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Expression of cystic fibrosis transmembrane conductance regulator during early human embryo development

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Formation of the blastocyst is one of the first morphological changes in early embryonic development. Ion transport has been shown to be crucial for blastocoele cavity formation and expansion, although the mechanisms that underlie this process are presently unknown. As a transmembrane Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR) may participate in ion transport and early blastocoele formation. CFTR mRNA was detected throughout preimplantation embryo development and in the unfertilized oocyte. Immunocytochemistry disclosed the presence of CFTR protein from the 8-cell stage, reaching maximum immunoreactivity at early blastocyst stage embryos. Patch clamp electrophysiology of morulae and blastocysts demonstrated typical CFTR Cl^- channel activities in the apical membrane of trophectoderm cells. Thus CFTR is expressed both at mRNA and protein levels in human morulae and blastocysts, and functions as a cAMP-regulated apical membrane Cl^- channel. These data suggest that CFTR may contribute to blastocoele formation in the early human embryo.

Key words: blastocoele/blastocyst/CFTR/cystic fibrosis/embryo cavitation

Introduction

Mammalian preimplantation embryonic development is characterized by a series of rapid cell divisions. Maternally derived products deposited in the oocyte during oogenesis control the first cleavage divisions. Further embryonic development, which in mammals requires activation of the embryonic genome, occurs between the 2- to 8-cell stages (Braude *et al*., 1988; Telford *et al*., 1990). Subsequent cleavage stages lead to the first differentiation process, which results in the creation of a blastocyst. The blastocyst contains two morphologically distinct cell types: trophectoderm, which gives rise to the placenta, and the inner cell mass (ICM), which is composed of relatively undifferentiated cells that will develop into the embryo (Cross, 2000).

Knowledge of the physiology of blastocoele fluid formation was initially obtained by extensive studies on the rabbit which has a conveniently large blastocyst, beginning with the independent work of Smith, Gamow and Prescott, and Daniel (Daniel, 1970; Gamow and Prescott, 1970; Smith, 1970). Later studies focused on the mouse, in which the genetic control of the proteins involved in this process could be studied in depth (Biggers *et al*., 1988; Watson *et al*., 1992). After the 8-cell stage, the mouse embryo contains tight junctions between the adjacent blastomeres and the cells become tightly sealed with each other. This cellular process, referred to as compaction, results in formation of the morula (Ducibella *et al.*, 1975; Fleming and Johnson, 1988). The next developmental stage requires the first obvious cellular differentiation, accompanied by creation of a liquid filled cavity—the blastocoele. During this process, external cells form the outside layer of trophectoderm, while internal cells establish the ICM. The trophectoderm cells, which represent the first differentiated cell type in development, form a polarized epithelium joined by tight junctions. This cell type represents the first functional epithelium in mammalian embryo development. Vectoral fluid transport by this epithelium into the intercellular space generates the blastocoele cavity (Manejwala *et al*., 1989) which is crucial for subsequent embryonic development.

Previous reports have shown that Na^+ and Cl^- play a major role in blastocoele formation (Cross, 1973; Borland *et al.*, 1976; Manejwala *et al*., 1989). Na/K-ATPase, located in the basolateral membrane of the trophectoderm cells, is necessary for fluid accumulation (Overstrom *et al*., 1989). However, further details regarding the molecular mechanisms involved in blastocoele function are unclear. Na^+ flux into mouse trophectoderm cells is reduced by inhibitors of Na^+/H^+ exchange and Na^+ channels (Manejwala *et al*., 1989). In rabbit trophectoderm, transepithelial Na^+ transport seems to involve amiloride-sensitive Na^+ channels and Na^+/H^+ exchangers (Robinson *et al*., 1991), as can be observed in other fluid-transporting epithelia. The pathways utilized by Cl^- are less well defined. In the mouse embryo, early reports suggested that Cl^- transport is passive and paracellular since some Cl^- channel inhibitors, including diisothiocyanatostilbene-disulphonic acid (DIDS) and 2-(3,4-dichlorbenzyl)-5-nitrobenzoic acid, had no effect on Cl^- uptake or blastocoele formation (Manejwala *et al*., 1989). In contrast, blastocoele formation

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was reduced by furosemide, DIDS and anthracene-9-carboxylic acid in rat (Brison and Leese, 1993) and rabbit embryos (Benos and Biggers, 1983). Interestingly, a previous report showed that both Cl^- efflux as well as re-uptake into Cl^- depleted blastocoele, occurs via a Cl^- channel-like mechanism (Zhao *et al*., 1997). However, the identity of this Cl^- channel remains unknown.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a protein encoded by the cystic fibrosis (CF) gene (Riordan *et al*., 1989). CFTR is a cAMP-regulated Cl^- channel (Bear *et al.*, 1992) which is expressed in epithelial cells in a number of adult tissues, including the pancreas, intestine, lungs and salivary glands (Kartner *et al*., 1992). It plays a role in transepithelial ion and fluid transport by providing a pathway for Cl^- across the apical membrane. CFTR has been detected in mid-trimester fetal tissue (Harris *et al.*, 1991; McCray *et al.*, 1992) and the CFTR mRNA transcript has been found in human fetuses as early as 10 weeks gestation (Tizzano *et al*., 1993). However, CFTR expression and its possible involvement in human preimplantation embryo development have not been established. We report here that CFTR is expressed during early human embryo development, and that it functions as a cAMP-regulated Cl^- channel in apical membranes of the human blastocyst. Moreover, maternally deposited mRNA for CFTR protein may explain the mechanism of how CFTR mutant blastocysts cavitate and are able to develop beyond the implantation stage.

