium to prevent carry-over affecting osmolarity. Control blastocysts were treated as above but only normosmotic medium was used. All treatments were performed in drops under oil at 37°C, 95% air and 5% CO₂. In some experiments, SP600125 (20 μ M; Sigma) was added to medium to inhibit JNK during treatments. Embryos were imaged throughout treatment using an inverted phase contrast microscope (Evos XL Core) and embryo diameter was measured using ImageJ software.

***In vitro* implantation model**

The *in vitro* implantation model was employed as described previously (Ruane *et al.* 2017). Briefly, confluent Ishikawa cells in 24-well plates were incubated with co-culture medium (1:1 DMEM:F12 containing 2 mM L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin) 24h prior to co-culture with three hatched mouse blastocysts per well and incubation at 37°C, 95% air and 5% CO₂. Blastocyst attachment stability was recorded at 4h intervals from E5.5-6 and at E6.5, using an inverted phase contrast microscope. Attachment stability was graded upon gentle and continuous agitation of the plate, and observation of blastocyst movements (not attached) or oscillations (weakly, intermediately or stably attached). After co-culture, samples were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 minutes.

Fluorescence staining and microscopy

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

Statistical Analysis

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

Results

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

Declaration of interest

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.

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

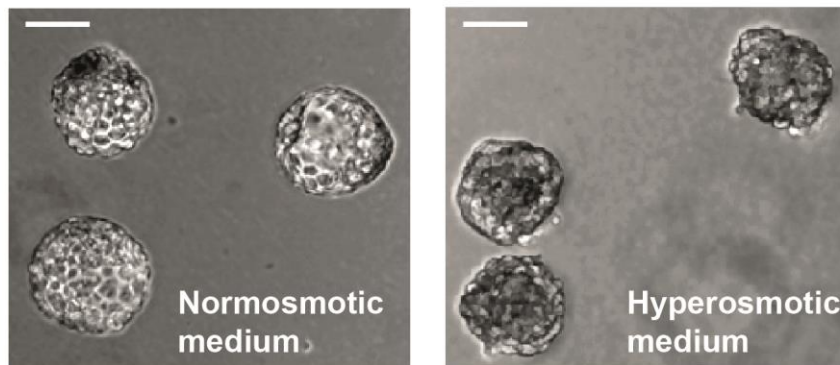
Figure 3 A E4.5 blastocysts were incubated in normosmotic or hyperosmotic medium for 2h in the presence or absence of 20µM JNK inhibitor SP600125 before co-culture with Ishikawa cells. Stability of embryo attachment was monitored from E5.5 to E6.5. A total of 134 blastocysts were used in 3 separate experiments. 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 20 μ M 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

