Changing expression of chloride channels during preimplantation mouse development

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Abstract

Plasma membrane chloride channels (ClCs) play important roles in a broad range of cellular processes including cell volume regulation, proliferation, and transepithelial transport, all of which are critical during preimplantation embryonic development. In this study, the molecular and functional expression of voltage-gated ClCs was analyzed throughout preimplantation development of the mouse conceptus. mRNA transcripts for all *Clcn* genes were detected. Only *Clcn1* mRNA showed differential expression in the blastocyst, being detected in the trophoblast but not in the inner cell mass. CLCN3 protein was detected at low levels in the cytoplasm and plasma membrane in 4-cell embryos and was localized to the apical plasma membrane of the trophoblasts in the blastocyst. Whole-cell patch-clamp recordings demonstrated the presence of a DIDS-sensitive, outwardly rectifying Cl[−] current throughout development, with this conductance being large at the 1-cell, morula and blastocyst stages. A second DIDS-insensitive Cl[−] current, which was inactivated by membrane depolarization, was present in cells differentiating into the trophoblast lineage and during blastocyst expansion. Inhibition of the DIDS-sensitive current and the DIDS-insensitive current, with 9-AC, prevented blastocyst expansion.

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Introduction

The ionic environment experienced by the conceptus is likely to exert a powerful effect on its development. The extent of this effect will depend on the ability of the conceptus to regulate its ionic composition in response to external ionic change, which will ultimately depend on the activity of ion channels and the ions transported at each developmental stage. Our knowledge of the ion transporters and channels functioning at early stages of development is limited and this deficit may hinder attempts to improve embryo culture media used in human-assisted reproductive technologies (Biggers 2002, Leese *et al.* 2007). A fairly detailed analysis of the mechanisms available to the conceptus for the handling of acid and alkaline load, including Na⁺–H⁺ exchangers, H⁺–monocarboxylate cotransporters, and Cl[−]–HCO₃[−] exchangers, has been described (Gibb *et al.* 1997, Harding *et al.* 1999). However, for ions other than H⁺, information is less systematic. It is clear that Ca²⁺ plays critical roles at various stages of preimplantation development, including fertilization (McGuinness *et al.* 1996, Day *et al.* 2000), and during signaling by embryotrophic factors during later stages (Li *et al.* 2007, O'Neill 2008). There is also evidence implicating monovalent cations (Na⁺ and K⁺) in the temporal

(Day *et al.* 1993, 1998) and spatial (Wiley 1984, Wiley *et al.* 1990, 1991) programming of early development.

Channels that selectively allow passage of Cl[−] ions across membranes are thought to play a number of roles in the physiology of somatic cells, and so an understanding of which channels are present and active during the preimplantation stages of development is likely to be important. Molecular studies have identified several genes expressing proteins with Cl[−] transport characteristics. In parallel, electrophysiological studies have identified Cl[−] channels and transporters with distinctive physiological characteristics and with involvement in a number of physiological activities, including volume regulation in response to osmotic stress (Strange *et al.* 1996), pH regulation in response to alkaline load, and passage through the cell cycle (Block & Moody 1990, Rutledge *et al.* 2001). We currently have only very limited information about patterns of chloride channel (ClC) gene expression and activity during preimplantation development. There is evidence for the activity of an osmo-sensitive ClC, which also transports organic osmolytes, and is active during the latter stages of oocyte maturation and the first two cell cycles of mouse development, declining at entry into second mitotic metaphase (Séguin & Baltz 1997, Kolajova & Baltz 1999, Kolajova *et al.* 2001). In addition, there are several

reports of the presence of a Cl^- - HCO_3^- exchanger in conceptuses from several species, which plays a role in the alkaline load response of the early conceptus (Lane *et al.* 1999a, 1999b, Phillips & Baltz 1999, Harding *et al.* 2002).

Here, we report on the profile of mRNA expression of the genes encoding the major voltage-gated ClCs and an electrophysiological study of ClC activity during pre-implantation mouse development. We find that at least two ClCs are detectable electrophysiologically and that the profile of the activities varies with developmental stage and emergent tissue lineages. In addition, we find that mRNAs encoding eight ClCs are detectable.

Results

Expression of *Clcn* mRNA in mouse oocytes and conceptuses

RT-PCR were carried out on RNA from all stages of development from the unfertilized oocyte through to the blastocyst stage. Transcripts for all the *Clcn* genes were detected (Fig. 1, Table 1). *Clcn1*, 2, 3, 4, 5, 6, 7, and *ka/b* mRNAs were detected in all stages of development examined, although expression levels of *Clcn2* and 5 appeared to dip during the 2-cell stage and those of *Clcnka/b* (*Clcnk1/2*) was weak before fertilization (Table 1, Figs 1 and 2). A more detailed examination of the expression of *Clcn3* mRNA showed that it was present in oocytes, 1-cell and early 2-cell stages, absent from mid 2-cell to late 2-cell stages, and present from the