Materials and methods

Human samples

Human cumulus cells, unfertilized oocytes and spare preimplantation embryos were obtained from the IVF programme at The Toronto Hospital, General Division. Patients who chose not to freeze their spare embryos for future transfers were asked to donate these embryos for research and their informed consent was obtained. This research was approved by the Committee for Research on Human Subjects of the Toronto Hospital. Donated spare embryos were incubated in HAM's-F10 media (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated human serum. Morphological assessment of the developing embryos was recorded daily until they reached the blastocyst stage, ~5–6 days after oocyte insemination.

Nucleic acid preparation from a small number of cells and RT–PCR

We used RT–PCR for a small number of cells as previously described (Brady and Iscove, 1993). To ensure adequate nucleic acid extraction, we used one blastocyst or morula, 3–5 embryos in early cleavage stages or ~100 cumulus cells, which were loaded into guanidinium thiocyanate solution. Total nucleic acid was recovered from each sample by ethanol precipitation using glycogen as a carrier. The pellet of nucleic acid obtained from the embryos and cumulus cells was reverse transcribed as previously described (Jurisicova *et al*., 1996). RNA from first trimester placenta or intestinal epithelium was used as a positive control (up to 1 μg total). The RT product was divided into two portions for PCR amplification of CFTR and β2-microglobulin (β2m) transcripts. Thirty-five cycles of PCR, which included denaturation at 95°C for 1 min, annealing at 61°C for 1.5 min and extension at 72°C for 2 min, were performed in a PCR Thermal Cycler (Perkin-Elmer). Following PCR, the amplified products were separated on a 2% agarose gel along with a 1 kb DNA ladder. To differentiate between the possible genomic contamination, primers were designed to span the two last exons (23 and 24) of the CFTR gene. The desired amplified product of the CFTR cDNA is only 278 bp. The sequence of the primers was as follows: CF1 5′-CACAGGATAGAAGC-AATGCTG-3′ and CF2 5′-CATGAGCAAATGTCCCATGAC-3′. The specificity of the CFTR cDNA amplified fragment was confirmed by restriction digestion with the Alu I enzyme, which was expected to cut the RT–PCR product into two 140 bp products. Primers for β2m were used as previously described (Jurisicova *et al*., 1996).

Quantitative analysis of CFTR mRNA expression

In order to quantitate expression of the CFTR transcript during preimplantation embryo development, we used a RT–PCR dot blot-based assay (Rambhatla *et al*., 1995). Three samples of cDNA at each developmental stage, derived from a single oocyte or embryo, were amplified as previously described (Brady and Iscove, 1993). The amplified material was then analysed by hybridization of dot blots with random prime radiolabelled cDNA probes, followed by quantitation of signals on a phosphorimager as described (Rambhatla *et al*., 1995). The cDNA probes used for analysis recognize the 3′ untranslated regions (3′ UTR) of β-actin and CFTR, and were either cloned from an oligo dT-primed human full term placenta cDNA library (ATCC, Rockville, USA) or amplified by RT–PCR using primers located within 3′ UTR (5610–6050 bp) of the CFTR transcript (accession no. M28668). The identity of both clones was confirmed by sequencing using a dsDNA Cycle Sequencing Kit (Gibco).

Immunocytochemistry

A mouse monoclonal CFTR antibody of isotype IgG1-*kappa* (M3A7) was used as the primary antibody. This antibody recognizes an intracytoplasmic domain of the human CFTR protein between amino acids 1195–1480 (Kartner *et al*., 1992). As a secondary antibody, we used biotinylated swine immunoglobulin (Multilink; Dako, Denmark). The complex was identified by streptavidin conjugated to Texas Red (Calbiochem, La-Jolla, CA, USA).

Morulae and blastocysts were washed twice with phosphate-buffered saline (PBS) and either fixed on slides with cold acetone or processed as whole mounts. Fixed samples were washed in PBS for 10 min at room temperature, preincubated in PBS + 0.1% Triton-X supplemented with 10% normal swine serum (PTS) for 10 min, and then incubated with M3A7 antibody (20 μg/ml; 4°C, overnight). Following two washes in PBS, the embryos were re-incubated in 1:150 dilution of Multilink for 30 min. A final incubation was performed at 4°C in Streptavidin–Texas Red in PBS for 45 min (1:200 dilution), followed by three washes in cold PBS (10 min each) and counterstaining with nuclear fluorochrome, DAPI. Control embryos were exposed to the same conditions omitting M3A7 antibody in the primary incubation or were exposed to non-specific IgG antibody as previously described (Kartner *et al*., 1992). All samples were examined using a Zeiss Axioplan Universal microscope and photographs were taken with a Zeiss microscope Camera MC 100.

