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Deletion of the Na/K-ATPase α1-subunit gene (*Atp1a1*) does not prevent cavitation of the preimplantation mouse embryo

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Abstract

Increases in Na/K-ATPase activity occur concurrently with the onset of cavitation and are associated with increases in Na^+-pump subunit mRNA and protein expression. We have hypothesized that the α1-isozyme of the Na/K-ATPase is required to mediate blastocyst formation. We have tested this hypothesis by characterizing preimplantation development in mice with a targeted disruption of the Na/K-ATPase α1-subunit (*Atp1a1*) using embryos acquired from matings between *Atp1a1* heterozygous mice. Mouse embryos homozygous for a null mutation in the Na/K-ATPase α1-subunit gene are able to undergo compaction and cavitation. These findings demonstrate that trophectoderm transport mechanisms are maintained in the absence of the predominant isozyme of the Na^+-pump that has previously been localized to the basolateral membranes of mammalian trophectoderm cells. The presence of multiple isoforms of Na/K-ATPase α- and β-subunits at the time of cavitation suggests that there may be a degree of genetic redundancy amongst isoforms of the catalytic α-subunit that allows blastocyst formation to progress in the absence of the α1-subunit.

Keywords: Blastocyst; *Atp1a1*; Trophectoderm

1. Introduction

Blastocyst formation (cavitation) is dependent on formation of the trophectoderm epithelium, and is initiated following the establishment of ion gradients and osmotic fluid accumulation across this cell layer (reviewed by Borland, 1977; Wiley, 1984; Biggers et al., 1988; Benos and Balaban, 1990; Watson, 1992; Watson et al., 1999; Watson and Barcroft, 2001). Transporting epithelia utilize the polarized localization of ion transporters and channels within apical and basolateral membrane domains to establish and maintain trans-cellular ion gradients necessary for ion and solute transport, energized by basolateral expression of the Na^+ and K^+ adenosine triphosphatase (Na/K-ATPase). The requirement for polarized expression and activity of the Na/K-ATPase in the trophectoderm has been demonstrated in studies examining cavitation and blastocyst re-expansion in the presence of the Na^+-pump specific inhibitor, ouabain (Powers and Tupper, 1975; DiZio and Tasca, 1977; Powers and Tupper, 1977; Biggers et al., 1978; Wiley, 1984; van Winkle and Campione, 1991; Betts et al., 1997; Baltz et al., 1997), or following disruption of trophectoderm cell polarity with blocking antisera against the cell–cell adhesion molecule E-cadherin (Watson et al., 1990a). Increases in Na/K-ATPase activity occur concurrently with the onset of cavitation (Powers and Tupper, 1975, 1977; Dumoulin et al., 1993; Betts et al., 1998) and are associated with increases in Na^+-pump subunit mRNA (Gardiner et al., 1990; Watson et al., 1990b; Betts et al., 1997) and protein expression (Benos, 1981; Watson and Kidder, 1988; Overstrom et al., 1989; Gardiner et al., 1990). Multiple isoforms of α- and β-subunits (as many as six

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isozymes) are expressed by murine (Watson and Kidder, 1988; Betts et al., 1997, 1998; Jones et al., 1997; Waelchli et al., 1997; MacPhee et al., 2000; Barcroft et al., 2002; Jones et al., 2001 reviewed by Watson and Barcroft, 2001; Kidder, 2002) and bovine (Betts et al., 1997, 1998) preimplantation embryos. The α1-subunit, however, appears to be the predominant isoform expressed within the basolateral membranes of the trophectoderm in all species examined (Watson and Kidder, 1988; Betts et al., 1998; MacPhee et al., 2000; Waelchli et al., 1997). The specific contribution that each expressed Na/K-ATPase subunit has in coordinating blastocyst formation has not yet been determined. Initial characterization of heterozygous mice that express only one copy of the Na/K-ATPase α1-subunit gene (*Atp1a1*$^{+/-}$) has demonstrated that these animals are fertile and generally healthy, whereas homozygous null mice do not survive to birth (James et al., 1999). Our hypothesis was that expression and activity of the α1-isozyme of the Na/K-ATPase within basolateral membranes of the trophectoderm is required to mediate blastocyst formation. We have tested this hypothesis by characterizing preimplantation development in embryos acquired from matings between mice heterozygous for the *Atp1a1* null allele.

2. Methods and materials

2.1. Generation of the Na/K-ATPase α1-subunit knockout mouse

Targeted disruption of the Na/K-ATPase α1-subunit gene was conducted at the University of Cincinnati, (Cincinnati Ohio, USA; James et al., 1999) employing methods of homologous recombination as described previously (Mansour et al., 1988; James et al., 1999). This gene targeting strategy resulted in removal of exons 15–18 of the murine Na/K-ATPase α1-subunit genomic sequence (James et al., 1999). In order to study the preimplantation phenotype of the *Atp1a1* null embryos, re-derivation of the *Atp1a1*$^{+/-}$ mouse line was conducted at the University of Western Ontario by the Robarts Research Institute Transgenics Barrier Facility (London, Ontario). Five pregnant FVBN female mice (mated to *Atp1a1*$^{+/-}$ males [129/Black Swiss]) were shipped to the University of Western Ontario from the University of Cincinnati for blastocyst recovery and embryo transfer into pseudopregnant CD-1 female mice (Robarts Transgenics Barrier Facility). From this re-derivation, two heterozygous founder mice (1 male and 1 female) were obtained. The colony size was increased by mating *Atp1a1*$^{+/-}$ mice onto a CD-1 background. A breeding colony was maintained in the Robarts Barrier Facility in addition to a conventionally housed experimental colony within the Collip Animal Care Facility (Department of Biology, University of Western Ontario). Experiments were conducted on mice of mixed genetic background (129/Black Swiss/FVBN) following 1–5 backcrosses onto the CD-1 background. The number of backcrosses onto the CD-1 background did not affect the outcomes.