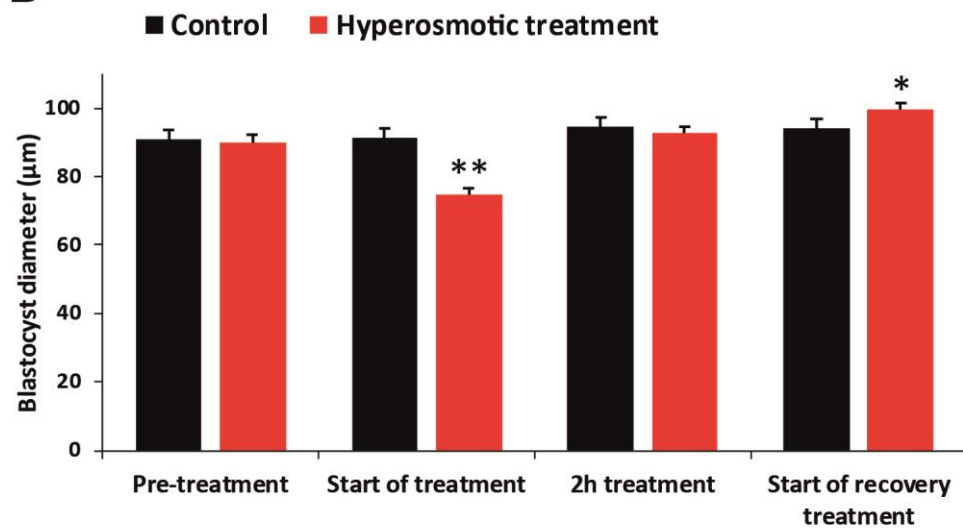


Figure 2

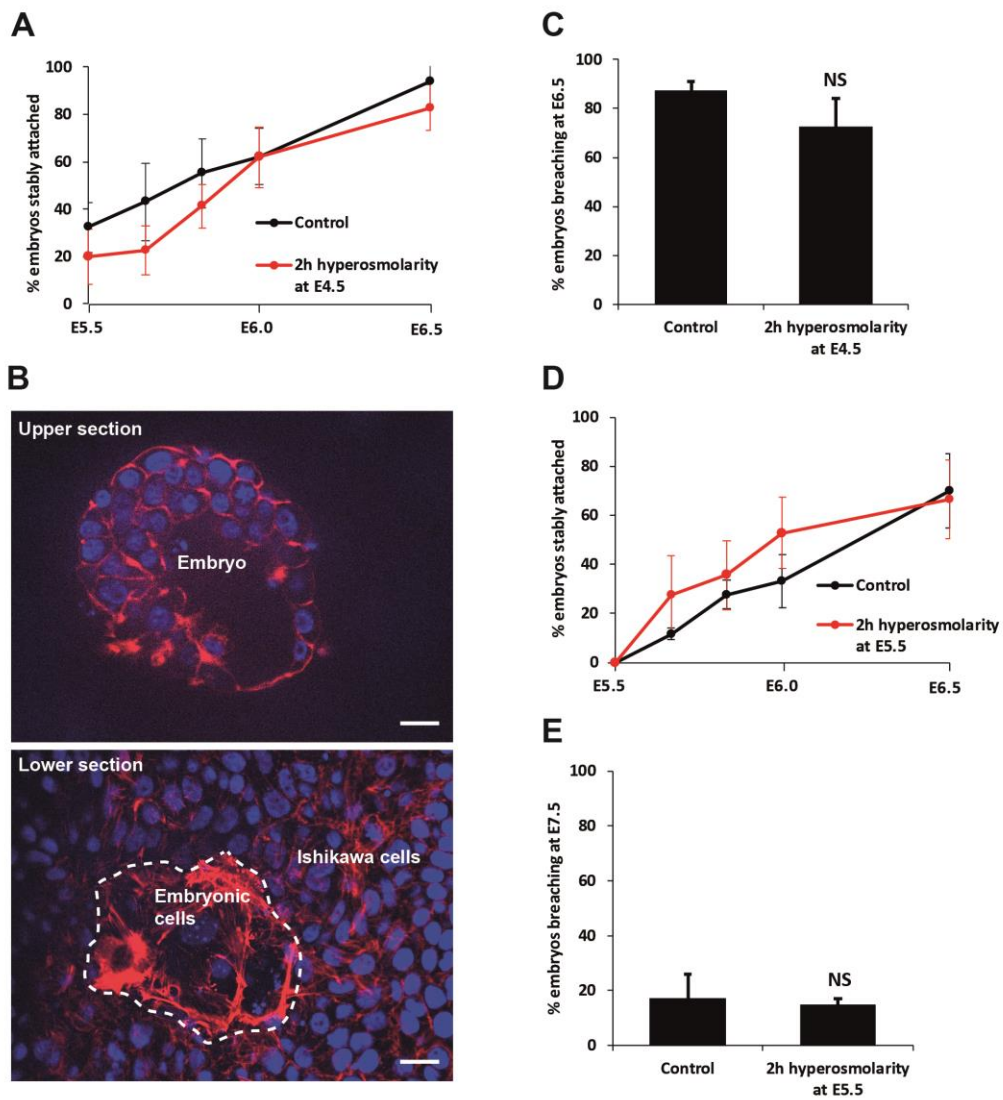
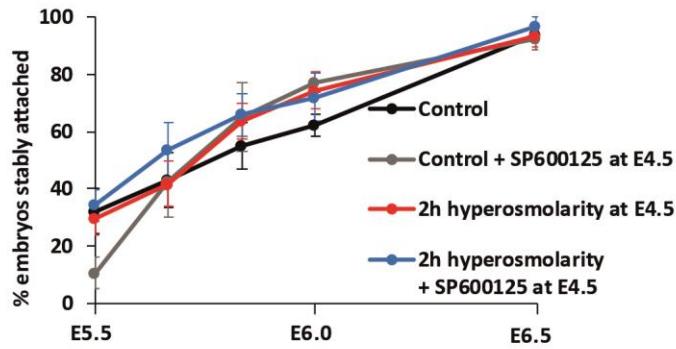
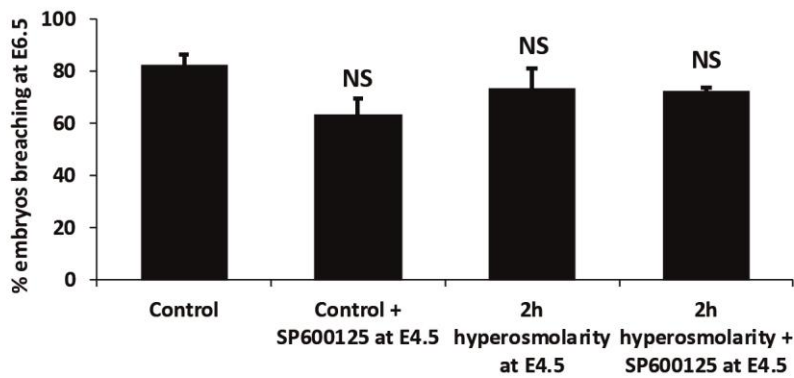