Electrophysiology

Oocytes or embryos were transferred onto a glass coverslip and allowed to stick on the stage of an inverted microscope mounted in a perfusion chamber, and visualized with video enhancement. Prior to transferring the samples to the chamber, the zona pellucida was removed using acid Tyrode's solution and the embryos were extensively washed in fresh culture medium.

The patch clamp technique was used to record single channel currents in cell-attached and excised (inside-out) patches. Recordings were usually carried out with identical solutions in the pipette and bath, using either (i) an NMDG-Cl solution (in mmol/l): 120 N-methyl-D-glutamate (NMDG), 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, 5 glucose, pH 7.4 with HCl; or (ii) a CsCl solution (in mmol/l): 120 CsCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, 5 glucose, pH 7.4 with CsOH. Data are shown for experiments performed with the NMDG solutions. Similar results were obtained with the Cs^+ solution (data not shown). In asymmetrical conditions, NMDG-Cl was reduced to 20 mmol/l in the bath and 200 mmol/l glucose was added to balance the osmotic pressure. The patch pipettes used (<0.5 μm diameter) had a resistance of 15–30 megaohms and were heat polished. In cell-attached recordings, intracellular levels of cAMP were elevated by addition to the bath of a cocktail (FIC) containing forskolin (1–10 μmol/l), 3-isobutyl-1-methylxanthine (IBMX; 10–100 μmol/l) and chlorophenylthio-cAMP (CPT-cAMP; 10–100 μmol/l). In excised patches, the catalytic subunit of protein kinase A (PKA; 90 nmol/l) and ATP (1 mmol/l) were applied to the bath solution. Treatments were added directly as a stock solution to achieve the desired concentration, or by perfusion (3–4 ml/min). All experiments were performed at room temperature. Currents were amplified (Axopatch-1D; Axon Instruments, Inc.), digitized (Macintosh computer via ITC-16 interface; Instrutech Corp.) and saved directly onto the hard disk (Pulse+PulseFit software; HEKA Electronik). Data were analysed using Pulse+PulseFit and MacTac (Skalar Inst.). Open probability (Po) of single channels was determined for 20 s periods of recording. Data were expressed as original values or mean ± SEM, and curves were fitted using simple regression analysis. X/Y indicates that X out of total Y patches responded to a drug.

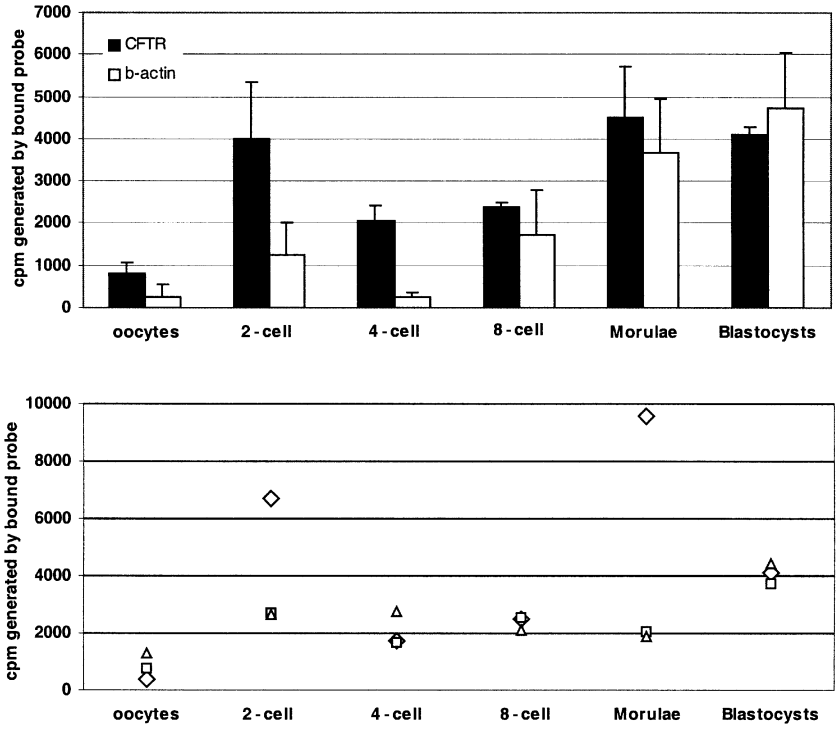

Figure 1. Expression of CFTR and β-actin transcripts in oocytes and normal human embryos. (**A**) This graph depicts the levels of transcripts expressed during early embryo development in units of bound counts per min (cpm). Embryo stage is shown below each graph. Bars denote the mean ± SEM of expression. Three samples of oocytes or embryos were analysed for each stage. (**B**) Scatter plot representation of the raw data obtained for levels of CFTR transcripts in units of bound cpm for oocytes and each preimplantation stage. The trend observed suggests that the blastocysts express the highest levels of CFTR mRNA.