3. PCR analysis of genotype

The genotype of 10 day-old pups and preimplantation stage embryos generated from heterozygous matings between *Atp1a1*$^{+/-}$ mice was performed using a polymerase chain reaction-based technique for amplification of genomic DNA. Toe samples or individual preimplantation embryos were lysed in 35 or 10 μl, respectively, of Proteinase K buffer (PKB: 10 mM Tris–HCl [pH 8.0]; 50 mM KCl; 2 mM $MgCl_2$; 0.5% Tween-20; 0.5 mM Proteinase K). Toe samples were digested at 56 °C for 4–5 h and diluted to 400 μl with sterile dH_2O and 2 μl of DNA was used for genotyping employing the Exon16 and HPRT/Exon19 PCR primers (PCR master mix = 1 × Qiagen™ PCR Buffer [1.5 mM $MgCl_2$], 1 × Q Solution™ [Qiagen], 0.2 mM of each dNTP, 0.7 μM each 4ASMS16 [5′-ATCAGAGCCAACAATCCC-3′]/4SMS16primers [5′-GGTTAAATGGGAGGAGAATATG-3′], 5 μM each 1ASMSEX19 [5′-TGGATCATACCTAAGTTGG-3′]/4SHPRT [5′-TACTCCCATTTGTCACGTC-3′] primers, 0.5 units *Taq* DNA poly-merase [Qiagen]) for 35 cycles of amplification consisting of template denaturation at 95 °C for 50 s, template annealing at 50 °C for 50 s and primer extension at 72 °C for 50 s. Preimplantation embryos were digested at 56 °C for 2 h in 10 ul of PKB prior to heat inactivation of the Proteinase K at 95 °C for 5 min. Embryo genotype was determined by employing a 'hot start' method using 2 ul of embryo DNA per reaction for 40 cycles of PCR amplification. PCR amplification products were visualized following electrophoresis on 2% agarose gels containing 0.5 mg/ml Ethidium Bromide by employing the Image Master VDS imaging package (Pharmacia BioTech).

3.1. Characterization of preimplantation development in *Atp1a1*$^{-/-}$ embryos

Male and female *Atp1a1*$^{+/-}$ mice (mixed genetic background: CD-1/FVBN/C57/Black Swiss) were maintained on a 10:14 h dark:light cycle. For production of preimplantation embryos, natural matings between *Atp1a1*$^{+/-}$ female mice (7–12 weeks old) and *Atp1a1*$^{+/-}$ males were conducted. Presence of a vaginal plug the following morning was used to assess successful mating. The morning post-coitum (p.c.) was assigned as embryonic day 0.5 (E0.5) and embryo stages where 8 cell, morula, blastocyst and hatched blastocysts were collected at E2, E2.5–3, E3.5 and E4, respectively. All embryos were retrieved employing the method of Spindle (1980) where flushing medium I (FMI) was used to retrieve 8 cell embryos

from oviducts and morula and blastocysts were recovered from uteri with flushing medium II (FMII) (Jones et al., 1997; MacPhee et al., 2000; Offenberg et al., 2000). The number of embryos retrieved varied between 9–15 embryos per pregnant female. In each experiment, images of individual embryos were collected using a 40 × objective on a Nikon TS100 inverted microscope equipped with a DXM1200 digital camera in conjunction with the Act-1 Image Capture system (Nikon; Version 2.0)

In the first experiment, blastocysts were collected from uteri on embryonic day 3.5. In total, 82 embryos were recovered from 7 pregnant *Atp1a1*$^{+/-}$ mice. Immediately following embryo retrieval, each embryo was washed 3 × in fresh FMII and then placed individually into KSOMaa medium drops (2 μl) under oil (Erbrach et al., 1994; Jones et al., 1997). KSOMaa medium was prepared as described in Ho et al. (1995). The amino acids used to supplement this medium where diluted to 1 × with KSOM medium and included both MEM essential and non-essential amino acid formulations (Gibco BRL). We consistently observe a high rate of development of normal zygotes to the blastocyst stage (80–85%) when this medium is used. Embryos were scored for morphology (presence of a blastocoel cavity), measurements of blastocoel diameter were made and images of each embryo were recorded. Following morphological assessment, embryos were lysed in PKB and embryo genotype was determined. Measurements of blastocoel diameter were analyzed by one-way ANOVA with Tukey–Kraemer test to determine whether differences in blastocoel diameter were significant ($P < 0.05$).

In the second experiment, embryos were collected at the compacting 8-cell stage (E2) from oviducts of *Atp1a1*$^{+/-}$ mice. In total 8 pregnant females were utilized, yielding 88 compacted 8-cell embryos. Immediately after embryo retrieval, embryos were washed 3X in fresh FMI. Individual embryos were placed into 2 μl drops of KSOMaa under oil. Initial images of each embryo were collected, and embryos were cultured for 36 h at 37 °C under a humidified 5% CO_2 in air atmosphere. Embryos were scored at 4 h intervals for a period of 24 h with a final image of each embryo collected at 36 h before embryos were lysed in 10 μl of PKB for analysis of genotype.

