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Michelle I Violette

Pavneesh Madan

Andrew J Watson

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Na^+/K^+-ATPase regulates tight junction formation and function during mouse preimplantation development

Michelle I. Violette, Pavneesh Madan, Andrew J. Watson *

Department of Physiology and Pharmacology, The University of Western Ontario, The Children's Health Research Institute-Victoria Research Laboratories, 800 Commissioners Road, London, Ontario, Canada N6A 4G5
Department of Obstetrics and Gynaecology, The University of Western Ontario, The Children's Health Research Institute-Victoria Research Laboratories, 800 Commissioners Road, London, Ontario, Canada N6A 4G5

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Abstract

Research applied to the early embryo is required to effectively treat human infertility and to understand the primary mechanisms controlling development to the blastocyst stage. The present study investigated whether the Na^+/K^+-ATPase regulates tight junction formation and function during blastocyst formation. To investigate this hypothesis, three experimental series were conducted. The first experiments defined the optimal dose and treatment time intervals for ouabain (a potent and specific inhibitor of the Na^+/K^+-ATPase) treatment. The results demonstrated that mouse embryos maintained a normal development to the blastocyst stage following a 6-h ouabain treatment. The second experiments investigated the effects of ouabain treatment on the distribution of ZO-1 and occludin (tight junction associated proteins). Ouabain treatment (up to 6 h) or culture in K^+-free medium (up to 6 h) resulted in the appearance of a discontinuous ZO-1 protein distribution and a loss of occludin immunofluorescence. The third set of experiments examined the influence of ouabain treatment on tight junction function. Ouabain treatment or culture in K^+-free medium affected tight junction permeability as indicated by an increase in the proportion of treated embryos accumulating both 4 kDa and 40 kDa fluorescein isothiocyanate (FITC)-dextran into their blastocyst cavities. The results indicate that the Na^+/K^+-ATPase is a potent regulator of tight junction formation and function during mouse preimplantation development.

Keywords: Ion transport; Cell junctions; In vitro fertilization; Cell polarity; Trophectoderm

Introduction

Unraveling the mechanisms that direct how embryonic cells segregate and differentiate is central to understanding normal tissue development, disease states and to providing insight into the mechanisms that govern early development (Rajasekaran et al., 2003a; Johnson and McConnell, 2004). The preimplantation mouse embryo is an elegant system to investigate the regulation of epithelial cell differentiation (Wiley, 1988; Wiley et al., 1990; Fleming et al., 2001; Watson and Barcroft, 2001; Johnson and McConnell, 2004). This process is of fundamental importance in biology and medicine, since epithelia are the most common cell type and alterations in their differentiative pathways occur in many disease states, including carcinomas (Contreras et al., 1995a). The present study was conducted to examine the role of Na^+/K^+-ATPase in regulating tight junction (TJ) formation and function in the mouse blastocyst.

The blastocyst is composed of the first differentiated cell type of mammalian development, the outer epithelial trophectoderm (TE), surrounding a large fluid-filled cavity and a small group of cells, called the inner cell mass (ICM), that are the progenitors of all embryonic cell lineages (Watson and Barcroft, 2001; Johnson and McConnell, 2004). Following blastocyst formation the embryo frees itself from its surrounding zona pellucida and initiates contact between the TE and the endometrium to mediate attachment and implantation into the uterus. Blastocyst formation is essential for implantation, establishment of pregnancy and is a principal determinant of embryo quality prior to embryo transfer (Watson and Barcroft, 2001).

* Corresponding author. Department of Physiology and Pharmacology and Department of Obstetrics and Gynaecology, The University of Western Ontario, The Children's Health Research Institute-Victoria Research Laboratories, 800 Commissioners Road, London, Ontario, Canada N6A 4G5. Fax: +1 519 685 8186.
E-mail address: awatson@uwo.ca (A.J. Watson).

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During compaction (which precedes blastocyst formation) the outside cells of the 8-cell mouse embryo begin to flatten against one another and eventually become attached by tight junctions (TJ), adherens junctions (AJ), desmosomes, and gap junctions (GJ) (Watson et al., 1990a,b; Kidder, 1992; Fleming et al., 2000a,b). The close cell-to-cell contacts that develop are due to the presence of the Ca^{2+}-mediated cell adhesion molecule E-cadherin, which progressively becomes distributed to areas of cell–cell contact and becomes absent from the free apical areas of the outer cells (Johnson and Ziomek, 1981; Johnson et al., 1986; Ohsugi et al., 1997; Torres et al., 1997). At the same time focal TJ appear which eventually divide the plasma membrane of the outer blastomeres into separate apical and basolateral membrane domains (Fleming et al., 2000a,b). The acquisition of cell polarity is accompanied by a reduction in cell totipotency as the outer polar cells of the embryo become committed to the TE cell lineage (Fleming et al., 2000a; Johnson and McConnell, 2004).

As TE cells differentiate Na^+/K^+-ATPase becomes confined to the basolateral membrane domain of the differentiating outer cells establishing trans-epithelial ion transport (Benos et al., 1985; Watson and Kidder, 1988; Biggers et al., 1988; MacPhee et al., 1994; Baltz et al., 1997; MacPhee et al., 2000; Houghton et al., 2003; Barcroft et al., 2004). This is accompanied by the formation of a TJ permeability seal that regulates paracellular transport and maintains the polarized Na^+/K^+-ATPase distribution (Watson et al., 1990a; Fleming et al., 2000a, 2001). The fluid movement across the TE is thought to flow down the Na^+ gradient (Benos et al., 1985; Watson and Barcroft, 2001). Although fluid accumulation during cavitation has been attributed to simple diffusion, blastocyst formation may occur in the absence of a steep osmotic gradient across the TE (Borland et al., 1977; Biggers et al., 1988; Baltz et al., 1997). Therefore, water movement across the TE could represent near iso-osmotic water transport, facilitated by water channels or aquaporins located in TE cell membranes (Offenberg et al., 2000; Barcroft et al., 2003).

Na^+/K^+-ATPase is specifically inhibited by the cardiac glycoside, ouabain (Schoner, 2002; Schneeberger and Lynch, 2004). Apart from the subtle differences in reactivity toward Na^+, K^+ and ATP, the most significant differences in Na^+/K^+-ATPase isozymes correspond to reactivity towards ouabain (Jewell and Lingrel, 1991; Mobasheri et al., 2000). The sheep and human enzymes are a thousand times more sensitive than the corresponding ones from rat and mouse (Price and Lingrel, 1988; Price et al., 1990; Contreras et al., 1995b). In addition results from gene targeting experiments have demonstrated that Na^+/K^+-ATPase α1-subunit null mutants are never born and suffer a developmental lethality, while Na^+/K^+-ATPase α2-subunit null mutants are born but do not survive the first day post partum (Lingrel et al., 2003). We have reported that mouse embryos homozygous for a null mutation in the Na^+/K^+-ATPase α1-subunit gene undergo compaction and cavitation but subsequently fail during the peri-implantation phase of development (Barcroft et al., 2004).

As we learn more about the ways in which epithelial cells regulate their paracellular permeability, questions arise as to the manner in which the preimplantation embryo maintains the fluid that accumulates within the blastocoel cavity and the regulation of TJ seals to maintain the basolateral location of Na^+/K^+-ATPase required for blastocyst development. Recent studies applied to MDCK cell cultures have demonstrated that assembly and maintenance of the TJ in epithelia is dependent on the phosphorylated state of the cadherin/catenin complex and also on Na^+/K^+-ATPase activity (Rajasekaran and Rajasekaran, 2003; Rajasekaran et al., 1996, 2001a,b, 2003a,b). Inhibition of the Na^+/K^+-ATPase in cell cultures by either ouabain treatment or culture in K^+ depleted medium prevents the formation of TJ, detaches cells from each other and from their substrate and inhibits the acquisition of cell polarity (Contreras et al., 1999; Rajasekaran et al., 2003a,b). A relationship between Na^+-pump and TJ formation within the blastocyst would reveal an enhanced role for the increased Na^+/K^+-ATPase expression and activity observed during the morula–blastocyst transition (Betts et al., 1997). For these reasons the current study was conducted to investigate whether the Na^+/K^+-ATPase is involved in the regulation of TJ formation and function during TE differentiation. The primary hypothesis of this study is that Na^+/K^+-ATPase mediates TE TJ formation and function during blastocyst development. Our results indicate that mouse embryos recover and develop normally to the blastocyst stage following ouabain treatment (up to 6 h). More importantly ouabain treatment or culture in K^+-free medium affects the distribution of TJ marker proteins (ZO-1 and occludin) and TJ permeability in mouse blastocysts indicating that Na^+/K^+-ATPase plays a role in the regulation of TJ formation and function during preimplantation development.