Results

RT–PCR detection of CFTR and β2m in human embryos

RT–PCR analysis of RNA isolated from intestine with primers CF1 and CF2 resulted in the expected 278 bp CFTR product. Cumulus cell samples obtained from five different patients, as well as first trimester placenta, were also positive for CFTR expression. A β2m specific product (123 bp) was amplified from all samples analysed. CFTR transcript was detected in all human embryo samples analysed from the 8- to 12-cell stage onwards ($n = 3$ for each stage; data not shown). Expression persisted throughout the morula and blastocyst stages. All these developmental stages were also positive for β2m expression, which served as the internal control. The specificity of the CFTR cDNA amplified fragment from human blastocysts was also determined by restriction digestion analysis with the Alu I enzyme, which yielded the expected 140 and 138 bp fragments.

In order to estimate the abundance of CFTR transcript throughout human preimplantation embryo development, we used quantitative RT–PCR dot blot analysis. Results are shown in Figure 1A and revealed that CFTR mRNA is abundantly stored in unfertilized oocytes and 2-cell stage embryos and later appears to be re-expressed after activation of the embryonic genome from the 8-cell stage onwards, with the highest levels observed in blastocysts. Figure 1B depicts the raw data obtained for the level of CFTR transcripts for three independent samples shown in Figure 1A. These results suggest that CFTR expression levels are highest in blastocysts.

Detection of CFTR protein during human preimplantation development

No specific CFTR fluorescence was observed in the cytoplasm of unfertilized oocytes, polypronuclear zygotes or early cleavage stage embryos (2- to 4-cell stage) using indirect immunocytochemistry with a CFTR-specific antibody (Figure 2A–D). In addition, no specific CFTR staining was found on the surface of either oocytes or embryos when non-specific IgG antibody was used or when the primary antibody was omitted (Figure 2G,H). The first developmental stage exhibiting cytoplasmic but no membrane staining of CFTR was at the 8-cell stage (Figure 2E). Strong staining persisted in an 8- to 16-cell embryo (Figure 2F).

At the early blastocyst stage, strong antibody-associated fluorescence was observed in both the trophectoderm and the ICM (Figure 3C). At the expanded blastocyst stage staining appeared to be predominately localized to membrane compartments (Figure 3D). In these cases, where the blastocoele was extensively filled with fluid, acetone fixation of the embryos affected the morphology and it was sometimes difficult to localize the ICM within the blastocoele cavity. However, in a few hatching blastocysts ($n = 3$) staining was observed in the ICM area and the overlaying polar trophectoderm with minimal to no staining in the mural trophectoderm (Figure 3F).

Activity of CFTR at the blastocyst stage

The patch clamp technique was used to determine whether CFTR functions as a regulated Cl^- channel in the apical membrane of trophectodermal cells. Single channel activities in the apical plasma membranes of human blastocysts were recorded in both cell-attached and inside-out patches. In unstimulated blastocysts, no channels were observed in the cell-attached configuration. Addition of a cocktail containing forskolin (1–10 μmol/l), IBMX and CPT-cAMP (each 10–100 μmol/l) activated low-conductance channels in 5/7 patches (Figure 4A). Upon excision of the patch the channel activities disappeared, but subsequent addition of the catalytic subunit of PKA (90 nmol/l) and ATP (1 mmol/l) to the bath activated similar channels in 5/5 inside-out patches (Figure 4A). The activities sometimes appeared as single channels, but more often multiple channels were