In the third experiment, embryos were flushed at E4.0 from 4 pregnant female *Atp1a1*$^{+/-}$ mice, and 41 in vivo hatched blastocysts were recovered. Washed blastocysts were transferred individually to 2 μl drops of KSOMaa under oil. Embryo morphology was scored and a time 0 post-flush image for each embryo was collected before embryos were cultured for 24 h at 37 °C under a humidified 5% CO_2 in air atmosphere. Images were also collected at 8, 12 and 24 h post embryo retrieval to assess: (1) blastocoel expansion (8 h); (2) presence of a blastocoel cavity (12 h); and (3) evidence of cell attachment and trophoblast outgrowth (24 h post embryo retrieval). Embryos were lysed at the end of the 24 h culture interval and embryo genotype was determined.

3.2. Whole-mount indirect immunofluorescence analysis

Female *Atp1a1*$^{+/-}$ mice (3–5 weeks old) were superovulated by administering 5 IU PMSG (Sigma) i.p. followed 48 h later by 5 IU hCG (Sigma) i.p. and mated to *Atp1a1*$^{+/-}$ males. Embryo stages were collected as follows: 1) morulae (80–85 h post-hCG) and 2) blastocysts (90 h post-hCG). All embryos were fixed in methanol and processed for application of whole-mount indirect immunofluorescence using a 1:100 dilution of mouse monoclonal $IgG_{1\kappa}$ anti-Na^+/K^+-ATPase α1 [Clone C464.6; Upstate Biotechnology] as described (Pietrini et al., 1992, 1994; Gottardi and Caplan, 1993a,b; Barcroft et al., 1998; Betts et al., 1998; MacPhee et al., 2000). Immunofluorescence localization of Na/K-ATPase α1 subunit protein was analyzed using either a Nikon Eclipse E1000 epifluorescence microscope equipped with a Nikon DXM1200 digital camera or a BioRad MRC-600 (BioRad) Confocal Laser Scanning Microscope (Department of Chemistry, The University of Western Ontario). Images were collected using the Act-1 (Version 2.0; Nikon) or COMOS (BioRad) image capture software packages, respectively.

3.3. Post-immunofluorescence genotyping of preimplantation embryos

To correlate protein expression patterns with preimplantation embryo genotype, individual murine embryos were processed for post-immunofluorescence genotype analysis as described previously (De Sousa et al., 1997).

4. Results

4.1. Early development of Na/K-ATPase α1-subunit-null embryos

Deletion of the Na/K-ATPase α1-subunit resulted in the absence of homozygous null offspring at birth and as early as E8.5 (James et al., 1999). In the first experiment, 82 morula/blastocyst stage embryos were flushed from the uteri of pregnant *Atp1a1*$^{+/-}$ mice on E3.5 to examine the ability of wildtype (+/+), heterozygous (+/−) and homozygous null (−/−) embryos to undergo cavitation in vivo. Genotype analysis of these embryos demonstrated the presence of 19 wildtype (23%), 44 heterozygous (54%) and 19 (23%) homozygous null embryos (Fig. 1a) representing a genotype distribution which did not vary significantly ($P < 0.05$) from the expected Mendelian distribution for independent sorting of one gene with two alleles ($\chi^2 = 0.44$; degrees of freedom [df] = 81). Of this pool of embryos, 89% (17/19) wildtype, 88% (39/44) heterozygous and 100% (19/19) homozygous null embryos were at the blastocyst stage at the time of embryo retrieval. Among blastocysts collected on embryonic day 3.5, there was no visible morphological difference between embryos