Materials and methods

Mouse embryo collection and culture

For collection of mouse preimplantation embryos CD1 females (4–6 weeks of age; Charles River Canada Ltd., St. Constant, QC) were superovulated by intraperitoneal injection of 5 i.u. pregnant mares serum gonadotropin (PMSG; Invitrogen Canada Inc. Burlington, ON) followed 46 h later by 5 i.u. human chorionic gonadotropin (hCG; Invitrogen Canada Inc. Burlington, ON). Following the hCG injection females were placed with $CB6F_1/J$ males (The Jackson Laboratory, Bar Harbor, ME). Successful mating was determined the following morning (day 1) by detection of a vaginal plug. Embryos were flushed from dissected oviducts and uteri using flushing medium-I (Spindle, 1980; 70–72 h post-hCG for 8-cell stage embryos; 82 h post-hCG for morula; 96 h post-hCG for blastocysts) and cultured in potassium simplex optimized medium with amino acids (KSOMaa) (Summers et al., 1995) under a 5% CO_2 in air atmosphere at 37°C until transferred into experimental treatment groups as defined below. All medium components were purchased from Sigma (St. Louis, MO) unless stated otherwise. KSOMaa medium was made fresh before each collection and was sterile filtered. The osmolarity of the medium was tested each time it was prepared and ranged between 288 and 298 mOsm. All experiments described in this study, maintained a treatment drop volume to embryo ratio of one embryo per μl of KSOMaa culture medium.

Optimization of ouabain dose and treatment time on development

Embryo development following long-term ouabain treatment

To assess the effects of ouabain treatment on development 8-cell stage embryos were placed into treatment groups consisting of a control group, KSOMaa alone (Summers et al., 1995), and treatment groups in KSOMaa

including 10^{-7} M, 10^{-5} M, and 10^{-3} M ouabain (10^{-3} M concentration known to block mouse blastocyst re-expansion following cytochalasin D induced collapse; DiZio and Tasca, 1977; Wiley, 1984; Betts et al., 1997). Each treatment group consisted of 25 8-cell stage embryos in 25 μl culture drops under mineral oil and incubated at 37°C, under a 5% CO_2 in air atmosphere for 48 h. For each experimental replicate, development was assessed at 24-h intervals. Blastocoel diameter was measured with an ocular micrometer at the end of the experimental period. Eight experimental replicates were conducted, and in total 800 mouse embryos were treated, measured, and scored for development.

Determination of cell number

To count blastocyst nuclei embryos from each treatment group were treated at the conclusion of each experiment with DAPI (4′,6-diamidino-2-phenylindole; 1 μg/μl in PBS) for 20 min at 38°C in a humidified chamber. Following DAPI treatment embryos were washed 2× in fresh PBS without DAPI for 20 min before being mounted onto glass slides, and examined with a Zeiss LSM 410 Confocal Laser Scanning Microscope. Embryos were scanned through 10 μm Z-plane sections.

Embryo development following short-term ouabain treatment

To determine embryo development following short-term ouabain treatment, morula (82 h post-hCG) stage mouse embryos were collected and divided into four treatment groups and cultured in KSOMaa, (15–20 morulae in 15–20 μl drops under oil at 37°C, under a 5% CO_2 in air atmosphere per replicate) as follows: (1) morulae cultured in KSOMaa for 3 h and 6 h, followed by 24 h of fresh KSOMaa medium (control); (2) morulae cultured for 3 h and 6 h in KSOMaa plus 10^{-5} M ouabain, followed by 24 h in ouabain-free KSOMaa; (3) morulae cultured for 3 h and 6 h in KSOMaa media plus 10^{-4} M ouabain, followed by 24 h in ouabain-free KSOMaa; (4) morulae cultured for 3 h and 6 h in KSOMaa plus 10^{-3} M ouabain, followed by 24 h in ouabain-free KSOMaa. Embryo development was assessed at 12 h, and 24 h following treatment. Five replicates were conducted, and in total 600 mouse morulae were treated and evaluated for blastocyst development. Sub-sets of treated embryos were fixed in 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (GIBCO® BRL Invitrogen Canada Inc. Burlington, ON) for 30 min and processed to assess the localization of the TJ protein, ZO-1, by immunofluorescence methods (see below).

Influence of ouabain treatment on the distribution of TJ associated ZO-1 protein

Recovery from cytochalasin D induced blastocyst formation is an established method for assessing TJ re-assembly and blastocyst formation (DiZio and Tasca, 1977; Betts et al., 1998). Embryos were collected at the compacting 8-cell (72 h post-hCG) and morula stages (82 h post-hCG) and allowed to progress to the blastocyst stage in KSOMaa (30 μl drops under oil at 37°C, 5% CO_2 in air atmosphere). Blastocysts were treated with 20 μg/ml cytochalasin D for 2 h disrupting the actin filamentous cytoskeleton and collapsing the blastocoel cavity (Cooper, 1987). A subset of blastocysts were left out of the cytochalasin treatment as untreated controls and were cultured in KSOMaa for the entire experimental interval. The cytochalasin D treated embryos were split into two treatment groups: (1) cytochalasin D treatment for 2 h followed by 3 h or 6 h in drug-free KSOMaa (control); (2) cytochalasin D treatment for 2 h followed by 3 h or 6 h in KSOMaa plus 10^{-3} M ouabain. At each time interval (3 h or 6 h), embryos were removed from treatment and fixed in 2% PFA in PBS and stored at 4°C in PBS until required. Three experimental replicates were conducted, and in total 360 mouse blastocysts were treated and evaluated for effects on the distribution of TJ ZO-1 by immunofluorescence.

Influence of Short term Na/K-ATPase blockade on the distribution of TJ associated proteins ZO-1 and occludin

To define the consequences of short-term Na/K-ATPase blockade on the distribution of TJ associated proteins blastocysts were collected and divided into four treatment groups (15–20 blastocysts in 15–20 μl drops under oil at 37°C, under a 5% CO_2 in air atmosphere per replicate) as follows: (1) blastocysts cultured in KSOMaa for 3 h and 6 h (control); (2) blastocysts cultured for 3 h and 6 h in KSOMaa plus 10^{-3} M ouabain; (3) blastocysts cultured for 3 h and 6 h in K^+-free KSOMaa media (K^+-free KSOMaa; KCl and KH_2PO_4 in KSOMaa were substituted with NaCl and NaH_2PO_4 of equal molarity; the osmolarity of the medium was tested each time it was prepared and ranged between 288 and 298 mOsm); and (4) blastocysts cultured for 3 h and 6 h in KSOMaa plus 2 mM EGTA (a positive control that disrupts tight junctions). In total, 160 murine blastocysts were fixed in 2% PFA in PBS immediately after treatment and stored at 4°C in PBS until required for analysis of TJ protein ZO-1 and occludin distribution by immunofluorescence methods (see below). To count blastocyst nuclei embryos from each treatment group were treated with DAPI (as above).

Antisera

The primary antisera used included a rat monoclonal IgG raised against the TJ protein ZO-1 from mouse liver (Chemicon International; Temecula, CA) and a mouse monoclonal IgG raised against human occludin (Zymed Laboratories, Inc., San Francisco, CA). These antisera have been extensively characterized by Western blot analysis (Stevenson et al., 1986) and immunofluorescence studies (Stevenson et al., 1986; Fleming et al., 1989) of mammalian tissues, cultured cells and mouse preimplantation embryos. Embryos were incubated with a 1:50 dilution of either ZO-1 or occludin antisera in Wash Buffer [PBS + 0.005% Triton X + 0.05% NDS]. A fluorescein isothiocyanate (FITC)-conjugated goat anti-rat (GARF) IgG was used as a secondary antibody for ZO-1 1:100 dilution (Sigma, St. Louis, MO). A FITC-conjugated donkey anti-mouse (DAMF) IgG was used as a secondary antibody for occludin 1:200 dilution (Jackson Labs, West Grove, PA).