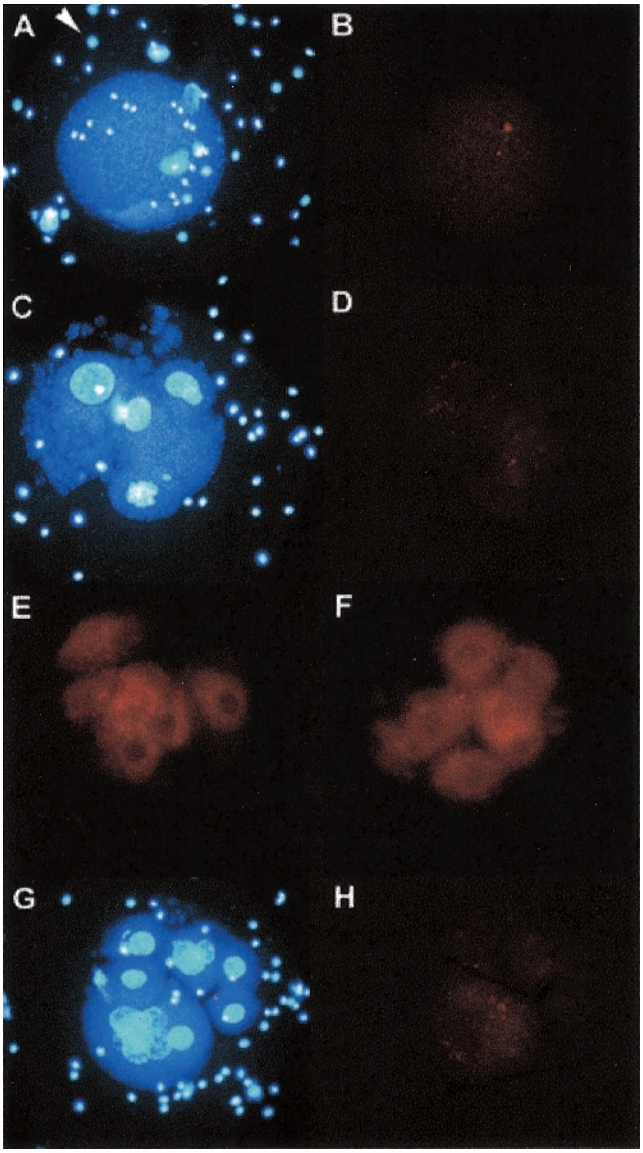

Figure 2. Detection of CFTR protein during human preimplantation embryo development. (**A**) DAPI staining of an unfertilized oocyte arrested at MII. Multiple sperm (arrowhead) could be seen attached to the zona pellucida. (**B**) CFTR protein could not be detected in the oocyte shown. (**C**) A 4-cell stage embryo stained with DAPI. (**D**) No CFTR associated staining could be observed at this developmental stage. (**E**,**F**) Six- to 8-cell stage embryos stained with M3A7 antibody. The staining is strictly localized to the cytoplasmic component of the blastomeres. Negative control showing lack of staining in a 8- to 16-cell stage human embryo (**G**,**H**) when the primary antibody was omitted. All images were viewed under 400× magnification.

observed (Figure 4C). Single channels had a conductance of 6–10 pS and a linear I-V relationship (Figure 4B). The channels were Cl^- selective, since imposing a Cl^- gradient across the patch (120 mmol/l Cl^-_{ext}/20 mmol/l Cl^-_{cyt}) shifted the I-V curve to negative voltages, as predicted for Cl^- selective channels (Figure 4B). The channel conductance was inhibited by the presence of 60 mmol/l iodine on the extracellular side of the channel (Figure 4C,D), whereas the channels were insensitive to the anion transporter inhibitor DIDS (Figure 4E). The open probability Po in single channel recordings was 0.58. Open and closed time histograms of single channel currents showed one open and one closed state (23 and 17 ms respectively) when measured at +60 mV. The characteristics of these channels resemble CFTR Cl^- channels observed in the plasma membranes of many cell types (Welsh *et al*., 1992).