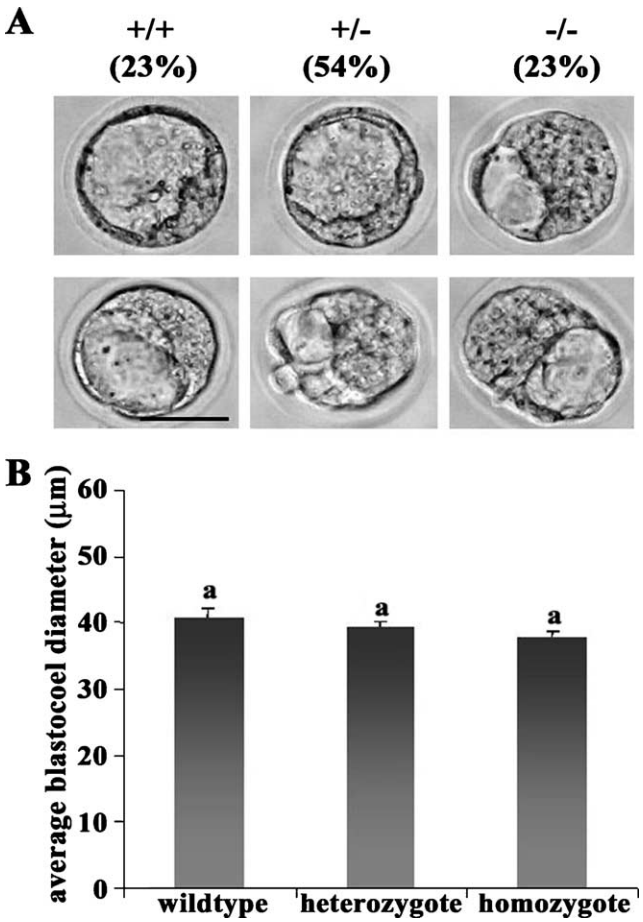

Fig. 1. Genotype distribution, morphology and average blastocyst diameter from populations of wildtype (+/+) heterozygous (+/−) and homozygous (−/− null) *Atp1a1* embryos collected at the blastocyst stage of development. (A) The genotype distribution of blastocysts collected on embryonic day 3.5 approximated the expected distribution for 1 gene with 2 alleles, demonstrating that null (−/−) embryos are able to progress to the blastocyst stage despite lacking both copies of wildtype α1-subunit alleles. Visual analysis of overall blastocyst morphology demonstrated no detectable difference in blastocyst appearance dependent on embryo genotype, and average blastocoel diameter at the time of embryo retrieval did not vary significantly ($P < 0.05$) between embryos of different genotypes (B).

of different genotypes (Fig. 1a) and no significant difference ($P < 0.05$) in mean blastocoel diameter between genotypes (Fig. 1b).

In the second experiment, embryos collected at the compacting 8-cell stage ($n = 88$) were cultured individually in KSOMaa medium for a total period of 36 h to observe genotype-dependent patterns of early embryo development in vitro. From these embryos 23 wildtype (26%), 44 heterozygous (50%), and 21 homozygous (24%) embryos were identified. The genotype distribution of this embryo population was not significantly different ($P < 0.05$) from the expected Mendelian distribution ($\chi^2 = 0.29$; df $= 87$). Embryos progressed to the compacted morula stage within 12–16 h of embryo retrieval independently of genotype (Fig. 2). By 24 h post-retrieval, 100% of wildtype, 87% of heterozygous and 80% of homozygous mutant embryos had initiated cavitation (Fig. 2) and all embryos had undergone cavitation by 26–27 h. In order to examine genotype-dependent maintenance of blastocyst morphology, embryos were cultured for an additional 12 h (36 h post embryo retrieval) before a final image was collected and PCR genotype analysis was conducted. At 36 h post-retrieval, 100% of wildtype and heterozygous embryos were expanded blastocysts (Fig. 2). In contrast, 0 of the 21 null embryos maintained a visible blastocoel cavity at this time. By 36 h post-retrieval, all *Atp1a1* homozygous null embryos demonstrated cell dissociation within the zona pellucida that was characterized by blastomere rounding/swelling and fragmentation (Fig. 2).

4.2. Embryos homozygous for the α1-subunit null mutation are unable to form trophoblast outgrowths in vitro

In order to determine whether *Atp1a1* null blastocysts were able to attach to plastic and form trophoblast outgrowths in vitro 41 in vivo hatched blastocysts were recovered on E4 (Dickson, 1967). The hatched blastocysts collapsed during recovery in all genotypes (Fig. 3). Embryos were cultured for a 24 h interval following retrieval to analyze blastocoel re-expansion (8 h), maintenance of a blastocoel cavity (12 h) and evidence of trophoblast outgrowth (24 h). The hatched blastocysts consisted of 9 wildtype (22%), 20 heterozygous (49%) and 12 homozygous null (29%) embryos, which did not vary significantly ($P < 0.05$) from the expected Mendelian distribution ($\chi^2 = 0.45$; df $= 40$). Within 8 h of embryo retrieval, 88.9% of wildtype, 90% of heterozygous and 75% of homozygous null embryos displayed the presence of a fluid cavity indicative of blastocoel re-expansion (Fig. 3). At 12 h post-retrieval, while all wildtype and heterozygous embryos maintained a visible blastocoel cavity, only 25% of null blastocysts still had a visible cavity. At this time, the majority of *Atp1a1* homozygous null embryos were characterized by blastomere rounding/swelling and cellular dissociation (Fig. 3). At 24 h of embryo culture, all *Atp1a1* null blastocysts demonstrated cell dissociation and dispersal of embryonic blastomeres across the bottom of the culture drop, while 100% of wildtype and 90% of heterozygous embryos had attached and developed into trophoblast outgrowths (Fig. 3).

4.3. Cavitation occurs in the absence of immuno-detectable levels of oogenetic α1-subunit protein

The possible presence of oogenetically derived α1-subunit protein in morula/blastocyst stage *Atp1a1*$^{-/-}$ embryos was assessed by whole-mount indirect immunofluorescence. Embryo recovery following immunofluorescence resulted in the retrieval of 83 embryos including 35 blastocysts and 27 morulae that were successfully genotyped (Fig. 4). With morulae, all wildtype (11/11) and heterozygous (7/7) embryos had immunodetectable α1-subunit protein. *Atp1a1*$^{-/-}$ morulae, however, did not display any