Figure 3

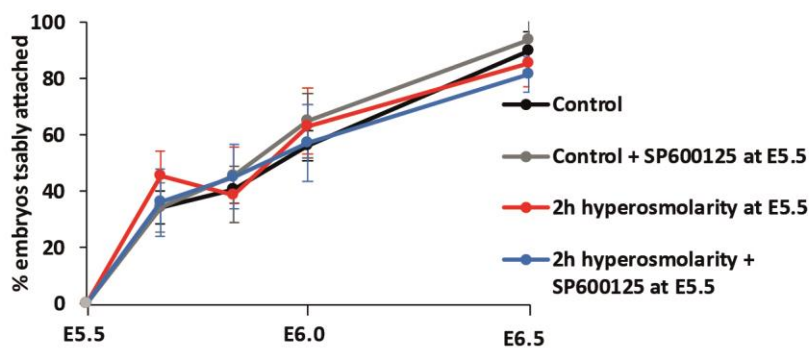
A



B



C



D

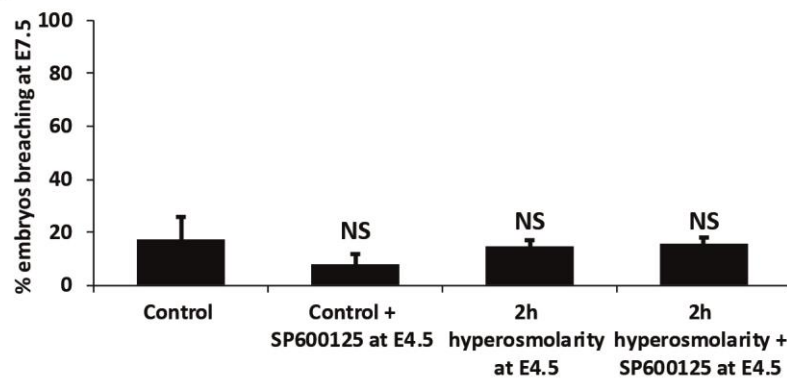
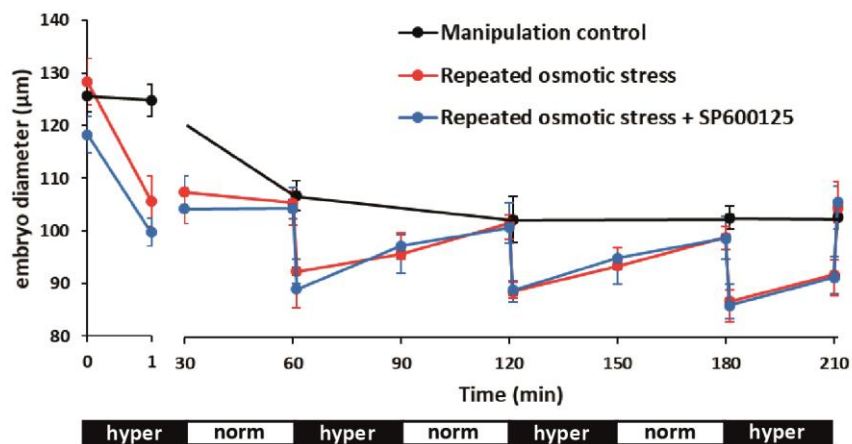
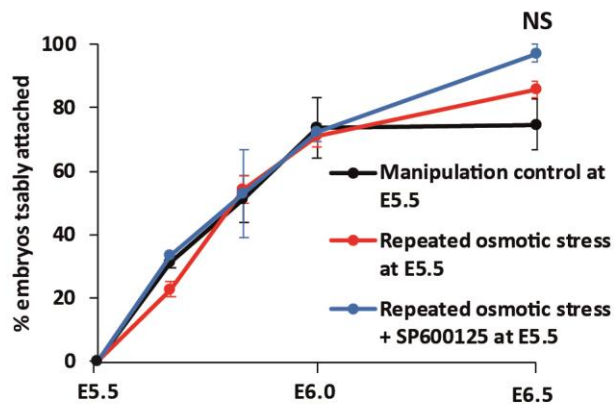


Figure 4

A



B



C