Figure 1 Expression of mRNA encoding *Clcn1*, *Clcn2*, *Clcn4-2* (*Clcn4*), *Clcn5*, *Clcn6*, *Clcn7*, and *Clcnka/b* in the preimplantation mouse embryo. Representative PCR products from unfertilized oocytes (O), 1-cell (1), 2-cell (2), 4-cell (4), 8-cell (8), morula (M), and blastocyst (B) stage embryos are shown. ICM and TE indicates inner cell mass and trophoblast respectively. E, M, and L indicate early, mid, and late stages respectively. Each reaction was repeated on two to eight separate cDNA samples.

early 4-cell stage through to the blastocyst stage (Fig. 2A). Within the blastocyst, *Clcn3* mRNA was found in both inner cell mass (ICM) and trophoblast (TE) cells (Fig. 2B). *Clcn1* mRNA was only detected in cells from the trophoblast of blastocysts but not in the ICM (Fig. 1).

Immunolocalization of CLCN3

Expression of CLCN3 protein, the product of the *Clcn3* gene, in mouse preimplantation stages was investigated by immunofluorescence, this being the only channel protein for which validated antibodies are available. In 4-cell embryos, weak staining for CLCN3 was distributed throughout the cytoplasm and membrane (Fig. 2C, panel a). In the blastocyst stage, there was strong staining of the apical membrane of the trophoblasts (Fig. 2C, panel d). There was also diffuse staining throughout the cytoplasm of both ICM and trophoblasts. When the antibody was preadsorbed with the peptide antigen (Fig. 2C, panels b and e) or when the secondary antibody only was used (Fig. 2C, panels c and f), very little staining was observed.

Cl^- currents in preimplantation embryonic cells

Whole-cell patch-clamp studies on 1-cell stage mouse zygotes under isotonic conditions using a Cl^- -rich bath solution identified an outwardly rectifying current ($n=6$; Fig. 3). Following replacement of extracellular Cl^- with glutamate (Cl^- free bath solution), the size of this conductance was reduced significantly (Fig. 3A, B and C) and the reversal potential shifted from -16.4 ± 2.6 mV ($n=6$) to 8.7 ± 4.1 mV ($n=6$; $P < 0.001$). Addition of 100 μM 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS), a broad specificity inhibitor of Cl^- channels, in the presence of the Cl^- containing extracellular solution also significantly decreased the size of the outward current ($n=5$; Fig. 3D), as expected if the current was due to movement of Cl^- through a channel.

The expression of this DIDS-sensitive outwardly rectifying Cl^- conductance in later stages of pre-implantation development was also investigated. At the 4-, 8-cell, and morula stages, an outward conductance with properties similar to that observed in the zygote was present in whole-cell recordings. This current was reduced significantly by removal of extracellular Cl^- (Fig. 4). Addition of 100 μM DIDS significantly reduced the conductance in 4-cell stage embryos and morulae (Fig. 4A and C) but had no significant effect on the conductance at the 8-cell stage (Fig. 4B).

In the mouse blastocyst, an outwardly rectifying Cl^- current was also observed in isolated ICMs and in the trophoblast of expanded, day 5 embryos (Fig. 5). Addition of DIDS (100 μM) to the bathing solution significantly reduced the current in both these cell types (Fig. 5A and B(iii)).

Table 1 Voltage-gated Cl^- channel transcript expression in the preimplantation mouse embryo.

Chloride channel	UF	1-Cell	2-Cell	4-Cell	8-Cell	Mor	BL
<i>Clcn1</i>	+	+	+	+	+	+	+
<i>Clcn2</i>	+	ND	+	+	ND	ND	+
<i>Clcn3</i>	+	+	+	+	+	+	+
<i>Clcn4-2</i>	+	ND	+	+	+	+	+
<i>Clcn5</i>	+	+	+	+	+	+	+
<i>Clcn6</i>	+	+	+	ND	+	+	+
<i>Clcn7</i>	+	+	+	+	+	+	+
<i>Clcnka/b</i>	ND	ND	+	+	+	+	+

ND, RT-PCR not done; +, band present; —, band absent. RNA from unfertilized oocytes (UF); 1-, 2-, 4-, and 8-cell, morula (mor), and blastocyst (BL) stages were used. Each reaction was repeated three to 16 times.