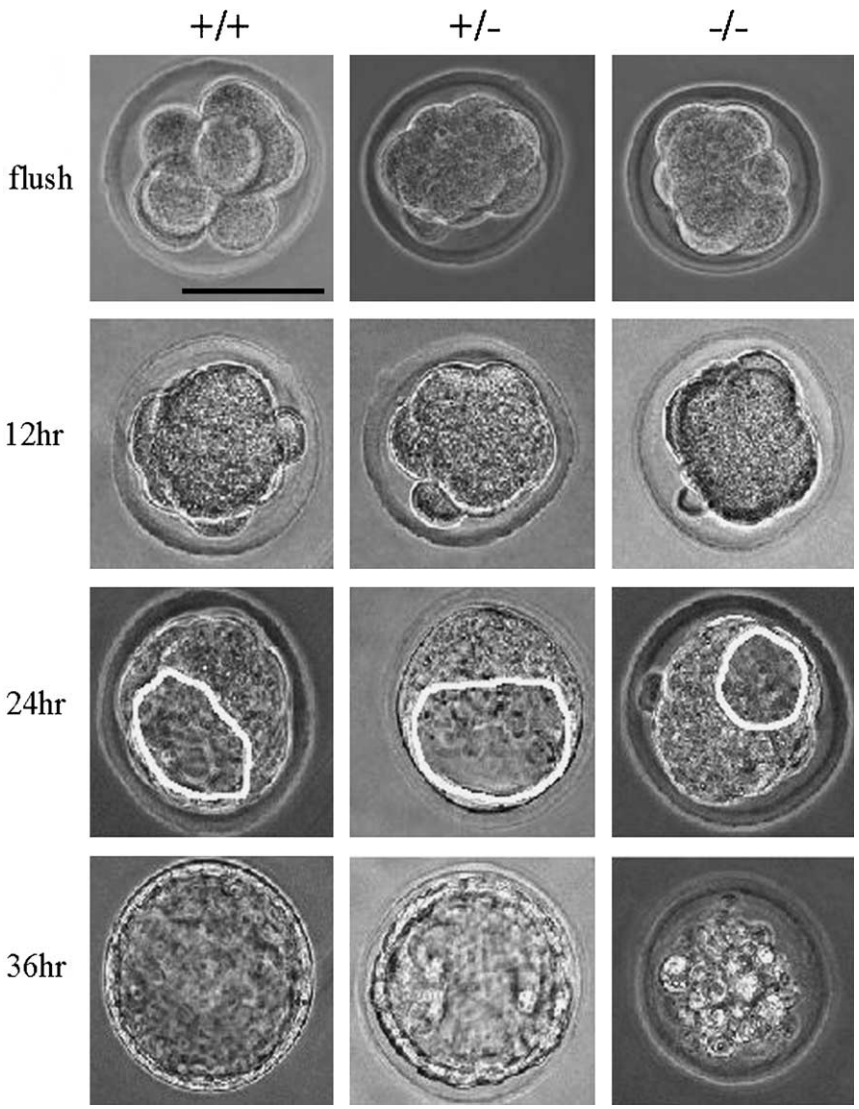

Fig. 2. In vitro development and morphology of *Atp1a1* embryos collected at the compacting 8-cell stage. Wildtype (+/+), heterozygous (+/−) and homozygous null (−/−) embryos all progress through compaction and cavitation. Representative embryos at the morula and blastocyst stage are shown following 12 and 24 h embryo culture, respectively, for each genotype (blastocoel cavities are indicated by the outline in the 24 h panels). Following an additional 12 h culture interval (36 h; time = post-embryo retrieval), null blastocysts had all undergone cell dissociation and loss of an expanded blastocyst morphology. Scale bar = 50 μm.

fluorescence attributable to α1-subunit immunofluorescence (0/9 embryos; Fig. 4). An identical pattern was observed with blastocysts, where 13/13 wildtype and 13/13 heterozygous blastocysts were immuno-positive for Na/K-ATPase α1-subunit within the basolateral cell margins of trophectoderm and all cell surfaces of ICM cells (Fig. 4). Once again, all identified null blastocysts (9/9) lacked detectable levels of α1-subunit immunoreactivity (Fig. 4).

5. Discussion

We hypothesized that the α1-subunit of the Na/K-ATPase is required to mediate blastocyst formation. We have tested this hypothesis by characterizing preimplantation development in a mouse line lacking both copies of the Na/K-ATPase α1-subunit (*Atp1a1*), using embryos acquired from matings between *Atp1a1* heterozygous mice (James et al., 1999). Animals heterozygous for this mutation appear healthy and are fertile, although changes in muscle contractile properties have been observed in adult animals (James et al., 1999; He et al., 2001). However, matings between heterozygous animals do not produce homozygous mutant offspring, indicating an essential function of the Na/K-ATPase α1-subunit during normal embryonic development (James et al., 1999). Several studies have provided evidence supporting the role of the Na/K-ATPase in establishing ion gradients across the trophectoderm cell layer to facilitate the accumulation of water within the blastocoel cavity. Treatment of mammalian preimplantation embryos with the Na^+-pump inhibitor, ouabain, blocks blastocyst formation (Wiley, 1984). Ouabain also blocks