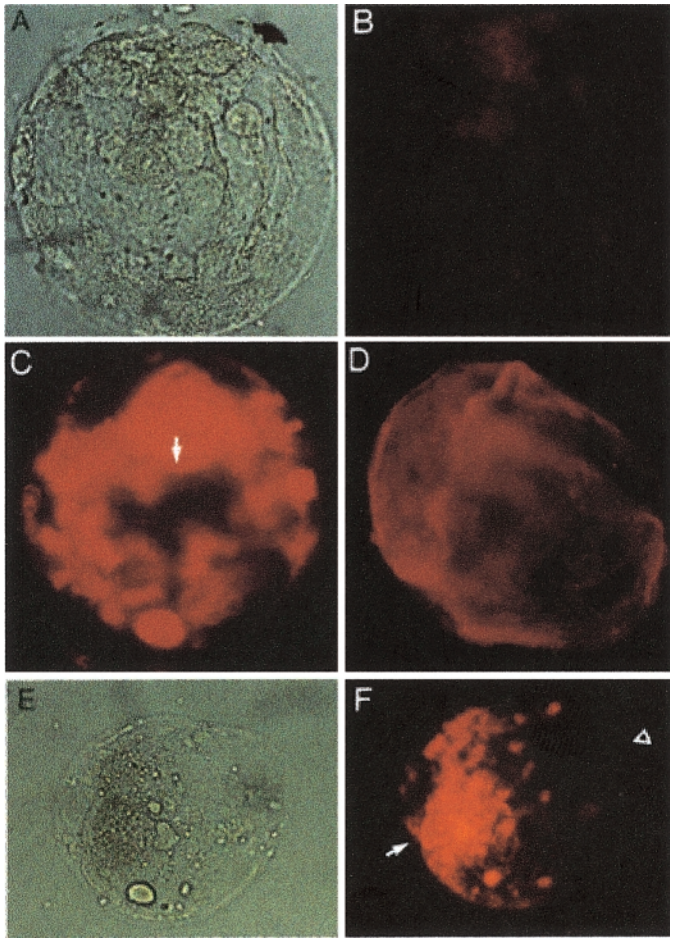

Figure 3. Detection of CFTR protein during human blastocyst development. (**A**) Light micrograph of a human blastocyst used as a control for CFTR staining. (**B**) No staining was observed with the omission of the primary antibody. A strong signal for CFTR protein was detected at the cavitating morula stage (**C**) in both trophectodermal cells as well as the ICM. The formation of the blastocoele cavity is clearly evident at this developmental stage (arrow). (**D**) An expanded blastocyst stained with CFTR. (**E**) Light micrograph of a hatching blastocyst (200× magnification). (**F**) CFTR staining was predominately observed in the ICM and polar trophectoderm (arrow) at this developmental stage (200× magnification). No positive staining was observed on the mural trophectoderm (arrowhead). All images were viewed under 400× magnification unless otherwise specified.

Discussion

Compaction of embryonic blastomeres at the 8-cell stage in mice and the 16-cell stage in humans results in the formation of external and internal blastomeres. The internal cells develop into the ICM which gives rise to the embryo proper. In contrast, the external cells become polarized, with apical membranes containing amino acid transport systems (DiZio and Tasca, 1977) and Na^+ channels (Manejwala *et al*., 1989; Robinson *et al*., 1991). In addition, the apical membranes have 'apposed' basolateral regions which are rich in gap and tight junctions and have high levels of Na^+-K^+-ATPase and the cell adhesion molecule uvomorulin (Vestweber *et al*., 1987). The differentiation of trophectoderm cells into polarized epithelial cells is hypothesized to result from asymmetric cell–cell contacts which lead to an ionic gradient across the blastomeres (Wiley *et al*., 1990). The development of trophectoderm polarity is in turn proposed to promote vectoral transport of solutes and water, with the consequent production of blastocoele fluid. Expansion of the embryo, and blastocoele formation, are essential for further differentiation of the ICM and for hatching of the embryo from the zona pellucida.

