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Na/K-ATPase beta1 subunit expression is required for blastocyst formation and normal assembly of trophectoderm tight junction-associated proteins.

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NA/K-ATPASE β1 SUBUNIT EXPRESSION IS REQUIRED FOR BLASTOCYST FORMATION AND NORMAL ASSEMBLY OF TROPHECTODERM TIGHT JUNCTION ASSOCIATED PROTEINS*

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Na/K-ATPase plays an important role in mediating blastocyst formation. Despite the expression of multiple Na/K-ATPase α and β isoforms during mouse preimplantation development, only the α1 and β1 isoforms have been localized to the basolateral membrane regions of the trophectoderm. The aim of the present study was to selectively down-regulate the Na/K-ATPase β1 subunit employing microinjection of mouse 1 cell zygotes with small interfering RNA (siRNA) oligos. Experiments comprised of non-injected controls and two groups microinjected with either Stealth™ Na/K-ATPase β1 subunit oligos or non-specific Stealth™ siRNA as control. Development to the 2-, 4-, 8-, 16-cell and morula stages did not vary between the three groups. However, only 2.3% of the embryos microinjected with Na/K-ATPase β1 subunit siRNA oligos developed to the blastocyst stage as compared to 73% for control injected and 91% for non-injected controls. Na/K-ATPase β1 subunit down regulation was validated by employing RT-PCR and whole-mount immunofluorescence methods to demonstrate that Na/K-ATPase β1 subunit mRNAs and protein were not detectable in β1 subunit siRNA microinjected embryos. Aggregation chimera experiments between β1 subunit siRNA microinjected embryos and controls demonstrated that blockade of blastocyst formation was reversible. The distribution of Na/K-ATPase α1 and tight junction associated proteins Occludin and ZO-1 were compared among the three treatment groups. No differences in protein distribution were observed between control groups however, all three polypeptides displayed an aberrant distribution in Na/K-ATPase β1 subunit siRNA microinjected embryos. Our results demonstrate that the β1 subunit of the Na/K-ATPase is required for blastocyst formation and that this subunit is also required to maintain a normal Na/K-ATPase distribution and localization of tight junction associated polypeptides during preimplantation development.

Research investigating the mechanisms directing blastocyst formation has demonstrated that: a) the Na/K-ATPase assumes a polarized distribution confined to the trophectoderm basolateral membrane regions just prior to the onset of cavitation (1,2); b) expression of Na/K-ATPase subunit genes are up-regulated during the morula to blastocyst transition (3-7); c) that Na/K-ATPase activity is significantly increased during the morula to blastocyst transition for a number of mammalian species (2,8-10); d) that treatment with ouabain (a potent and specific inhibitor of the Na/K-ATPase) affects cavitation and blastocyst formation in a number of mammalian species (5,11-15); e) that deletion of the Na/K-ATPase α1 subunit gene product is linked to aberrant blastocyst formation in vitro and likely peri-implantation lethality in vivo (16); and f) that Na/K-ATPase also regulates the formation and function of trophectoderm tight junctions (17).

Taken together, these data support the hypothesis that the Na/K-ATPase contributes directly to the mechanism that regulates fluid movement across the trophectoderm resulting in the formation of the fluid-filled blastocoelic cavity. Our most recent efforts have initiated

studies to investigate the individual roles of each Na/K-ATPase subunit during preimplantation development (16) and to explore whether the Na+-pump also regulates tight junction formation and function during blastocyst formation (17). Much to our initial surprise we discovered that it was possible to collect quite normal looking day 3.5 post-hCG Na/K-ATPase α1 null blastocysts from the reproductive tracts of heterozygous mice (16). However these α1 null embryos struggle to attach and form trophoblast outgrowths in culture and do not form fully expanded blastocysts if collected and placed into culture at the 8-cell stage for determination of their developmental potential (16). Therefore we have concluded that the α1 subunit of the Na/K-ATPase is required for normal development and initiation of pregnancy and that limited compensation perhaps by an alternative Na/K-ATPase α subunit isoform is able to allow for short term development of α1 null embryos to the blastocyst stage in vivo.

In the continuation of our pursuit of an understanding of the roles of each Na/K-ATPase subunit during preimplantation development, we have conducted the present study to characterize the consequences to early development and blastocyst and tight junction formation following the selective down-regulation of the Na/K-ATPase β1 subunit by employing microinjection of 1 cell mouse zygotes with small interfering RNA (siRNA) oligos. Our results demonstrate that the β1 subunit of the Na/K-ATPase is required for blastocyst formation and that this subunit is required to maintain a normal Na/K-ATPase distribution and localization of tight junction associated polypeptides during preimplantation development.

Experimental Procedures

Super-ovulation and embryo collection - Female CD-1 mice (Charles River, Canada), 4-5 weeks of age, were injected with 10 IU of PMSG (pregnant mare's serum gonadotrophin; Intervet, Whitby, Canada), followed by 10 IU of hCG (human chorionic gonadotropin; Intervet, Whitby, Canada) 48 h later and just before mating with CD-1 males. Successful mating was determined the next morning by the presence of a vaginal plug and was considered day 0.5 of development. One-cell stage embryos were flushed from oviducts of female mice using flushing medium I (1.71 mM calcium lactate, 0.25 mM sodium pyruvate, 3 mg/ml BSA and 10x Leibovitz-modified Hanks balanced salt solution, all diluted with water to 1x) (Spindle, 1980) containing hyaluronidase (1 mg/ml, Sigma, St Louis, Mo.). The embryos were washed 3x in potassium simplex optimized medium (KSOM) media (Summers et al., 1995) under paraffin oil in sterile culture dishes and subsequently cultured in KSOM medium plus amino acids (KSOMaa) (Summers et al., 1995) under a 5% CO_2 in air atmosphere at 37^{o}C until transferred into experimental treatment groups as defined below. All medium components were purchased from Sigma 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 288-298 mOsm. All experiments described in this study, maintained a treatment drop volume to embryo ratio of one embryo per μl of KSOMaa culture medium. Animal care and handling was according to the guidelines of the University of Western Ontario Animal Care Committee.