Comparison of the Cl^- - and DIDS-sensitive components of the outwardly rectifying conductance throughout development

The sensitivity of different voltage-gated Cl^- channels to inhibition by DIDS varies. Therefore, to determine whether all the Cl^- conductance measured at each embryonic stage of development was blocked by DIDS, the size of the Cl^- -sensitive conductance was compared with the size of the conductance that was blocked by DIDS. The Cl^- -sensitive conductance was calculated from the change in conductance before and after removal of extracellular Cl^- . Similarly, the DIDS-sensitive conductance was calculated from the change in conductance before and after the addition of 100 μM DIDS, in the presence of extracellular Cl^- . The size of the Cl^- -sensitive conductance was significantly smaller at the 4- and 8-cell stages compared with that measured in the zygote. It increased again at the

morula stage before decreasing in the ICM and trophoblast of the blastocyst (Fig. 6). The sizes of the Cl^- -sensitive and DIDS-sensitive conductances were the same for all stages except for the morula and trophoblast of expanded blastocysts, in which the size of the DIDS-sensitive conductance was smaller than the total Cl^- -sensitive conductance. Following blastocyst collapse, this difference between the conductances in trophoblasts was abolished. These data suggest that more than one type of Cl^- channel was expressed in morulae and trophoblasts, one channel that was inhibited by DIDS and another that was not inhibited by DIDS.

Voltage-inactivated Cl^- currents in preimplantation stages

A second type of Cl^- current, which showed voltage-sensitive, slow inactivation at higher positive voltages

Figure 2 Expression of *Clcn3* mRNA and protein in mouse preimplantation embryos. (A) Representative RT-PCR products from amplification of *Clcn3* and *B-actin* in unfertilized oocytes (UF), 1-cell (1C), early 2-cell (E2C), mid 2-cell (M2C), late 2-cell (L2C), early 4-cell (E4C), late 4-cell (L4C), early 8-cell (E8C), late 8-cell (L8C), and blastocyst (BL) stage embryos. (B) Representative RT-PCR products from amplification of *Clcn3* in inner cell mass (ICM) and mural trophoblast (TE) cells of blastocyst stage embryos. Each reaction was repeated two to eight times. Sizes of PCR products are indicated. (C) Distribution of CLCN3 in the 4-cell (a, b and c) and blastocyst (d, e and f) stages stained with anti-CLCN3 antibody (a and d), anti-CLCN3 preadsorbed with the peptide antigen (b and e), and secondary antibody only (c and f). Green indicates antibody staining and blue indicates DAPI staining of the nuclei. Each image is representative of staining observed in three separate experiments.

Figure 3 Characteristics of the outwardly rectifying Cl⁻ current in 1-cell stage embryos. (A) Representative current tracings obtained using (i) Cl⁻-rich and (ii) Cl⁻-free (Na-glutamate) bath solutions. (B) Mean current-voltage (I-V) relationship for the steady-state current recorded in the Cl⁻-rich (filled circle) and Cl⁻-free (open circle) bath solutions from six embryos. Normalized chord conductance of the outwardly rectifying Cl⁻ current in 1-cell stage embryos measured at 100 mV (C) in Cl⁻-rich and Cl⁻-free bath solutions and (D) in the presence of 100 μM DIDS. Conductance was normalized to Cl⁻-rich or control values. Data are shown as mean ± S.E.M. with (n) the number of recordings. ***P* < 0.01.

was also observed in preimplantation stages (Fig. 7). DIDS (100 μM) did not inhibit this voltage-inactivated current in day 4 blastocysts, although the total outward Cl⁻ conductance was reduced (Fig. 7B), consistent with DIDS having an inhibitory effect on the underlying outwardly rectifying Cl⁻ current described in the previous sections. The voltage-inactivated current was observed in morula and blastocyst stages, but rarely if ever at earlier cleavage stages or in the ICM (Fig. 7C). Moreover, when isolated embryogenic cells were examined (Fig. 7D), this voltage-inactivated current was absent in apolar 1/16 cells (*n*=5), observed in 6% of polar 1/16 cells (*n*=16), 38% of polar 1/32 cells (*n*=21), and 57% of 1/64 trophoblast cells (*n*=28). This current was absent from ICM cells (*n*=5) but observed in a significantly greater proportion of trophoblasts in collapsed than in expanded blastocysts (Fig. 7E).

Effect of Cl⁻ channel inhibitors on blastocoel cavity formation

Previous studies have shown that Cl⁻ transport plays an important role in blastocyst expansion. In order to determine whether the Cl⁻-sensitive conductances observed in this study played a role in blastocyst expansion, the effect of Cl⁻ channel blockers on blastocoel cavity formation was investigated. Culture of morulae in medium containing 100 μM DIDS significantly reduced the proportion of embryos that formed a fluid-filled cavity (Fig. 8). As *Clcn1* mRNA showed a similar pattern of expression to the voltage-inactivated

Cl⁻ current observed in the trophoblast lineage, we investigated whether the ClC1 channel blocker 9-anthracenecarboxylic acid (9-AC) had any effect on cavity formation. Morulae exposed to 1 mM 9-AC showed a significantly reduced rate of cavitation compared with control morulae, which formed expanded blastocysts (Fig. 8). As we hypothesized that DIDS and 9-AC were blocking different Cl⁻ channels, we also examined the effect of the drugs in combination. Culture of morulae in medium containing 100 μM DIDS + 1 mM 9-AC reduced cavity formation to a greater extent than either drug alone.