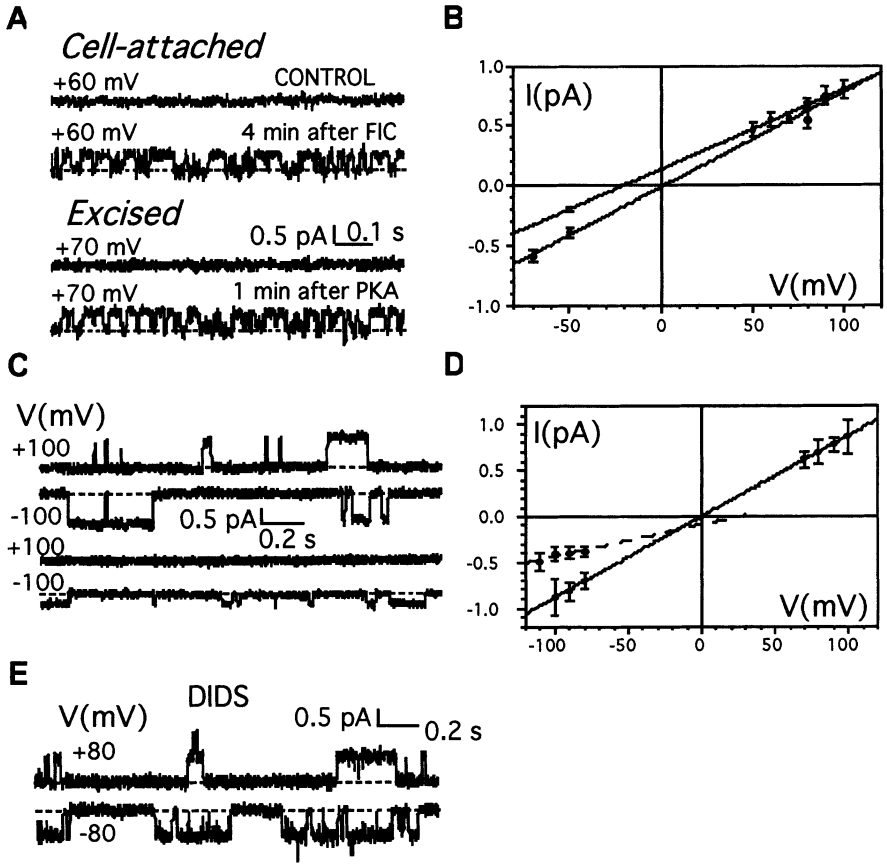

Figure 4. CFTR Cl^- channel activities in human blastocyst. (**A**) Single CFTR Cl^- channels recorded from the same patch in cell-attached and subsequently in excised (inside-out) patch configurations. Symmetrical NMDG-Cl solutions were present in bath and pipette. A cocktail (FIC) containing forskolin (10 µmol/l), IBMX (100 µmol/l) and 8-bromo-cAMP (10 µmol/l) activated single Cl^- channel activities in the cell-attached mode 4 min after its application. After excision, protein kinase-A (PKA) (90 nmol/l) in the presence of ATP (1 mmol/l) stimulated single Cl^- channels 1 min after addition. Membrane potentials were as indicated. Dashed lines represent closed state. Results are representative of five experiments. (**B**) I-V relationships for activities recorded in the inside-out mode in symmetrical (120 mmol/l NMDG-Cl; open circles) and asymmetrical (120 mmol/l NMDG-Cl in pipette, 20 mmol/l NMDG-Cl in bath; closed circles) solutions. Slope conductance in symmetrical conditions was 7.9 ± 0.3 pS (three patches). Under asymmetrical conditions, the I-V curve was shifted to the left, indicating Cl^- selectivity (three patches). Data filtered at 300 Hz. (**C**) Block by iodide in inside-out patch. In two upper traces, pipette and bath solutions contained (in mmol/l): 120 KCl, 3 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, 5 glucose, pH 7.3 with KOH (five patches). In two lower traces, the pipette solution contained 60 mmol/l I^-, replacing Cl^- (four patches). PKA (90 nmol/l) and ATP (1 mmol/l) were present in the bath. Membrane potentials (V) were as indicated. (**D**) I-V relationship for currents shown in (**C**). The slope conductance under symmetrical conditions (closed circles) was 8.7 ± 1.9 pS (five patches). With I^- on the extracellular side (open circles), conductance was reduced to 3.4 ± 0.4 pS (four patches). The shift of extrapolated I-V curve (dashed line) toward positive potentials indicates the channel is selective for Cl^- over I^-, though currents at positive voltages could not be resolved. (**E**) Insensitivity of CFTR Cl^- channels to DIDS (0.3 mmol/l). Results are representative of six experiments. Data were filtered at 300 Hz.

Recently it was proposed that water transport via aquaporins, major intrinsic membrane proteins that are present in the trophoblast cells of the preimplantation embryo, are involved in blastocyst formation (Offenberg *et al*., 2000). These proteins, 10 of which have been identified to date in mammals, function as molecular water channels in somatic epithelia that allow for the rapid flow of water across plasma membranes. Preliminary results of aquaporin inhibitors strongly suggest a role for these water channels in blastocyst formation (Watson and Barcroft, 2001). Thus, if CFTR facilitates water and chloride transport, clear evidence exists regarding the redundancy of molecular pathways in this system.

Previous studies in rabbits have shown that fluid accumulation in the blastocoele is secondary to active transport of Na^+ and Cl^- (Cross, 1973; Borland *et al.*, 1976), although the exact mechanisms responsible for blastocoele formation remain unclear. This ionic transport necessitates entry of the ions through the apical side of the cells and extrusion from the basolateral side mediated by Na^+-K^+-ATPase (DiZio and Tasca, 1977; Benos, 1981; Wiley, 1984). Manejwala *et al.* confirmed the crucial role of Na^+ and Cl^-, but not K^+ in mouse blastocoele formation by demonstrating that elimination of Na^+ or Cl^- from the medium resulted in a 2-fold reduction in the rate of blastocoele expansion, while elimination of K^+ had no effect (Manejwala *et al.*, 1989). Furthermore, the use of Na^+ transport inhibitors indicated that Na^+ transport is mediated by several routes, including Na^+ channels, a Na^+/H^+ exchanger and the ouabain-sensitive Na^+-K^+-ATPase. Also present throughout preimplantation mouse embryo development are bicarbonate/chloride exchangers which regulate intracellular pH and which are necessary for embryo viability (Zhao *et al*., 1995; Zhao and Baltz, 1996). Swell-activated anion channel activity has also been characterized in early preimplantation mouse embryos (Kolajova *et al*., 2001).

Cl^- depleted medium strongly inhibits mouse blastocyst formation (Manejwala *et al.*, 1989; Brison and Leese, 1993). The use of Cl^- transport inhibitors has yielded conflicting results, and it is still not clear whether Cl^- transport is transcellular or paracellular. The results of our study suggest that CFTR may play a role in fluid transport in the human blastocoele by providing a transcellular pathway for Cl^- flux. Furthermore, the role of cAMP in blastocoele formation has been investigated in mouse embryos treated with forskolin and cholera toxin. Early cavitating embryos treated with these agents displayed

an increase in the rate of fluid accumulation, providing evidence that cAMP may have a physiological role in blastocoele formation (Manejwala *et al*., 1986). Whether the increased rate of blastocoele formation stimulated by cAMP could be mediated in part by CFTR channels remains to be elucidated.