Microinjection of siRNAs - Microinjection was performed under an inverted microscope using a mechanical micromanipulator (Leica) attached to Picoinjector PLI-100 (Harvard Apparatus, Saint-Laurent, Quebec, CA). Each injection delivered 10 pl of 20 μM siRNA duplexes into the cytoplasm of one-cell stage embryos. Microinjection of embryos was performed according to a standard procedure. One cell embryos were placed in KSOMaa medium under light mineral oil. A holding pipette (Conception Technologies, Colorado, USA) was used to keep the 1-cell embryos stationary during manipulation. An injection pipette (Conception Technologies, Colorado,

USA) loaded with dsRNA solution was inserted into the cytoplasm of each zygote followed by the microinjection of ~10 pl dsRNA. After microinjection, embryos were cultured in KSOMaa medium as described above for up to 4 days to allow for an assessment of developmental capacity to the blastocyst stage. About 300 embryos were used for each experimental replicate and in total a set of three replicates were conducted.

RNA extraction, reverse transcription and polymerase chain reaction (RT-PCR) - Total RNA was extracted from murine embryos (pools of 20 embryos/stage at 1-, 2-, 4-, 8-cell, morula and blastocyst stages) using the phenol chloroform method of Chomczynski and Sacchi (18). The total RNA extracts were digested with deoxyribonuclease (DNAse)-1 to eliminate possible contamination from genomic DNA. The reverse transcription (RT) reactions were conducted using oligo-dT primers (Gibco BRL) as previously described (19,20). Briefly, samples were incubated for 90 min at 42^{o}C in a total volume of 20 µl consisting of 50mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, and 200 units of Superscript II (Invitrogen, Mississauga, Ontario) followed by heating the samples to 95^{o}C for 5 min for termination of reaction.

Polymerase chain reaction (PCR) was conducted using a previously described protocol (21). Briefly, two embryo equivalents for each stage of development under investigation were used per PCR reaction, which was repeated a minimum of three times from pools of three different developmental series of embryos. The conditions for each PCR reaction are given in Table 1. PCR products were resolved on 2.0% agarose gels containing 0.5 µg/ml ethidium bromide (Invitrogen, Burlington, ON). To confirm the specificity of each PCR product, representative amplicons were extracted from the gels and purified using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON) and submitted for nucleotide sequencing (DNA Sequencing Facility, Robarts Research Institute, London, ON, Canada). The nucleotide sequence was subsequently compared to sequences available in GenBank nucleotide sequence database and in all cases the specificity of each PCR product was confirmed. There was 98% sequence identity to mouse Na/K-ATPase β1 subunit sequence.

Embryo fixation and whole-mount indirect immunofluorescence - To analyze the distribution of Na/K-ATPase α and β isoforms, ZO-1 and Occludin polypeptides during murine preimplantation development, a whole-mount immunofluorescence method previously described (21) was employed. Immunofluorescence was detected using laser scanning confocal microscopy as described (19). Briefly, embryos at timed stages of development (1-cell zygotes, 2-,4-,8-cell, morula and blastocyst stages) were washed in 1X phosphate buffer saline (PBS) and then fixed in 2% paraformaldehyde (PFA) in PBS for 20 min at room temperature. These fixed embryos were washed in 1X PBS and either processed immediately for immuno-staining or stored at 4^{o}C in PBS + PHEM for a maximum of 4 weeks.

For immuno-staining, fixed embryos were permeabilized and blocked in 1X PBS + 5% Donkey Serum + 0.01% Triton X-100 for 1 hour at room temperature. Embryos were washed in 1X PBS and incubated with primary antibody diluted 1:100 in 1X PBS +1% Donkey Serum + 0.005% Triton X-100 for one hour at room temperature followed by additional washes totaling 1 hour at 37^{o}C. Primary antibodies were labeled by incubation for one hour with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Jackson Immuno Labs, MA, USA) diluted 1:200. Embryos were then treated with rhodamine-conjugated phalloidin (5µg/ml; 1:20) and 4,6-diamidino-2-phenyl-indole (DAPI) (1mg/ml; 1:2000) for 30 minutes at 37^{o}C followed by 2 washes for 2 hours each at

37°C. Embryos were mounted in Fluoro-Guard Antifade Mounting Reagent (BioRad, Mississauga, ON, Canada). Fluorescence patterns were examined using a Zeiss LSM 410 (laser scanning microscope) with an inverted Axiovert 100 microscope under 20-40X magnification. The images were then captured and stored as TIFF files by the Zeiss LSM software package.

Primary antibodies for Na/K-ATPase α and β subunits and Z0-1 were obtained from Upstate Cell Signaling Solutions, Charlottesville, VA. Primary antibody for Occludin was obtained from Zymed, San Francisco, CA.

siRNA oligo preparation - Double stranded siRNA oligos were designed using BLOCK-iT™ RNAi Designer software (Invitrogen, Mississauga, ON). Stealth small interfering RNA (siRNA) duplex oligoribonucleotides against β1 subunit of Na/K-ATPase (GenBank Accession no: NM_009721) were synthesized by Invitrogen. The sequences were as follows: (i) sense 5'- CCC AAG AAU GAA UCC UUG GAG ACU U-3', antisense 5'- AAG UCU CCA AGG AUU CAU UCU UGG G-3'; (ii) Control sense 5'- CCC AAG AGU CUA UUC AGG AGA ACU U -3', antisense 5' AAG UUC UCC UGA AUA GAC UCU UGG G-3'. The duplex oligoribonucleotides were re-suspended in DEPC-treated water to make a 20 μM solution and stored at -20°C till further use.

Reversal of developmental blockade by aggregation chimeras – Embryos from the three treatment groups (β1 subunit siRNA microinjected, random sequence siRNA injected control and non-injected control) were collected at the non-compacted 8-cell stage. These embryos were treated with acid Tyrode's solution (pH 2.5) to remove zona pellucida. Denuded 8-cell embryos were washed 3 times in fresh KSOMaa drops and aggregated together for chimera production. The β1 subunit siRNA microinjected embryos were either paired with self like (A:A) or with uninjected controls (A:C) or random sequence siRNA injected controls (A:B). In addition, injected controls were also paired together (B:B) to serve as additional control. In total, 76 chimeras were produced from 152 embryos over three replicates. Development of each aggregation chimera combination to the blastocyst stage was assessed. In addition chimeras from each group were processed for immunofluorescence localization of Na/K-ATPase β subunit polypeptides as described.