Discussion

There is considerable evidence that even quite short periods of *in vitro* culture can induce a number of changes in the conceptus, which can affect developmental potential and even survival (reviewed in Thompson *et al.* (2007)). Understanding how the composition of the culture medium influences development thus has potentially important practical consequences. Ions are known to exert important influences through their effects on cell physiology (Leese 1995, Biggers 1998), so understanding the nature and properties of the ion transport systems available to the conceptus is of particular significance.

This study investigated the expression of members of the voltage-gated Cl⁻ channel family during mouse preimplantation development. mRNAs encoding all the *Clcn* channels were detected throughout development

Figure 4 The outwardly rectifying Cl⁻ current in 4-, 8-cell, and morula stage embryos. (i) Mean I-V relationship for the steady-state current recorded in 4-cell (A), 8-cell (B), and morula (C) stages in isotonic Cl⁻-rich (filled circle) and Cl⁻-free (open circle) bath solutions. Normalized chord conductance of the outward Cl⁻ current (ii) in the presence and in the absence of extracellular Cl⁻ and (iii) in the presence of 100 μ M DIDS. Data are shown as mean $\pm$ S.E.M. with (n) the number of recordings. *P < 0.05 and ***P < 0.001.

extending a previous report of *Clcn* expression in zygotes (Sonoda *et al.* 2003). Two types of ClC activities were detected using whole-cell voltage clamp methods. Both these currents had reversal potentials consistent with chloride being the main permeable ion, the removal of extracellular chloride reducing the size of the outward current, and causing a positive shift in the reversal potential as expected for a Cl⁻ conductance. One of these Cl⁻ currents was outwardly rectifying and was inhibited by DIDS. This current was constitutively active throughout the preimplantation stages of embryonic development, being large in the 1-cell and morula stages. In the 1-, 4-, and 8-cell stages, the DIDS-sensitive current constituted all the Cl⁻-sensitive current. The electrophysiological characteristics of this outwardly rectifying Cl⁻ current were similar to that of a current described previously in the preimplantation mouse embryo under both isotonic and hypotonic conditions (Kolajova & Baltz 1999, Kolajova *et al.* 2001, Sonoda

et al. 2003). In the blastocyst, this DIDS-sensitive current was present in both the TE and the ICM. When blastocysts were collapsed by disruption of the tight junctions with cytochalasin D, the inhibition of the outwardly rectifying Cl⁻ current by DIDS was increased. This suggests that either the Cl⁻ channel was only active during the process of expansion or that the channel was located on the basolateral surface and that DIDS could not access the interior of the trophoblast cells due to the presence of tight junctions between the cells. DIDS reduced cavity formation supporting a role for this conductance in the process of blastocyst expansion.

The second type of Cl⁻ conductance observed in embryos was inactivated at voltages > +60 mV and was not inhibited by DIDS. This conductance was detected predominantly from the morula stage of development. Compaction of embryonic blastomeres at the 8-cell stage in mice results in the formation of two separate cell types, the ICM and the TE. The appearance of the

A Inner cell mass

B Trophoblast

Figure 5 The outwardly rectifying Cl⁻ current in the inner cell mass and trophoblast of the blastocyst. (i) Mean I-V relationship for the steady-state current recorded in inner cell mass (A) and trophoblast (B) in isotonic Cl⁻-rich (filled circle) and Cl⁻-free (open circle) bath solutions. Normalized chord conductance of the outward Cl⁻ current in (ii) the presence and absence of extracellular Cl⁻ and (iii) in the presence of 100 μM DIDS. Data are shown as mean ± s.e.m. with (n) the number of recordings. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

voltage-inactivated Cl⁻ conductance was associated with the emergence of this trophoblast lineage (Johnson 1986, 2009), suggesting a role for this conductance in the vectorial movement of electrolytes and water into the blastocoel cavity. Expansion of the blastocoel cavity in the mouse blastocyst requires extracellular Cl⁻, is dependent on both paracellular and transcellular movement of Cl⁻ across the TE (Manejwala *et al.* 1989, Brison & Leese 1993, Zhao *et al.* 1997), and is driven by the basolaterally localized Na⁺-K⁺-ATPase (DiZio & Tasca 1977, Biggers *et al.* 1978, Watson & Kidder 1988). In this study, DIDS did not completely prevent blastocyst expansion, as observed previously (Zhao *et al.* 1997), an observation that suggested a role for other pathways for Cl⁻ movement such as the paracellular pathway or the DIDS-insensitive, voltage-inactivated current described here. In the rat, DIDS reduces the rate of blastocyst re-expansion after collapse (Brison & Leese 1993), and in this study on mouse blastocysts, DIDS was only able to completely block Cl⁻ conductance when blastocysts were collapsed, supporting the possibility that a basolaterally located Cl⁻ channel is important for expansion.