In humans, we observed that mRNA for the CFTR gene was maternally inherited via storage of this transcript in the unfertilized oocytes. Similarly, Xenopus oocytes and early cleavage stage embryos express CFTR mRNA as detected by RT–PCR and by RNase protection assays (Tucker *et al*., 1992), suggesting conservation of CFTR biological function during early embryonic stages. Interestingly, no expression of the CFTR transcript could be observed in rodent ovaries using in-situ hybridization (Trezise *et al*., 1993), either reflecting differences in species or in the sensitivity of techniques used to detect expression. Species differences in the abundance and pattern of CFTR expression have been previously described in rodents and humans (Tizzano *et al.*, 1993; Trezise *et al.*, 1993; White *et al.*, 1998). In humans, no information is available about localization of CFTR protein during early stages of embryonic development. In our study, CFTR protein could be detected from the 8-cell stage onwards, and appeared to localize to the cytoplasm of blastomeres. However, later in development, staining was observed in both membranes and cytoplasmic compartments. Using patch clamp electrophysiology, Cl^- channels could easily be detected on the apical membrane of trophectodermal cells, confirming localization observed by immunocytochemistry. The properties of the detected channels are consistent with the activities of CFTR, confirming that CFTR is present during blastocyst formation and its protein functions as a cAMP-regulated Cl^- channel.

At the early-expanded blastocyst stage, CFTR protein appeared to be localized to all trophectodermal cells. In contrast, fully expanded hatching blastocysts only showed expression within the ICM and the proliferative cells that overlie the ICM—the polar trophectoderm. Down-regulation of expression was observed in cells that were in direct contact with the blastocoele cavity—the mural trophectoderm. Also, in preliminary patch clamp studies, we were not able to detect any cAMP-activated Cl^- channel activities in trophectoderm of hatched blastocysts, unlike in earlier blastocyst stages or morulae. These observations suggest that early embryonic expression reflects an involvement of CFTR protein in cavitation and blastocyst formation. Once the blastocyst is formed, the most differentiated cells of the blastocyst, the mural trophectoderm (Ziomek, 1987), seem to down-regulate their CFTR expression, most probably as a part of their differentiation pathway into primary giant cells (Cross, 2000). Thus, it is likely that activity of these Cl^- channels is no longer required within these cells.

In contrast to the mural trophectoderm, cells of the ICM and polar trophectoderm—cells that give rise to the embryo proper, extra-embryonic and placental tissues—maintain CFTR protein expression. This observation correlates with the presence of CFTR transcript in a variety of embryonic tissues at 10 weeks gestation (Tizzano *et al*., 1993). We were also able to detect CFTR transcript by RT–PCR in first trimester placenta (8 weeks gestation). However, the expression is likely to be not very strong, since Northern blot analysis as well as in-situ hybridization experiments have failed to detect the CFTR transcript in this tissue (Riordan *et al.*, 1989; E.Tizziano, personal communication). Consistent with this observation, chloride transport occurs in the syncytiotrophoblast membrane in both first and third trimester placenta via several mechanisms (Doughty *et al*., 1998).

If CFTR plays a role in blastocoele formation, how can embryos homozygous for CFTR mutations develop to term and be delivered? Our results suggest that mothers heterozygous for the CFTR mutation accumulate mRNA in the oocyte. Once the embryonic genome is activated, these maternally-derived CFTR transcripts may be translated into protein, which functions to produce at least some functional Cl^- channels and to rescue the embryo by promoting blastocoele formation. This same 'stockpile' mechanism has been proposed to be responsible for rescue of embryos from death caused by several targeted mutations during mouse preimplantation embryo development. One such example is disruption of β-catenin, where maternally-derived proteins persisted until the expanded blastocyst stage (Haegel *et al*., 1995). On the other hand, when a homozygous affected mother conceives a heterozygous baby, the functional paternal allele may allow for sufficient production of CFTR protein. Reduced fertility of CF women (Kotloff *et al*., 1992) has always been attributed to pathological changes in the secretions of the uterus, cervix and Fallopian tubes (Tizzano *et al.*, 1993; Trezise *et al.*, 1993), rather than to abnormal embryo development. We are not aware of any report in the literature where a CFTR homozygous-affected mother has given birth to a CF-affected child, suggesting that CFTR protein may be required for proper embryo cavitation in humans.

Alternatively, as previously indicated, other biologically redundant mechanisms may play a role in proper water and Cl^- transport during formation of the blastocoele. Other Cl^- channels may be present on the apical membranes of trophectodermal cells. These channels may be able to participate in blastocoele development in the absence of functional CFTR protein in CF defective embryos or might even be up-regulated to compensate for lack of CFTR function. Alternatively, trophectodermal transepithelial Cl^- entry may not be carrier-mediated and may occur through a paracellular route between the trophectodermal cells (Manejwala *et al*., 1989). In this case, CFTR may provide an additional pathway for transepithelial Cl^- flux, or it may be involved in other functions that do not affect early embryogenesis.

In conclusion, the CFTR transcript is abundantly stored in human oocytes, but it appears to be translated only from the 8-cell stage onwards. CFTR mRNA and its protein product is expressed at highest levels during blastocyst formation and functions as a regulated Cl^- channel in the apical membrane of trophectodermal cells at these embryonic stages. Our data suggest that the CFTR channel may contribute to blastocoele cavity formation in the normal human embryo and that its expression is tightly linked with the formation of the blastocyst.

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