Statistical analysis - The results are presented as the means ± S. E. M. from three independent experiments. Statistical differences between time points were assessed by analysis of variance (ANOVA). Differences were considered significant when $p < 0.05$. Significant differences between the means were determined using the least-significant-difference test.

RESULTS

To investigate the consequences of Na/K-ATPase β1 subunit down regulation on preimplantation development we employed microinjection of β1 siRNAs into 1-cell zygotes. Our control groups consisted of non-injected zygotes and those injected with a random sequence siRNA. In total over 300 zygotes were placed into each treatment group for evaluation of developmental outcomes. The experiment was repeated 3 times using zygotes collected from different mouse populations each time. The morphology of zygotes in each group four days after injection is displayed in (Figure 1A). Zygotes injected with the Na/K-ATPase β1 siRNA developed through to the morula stage (Figure 1Aa and B). In contrast zygotes injected with control siRNA or non-injected controls displayed a high frequency of development through to the blastocyst stage after injection (Figure 1Ab and c). In both injected groups we observed a nearly equal number of zygotes that did not develop likely due to damage caused by the

microinjection procedure (Figure 1Aa and b). When the data was plotted and analyzed we clearly observed that injection with the Na/K-ATPase β1 subunit siRNA resulted in a significant reduction in the proportion of zygotes that completed development to the blastocyst stage (Figure 1B). Control injected and control non-injected zygotes displayed a comparable blastocyst developmental frequency (Figure 1B). The majority of β1 siRNA injected zygotes attained and then remained at the morula stage 4 days after injection (Figure 1B). Attempts to provide morulae in the Na/K-ATPase β1 siRNA treatment with additional time to progress beyond the morula stage by extending their culture interval for an additional 24 hours did not increase the develop of embryos in this group to the blastocyst stage (data not shown).

To ensure that the Na/K-ATPase β1 siRNAs we employed were effective at down regulating Na/K-ATPase β1 gene expression during early development we applied RT-PCR methods to detect Na/K-ATPase β1 mRNAs and also whole mount immunofluorescence methods to map out Na/K-ATPase β1 protein distribution. It was readily possible to detect transcripts encoding the Na/K-ATPase β1 subunit in control injected and non-injected control groups as indicated by the appearance of an expected size RT-PCR product in samples from both groups (Figure 1C). In contrast it was not possible to detect the Na/K-ATPase β1 RT-PCR product in samples prepared from Na/K-ATPase β1 siRNA injected zygotes (Figure 1C). To ensure that Na/K-ATPase β1 siRNA prepared samples still retained intact RNA, we employed RT-PCR to amplify transcripts encoding β-actin. In all samples from all treatment groups it was readily possible to detect the expected size β-actin cDNA product. The application of whole-mount immunofluorescence methods employing a Na/K-ATPase β1 subunit specific antiserum revealed a complete absence of detectable Na/K-ATPase β1 subunit protein in β1 siRNA injected zygotes (Figure 2 a,b,c). In contrast the Na/K-ATPase β1 subunit was detected in both control injected and non-injected controls beginning at the 8-16 cell stage and also in both morulae and blastocyst stages as reported earlier (Figure 2 e,f,g,h,; (7)). In control blastocysts the Na/K-ATPase β1 subunit immunofluorescence maintained the expected polarized distribution in the trophectoderm and an apolar distribution in the inner cell mass (Figure 2h). When we examined the few blastocysts that formed in the Na/K-ATPase β1 siRNA injected treatment group, no organized Na/K-ATPase β1 subunit protein distribution could be detected (Figure 2d), instead a few small indistinct patches of green fluorescence were observed in these embryos (Figure 2d). In total, these outcomes demonstrate that the Na/K-ATPase β1 siRNA we employed in this study effectively down regulated Na/K-ATPase β1 subunit expression for at least 5 days during mouse preimplantation development.

We investigated the reversibility of the developmental blockade displayed by β1 subunit siRNA microinjected embryos by aggregating together 8-cell embryos from all three treatment groups to measure chimera formation and progression to the blastocyst stage. The β1 subunit siRNA microinjected embryos (Figure 4 A:A; Panel A) did not progress to the blastocyst stage. In contrast, β1 subunit siRNA microinjected embryos which were paired with either random sequence siRNA microinjected (A:B), uninjected controls (A:C) or even injected controls paired together (B:B) all displayed a normal progression to the blastocyst stage (Figure 4 Panel A). In addition localization of Na/K-ATPase β subunit polypeptides in these chimeras by the application of immunofluorescence methods confirmed the complete absence of β subunit immunofluorescence in A:A (β subunit siRNA

injected) pairs (Figure 5a), normal β subunit immunofluorescence in B:B pairs (Figure 5c) and reduced but obvious β subunit immunofluorescence in A:B pairs (Figure 5i).We would conclude that development of the A:B and A:C chimeras to the blastocyst stage (Figure 4) was achieved by expression of Na/K-ATPase β subunits by cells derived from the B or C embryo. This experiment therefore supports our initial conclusion that Na/K-ATPase β subunit expression is required for progression to the blastocyst stage.

These studies clearly indicated that down regulation of Na/K-ATPase β1 subunit expression resulted in a blockade of blastocyst formation. To begin to define the Na/K-ATPase β1 subunit's role in mediating blastocyst formation, we investigated the effects of β1 subunit down regulation on the distribution of Na/K-ATPase α1 polypeptides and tight junction associated polypeptides, Occludin and ZO-1, since these polypeptides are among the best characterized markers of trophectoderm differentiation and blastocyst formation. We confined our analysis to morula stage embryos since it was not possible to generate blastocysts following microinjection of Na/K-ATPase β1 subunit siRNAs. In control injected and also control non-injected morulae we observed normal patterns of Na/K-ATPase α and ZO-1 and Occludin protein distribution (Figure 3 b,d,f). For Na/K-ATPase α1 protein the fluorescence extended in an apolar fashion surrounding each morula blastomere (Figure 3b). Likewise both ZO-1 and Occludin fluorescence maintained a tight cortical pattern surrounding the apical regions of each blastomere (Figure 3 d, f). In complete contrast Na/K-ATPase β1 siRNA injected morulae displayed a very aberrant Na/K-ATPase α1 fluorescence pattern in which the fluorescence became "discontinuous" around each cell periphery, consisting more of a general cytoplasmic distribution rather than a cortical distribution (Figure 3a). The impact on ZO-1 and Occludin protein distribution was even more dramatic and the fluorescence pattern for both of these tight junction associated proteins became very diffuse and cytoplasmic in nature (Figure 3 c, e). Injection of Na/K-ATPase β1 siRNAs resulted in a complete loss of the tight "continuous" cortical distribution that is so typical for both ZO-1 and Occludin protein distributions in developing epithelia such as the trophectoderm.