The voltage-gated Cl⁻ channel gene family encodes Cl⁻ channels and Cl⁻/H⁺ exchangers that are expressed in the plasma membrane and intracellular vesicles (for review, see Jentsch (2008)). Members of this family that are localized to the plasma membrane

include CLIC1, 2, 3, k1, and k2, all of which were detected at the RNA level in embryos. Of these, CLIC2, 3, K1, and K2 are inhibited by DIDS; however, only CLCN3 is outwardly rectifying. The current elicited by CLCN3 under isotonic conditions in other cell types (Guan *et al.* 2006, Mitchell *et al.* 2008, Cuddapah & Sontheimer 2010, Yang *et al.* 2011) has properties similar to the DIDS-sensitive current we have found throughout

Figure 6 Plots of the conductance of the Cl⁻ (white bars) and DIDS-sensitive (black bars) currents during preimplantation development and in trophoblasts of expanded (exp BL) and collapsed (coll BL) blastocysts. Data are shown as mean ± s.e.m. with (n) the number of recordings. *Significant difference (*P* < 0.05) from the conductance at the 1-cell stage. #Significant difference (*P* < 0.05) between the Cl⁻ and DIDS-sensitive currents at a particular stage.

Figure 7 Expression of a voltage-inactivated Cl^- current in mouse preimplantation stages of development. (A) Representative current tracings obtained from a trophoblast cell in an expanded blastocyst. (B) Representative I-V relationships obtained in isotonic Cl^- -rich (filled circle) and Cl^- -free (filled square) bath solutions and in the presence of 100 μM DIDS (open circle). (C) Incidence of the voltage-inactivated current throughout development in 1-cell (1C; $n=15$), 2-cell (2C; $n=5$), 4-cell (4C; $n=26$), 8-cell (8C; $n=13$), morula (M; $n=5$) stages, expanding blastocysts (day 4 BL; $n=22$), expanded blastocysts (day 5 BL; $n=12$), and inner cell mass (ICM; $n=5$); (D) in isolated single cells and small clusters of apolar 1/16 cells ($n=5$) and polar 1/16 ($n=16$), 1/32 ($n=21$) and 1/64 ($n=28$) cells; and in (E) trophoblasts of collapsed ($n=5$) and expanded ($n=12$) blastocysts. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

preimplantation embryo development. Furthermore, mRNA encoding *Clcn3* was detected throughout development and CLCN3 protein was present in the plasma membrane at the 4-cell stage and localized to the apical membrane of trophoblasts. CLCN3 is also a candidate for the volume-sensitive outwardly rectifying anion channel (VSOAC) seen in many cell types (Duan *et al.* 1997, Yamamoto-Mizuma *et al.* 2004) as well as mouse embryos (Kolajova & Baltz 1999, Kolajova *et al.* 2001). However, the molecular identity of VSOAC has not been firmly established to be CLCN3. There is also evidence in other cell types for the involvement of CLCN3 in Cl^- currents observed under isotonic conditions (Yang *et al.* 2011) and for these currents being required for cell proliferation (Habela *et al.* 2008, Tang *et al.* 2008, Zhu *et al.* 2012). In mouse embryos, VSOAC is only present in the oocyte, 1-, and 2-cell stages in which its activation by hypotonicity is cell cycle dependent (Kolajova & Baltz 1999, Kolajova *et al.* 2001). DIDS prevents cell proliferation and development of mouse embryos (Sonoda *et al.* 2003, Li *et al.* 2009), suggesting that CLCN3 may play a role in regulation of the resting cell volume, and thereby cell proliferation, throughout development.

The molecular identity of the voltage-inactivated Cl^- current remains unclear. This current is unlikely to be carried by CIC2, which produces currents that are activated by membrane hyperpolarization and show inward rectification (Thiemann *et al.* 1992), or by ClCKa/b as these channels are inhibited by DIDS (Piccolo *et al.*

2004). On the other hand, *Clcn1* mRNA showed a similar expression pattern to the voltage-inactivated Cl^- current in embryos, being only expressed in the trophoblast and not the ICM. There is no report of voltage inactivation of currents elicited by CIC1 in other cell types, although there is evidence that CIC1 forms heteromultimers with other CIC subtypes and this alters the electrophysiological characteristics of the resulting current (Lorenz *et al.* 1996). Furthermore, like the voltage-inactivated current in embryos, CIC1 is not blocked by DIDS (Steinmeyer *et al.* 1995, Jentsch 2008) but is inhibited by 9-AC, which has been shown to decrease expansion of rat blastocysts (Brison & Leese 1993) and mouse blastocysts (this study). Taken together, these data suggest that the voltage-inactivated Cl^- current is composed of CIC1 and expressed in the mouse trophoblast lineage where it plays a role in blastocyst expansion.