Immunofluorescence methods and confocal microscopy

Following each experiment embryos from each treatment were washed in PBS and fixed in 2% PFA in PBS at room temperature for 30 min for application of immunofluorescence methods. Fixed embryos were washed once in PBS and either used immediately or stored at 4°C in Embryo Storage Buffer [PBS + 0.9% sodium azide] for up to 1 week before processing for whole-mount immunofluorescence. Fixed embryos were permeabilized and blocked for 30 min in Immuno-Block [PBS + 0.01% Triton X + 5% NDS] and then washed in Antibody Dilution Buffer [PBS + 0.005% Triton X + 0.05% NDS]. Embryos were incubated with primary antiserum prepared in and subsequently washed in Antibody Dilution Buffer overnight at 4°C in a humidified chamber. Excess primary antibody was removed by washing embryos 3×, the first 2 washes 20 min, and the third wash 30 min at 37°C in a 9-well Pyrex plate placed in a humidified box. Embryos were then incubated with FITC-conjugated secondary antiserum. Excess secondary antibody was removed by washing embryos 3×, the first wash containing DAPI (1 μg/μl) for nuclear staining for 20 min at 38°C; the final 2 washes were 20 min in Antibody Dilution Buffer. Non-specific fluorescence staining contributed by the secondary antiserum was determined by treating a small pool of embryos with only the FITC-conjugated secondary IgG. These embryos were subjected to the same incubation and wash protocol as described. Embryos were mounted in 20 μl Fluoro-Guard [to prevent photobleaching] (Bio-Rad Laboratories (Canada) Ltd.; Mississauga, ON) on glass slides under glass cover slips with vaseline on each corner of the coverslip (to prevent crushing embryos), and sealed with nail polish. The distribution of ZO-1 or occludin protein within the embryos was visualized using a Zeiss LSM 410 Confocal Laser Scanning Microscope. The ZO-1 and occludin fluorescence distribution was judged as *negative* if no fluorescence was detected within cell margin regions, *discontinuous* if marginal areas displayed a disrupted linear fluorescence pattern, or *continuous* if all marginal areas displayed a linear ‘belt-like’ fluorescence (Eckert et al., 2004).

Influence of ouabain treatment on TJ permeability in mouse blastocysts

To investigate the effects of ouabain treatment on TJ permeability a series of experiments were conducted to examine effects of treatment on accumulation of FITC-dextran (4 kDa, 40 kDa and 70 kDa) into the blastocyst cavity of

treated embryos. For each experiment treatment groups included: (1) KSOMaa medium (control); (2) KSOMaa medium plus 2 mM EGTA; (3) KSOMaa medium plus 10^{-3} M ouabain; and (4) K^+-free KSOMaa medium. Blastocysts were placed into each treatment group for 3 h and 6 h and were cultured in 10–15 μl drops under standard conditions as described. Following treatment blastocysts were incubated in KSOMaa containing 1 mg/ml fluorescein isothiocyanate (FITC)-dextran of size 4 kDa (Sigma, St. Louis, MO) for 30 min, washed 3× in dextran-free KSOMaa and viewed immediately by fluorescence microscopy. Each embryo was scored for the presence or absence of FITC-dextran within the blastocoel cavity. Four replicates were conducted, and in total 321 blastocysts were assessed for 4 kDa FITC-dextran permeability. Sixteen additional replicates of this experimental design were conducted to investigate TJ permeability to 40 kDa and 70 kDa FITC-dextran. Each experimental replicate in these series included an additional 10^{-3} M ouabain treatment group (positive control incubated in 4 kDa FITC-dextran) to ensure consistency of outcomes between experiments. In total, 821 blastocysts were assessed for 40 kDa and 70 kDa FITC-dextran TJ permeability after ouabain treatment or exposure to K^+-free medium. In a final experiment, eight experimental replicates (639 blastocysts) were conducted to investigate whether ouabain treatment with 10^{-5} M and 10^{-4} M ouabain for 6 h also affected TJ permeability to 4 kDa FITC-dextran.

Statistical analysis

Results are presented as an average mean ± SEM for the number of embryos indicated. Statistical analysis was performed using one-way ANOVA and Dunn's multiple comparison test or two-way ANOVA followed by Tukey's post hoc test where appropriate. The non-parametrical equivalent test (Kruskal–Wallis) was used when samples did not meet the assumption of normal distribution or homogeneity of variance. Data calculations were preformed using SigmaStat™ software (Jandel Scientific, San Rafael, CA, USA) and means were considered statistically significant when $P < 0.05$.

Results

Embryo development and cell number following long-term ouabain treatment

To define the optimal levels for ouabain treatment, dose response experiments were conducted by collecting 8-cell stage embryos and monitoring effects to development for up to 48 h. In the first experiment the concentrations of ouabain employed were 10^{-7} M, 10^{-5} M, and 10^{-3} M. Ouabain concentration had a significant influence on both development to the blastocyst stage and blastocoel diameter (Figs. 1A and B). Embryos treated with 10^{-3} M ouabain did not progress beyond the 8-cell stage (Figs. 1A and B). In contrast, embryos treated with 10^{-7} M and 10^{-5} M ouabain or drug-free KSOMaa all displayed a normal development to the blastocyst stage (Figs. 1A and B). Embryo cell number decreased as concentrations of ouabain increased (Fig. 1C). There was a significant difference in cell number between the 10^{-3} M ouabain treatment group and the other 3 treatment groups as well as between the 10^{-5} M ouabain treatment group and control group ($P \leq 0.05$). No significant difference in cell number was detected between the control and 10^{-7} M ouabain treatment group or between the 10^{-7} M and 10^{-5} M ouabain treatment groups (Fig. 1C). The results demonstrated that 8-cell mouse embryos subjected to a long-term treatment (>24 h) with 10^{-7} M or 10^{-5} M ouabain survive and progress to the blastocyst stage. Eight-cell embryos treated long term with 10^{-3} M ouabain are unable to progress beyond the 8-cell stage of development.

Embryo development following short-term ouabain treatment

Since the long-term treatment experiment determined that treatment with 10^{-3} M ouabain blocked embryo development we next characterized the outcomes from shorter term ouabain treatments. For this experiment, ouabain concentrations of 10^{-5} M, 10^{-4} M, and 10^{-3} M and treatment times of 3 h and 6 h applied to mouse morulae were investigated. The results demonstrated that there were no significant differences in blastocyst development in any of these ouabain concentration or treatment time groups (Fig. 1D). Therefore, treatment of mouse morulae for up to 6 h with 10^{-3} M ouabain does not impair development to the blastocyst stage.

Influence of ouabain treatment on the distribution of TJ associated ZO-1 protein

Our first experiments also included an examination of ZO-1 distribution following long-term culture in the presence of ouabain. Long-term culture in the presence of 10^{-7} M or 10^{-5} M ouabain did not affect ZO-1 distribution (data not shown). Since the short-term experiments defined a treatment interval (up to 6 h) and ouabain concentration (10^{-3} M) where embryo viability was not affected subsequent experiments employed this short time course (3 and 6 h) followed by 24 h culture in drug-free KSOMaa to analyze effects to TJ formation, re-assembly and distribution of TJ-associated proteins, ZO-1 and occludin. Experiments examined the influence of ouabain treatment on ZO-1 distribution following TJ formation and also TJ re-assembly following cytochalasin-D induced blastocyst collapse. ZO-1 normally displays a fluorescence pattern that is confined to the apico-lateral margins between cells of the outer TE layer (Fleming et al., 1989). ZO-1 fluorescence forms an uninterrupted belt at the perimeter of each cell corresponding to the region where mature TJs form (Fleming et al., 1989). Our results confirmed these findings as the ZO-1 distribution in embryos cultured in KSOMaa (control) medium appeared as a continuous linear 'belt-like' fluorescence encircling the apico-lateral periphery of each TE cell (Fig. 2ACon; and BCon).

Embryos treated with 10^{-5} M and 10^{-4} M ouabain for 3 h and 6 h displayed an identical ZO-1 fluorescence pattern as controls (Fig. 2A 10^{-5} and 10^{-4} and 2B 10^{-5} and 10^{-4}). In contrast, all embryos (>50 embryos per group) treated with 10^{-3} M ouabain for 3 h (A 10^{-3} M) and 6 h (B 10^{-3} M) followed by 24 h in drug-free medium displayed a ZO-1 fluorescence pattern that transitioned from continuous to discontinuous by the 6 h treatment time. Following 6 h of treatment there was an obvious decline in the intensity of Z0-1 immunofluorescence (B 10^{-3} M).