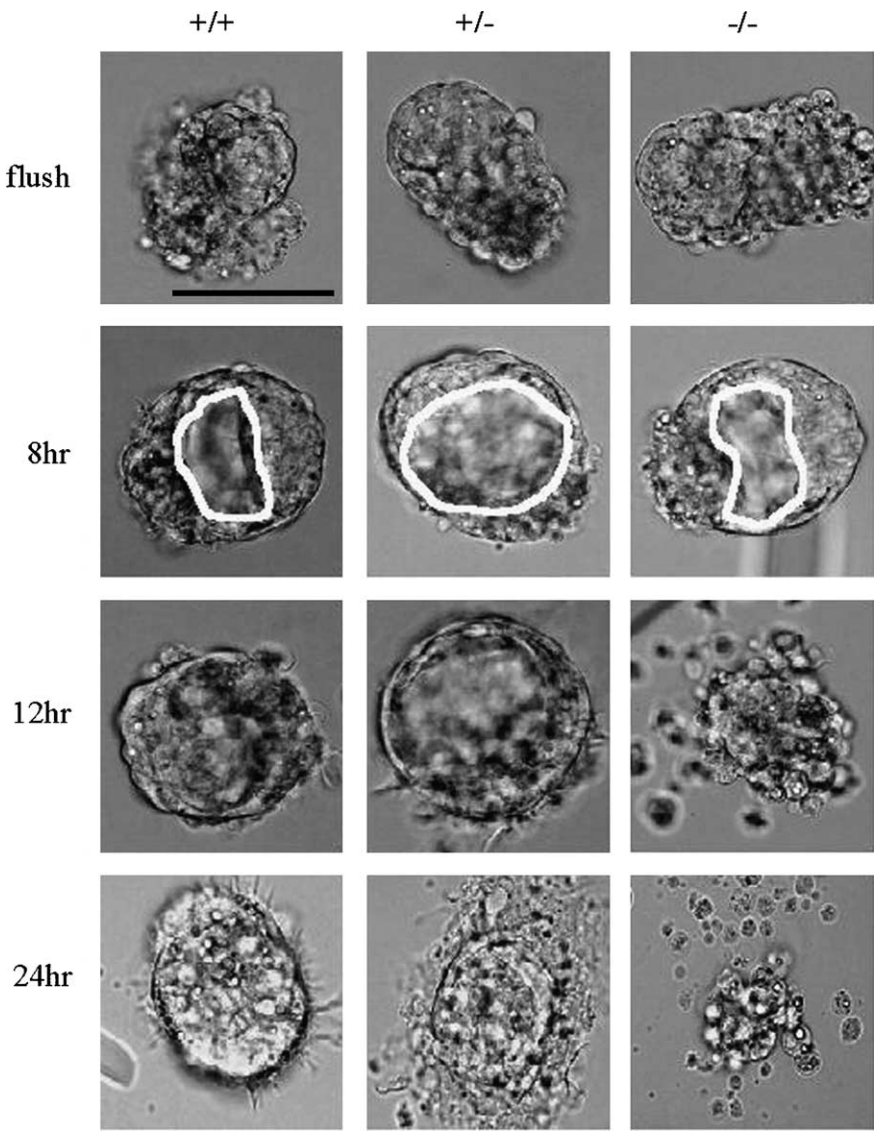

Fig. 3. Morphology of in vivo hatched *Atp1a1* blastocysts at various time points from embryo retrieval. Hatched blastocysts recovered from uteri at embryonic day 4 were cultured in 2 μl drops of KSOMaa for 24 h. At retrieval (flush), blastocysts of each genotype demonstrated collapsed blastocoel cavities. Following 8 h culture (8 h) greater than 90% of blastocysts from wildtype (+/+), heterozygous (+/−) and homozygous null (−/−) populations demonstrated re-expansion of the blastocoel cavity (blastocoel cavities outlines). By 12 h post embryo retrieval (12 h), null blastocysts demonstrated cell dissociation. By 24 h post retrieval (24 h), while wildtype and heterozygous embryos demonstrated evidence of attachment and trophoblast outgrowth, all null blastocysts had undergone cellular dissociation. Scale bar = 50 μm.

re-expansion of the blastocoel cavity following cytochalasin induced collapse in expanded blastocysts (DiZio and Tasca, 1977; Manejwala et al., 1989; Betts et al., 1997), as does disruption of the polarity of Na^+-pump expression within the trophectoderm (Watson et al., 1990a; Wiley et al., 1990). Additionally Na^+-pump activity undergoes a significant increase at the morula-blastocyst transition as measured by ouabain-sensitive $^{86}Rb^+$ transport (van Winkle and Campione, 1991; Betts et al., 1998). These past studies have formed the basis for our underlying hypothesis regarding the role of the α1-subunit in mediating cavitation.

Surprisingly, initial analysis of preimplantation embryo development following mating of *Atp1a1*$^{+/-}$ animals demonstrated that homozygous null embryos progress to the blastocyst stage and are indistinguishable from their wildtype and heterozygous counterparts. In vitro analysis of blastocyst stage embryos demonstrated that loss of α1-subunit expression is associated with subsequent loss of cell–cell association, cell rounding and fragmentation suggesting a loss of intracellular ion homeostasis. Most mammalian cells possess an intracellular ionic milieu characterized by high $[K^+]_i$ and low $[Na^+]_i$ which is maintained by the Na/K-ATPase (Lechene, 1988; Skou, 1992). The electrochemical gradients established and maintained as a result of Na^+-pump activity drive co- and counter transport processes that effect membrane potential, nutrient uptake, intracellular pH regulation, maintenance of normal cell volume, osmotic pressure and normal rates of