In summary, all members of the voltage-gated Cl^- channel gene family are expressed at the RNA level in the preimplantation mouse conceptus. Two types of voltage-gated CIC activities were detected using whole-cell voltage clamp methods, one of which was outwardly rectifying, inhibited by DIDS and present at all stages of development from oocyte to blastocyst and may be composed of CLCN3 channel proteins. A second Cl^- conductance, which was inactivated by membrane depolarization, was only expressed in the trophoblast lineage. Inhibition of both these conductances was required to prevent blastocyst expansion.

Figure 8 Effect of Cl^- channel inhibitors on blastocoel cavity formation. (A) Representative photomicrographs of embryos following 40–48 h of culture in (i) control medium (containing 0.2% DMSO), (ii) 100 μM DIDS, (iii) 1 mM 9-AC, or (iv) 100 μM DIDS + 1 mM 9-AC. (B) Proportion of embryos that formed an expanded, fluid-filled cavity in control medium (containing 0.2% DMSO), 100 μM DIDS, 1 mM 9-AC, or 100 μM DIDS + 1 mM 9-AC. The numbers of embryos in each treatment are shown in brackets and were from three to six separate culture experiments. Treatments with the same letter are significantly different ($P < 0.01$).

Materials and Methods

Collection and culture of oocytes and conceptuses

Quackenbush strain (QS) female mice (3–5 weeks old) on a fixed 12 h light:12 h darkness cycle were superovulated by i.p. injections of 10 intrauterine pregnant mares' serum gonadotropin (Intervet, Bendigo East, Victoria, Australia), followed ~48 h later by 10 intrauterine human chorionic gonadotropin (hCG; Intervet). Some of the female mice were then paired overnight with male QS mice (>12 weeks old) and mating ascertained by the presence of a vaginal plug. The use of animals was in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the Institutional Animal Care and Ethics

Committee. Unfertilized oocytes, 1-cell zygotes, 2-, 4-, and 8-cell stages, morula, and blastocysts were obtained from female mice killed by cervical dislocation at 12.5–13, 18–24, 39–44, 47–48, 69–72, 81–83, and 93–95 h post-hCG injection respectively. Unfertilized oocytes and zygotes were teased or flushed from the oviducts into M2 medium (Fulton & Whittingham 1978) containing 4 mg/ml BSA (M2 + BSA). Cumulus cells were removed by brief exposure to hyaluronidase (0.2 mg/ml; Type II, Sigma) and oocytes stored in M2 + BSA until used. Zygotes were cultured in medium M16 (Chatot *et al.* 1989) or KSOM (Erbach *et al.* 1994) containing 4 mg/ml BSA under liquid paraffin oil (Sigma) in 5% CO_2 at 37 °C. The media contained no phenol red or antibiotics at any stage. The 2-, 4-, and 8-cell conceptuses and morulae were flushed from the oviducts and blastocysts from the uteri and cultured until use. The zona pellucida was removed by brief exposure to acid Tyrode's solution, and embryos were allowed to recover for 30 min before use.

In addition to the use of whole concept, chloride current activities in later stage embryonic cells were also examined in isolated individual cells or small cell clusters. These were prepared from the conceptus according to well-established and validated procedures (for details, see Johnson & Ziomek (1982, 1983)). Briefly, 8- to 16-cell stages were rinsed through Ca^{2+} -free M2 medium, after which they were incubated in the same medium for 10 min at 37 °C. The cells were then disaggregated using a fine flame-polished micropipette. Individual 1/8, 1/16, and 2/16 blastomeres were then cultured under oil until their use either as single cells, pairs, or, when division had occurred, as small cell clusters (2–4/16) or 'mini-blastocysts' (2–8/32 and 4–8/64). The smaller cells isolated from 16-cell concepti were apolar, inner cells (Ziomek *et al.* 1982).

ICMs were isolated by immunosurgery (Solter & Knowles 1975). Fully expanded zona-intact blastocysts were incubated in M2 + BSA containing heat-inactivated rabbit anti-mouse spleen cell serum (kindly provided by Prof. Peter Kaye, University of Queensland) at 37 °C for 10 min. Blastocysts were then washed in M2 + BSA and incubated for 20 min in M2 + BSA containing guinea pig complement (ICN Biochemicals, Irvine, CA, USA) at 37 °C. The ICMs were separated from the dead trophoblasts by aspiration through a narrow bore pipette. The ICM was then washed and incubated in M16 + BSA under oil in 5% CO_2 at 37 °C for 30 min in order to ensure absence of contaminating fluid accumulating cells. TE cells were isolated from blastocysts by embryo bisection, performed in M2 + BSA at 37 °C on an agar-coated Petri dish (1% agar in 0.9% NaCl; Day *et al.* 1998). The cutting was accomplished by pressing a fine glass pipette down onto individual zona-free blastocysts so that the ICM was pushed to one side. The two embryo fragments were separated by gentle pipetting, thereby producing a fragment that contained the ICM, surrounded by trophoblasts, which was discarded, and a fragment composed of trophoblasts only, which was used for RT-PCR.