DISCUSSION

Our results demonstrate that the Na/K-ATPase β1 subunit protein is required for blastocyst formation. In addition, our results indicate that the Na/K-ATPase β1 subunit oversees the proper localization of Na/K-ATPase α1 subunit to the cortical membrane regions of each blastomere and also the proper distribution and assembly of tight junction associated polypeptides (ZO-1 and Occludin) to the apical membrane regions between differentiating trophectoderm cells. Interestingly, our results do not however indicate that the Na/K-ATPase β1 subunit is required to support early development to the morula (16-32 cell) stage of mouse development. We conclude that the Na/K-ATPase β1 subunit is instrumental in coordinating the proper insertion of Na/K-ATPase α/β subunits to appropriate membrane domains and also the formation and establishment of trophectoderm tight junctions. Thus the Na/K-ATPase β1 subunit is an important mediator of cell polarity, trophectoderm differentiation and blastocyst formation during mouse preimplantation development.

The Na/K-ATPase β subunit has been generally prescribed to serve two primary roles including; 1) a chaperone role that directs the insertion of the α subunit and functional enzyme unit to the appropriate membrane domain (22-25); and 2) the regulation of cation sensitivity of the Na/K-

ATPase (26-28). Most recently however, an additional role in regulating epithelial cell phenotype and cell motility has been defined for the Na/K-ATPase β subunit (29-35). We would suggest that the results from our study support the likelihood that the Na/K-ATPase β1 subunit contributes to all three roles during blastocyst formation in the mouse.

Our results certainly indicate that we were able to specifically down regulate Na/K-ATPase β1 subunit expression by the microinjection of β1 subunit specific siRNAs as we were unable to detect the presence of either Na/K-ATPase β1 subunit mRNAs or polypeptides in β1 subunit siRNA microinjected zygotes. This result coupled with the aberrant Na/K-ATPase α1 subunit distribution that we observed in the Na/K-ATPase β1 subunit siRNA microinjected embryos has lead us to conclude that the Na/K-ATPase β1 subunit is required to direct the proper targeting and insertion of the Na/K-ATPase α1 subunit into the trophectoderm cell membranes and without this occurring blastocyst formation is blocked.

But how might the Na/K-ATPase β1 subunit microinjected zygotes survive to the morula stage? We have known for some time that the Na/K-ATPase β1 subunit gene products display a very dramatic upregulation just prior to blastocyst formation suggesting that the upregulation of this gene is required for cavitation to occur (3-5). Na/K-ATPase α subunit mRNAs are present throughout preimplantation development and display a much more gradual increase as the embryo progresses to the blastocyst stage (3-5). It is very likely that Na/K-ATPase α subunits outnumber β subunits by a wide margin in early embryo stages and thus the full upregulation of the Na/K-ATPase required to support cavitation cannot occur until Na/K-ATPase β1 subunits increase to more closely match α subunit levels (3-5). As well mRNAs and polypeptides encoding the other Na/K-ATPase β subunits (β2, β3) are also present in preimplantation embryos (5,7). We would suggest that the "housekeeping" and early cleavage stage (1-cell to morula) requirements for the Na/K-ATPase may be handled by primarily α1/β2 or α1/β3 isoenzymes but that at the morula stage the upregulation of Na/K-ATPase β1 subunit gene products provides for the overall increase in enzyme (α1/β1 isozymes) that is required to drive blastocyst formation. Alternatively despite not detecting mRNAs and polypeptides encoding the Na/K-ATPase β1 subunit within β1 subunit siRNA microinjected embryos there may still be sufficient protein present that is below the level of immunofluorescence detection but is sufficient to pair up with Na/K-ATPase α1 subunits to satisfy the "housekeeping" requirement of the enzyme up to the morula stage. Our results clearly indicate that the Na/K-ATPase β1 subunit is required to support blastocyst formation and proper insertion of Na/K-ATPase α1 subunits into trophectoderm membranes.

We have employed the 8-cell aggregation chimera method (36) to investigate the reversibility of the developmental blockade produced by the loss of Na/K-ATPase β1 subunit embryos and confirm the presence of β subunit protein with development to the blastocyst stage. Our results have demonstrated that when β1 subunit siRNA microinjected 8-cell embryos are paired with either random sequence siRNA or uninjected controls they go on to form blastocysts in normal proportions, while those β1 subunit embryos which were paired together failed to develop to the blastocyst stage. This finding is consistent with our previous observation that down regulation of β1 subunit expression is associated with failure to support development to the blastocyst stage.

More recently, a series of important studies primarily from Dr. Rajasekaran's laboratory have demonstrated that the Na/K-

ATPase also has roles in regulating cell motility, oncogenic transformation, epithelial to mesenchymal cell transition and tight junction formation and function (29-31,37). Madin Darby Canine Kidney cells (MDCK) transformed by Moloney virus (MSV-MDCK) express reduced levels of the Na/K-ATPase β1 subunit compared to non-transformed MDCK cells (31). When the Na/K-ATPase β1 subunit levels are increased in MSV-MDCK cells one observes a corresponding increase in Na/K-ATPase α1 subunit protein due to enhanced α1 subunit translation (31). In addition, MSV-MDCK cells display a reduced cell motility (correlated with reduced Na/K-ATPase β1 levels) that is overcome by also increasing Na/K-ATPase β1 subunit expression in these cells (32,34). Further studies have indicated that Na/K-ATPase β1 subunit levels may be regulated by the transcription factor, "snail" as increased snail levels result in reduced Na/K-ATPase β1 subunit levels and blockade of snail levels increased Na/K-ATPase β1 levels (37). Collectively these studies demonstrate that the Na/K-ATPase β1 subunit is a critical regulator of Na/K-ATPase α1 subunits and thus enzyme function and that Na/K-ATPase subunit levels are important mediators of the epithelial cell phenotype and cell motility.