Influence of ouabain treatment and culture in K^+-free KSOMaa on the distribution of ZO-1 and Occludin

To establish the effects of Na/K-ATPase blockade on blastocyst TJ, we next contrasted the effects of both ouabain treatment and culture in K^+-free KSOMaa medium on the distribution of ZO-1 and also occludin, a core TJ protein. The

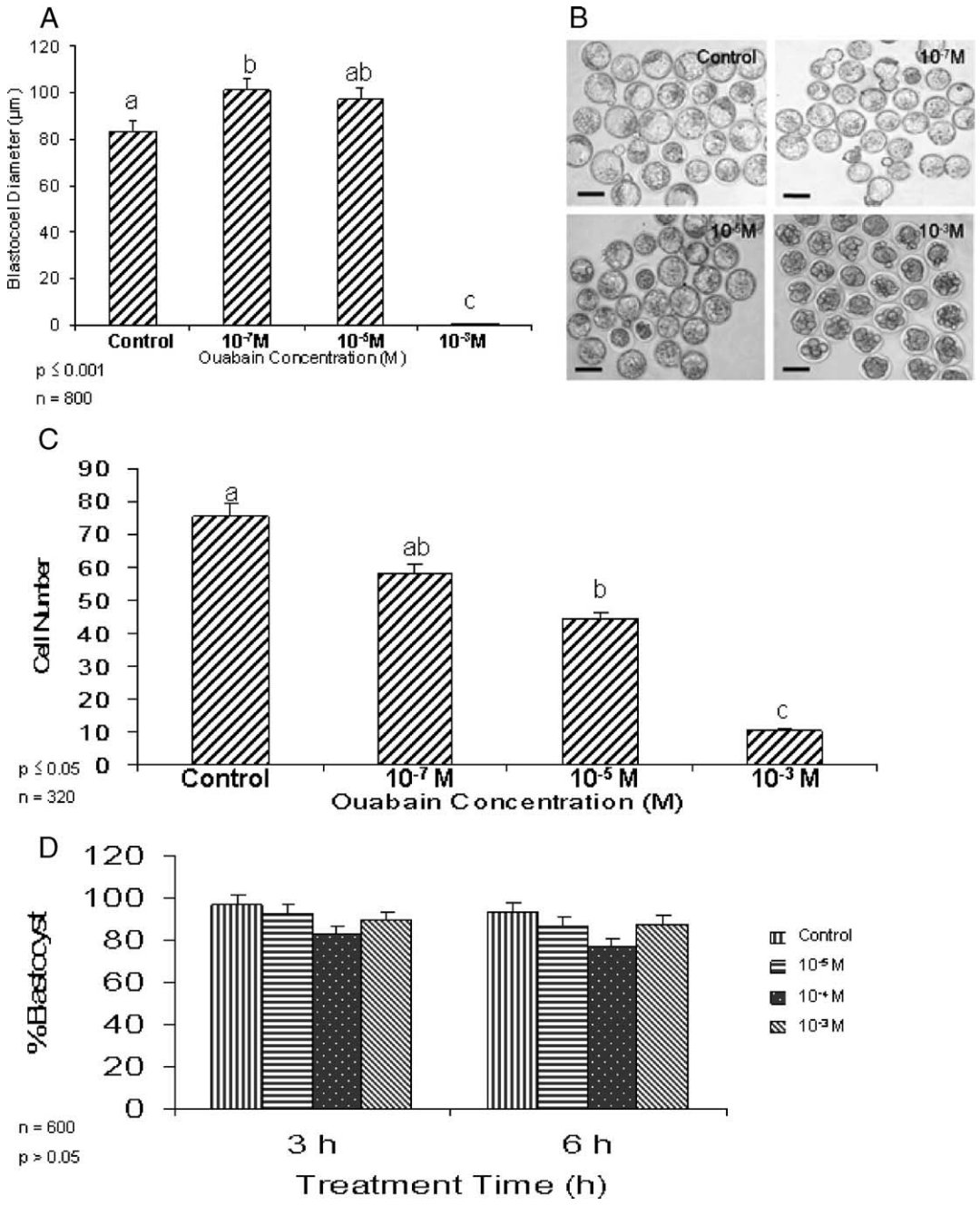

Fig. 1. Development of Mouse Embryos Following Ouabain treatment. (A) Influence of ouabain treatment at the 8 cell stage on blastocoel diameter. Embryos were placed into control medium (KSOMaa), or KSOMaa plus ouabain at 10^{-7} M, 10^{-5} M or 10^{-3} M. Embryos cultured in 10^{-7} M ouabain displayed a significant ($P \leq 0.05$) increase in blastocoel diameter compared to embryos cultured in KSOMaa medium alone or KSOMaa plus 10^{-3} M ouabain. Embryos cultured in 10^{-3} M ouabain from the 8-cell stage remained at the 8-cell stage. (B) Morphology of embryos cultured from the 8-cell stage in KSOMaa or KSOMaa medium with 10^{-7} M, 10^{-5} M and 10^{-3} M ouabain. Scale bar = 100 µm. (C) Effects of long-term ouabain treatment on blastocyst cell number. Embryos cultured in 10^{-3} M ouabain displayed a significant ($P \leq 0.05$) decrease in cell number compared to those cultured in KSOMaa, or 10^{-7} M and 10^{-5} M ouabain. Embryos cultured in 10^{-5} M ouabain displayed a significant ($P \leq 0.05$) decrease in cell number compared to those cultured in drug-free KSOMaa. (D) Influence of short-term ouabain treatment. Embryos at the morulae stage were treated for 3 h (3 h) or 6 h (6 h) in KSOMaa, or KSOMaa plus 10^{-5} M, 10^{-4} M or 10^{-3} M ouabain. Blastocyst development or cell number (data not shown) did not vary significantly ($P \leq 0.05$) among any of the treatment groups for either a 3 or 6 h treatment interval.

ZO-1 distribution in all embryos cultured in KSOMaa medium once again appeared as a continuous linear 'belt-like' fluorescence encircling the apico-lateral periphery of each TE cell (Fig. 3B KSOM). Occludin fluorescence mimicked the ZO-1 pattern in all KSOM control blastocysts (Fig. 3A KSOM). In contrast to these results but consistent with earlier experiments all blastocysts cultured in the presence of 10^{-3} M ouabain displayed a discontinuous fluorescence pattern for ZO-1 (Fig. 3B ouabain). Occludin fluorescence was reduced to minimal levels in all embryos in the 10^{-3} M ouabain treatment group (Fig. 3A ouabain). All blastocysts cultured in K^+-free KSOMaa medium displayed identical discontinuous