The effect of Cl^- channel inhibitors on blastocyst expansion was examined by culturing morulae in groups of 15–20 in 20 μl KSOM containing either 0.2% DMSO (control), 1 mM 9-AC, 100 μM DIDS, or 1 mM 9-AC and 100 μM DIDS. Embryos were analyzed visually for the presence of an expanded, fluid-filled blastocoel after 40–48 h in culture. Embryos were imaged

using a Leica DFC450C digital camera attached to a stereo microscope (Leica MZ FLIII).

Patch-clamping

Standard whole-cell patch-clamp techniques were used to study Cl^- currents as described previously (Day *et al.* 1998) using a List EPC-7 patch-clamp amplifier (List, Darmstadt, Germany). All patch-clamp experiments were performed at room temperature. Patch-clamp pipettes were manufactured from borosilicate microhematocrit tubes (Modulohm, Herlev, Denmark) and had a resistance of 2–5 M Ω when filled with N-methyl-D-glucamine (NMDG)-glutamate pipette solution, which had the following composition (in mM): NMDG-glutamate (130), MgCl_2 (1), H-HEPES (10), glucose (10), H-EGTA (0.5), H-EDTA (0.01), pH adjusted to 7.2 with HCl (~23 mM), with an osmolality of 290–300 mOsm/kg. Use of this pipette solution eliminated currents due to outward K^+ movement through K^+ channels and therefore enabled the analysis of Cl^- movement into the cell in isolation. The membrane potential was held at 0 mV and square-wave voltage pulses of 1 s duration between –120 and +120 mV were then applied. Currents were low-pass filtered, sampled, and digitized at 0.2 kHz with a MacLab-4 data acquisition interface (AD Instruments, Sydney, NSW, Australia) attached to a Macintosh computer. By convention, outward current is the movement of positive charge out of the pipette (i.e. out of the cell in whole-cell recording mode) or movement of negative charge into the pipette/cell. An agar bridge (1% (w/v) agar in 150 mM KCl) was used to connect the reference electrode to the bath solution when necessary. Liquid junction potentials between the bath and pipette electrodes were calculated using the Henderson equation (Barry & Lynch 1991) and were used to correct holding potentials. Currents are expressed relative to the cell capacitance (pA/pF) calculated from a voltage pulse to –10 mV in order to adjust for differences in cell size between each embryonic stage. Current–voltage relationships

(I–V curves) were plotted and the chord conductance was calculated between +100 mV and the voltage at which current is zero (i.e. the reversal potential). The chord conductance is the slope of the line on the I–V curve between the current at +100 mV and the reversal potential and is used when the I–V curve is not linear. The chord conductance eliminates the effect of any changes in reversal potential on the calculation of conductance. The Cl^- sensitive conductance was calculated by subtracting the conductance remaining after removal of extracellular Cl^- from the conductance obtained in the presence of extracellular Cl^- . Similarly, the DIDS-sensitive conductance was calculated by subtracting the conductance obtained in the presence of DIDS from the conductance in the absence of DIDS, with both these recordings made in the presence of extracellular Cl^- .

The standard bathing solution used was protein-free M2 medium (Fulton & Whittingham 1978) containing (in mM) NaCl (140), MgCl_2 (1.19), CaCl_2 (1.71), H-HEPES (10), and glucose (5.56). The chloride-free solution contained Na-glutamate (140), MgCl_2 (1.19), CaCl_2 (1.71), H-HEPES (10), and glucose (5.56). The osmolality of all solutions was 290–300 mOsm/kg and the pH was adjusted to 7.40.

RT-PCR of embryonic mRNA

Freshly recovered conceptuses were rinsed and sorted into homogeneously staged cohorts of 50. Each cohort was transferred to a PCR tube in minimal volume (~0.1 μl) of medium and the tube was immediately immersed in liquid nitrogen. Tubes were stored at –80 °C until used in RT-PCR. Alternatively, embryos were collected into 100 μl Tri-reagent (Sigma), 50 $\mu\text{g}/\text{ml}$ glycogen was added to aid in forming the RNA pellet. Tubes were stored at 4 °C until used in RNA extraction. RNA extraction was carried out using Tri-reagent as per manufacturer's instructions (Sigma).

A RT mix containing 1 mM dNTPs (Invitrogen), 20 ng/ml random hexamers (Invitrogen), 10 mM dithiothreitol

Table 2 Primers used for RT-PCR amplification of mouse *Clcn* transcripts.