In addition, recent studies have also demonstrated that the Na/K-ATPase has an important role in regulating tight junction formation and function (32-35). Tight junctions are composed of several trans-membrane proteins including Claudins and Occludin and several associated proteins including ZO-1, ZO-2, Cingulin, and 7H6 that link the junctional unit to the cell's actin cytoskeleton (38-41). Inhibition of Na/K-ATPase by ouabain treatment is associated with aberrant distribution of tight junction associated proteins and also tight junction function, as indicated by increased permeability to FITC labeled dextran molecules in both cell lines (32,34) and mouse blastocysts (17). We discovered that the disruption of ZO-1 tight junction protein distribution following ouabain treatment of early mouse embryos was correlated with re-arrangements in Occludin distribution and these effects provided a basis for understanding the increased permeability to FITC-Dextran that ouabain treated embryo displayed (17). We have therefore concluded that Na^+/K^+-ATPase is a regulator of tight junction paracellular permeability in mouse blastocysts as well as epithelial cell lines. The outcomes from the present study also support this conclusion as we observed a dramatic shift in normal protein distribution for both ZO-1 and Occludin in all Na/K-ATPase β1 subunit siRNA microinjected embryos. It is our expectation that the aberrant protein distribution patterns we observed in these embryos are indicative of a disrupted tight junction formation in Na/K-ATPase β1 subunit siRNA microinjected embryos that would result in a failure in establishing a proper trophectoderm tight junction seal that is required to allow for expansion of the blastocyst cavity during cavitation. Thus the failure of Na/K-ATPase β1 subunit siRNA microinjected embryos to form blastocysts could have resulted from both a mis-targeting and proper insertion of Na/K-ATPase α subunits into the appropriate trophectoderm domains and also due to a disruption of tight junction assembly and establishment of the trophectoderm tight junctional seal.

How might the Na/K-ATPase regulate tight junction formation? It seems likely that influences are mediated via regulation of the actin cytoskeleton. A model proposed by Barwe et. al., (29) suggests that the Na/K-ATPase α subunit is able to phosphorylate p85 which recruits this subunit to the plasma membrane and results in activation of PI3 kinase and the generation of PIP3 which induces the binding of annexin II to the Na/K-ATPase β subunit cytoplasmic tail, activation of the Rac1 RhoGTPase and modifications to

the actin cytoskeleton. Studies have shown that loss of tight junction structure and increased paracellular permeability is often linked to the disruption of the circumferential actin ring that is localized at the apical pole of polarized epithelial cells (42-44). In MDCK cells, loss of tight junction permeability upon Na^+/K^+-ATPase inhibition also is associated with reduced stress fiber content and reduced RhoA activity (34). Overexpression of RhoA can bypass the inhibitory effect of Na^+/K^+-ATPase inhibition on tight junction formation, indicating that RhoA is essential and may be downstream of Na^+/K^+-ATPase in regulating tight junction function (34). These possibilities certainly offer exciting directions to follow in our efforts to understand Na/K-ATPase function during early development and in particular its role in regulating tight junction formation and function in the early embryo.

In conclusion, blockade of Na/K-ATPase β1 subunit expression by microinjection of Na/K-ATPase β1 siRNAs resulted in a failure of microinjected embryos to develop to the blastocyst stage which was associated with aberrant expression of Na/K-ATPase α1 and tight junction ZO-1 and Occludin polypeptides indicating that the Na/K-ATPase β1 subunit is a potent regulator of both Na/K-ATPase α1 subunit membrane insertion and also tight junction protein assembly during preimplantation development.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Morphology of Na/K-ATPase β1 subunit siRNA microinjected embryos. *Panel A*, Morphological changes in mouse preimplantation development following Na/K-ATPase β1 subunit siRNA microinjection into zygotes. Note a lack of complete blastocyst formation in Na/K-ATPase β1 subunit siRNA microinjected embryos, however a few (2.3%) of the embryos did have multiple and small blastocoel cavities (a). The group of embryos microinjected with control siRNA or those representing non-injected controls developed normally to the blastocyst stage (b and c). *Panel B*, The percentage of embryos that developed into blastocysts following Na/K-ATPase β1 subunit siRNA microinjection or control siRNA microinjection (CI) or non injected controls (C) are shown. Only 2.3% of Na/K-ATPase β1 subunit siRNA injected zygotes progressed to the blastocyst stage compared to 73% (injected controls) or 91% (non injected controls). N= 3 independent experiments. Values with different symbols represent significant differences. P value = < 0.05. *Panel C*, Validation of Na/K-ATPase β1 siRNA efficacy at down regulating β1 gene expression. Transcripts encoding Na/K-ATPase β1 subunit were not detected in embryos injected with β1 siRNAs 4 days after microinjection. Embryos microinjected with control siRNA or non injected embryos displayed the appropriate Na/K-ATPase β1 mRNA product. Transcripts encoding β-actin were readily detected in all three treatment groups. The negative control represents no cDNA sample while the positive control is a mouse kidney cDNA sample.

Fig. 2. Na/K-ATPase β1 subunit protein levels following microinjection of β1 siRNAs. The distribution of Na/K-ATPase β1 subunit polypeptides during murine preimplantation development was assessed (Panel e, f, g and h). Green, red and blue colors in each representative photomicrograph indicate positive staining for the respective primary antibody, F-actin (rhodamine phalloidin), and nuclei (DAPI) respectively. Panels a, b, c display a marked reduction in Na/K-ATPase β1 subunit fluorescence in embryos microinjected with β1 subunit siRNAs. Panel d shows the presence of multiple blastocoel cavities, which failed to coalesce to form a single blastocoel cavity in one of the rare (i.e. 2.3%) of Na/K-ATPase β1 siRNA injected zygotes that progressed to the blastocyst stage.

Fig. 3. Distribution of Na/K-ATPase α1 subunit (a, b), ZO-1 (c, d) and Occludin (e, f) proteins in Na/K-ATPase β1 subunit siRNA microinjected embryos or control microinjected embryos. Note the discontinuous distribution of Na/K-ATPase α1 subunit in the Na/K-ATPase β1 subunit siRNA injected embryos. Loss of Na/K-ATPase β1 subunit also resulted in the shift from a "continuous" to "discontinuous" distribution for both ZO-1 and Occludin polypeptides. In addition both ZO-1 and Occludin immunofluorescence transitioned from a very tight focused signal adjacent to cell margins to become predominantly "discontinuous"and cytoplasmic in distribution.