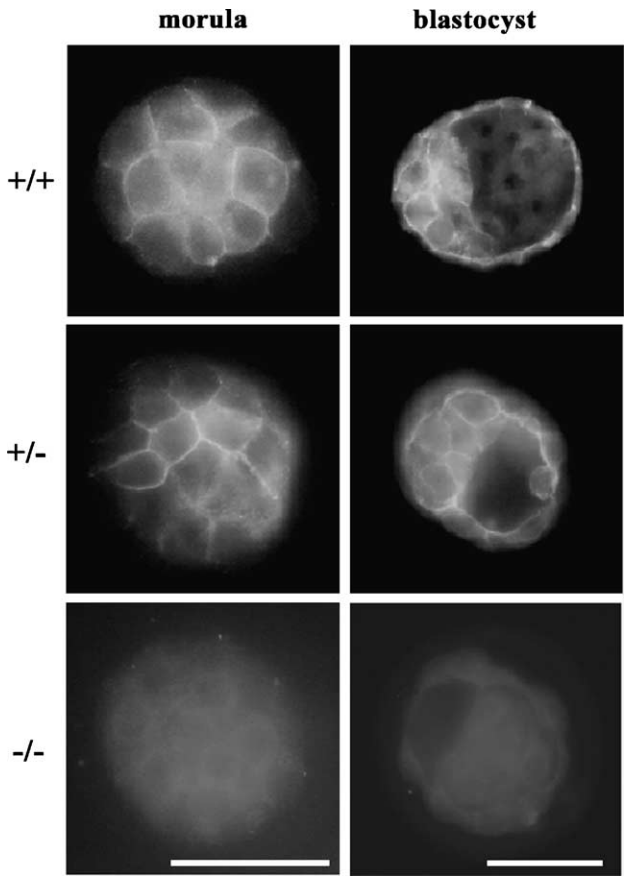

Fig. 4. Immunolocalization of α1-subunit protein in morula and blastocyst stage embryos from *Atp1a1*$^{+/-}$ crosses. Embryos were collected at 80 h (morula) or 90 h (blastocyst) post hCG. Numbers in parenthesis represent the number of embryos that were immunopositive for α1-subunit for each of wildtype (+/+), heterozygote (+/−) and homozygous null (−/−) in relation to the number of embryos recovered for each genotype. Null embryos at both developmental stages all lacked detectable levels of α1-subunit immunoreactivity. Scale bar = 50 μm.

protein synthesis (Tosteson, 1964; Schwartz et al., 1972; DiZio and Tasca, 1977; Ledbetter and Lubin, 1977; Lechene, 1988; Van Winkle et al., 1988; Cohen and Lechene, 1989; Petronini et al., 1989; van Winkle and Campione, 1991; Skou, 1992). Baltz et al. (1997) demonstrated that ouabain inhibition of Na^+-pump activity in morulae results in replacement of approximately 50% of initial $[K^+]_i$ (initial $[K^+]_i = 132$ mM) with Na^+ within a 5 h interval ($[Na^+]_i$ increasing from 15 to ~80 mM) when embryos were cultured in K^+-free medium in the presence of 5 mM ouabain. The rate of Na^+ exchange for K^+ observed in embryos is considerably slower than that observed for other cells (Lechene, 1988; van Winkle and Campione, 1991) where $[K^+]_i$ is reduced by 50% over a period of less than 2 h in the presence of ouabain. Baltz et al. (1997) suggested that this slow rate of exchange implies that embryos do not require a particularly high level of Na/K-ATPase activity prior to the blastocyst stage to maintain intracellular Na^+ and K^+ concentrations. Following culture of compacting 8-cell embryos to the blastocyst stage we have demonstrated that *Atp1a1* homozygous null embryos undergo compaction and cavitation in a manner similar to their littermates. Within 12 h from the onset of fluid accumulation, however, null embryos all demonstrate morphological characteristics associated with loss of ionic homeostasis, cell volume dysregulation and cellular necrosis. Clearly, the outcomes from the first and second experiments suggest that the developmental potential of the α1-subunit nulls is greater in in vivo derived embryos. This would suggest that the impact of deleting the α1-subunit is more extreme in cultured embryos as this could reflect the additional stress that the culture environment places on the embryo. Additional studies are required to determine if this is so.

Assessment of *Atp1a1*$^{-/-}$ embryos demonstrated a lack of immuno-detectable levels of Na/K-ATPase α1-subunit protein as early as the morula stage of development, suggesting that the processes of compaction and cavitation are able to proceed in the absence of this Na^+-pump isoform. Unlike E-cadherin (Larue et al., 1994; Riethmacher et al., 1995) and α-catenin (Torres et al., 1997), oogenetically derived α1-subunit protein does not appear to persist to the morula stage and thus may not contribute to the initiation of cavitation. Both of the Na/K-ATPase α1- and α3- subunit proteins are present in murine blastocysts within the basolateral cell margins and cytoplasm of the trophectoderm, respectively, while the α1 subunit is also present within the cell margins of the inner cell mass (MacPhee et al., 2000). Although α-subunit content does not change dramatically over the course of mouse development from the 1-cell to blastocyst stage on a per cell basis, β-subunit protein expression increases 9-fold between day 1 and day 4 of development (Gardiner et al., 1990). The profile of β-subunit accumulation during this interval (Gardiner et al., 1990; Watson et al., 1990b) has led to the hypothesis that increased β-subunit expression at the morula stage triggers insertion of Na^+-pumps into the trophectoderm plasma membrane driving the establishment of trans-trophectodermal ion gradients and initiation of trans-trophectoderm fluid accumulation.