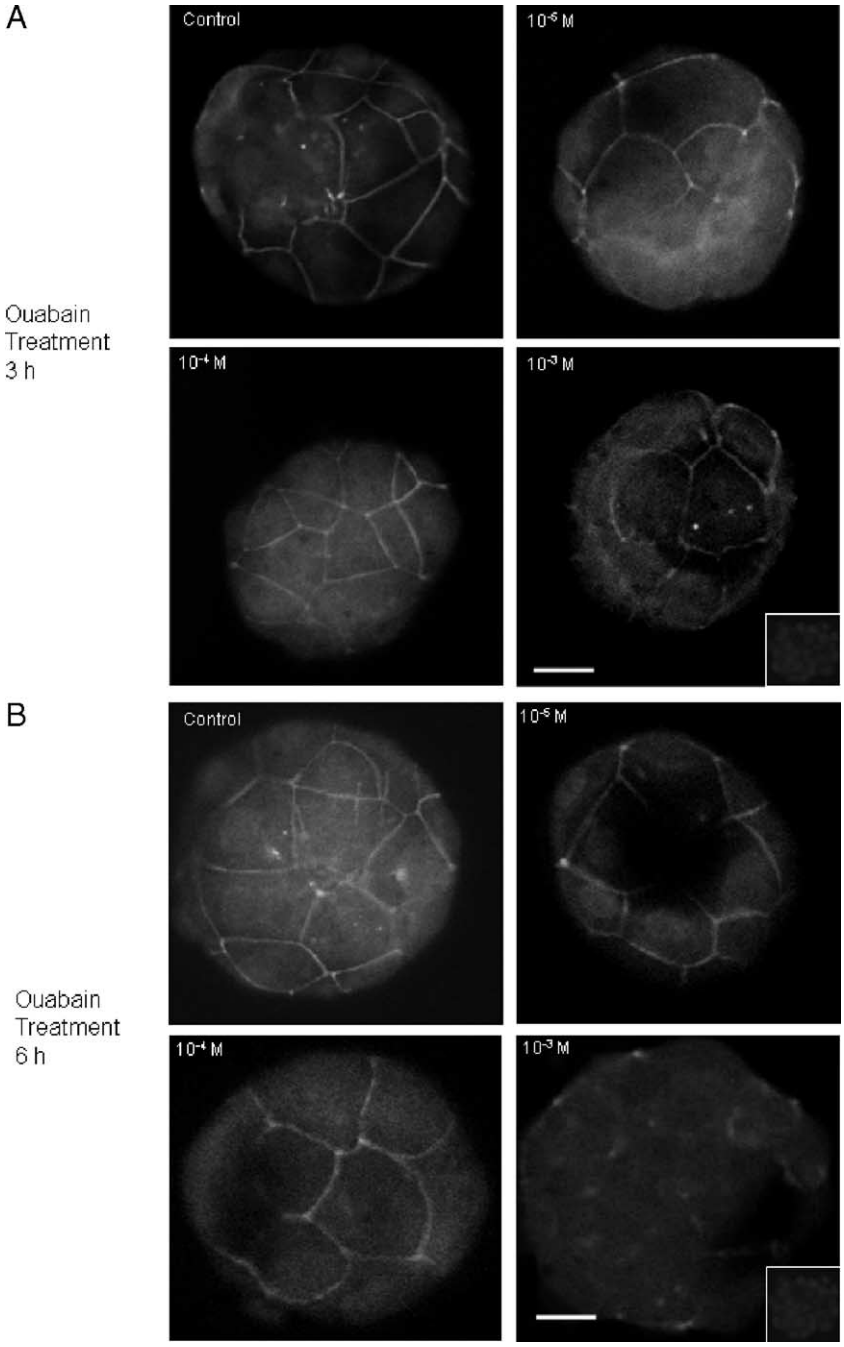

Fig. 2. Effects of 3 h and 6 h Ouabain Treatment on ZO-1 Distribution. Control blastocysts displayed a continuous linear 'belt-like' ZO-1 fluorescence pattern confined to the apical surface of the developing TE (A Control, B Control).Embryos treated with 10^{-5} M and 10^{-4} M ouabain for 3 h (A 10^{-5} M and A 10^{-4} M) and 6 h (B 10^{-5} M and B 10^{-4} M) displayed an identical ZO-1 fluorescence pattern as untreated controls. In contrast, embryos treated with 10^{-3} M ouabain for 3 h (A 10^{-3} M) and 6 h (B 10^{-3} M) displayed a ZO-1 fluorescence pattern that transitioned from continuous to discontinuous by the 6 h treatment time. No fluorescence was observed in embryos treated with secondary antibody alone (A 10^{-3} M inset; and B 10^{-3} M inset). All images are 1 μm thick confocal laser scanning projections. Scale bars = 25 μm.

ZO-1 and occludin fluorescence patterns to that observed for blastocysts cultured in medium with 10 M ouabain (Fig. 3A K^+-free KSOMaa and 3B K^+-free KSOMaa). Blastocysts cultured in medium with 2 mM EGTA displayed normal ZO-1 and occludin fluorescence patterns (Fig. 3A EGTA and 3B EGTA). No difference in blastocyst cell number was observed in any of these treatment groups following the 6 h treatment interval indicating that a short term treatment with 10^{-3} M ouabain did not affect overall blastocyst cell number (data not shown).

Influence of ouabain treatment on the re-distribution of ZO-1 following TJ disruption

Now that it was apparent that ouabain treatment, for as short an interval as 6 h affected the distribution of TJ associated

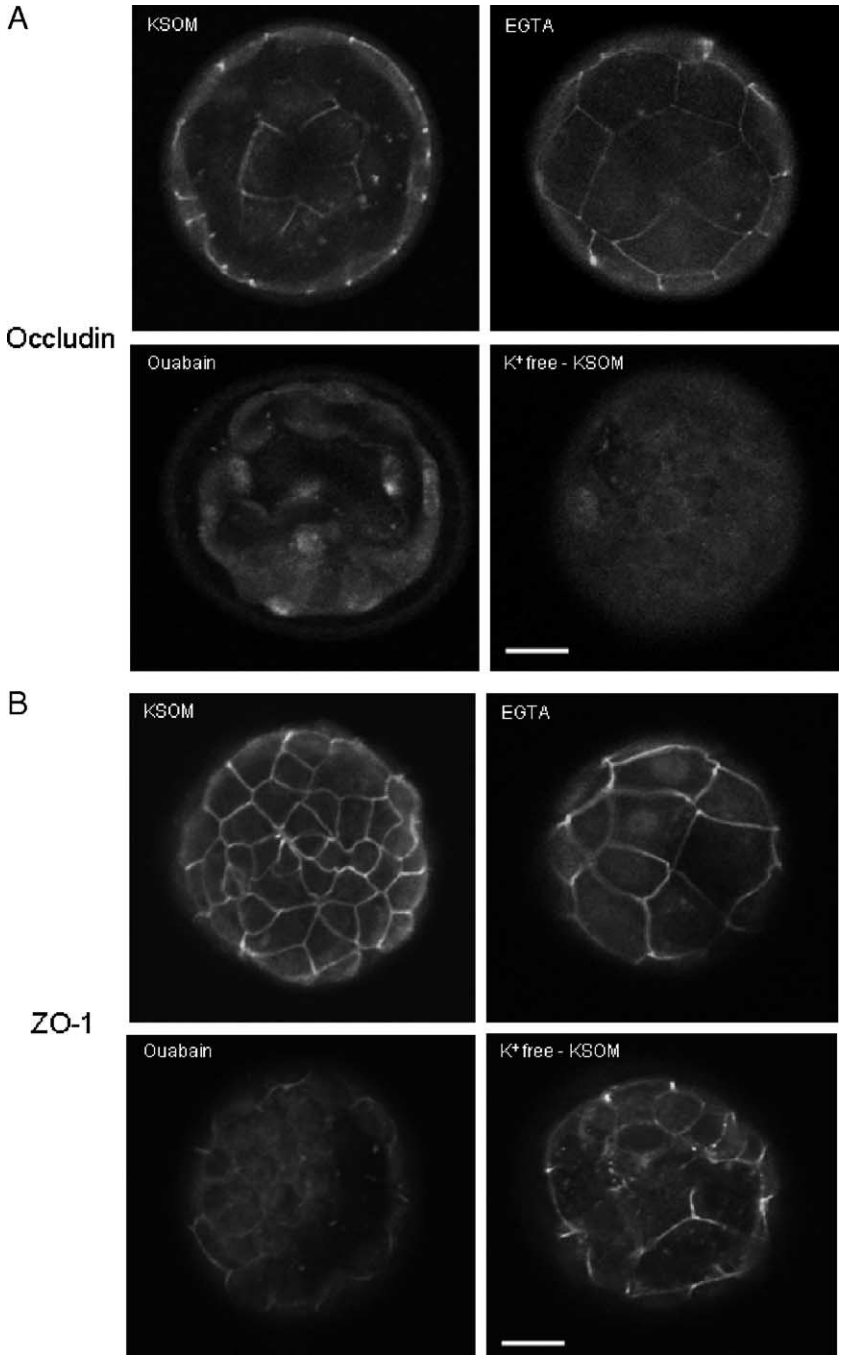

Fig. 3. Effects of Ouabain Treatment on ZO-1 and Occludin Distribution. Control blastocysts displayed a continuous ZO-1 and occludin fluorescence pattern confined to the apical surface of developing TE (A, Control; B, Control). Blastocysts cultured in medium plus 2 mM EGTA displayed identical ZO-1 and occludin fluorescence patterns to that observed in controls (A, EGTA and B, EGTA). In contrast the ZO-1 fluorescence pattern transitioned to become discontinuous for blastocysts cultured in the presence of 10^{-3} M ouabain for 6 h (B, ouabain) and also for blastocysts cultured in K^+-free KSOMaa for 6 h (B, K^+-free KSOMaa). In addition to becoming discontinuous, the occludin fluorescence pattern was also markedly reduced for blastocysts cultured in 10^{-3} M ouabain (A, ouabain) and K^+-free KSOMaa for 6 h (B, K^+-free KSOMaa). All images are 1 μm thick confocal laser scanning projections. Scale bars = 25 μm.

proteins, we next investigated whether ouabain treatment could also affect TJ ZO-1 distribution during junctional re-assembly following cytochalasin-D induced blastocyst collapse. Following 2 h of cytochalasin-D treatment, ZO-1 fluorescence was primarily cytoplasmic and discontinuous running along the outer margins of each blastomere in all embryos (Figs. 4D and G). In control untreated blastocysts, ZO-1 displayed the expected continuous belt-like fluorescence pattern along the cell margins of each TE cell (Figs. 4A, B and C). At 3 h following cytochalasin-D treatment, embryos placed into drug-free KSOMaa medium still displayed a discontinuous linear ZO-1 fluorescence pattern (Fig. 4E). These embryos often revealed the re-appearance of fluid-filled cavities (not shown). By 6 h in drug-free KSOMaa medium a continuous belt-like ZO-1 fluorescence pattern was restored and all of these embryos displayed the appearance of a fluid-filled cavity