Gene (accession number)	Direction	Sequence (5'–3')	Location (nucleotide)	Annealing temperature (°C)	Amplicon size (bp)
<i>Clcn1</i> ^a (NM_013491)	S	CTGCATTGGAAGGCTGGTAGGAG	1557–1717	62	161
	AS	AATGACGGCTGTGGAGACTGTGTG			
<i>Clcn2</i> (NM_009900)	S	TGAGTCCATGATCCTACTG	2519–2827	55	309
	AS	CCTGCTGACTCCATGTTG			
<i>Clcn3</i> (NM_007711)	S	CCTCTTTCCAAAGTATAGCAC	1270–1818	55	549
	AS	CTGGCATTGATGTCATTTT			
<i>Clcn4-2</i> (NM_011334)	S	GAGGACTTCCACACCATA	509–919	55	411
	AS	TGCAAACAGCAACGCCATA			
<i>Clcn5</i> (NM_016691)	S	GGAACATCTTGTCCTACTG	895–1457	55	563
	AS	TGTGTTGAAGTGGTTCTC			
<i>Clcn6</i> (NM_011929)	S	TCTTCCACGAGTCAAACC	1207–1612	55	406
	AS	TCATCCTTACAACCCAC			
<i>Clcn7</i> (NM_011930)	S	CGTGAGAATGACCCTTAGCC	1754–2084	60	331
	AS	GTCTCCTACATCTCCACCAC			
<i>Clcnka</i> and <i>b</i> (NM_024412/ NM_019701)	S	CATCACCTCCATCTACAAGACC	841–1299 (k1)	55	459
	AS	AGAATCAGCATCCAGAAC	834–1292 (k2)		
<i>B-actin</i> (NM_007393)	S	CATGTACGTAGCCATCCAG	472–1231	52	760
	AS	AAACGCAGCTCAGTAACAG			

S, sense primer; AS, antisense primer.

^aYamamoto-Mizuma *et al.* (2004).

(Invitrogen), 0.1 mg/ml BSA (Amersham Pharmacia Biotech), and 16 units RNasin (Promega) was prepared and the volume adjusted to 8.9 µl with double-distilled water (Baxter, Toongabbie, NSW, Australia). Before addition of reverse transcriptase, the reaction mix was added to the PCR tube containing frozen conceptuses and heated to 65 °C for 1 min. Two hundred units of reverse transcriptase (superscript II, Invitrogen) was then added and the RNA was reverse transcribed at 37 °C for 60 min. The RT reaction was terminated by heating to 95 °C for 10 min. The resulting cDNA samples were stored at –20 °C until use.

PCR primers were designed using GCG (ANGIS) based on cDNA sequence data available in GenBank. The primers used and the expected PCR product sizes are shown in Table 2. A PCR mix containing PCR buffer (Promega), 25 µM MgCl₂ (Promega), 2.5 units Taq DNA polymerase (Promega), and 0.5 µM of 5' and 3' gene-specific primers was prepared and made up to 40 µl with double-distilled water. This reaction mix was then added to 10 µl cDNA. The PCR was run on a GeneAmp PCR system 2400 (PE Applied Biosystems) at 94 °C for 1 min followed by 30–40 cycles of amplification (94 °C for 30 s, 30 s at annealing temperatures of 52–60 °C depending on the primer set used (Table 2), and 68 °C for 4 min) and a final extension at 72 °C for 7 min.

PCR products were run on a 2% (w/v) agarose gel, the correct sized bands were cut from the agarose gel, and the DNA was purified using a gel extraction kit according to the manufacturer's instructions (Qiagen). PCR products were cloned into the pGEM-Teasy vector and sequenced by the Australian Genome Research Facility (AGRF).

Immunostaining

Zona-free embryos were fixed by incubation in 4% (w/v) paraformaldehyde in PBS containing 1 mg/ml polyvinyl alcohol (PBS/PVA) for 30 min. Embryos were then permeabilized by incubation in the same solution containing 0.3% Triton X-100 for 30 min. The embryos were then washed in PBS+PVA and then incubated in PBS/PVA containing 0.7% (w/v) BSA (PBS/PVA/BSA) for 30 min at room temperature before incubation in 8 µg/ml anti-CLCN3 antibody (Alomone Labs, Jerusalem, Israel) in PBS/PVA/BSA for 2 h at room temperature. Embryos were then washed three times in PBS/PVA/BSA and incubated in 10 µg/ml Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) in PBS/PVA/BSA for 1 h. After washing three times in PBS/PVA/BSA, the embryos were mounted in vectashield containing 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) on a slide. All procedures were performed at room temperature. A confocal microscope (TCS NT, Leica) was used to observe the stained embryos (Bosch Institute, Advanced Microscopy Facility, University of Sydney). Images were captured using Leica confocal Assistant Software (version 4.02).

Statistical analysis

These data are expressed as the mean $\pm$ S.E.M. with the number of experiments (*n*) in parentheses. Normality was tested using a

Shapiro–Wilk test and data were compared using ANOVA followed by Tukey–Kramer *post hoc* test or paired Student's *t*-test (StatPlus, AnalystSoft, www.analystsoft.com) when appropriate. χ^2 Test was used to compare proportions. *P* values < 0.05 were considered statistically significant.

Chemicals

Culture solutions were made from cell culture grade reagents obtained from Sigma. DIDS and 9-AC (both from Sigma) were prepared as 100 mM and 1 M stock solutions in DMSO on the day of use respectively.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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