Fig. 4. Morphology of chimeric embryos. *Panel A*, Representative figures of chimeric embryos produced by pairing either β1 subunit deficient 8-cell embryos together (A:A), or to siRNA random sequence injected controls (A:B), or to uninjected controls (A:C) or sequence siRNA injected controls together (B:B). While A:A embryos demonstrated a developmental blockade

following compaction, A:B, A:C and B:B embryos all progressed normally to the blastocyst stage. *Panel B*, The percentage of embryos that developed into blastocysts following 8-cell aggregation is shown. Values with different symbols represent significant differences. P value < 0.05.

Fig. 5. Na/K-ATPase β1 subunit protein levels following chimera production. The distribution of Na/K-ATPase β1 subunit polypeptides was assessed between chimeric embryos produced by pairing either β1 subunit deficient 8-cell embryos together (A:A), or to siRNA random sequence injected controls (A:B), or pairing random sequence siRNA injected controls together (B:B). Green, red and blue colors in each representative photomicrograph indicate positive staining for the respective primary antibody (Panels a, e and i) , F-actin (rhodamine phalloidin) (Panels b, f and j), and nuclei (DAPI) (Panels c, g and k) respectively. Panels d, h and l show a composite image of all three channels.

Figure 1

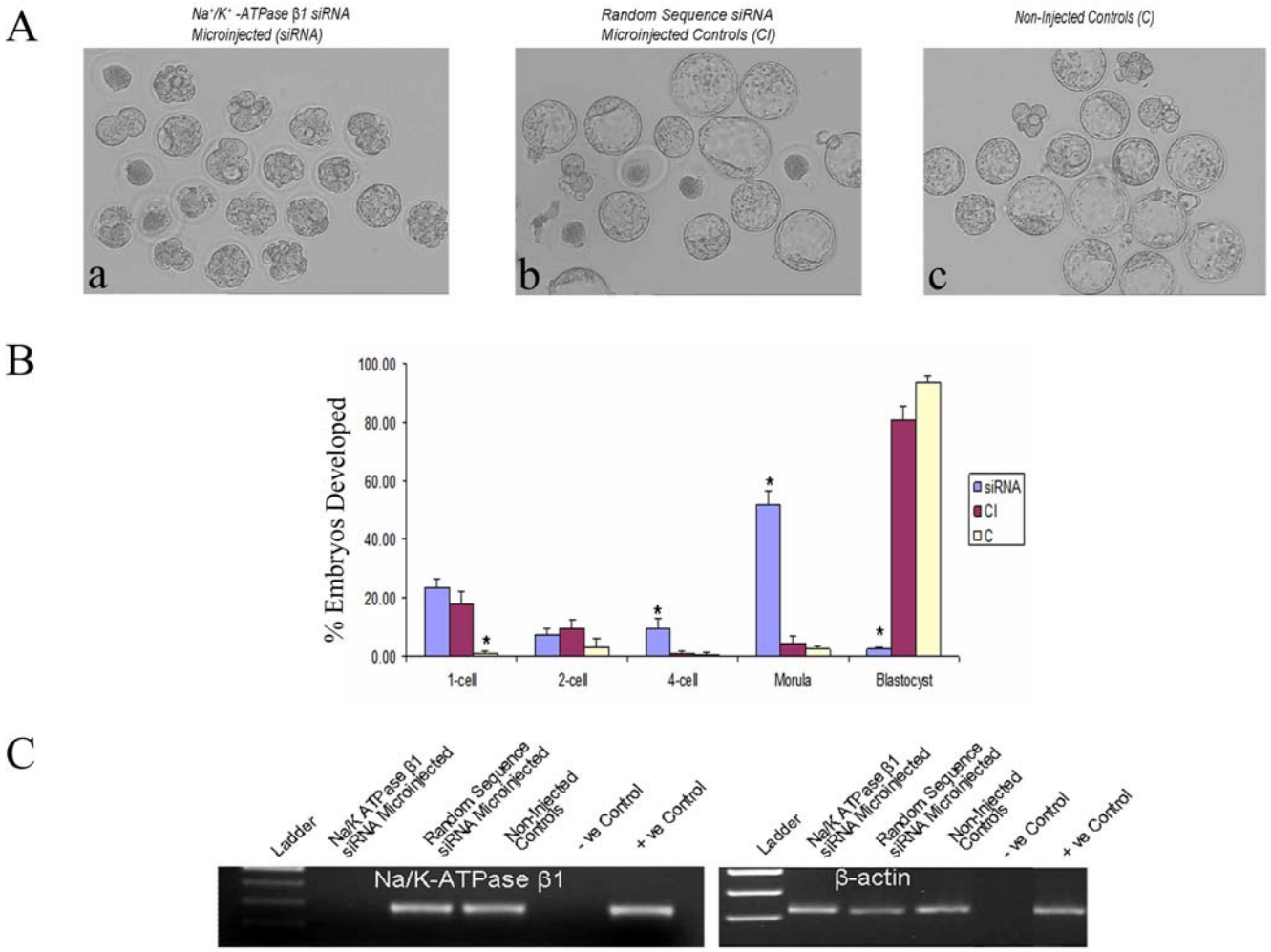
A
Na+/K+ -ATPase β1 siRNA Microinjected (siRNA)
Random Sequence siRNA Microinjected Controls (CI)
Non-Injected Controls (C)
a
b
c
B
% Embryos Developed
100.00
80.00
60.00
40.00
20.00
0.00
1-cell
2-cell
4-cell
Morula
Blastocyst
siRNA
CI
C
C
Ladder
Na/K ATPase β1 siRNA Microinjected
Random Sequence siRNA Microinjected
Non-Injected Controls
- ve Control
+ ve Control
Na/K-ATPase β1
β-actin