Isoforms of the α-subunit posess different Na^+, K^+ and ATP affinities (Jewell and Lingrel, 1991; Munzer et al., 1994; Daly et al., 1996; Blanco and Mercer, 1998). In particular, the α3-subunit has been shown to have a four-fold lower affinity for Na^+ than that observed for the α1-subunit (Munzer et al., 1994). Since each of the 4 α-subunit isoforms are capable of associating with any of the 3 β-subunit isoforms (Blanco et al., 1993, 1995a,b; Crambert et al., 2000) it is possible that cytoplasmic α3-protein within the trophectoderm of the mouse embryo may be shuttled to the plasma membrane as β-subunit expression increases, providing an avenue for basolateral transport of Na^+ into the nascent blastocoel of *Atp1a1*$^{-/-}$ embryos. We have previously suggested that apical localization of the α3-subunit within the trophectoderm of bovine blastocysts may serve to modulate intracellular Na^+ concentrations during

cavitation (Betts et al., 1998), similar to its role in other cell systems that deal with high intracellular Na^+ loads (Zahler et al., 1996). In this model, Na^+-pump α1-subunit is considered to be the ubiquitous 'house keeping' enzyme which functions at near saturation under conditions of basal and elevated $[Na^+]_i$, whereas the lower affinity α3-isoform becomes relevant as $[Na^+]_i$ increases beyond the turnover capacity of α1-isozymes preventing disruption to cellular processes inhibited by high [Na]. The trophectoderm possesses several mechanisms for apical entry of Na^+ (Miller and Schultz, 1985; Manejwala et al., 1989; Robinson et al., 1991; Barr et al., 1998) that would result in cellular accumulation of Na^+ in the absence of basolateral α1-subunit expression, resulting in elevations of intracellular $[Na^+]$. These elevations in Na^+ concentration may provide conditions under which basolateral transport of Na^+ by an α3/β1 isozyme could generate a sufficient osmotic gradient to drive cavitation. Subsequent embryo failure may then be attributed to continue accumulation of Na^+ within cells of the trophoblast and eventual disruption of membrane potential, cell volume regulation and Na^+-sensitive cellular processes such as protein synthesis. Future studies will investigate the possibility that assembly and insertion into the membrane of an α3/β1 isozyme of the Na/K-ATPase underlies the ability of *Atp1a1*$^{-/-}$ embryos to undergo cavitation.

Identification of all three embryo genotypes within populations of flushed in vivo hatched blastocysts clearly demonstrates that failure of *Atp1a1*$^{-/-}$ embryos occurs well after initiation of cavitation and suggests that these embryos persist until the peri-implantation phase of development. The third experiment examined whether it was possible for zona hatched null embryos to attach and form outgrowths in vitro. Although the genotype ratio of the embryos recovered following hatching of blastocysts in vivo was very close to the expected 1:2:1 ratio observed earlier for E3.5 blastocysts, there were slightly fewer (22%) wildtype hatched blastocysts recovered at this time point. This reduction may reflect potential differences in the timing of initiation of interactions between trophoblast and uterine epithelia between wildtype and heterozygous embryos. Morphological analysis of homozygous null embryos at the 24 h time point following embryo retrieval demonstrated that dissociated embryonic blastomeres were loosely distributed over the bottom of the culture drop with no evidence of cell attachment to the culture dish surface unlike cells of wildtype and heterozygous counterparts. While this assay is not a definitive test of the implantation ability of these embryos it clearly demonstrated that the ability of null blastocysts to attach and form outgrowths is compromised compared to heterozygous and wildtype littermates. A role for Na/K-ATPase expression and activity in the ability of cells to attach to fibronectin has been demonstrated (Belusa et al., 2002). These findings suggest that not only epithelial cell–cell adhesion and polarity (Contreras et al., 1999; Rajasekaran et al., 2002) but also the ability of cells to interact with the extracellular matrix are influenced by signaling mechanisms linked to the Na/K-ATPase (Belusa et al., 2002). In addition to resulting in eventual death of embryonic blastomeres as a result of loss of cellular ionic homeostasis, loss of the α1-subunit may also affect the ability of *Atp1a1* null trophoblast cells to initiate interactions with the uterine epithelium, ensuring a peri-implantation lethality as a result of this null mutation.

In summary, we have demonstrated that mouse embryos homozygous for a null mutation in the Na/K-ATPase α1-subunit gene are able to undergo compaction and cavitation, but subsequently fail during the peri-implantation phase of development. These findings demonstrate that trophectoderm transport mechanisms are maintained in the absence of the predominant isozyme of Na^+-pump localized in the basolateral membranes of mammalian trophectoderm cells (Benos, 1981; Watson and Kidder, 1988; Watson et al., 1990b; Waelchli et al., 1997; Betts et al., 1998; MacPhee et al., 2000). The observation that mammalian blastocysts express multiple isoforms of α- and β-subunits at the time of cavitation (reviewed by Watson and Barcroft, 2001; Kidder, 2002) suggests that there may be a degree of genetic redundancy amongst α-subunit isoforms that allows blastocyst formation to progress in the absence of the α1-isoform. It is clear, however, that continued expression of the α1/β-isozyme of the Na/K-ATPase is required to support mammalian development beyond the preimplantation phase of development.

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