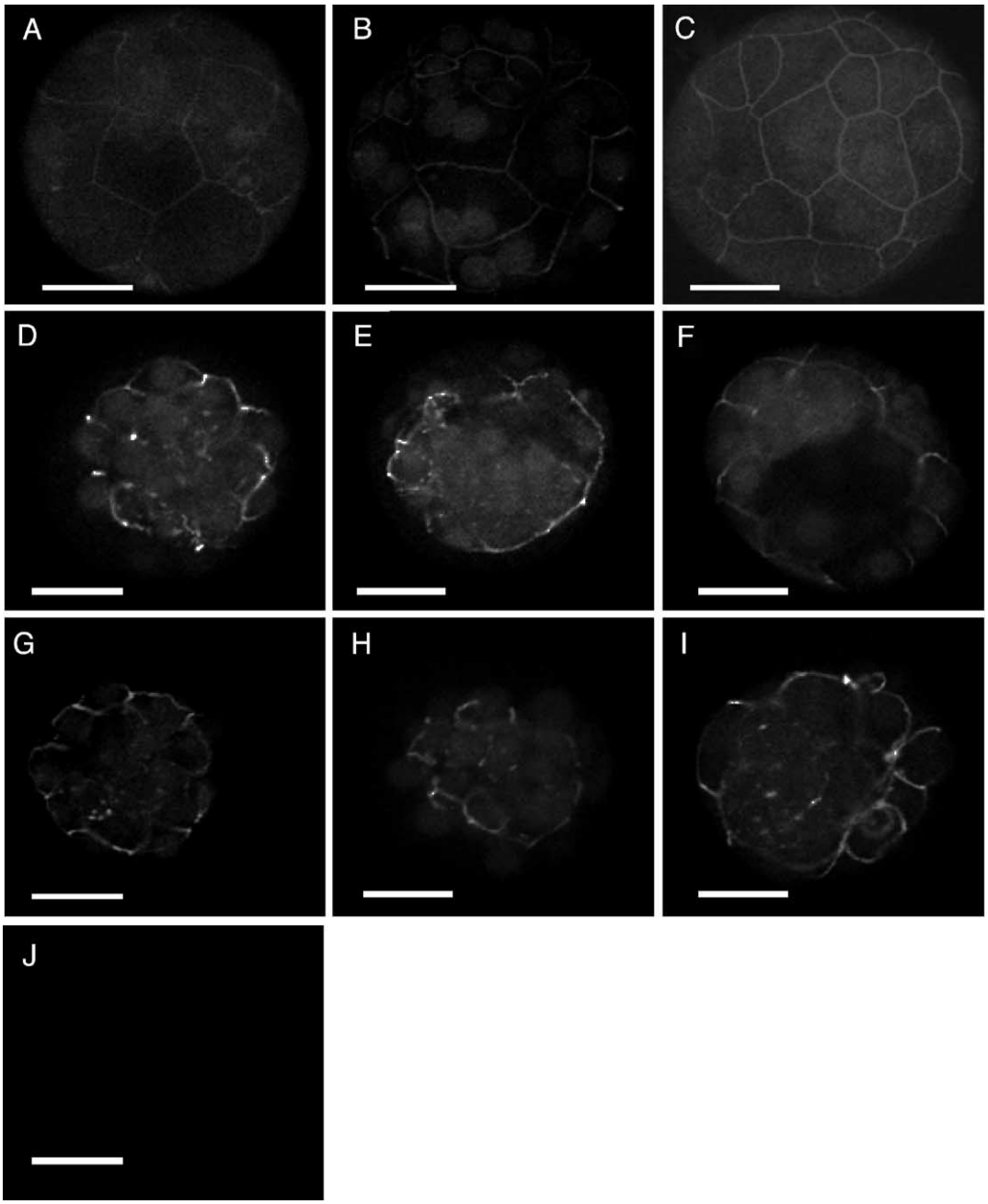

Fig. 4. Effects of Ouabain Inhibition on TJ Re-assembly. Blastocysts were treated in cytochalasin D for 2 h (D and G). Control embryos not exposed to cytochalasin D displayed the expected "continuous" belt-like ZO-1 fluorescence pattern along the TE cell margins at all time points (0 h A; 3 h B; 6 h C). At 3 h following cytochalasin D treatment embryos placed into drug-free KSOMaa still displayed a discontinuous linear ZO-1 fluorescence pattern (E). However, a continuous belt-like ZO-1 fluorescence pattern was established by 6 h in drug-free KSOMaa (F). The ouabain treated embryos maintained a discontinuous ZO-1 fluorescence patterns at all time points (3 h, H; 6 h, I). No fluorescence was observed in embryos treated with secondary antibody alone (J). All images are 1 μm thick confocal laser scanning projections. Scale bars = 25 μm.

(Fig. 4F). In contrast, re-expansion of mouse blastocysts was completely blocked in all embryos (>50 embryos) placed into the ouabain treatment group following cytochalasin D treatment (Figs. 4G–I). The ouabain treated embryos displayed a discontinuous ZO-1 fluorescence pattern in both 3 h and 6 h treatment time points (Figs. 4H and I). Therefore treatment with 10^{-3} M ouabain prevented recovery from cyctochalasin-D collapse and more importantly was associated with the retention of a discontinuous ZO-1 distribution.

Influence of ouabain treatment on TJ permeability in mouse blastocysts

The results to this point indicated that early mouse embryos can survive a 6 h treatment with 10^{-3} M ouabain and this treatment or culture in K^+-free KSOMaa medium are associated with an abnormal ZO-1 and occludin protein distribution. The final experimental series investigated whether Na/K-ATPase blockade by either ouabain treatment or culture in

Table 1
FITC-dextran permeability in embryos treated with ouabain for 3 h

3 h Treatment time						
FITC-dextran size	4 kDa		40 kDa		70 kDa	
	No. of embryos examined	No. of permeable embryos	No. of embryos examined	No. of permeable embryos	No. of embryos examined	No. of permeable embryos
Treatment group						
KSOMaa	42	6 (14.2%)	49	5 (10.2%)	40	1 (2.5%)
10^{-3} M Ouabain	43	20 (46.5%)*	58	18 (31.0%)*	39	2 (5.1%)
K+-free	40	17 (42.5%)*	50	22 (44.0%)*	39	1 (2.5%)
EGTA	39	23 (58.9%)*	53	26 (49.0%)*	38	13 (34.2%)*

* Significantly different from control by Dunn's Test $P < 0.05$.

K^+-free KSOMaa medium affected TJ function as assessed by examining FITC-dextran accumulation in treated blastocysts.

We first examined effects to 4 kDa FITC dextran accumulation. Table 1 shows that following 3 h, all three treatment groups, KSOM plus 10^{-3} M ouabain, KSOMaa plus EGTA, and K^+-free KSOMaa medium displayed a significant increase over untreated controls in the number of embryos that accumulated 4 kDa FITC-dextran into their blastocyst cavities ($P < 0.05$). Table 2 demonstrates that by 6 h of treatment, the proportion of embryos that displayed 4 kDa FITC-dextran fluorescence in their blastocyst cavities had increased in each group but was still significantly greater in the three treatment groups over that observed for untreated controls.

The next experimental series examined the effects of treatment on accumulation of 40 kDa and 70 kDa FITC-dextran. Tables 1 and 2 show that for accumulation of 40 kDa FITC-dextran all three treatment groups (10^{-3} M ouabain, K^+-free group and EGTA group) displayed a significant difference ($P < 0.05$) over untreated controls in the proportion of embryos that displayed FITC-dextran in their blastocyst cavities. Representative images of FITC-dextran fluorescence within the blastocoel of treated embryos at 3 h (Figs. 5A–D) and 6 h (Figs. 5 E–H) are displayed in Fig. 5. However, in contrast to these, results Tables 1 and 2 reveal that accumulation of 70 kDa FITC-dextran was only significantly greater in the EGTA treated control group for both 3 h and 6 h treatments. Thus ouabain treatment or culture in K^+-free KSOMaa medium had no effect on accumulation of 70 kDa FITC-dextran.

The final experiment investigated whether treatment with lower ouabain concentrations would also affect FITC dextran accumulation. Table 3 indicates that treatment with 10^{-5} M and 10^{-4} M ouabain for 6 h had no significant influence on accumulation of 4 kDa FITC-dextran. In contrast, Table 3 shows that the EGTA, 10^{-3} M ouabain and K^+-free treatment groups displayed a significant increase in the proportion of embryos accumulating 4 kDa FITC-dextran into their blastocyst cavities ($P < 0.05$).