Figure 2

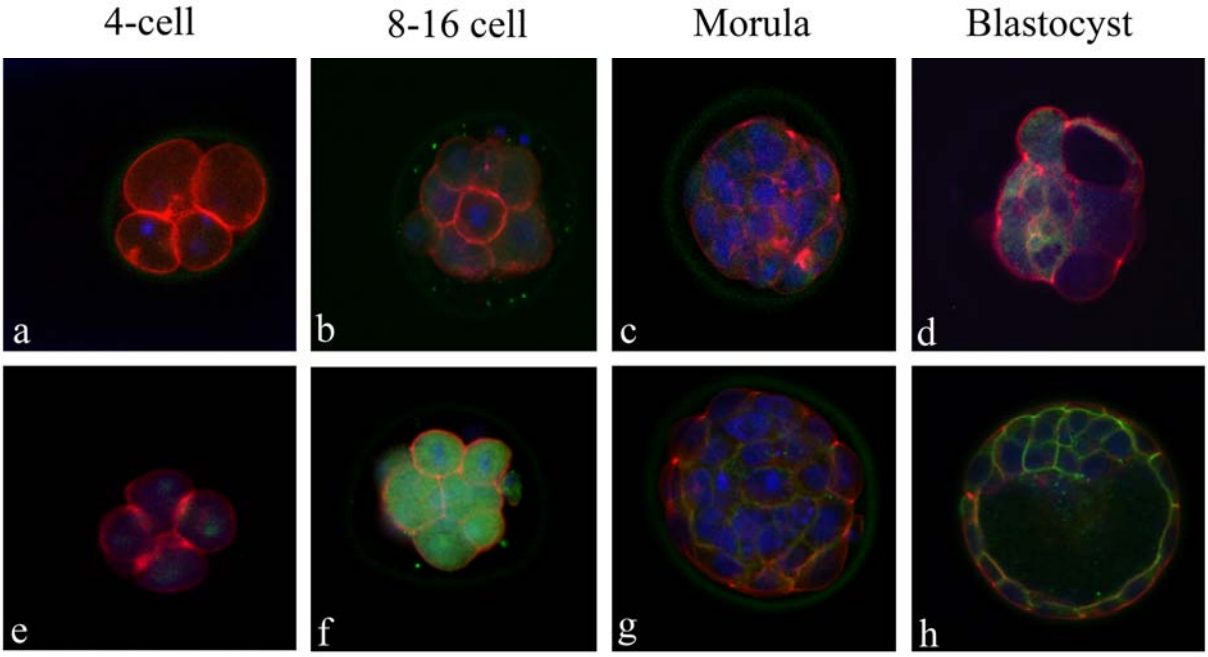

Figure 3

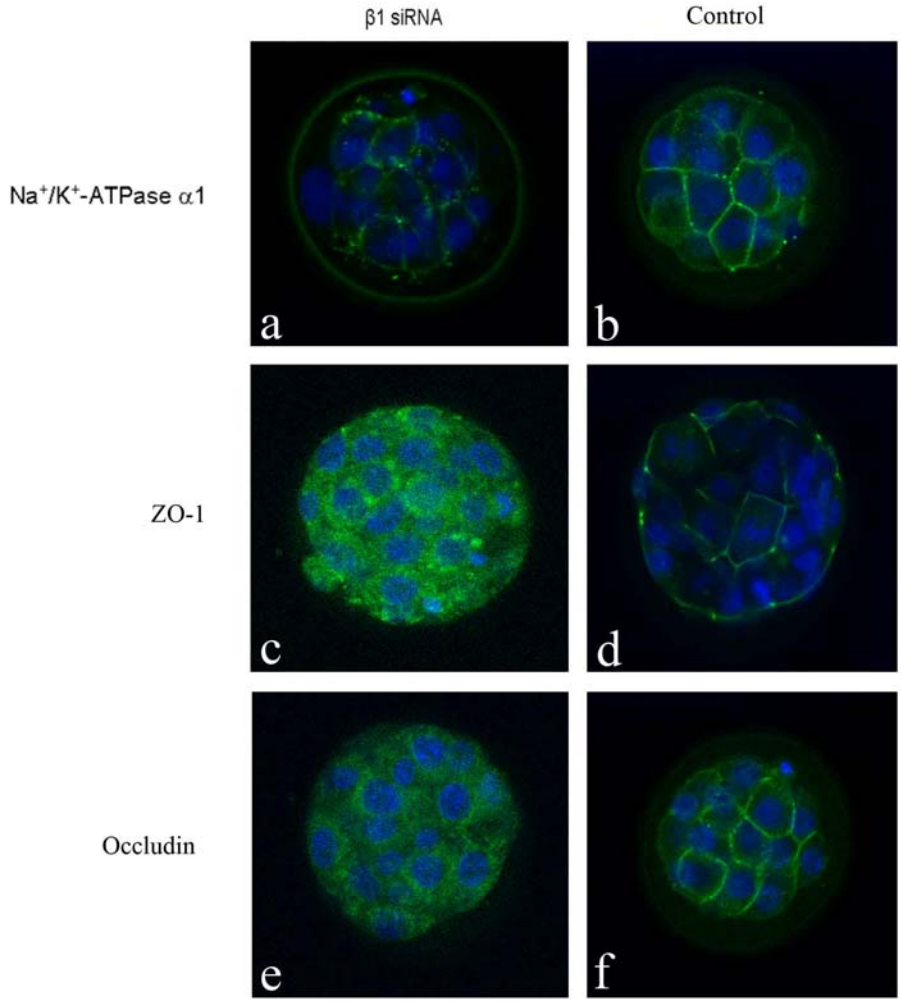

Figure 4

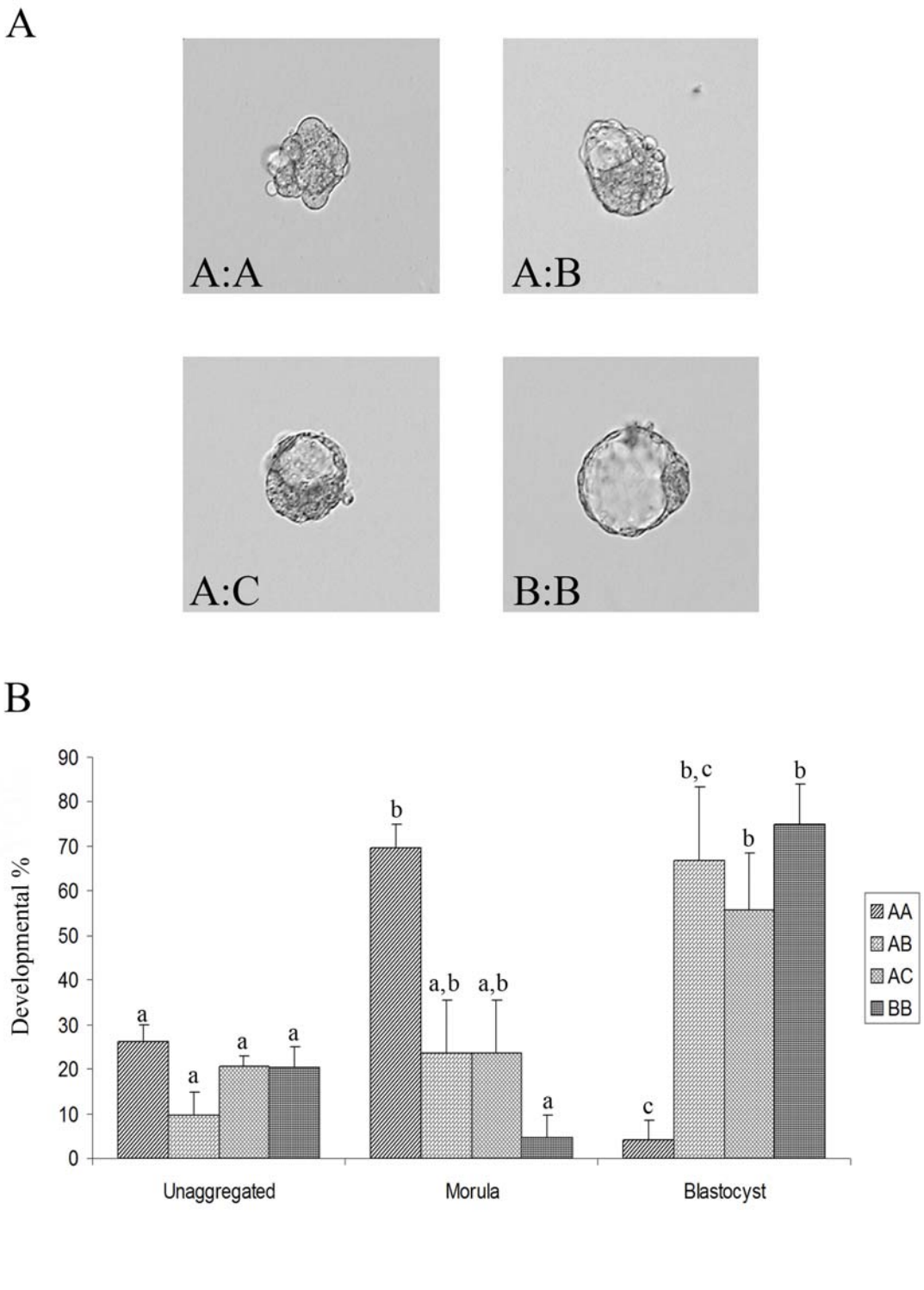
A
A:A
A:B
A:C
B:B
B
Developmental %
90
80
70
60
50
40
30
20
10
0
a
a
a
a
b
a,b
a,b
a
c
b,c
b
b
Unaggregated
Morula
Blastocyst
AA
AB
AC
BB

Figure 5

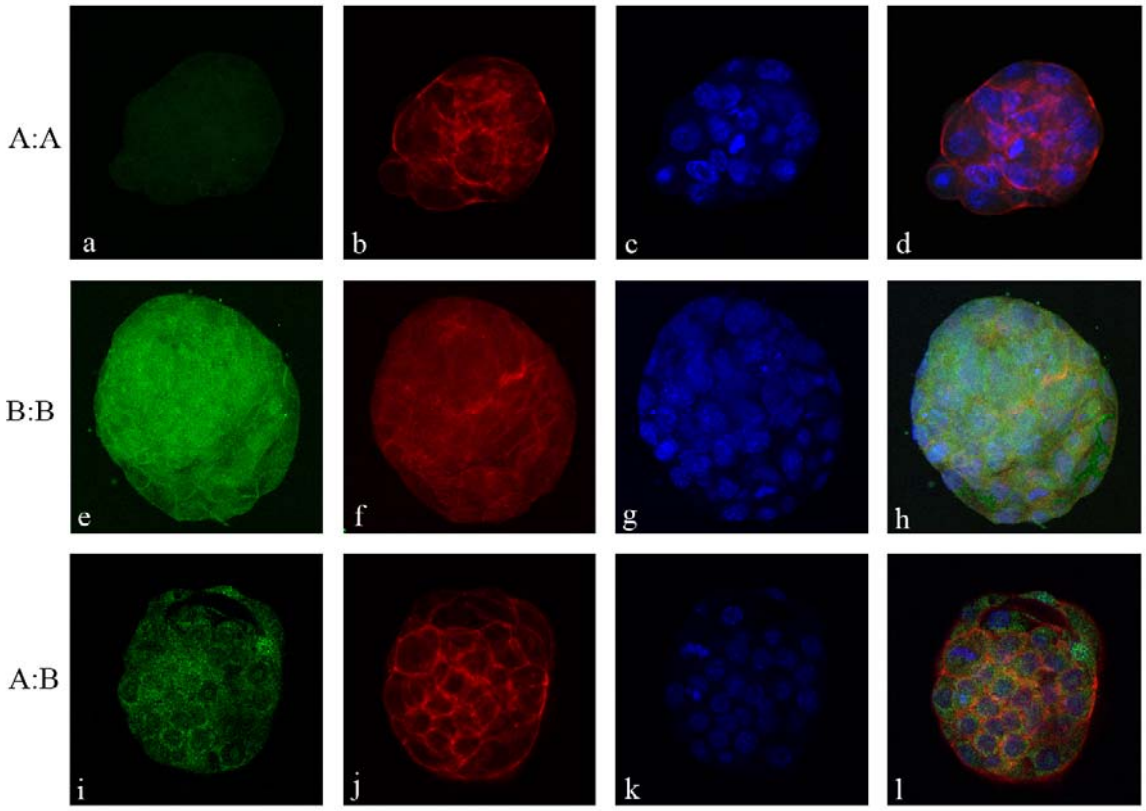

Table 1 Nucleotide sequences for polymerase chain reaction amplification reactions

Gene Product	Primer	Primer sequence	Size (bp)
Na/K-ATPase β1 subunit (5)	5’ 3’	TTCAGCCCAGAAGGACGACATG AGGGAAGCCGTAGTATCCGCCCA	378
β -actin	5’ 3’	CGTGGGCCGCCCTAGGCACCA GGGGGGACTTGGGATTCCGGTT	248