Discussion

In this study we have demonstrated that treatment of mouse embryos with a potent Na^+/K^+-ATPase inhibitor or culture in K^+-free KSOMaa medium affects the maintenance of TJ structure and function during preimplantation development. We have demonstrated that mouse embryos can be treated with ouabain at concentrations as high as 10^{-3} M for up to 6 h without apparent detriment as indicated by their continued development to the blastocyst stage and their maintenance of a normal cell number. Of even greater significance, the results indicate that ouabain treatment or culture in K^+-free KSOMaa medium both result in an increase in TE TJ permeability to 4 kDa and 40 kDa FITC-dextran. This increased TJ permeability was accompanied by a re-arrangement of the distribution of ZO-1 and occludin, both important TJ associated proteins. Collectively the results suggest that Na^+/K^+-ATPase is a potent regulator of TE tight junction function during murine preimplantation development.

Blastocyst formation is dependent upon the expression of several gene products including Na^+/K^+-ATPase, E-cadherin, and TJ components (Fleming et al., 1989; Ohsugi et al., 1997; Fleming et al., 2000a,b; Barcroft et al., 2004). This morphogenetic event is initiated during compaction as stable E-

Table 2
FITC-dextran permeability in embryos treated with ouabain for 6 h

6 h Treatment time						
FITC-dextran size	4 kDa		40 kDa		70 kDa	
	No. of embryos examined	No. of permeable embryos	No. of embryos examined	No. of permeable embryos	No. of embryos examined	No. of permeable embryos
Treatment group						
KSOMaa	39	6 (15.3%)	58	5 (8.6%)	45	3 (6.6%)
10^{-3} M Ouabain	40	21 (52.5%)*	63	17 (26.9%)*	48	6 (12.5%)
K+-free	39	20 (51.2%)*	79	25 (31.6%)*	48	5 (10.4%)
EGTA	39	29 (74.3%)*	63	35 (55.5%)*	51	14 (27.4%)*

* Significantly different from control by Dunn's Test $P < 0.05$.

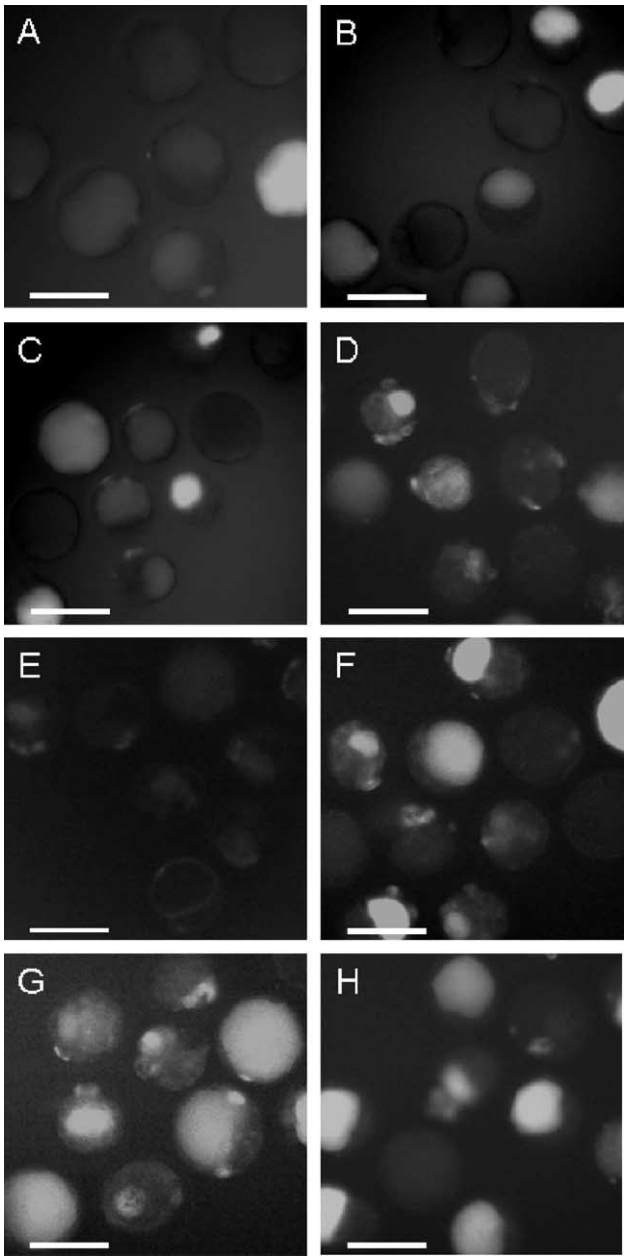

Fig. 5. FITC-Dextran Fluorescence. Representative images of FITC-dextran fluorescence within the blastocyst cavity of ouabain treated embryos at 3 h (A–D) and 6 h (E–H). Figures represent non treated controls cultured in KSOMaa medium (A, E), KSOMaa plus 10^{-3} M ouabain (B, F), K^+-free KSOMaa (C, G), and KSOMaa plus EGTA (D, H). Treated embryos displayed a significant increase in the number of embryos permeable to 40 kDa FITC-dextran compared to controls [KSOMaa] (A, E). Scale bars = 100 μm.

cadherin/catenin cell-to-cell AJs form as a prelude to focal TJ formation (Fleming et al., 2000a,b). TE differentiation proceeds as TJs mature and assist in the establishment of distinct apical and basolateral cell membrane domains. By the late morula stage, the α1 subunit of the Na^+/K^+-ATPase is up-regulated and this occurs in combination with the polarized localization of Na^+/K^+-ATPase to the basolateral domains of the outer TE, at the onset of fluid accumulation (Watson and Kidder, 1988; Watson et al., 1990b; Betts et al., 1998). It is hypothesized that the formation of the blastocyst cavity is facilitated by a trans-trophectodermal ion gradient established by a polarized distribution of Na^+/K^+-ATPase, which facilitates the movement of water into the extracellular space to form the fluid-filled cavity of the blastocyst (Benos et al., 1985; Biggers et al., 1988; Watson and Barcroft, 2001; Barcroft et al., 2004). However, blastocyst expansion only occurs after the TJ permeability seal fully forms to restrict the leakage of fluid via paracellular routes, ensuring the expansion of the cavity as fluid accumulates (McLaren and Smith, 1977; Sheth et al., 2000a).

Several studies have implicated Na^+/K^+ATPase activity and cell attachment as requirements for TJ formation in epithelial cells and TE cell differentiation (Wiley, 1984; Contreras et al., 1999; Rajasekaran et al., 2001a,b). Treatment of mammalian preimplantation embryos with the Na^+-pump inhibitor ouabain blocked blastocyst formation (Wiley, 1984). Prolonged ouabain blockade of Na^+/K^+-ATPase activity detached cultured cell lines from each other and their substrate (Contreras et al., 1999). Inhibition of Na^+/K^+-ATPase either with ouabain or K^+ depleted medium prevented TJ and desmosome formation in MDCK cells (Rajasekaran et al., 2001a,b). In cultures of human retinal pigment epithelial cells, inhibition of the Na^+-pump by ouabain treatment or exposure to K^+ depleted medium decreased trans-epithelial cell resistance and increased permeability of TJs to mannitol and insulin (Rajasekaran et al., 2003a,b). These studies have provided a foundation for our hypothesis, that Na^+/K^+-ATPase is an important mediator of TE TJ formation and function during blastocyst development.

The results demonstrated that 10^{-7} M ouabain did not affect blastocyst development, while at higher concentrations (10^{-3} M), long-term ouabain treatment blocked development at the 8-cell stage. Ouabain specifically inhibits Na^+/K^+-ATPase activity by binding to a domain located on the α-subunit (Kawamura et al., 1999). K^+ competes with ouabain for binding sites on the Na^+/K^+-ATPase (Price and Lingrel, 1988; Johnson et al., 1995). Ouabain can stimulate enzyme activity at lower concentrations and inhibit enzyme activity at higher concentrations (Wiley, 1984; Schoner, 2002). The cation gradients maintained by the Na^+/K^+-ATPase are needed in many cells to maintain protein synthesis. Ouabain (10^{-3} M) treatment slows or blocks formation of the mouse blastocyst cavity (DiZio and Tasca, 1977; Wiley, 1984; Manejwala et al., 1989; Betts et al., 1998). In addition, rodent cells are resistant

Table 3
FITC-dextran permeability in embryos treated with low ouabain concentrations

6 h Ouabain treatment 4 kDa FITC-dextran		
Treatment group	No. of embryos examined	No. of permeable embryos (5)
KSOMaa	109	18 (16.5%)
10^{-3} M Ouabain	110	49 (44.5%)*
K^+-free	95	31 (32.6%)*
EGTA	104	57 (54.8%)*
10^{-4} M Ouabain	111	24 (21.6%)
10^{-5} M Ouabain	97	24 (24.7%)

* Significantly different from control by Dunn's.

to ouabain treatment and are typically unresponsive to concentrations of ouabain less than 10^{-3} M, which explains why this ouabain concentration has been characterized for use in studies directed at defining the role of the Na^+/K^+-ATPase in mouse blastocyst formation (DiZio and Tasca, 1977; Betts et al., 1998; Mercer, 1993; Lingrel and Kuntzweiler, 1994). Despite having an influence on overall cell number, treatment with 10^{-5} M ouabain also did not affect blastocyst development. Since 10^{-7} M ouabain did not have any measurable effect it was removed from succeeding experiments that instead employed 10^{-5} M, 10^{-4} M and 10^{-3} M ouabain concentrations. In contrast to the findings of the long-term treatment experiment, short term treatment with 10^{-3} M ouabain for up to 6 h did not affect development to the blastocyst stage or embryo cell number.

DiZio and Tasca (1977) employed cytochalasin-D treatment for the purpose of disrupting TE TJ and to enable the investigation of the effects of ouabain treatment on blastocyst formation. Cytochalasin-D disrupts actin microfilaments, which results in the breakdown of a cortical microfilamentous cytoskeleton (Cooper, 1987). This causes a disruption of the TJ seal which is anchored to the actin cytoskeleton through associated cytoplasmic proteins (Fleming and Hay, 1991). In the present study, removal of cytochalasin from the medium and its replacement with drug-free medium resulted in a re-expansion of all embryos and a return to a normal continuous ZO-1 protein distribution associated with the apical contact points between adjacent TE cells. In contrast, neither blastocyst re-expansion nor return to a normal ZO-1 protein distribution was observed when cytochalasin-D collapsed embryos were transferred to medium containing 10^{-3} M ouabain. These results support our primary hypothesis by suggesting that Na^+/K^+-ATPase is required to promote the reformation of TJ following cytochalasin-D collapse. Furthermore, the results indicated that ZO-1 following 3 h of ouabain treatment was detected as a discontinuous fluorescence line along the TE cell margins. By 6 h, ZO-1 fluorescence was decreased in the 10^{-3} M ouabain group, and there was little presence of a continuous linear pattern as seen in the control groups. ZO-1 protein in preimplantation embryos is first observed at the compacting 8-cell stage, coincident with or just after development of basolateral cell adhesion and the appearance of apical microvillous polarity (Fleming et al., 1989). Initially, ZO-1 is present as a series of spots along the boundary between free and apposed cell surfaces in intact embryos, but becomes more linear with blastocyst TE cells becoming surrounded by a continuous apical ZO-1 fluorescent belt (Fleming et al., 1989). During compaction and the early phases of apical junction formation, ZO-1 transiently co-localizes with the catenin proteins in a single, permeable, apicolateral "intermediate" junctional complex (Fleming et al., 2000a,b). Subsequently, after occludin assembly in the late morula, the apical junctional complex segregates into two distinct structural domains (apical TJs; subjacent AJs) coincident with the apical separation of ZO-1 together with other TJs associated proteins from the E-cadherin complex thus establishing a permeability seal (Sheth et al., 2000b). Once this permeability seal forms the blastocyst cavity begins to expand (Sheth et al., 2000a).

One of the most dramatic outcomes we observed were that both treatment with 10^{-3} M ouabain and culture in K^+-free KSOMaa medium resulted in a marked reduction in occludin immunofluorescence. Occludin is a integral TJ protein that has four transmembrane regions and a long terminal cytoplasmic domain (Furuse et al., 1994). Experiments have determined that ZO-1 is directly associated with occludin and this association is required to maintain occludin's position within the TJ (Furuse et al., 1994; Umeda et al., 2004). Based upon our outcomes, we would propose that blockade of Na/K-ATPase results in disruption of ZO-1 distribution which is then followed by affects to occludin distribution. However, despite their compelling nature, our ZO-1 and occludin immunofluorescence studies do not reveal the functional consequences of ouabain treatment on the TE TJ. To investigate the functional links, we proceeded with experiments to investigate the influence of ouabain treatment on TJ permeability.

The results from the FITC-dextran permeability experiments demonstrated that blastocysts treated with 10^{-3} M ouabain for up to 6 h and cultured in K^+-free KSOMaa significantly increased their permeability to 4 kDa and 40 kDa FITC-dextran. In contrast, these embryos did not display a high degree of permeability to 70 kDa FITC-dextran. Thus we have demonstrated that ouabain treatment or culture in K^+-free KSOMaa results in a clear shift in the proportion of embryos that maintain an impermeable TJ seal. Interestingly, the seal does not become completely permeable as it is still capable of excluding movement of 70 kDa dextran. Thus, the effect of ouabain treatment or culture in K^+-free KSOMaa on TJ function is selective, suggesting that TJ function in the early embryo is subject to multiple modes of regulation.

TJs consist of a complex of several transmembrane proteins including the claudin family in addition to occludin; the increased permeability may result from a shift of major TJ proteins during the transition from the morula to the late expanding blastocyst (Fleming et al., 1993; Tsukita and Furuse, 2000; Furuse et al., 2002; Gonzalez-Mariscal et al., 2003). Occludin is involved in the establishment and maintenance of the permeability seal in early stage blastocysts (Sheth et al., 2000b). Post-translational modification (i.e., phosphorylation) of integral components of the TJ, in addition to protein interactions within the TJ complex, are important for maintaining the blastocyst TJ permeability barrier (Sheth et al., 2000a,b). We would conclude that the disruption of ZO-1 TJ protein distribution observed in the present study signals structural re-arrangements of TJ proteins such as we observed for occludin following ouabain treatment that could result in the increased permeability to FITC-Dextran. Support for this conclusion comes from studies that have examined affects to TJ permeability following knockdown of occludin (Yu et al., 2005; Matlin, 2005). This possibility is also consistent with the proposed mechanisms underlying an increase in paracellular ionic permeability caused by Na^+/K^+-ATPase inhibition in cultured cell lines (Contreras et al., 1999; Rajasekaran et al.,

2001a,b, 2003a,b). The results therefore support that Na^+/K^+-ATPase is a regulator of TJ paracellular permeability in mouse blastocysts.

Previous studies demonstrated that loss of TJ structure and increased paracellular permeability is linked to the disruption of the circumferential actin ring that is localized at the apical pole of polarized epithelial cells (Cooper, 1987; Fleming et al., 1989; Sheth et al., 2000a,b). A reduction in stress fibers projecting from the pre-junctional actin ring, is associated with the loss of TJ membrane contact points and increased TJ permeability in human retinal pigment epithelial cells (Rajasekaran et al., 2003a,b). In MDCK cells, loss of TJ permeability upon Na^+/K^+-ATPase inhibition also is associated with reduced stress fiber content and reduced RhoA GTPase activity (Rajasekaran et al., 2001a,b). Several studies have shown that RhoA GTPase function is also involved in the modulation of TJ structure and function (Rajasekaran et al., 2001a,b) and now even with regulation of occludin function (Yu et al., 2005; Matlin, 2005). Overexpression of wild-type RhoA GTPase bypassed the inhibitory effect of Na^+/K^+-ATPase inhibition on TJ formation, indicating that RhoA GTPase is an essential component downstream of Na^+/K^+-ATPase and also linking Na^+/K^+-ATPase to the formation of functional TJs (Rajasekaran et al., 2001a,b). Other mechanisms that cannot be ruled out include changes in signaling pathways by the intracellular Na^+ increase or K^+ decrease caused by inhibition of Na^+/K^+-ATPase that might alter TJ permeability (Rajasekaran and Rajasekaran, 2003). Na^+/K^+-ATPase-mediated signaling mechanisms involved in the regulation of RhoA GTPase and actin assembly will be an important area of future research that will provide more insight into how ion homeostasis might regulate the role of stress fibers in the assembly and function of TJs.

In conclusion, inhibition of Na^+/K^+-ATPase with ouabain or K^+-free medium may randomize the site of ZO-1 membrane assembly and delay its occurrence. Therefore, while E-cadherin clearly is essential for initial events that trigger the translocation of ZO-1 to the plasma membrane (Rajasekaran et al., 1996). Na^+/K^+-ATPase may be crucial for events that regulate formation of an undisrupted ZO-1 ring, ZO-1-occludin interactions and thus a functional TJ, and consequently completion of TE differentiation. Functional studies to determine the signaling mechanisms that control TJ formation will be required to clarify the role of Na^+/K^+-ATPase in TJ maintenance in the apical trophectodermal membrane domain of mouse embryos.

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