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Generation of SV40-transformed rabbit tracheal-epithelial-cell-derived blastocyst by somatic cell nuclear transfer

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Abstract

The prospect of developing large animal models for the study of inherited diseases, such as cystic fibrosis (CF), through somatic cell nuclear transfer (SCNT) has opened up new opportunities for enhancing our understanding of disease pathology and for identifying new therapies. Thus, the development of species-specific in vitro cell systems that will provide broader insight into organ- and cell-type-specific functions relevant to the pathology of the disease is crucial. Studies have been undertaken to establish transformed rabbit airway epithelial cell lines that display differentiated features characteristic of the primary airway epithelium. This study describes the successful establishment and characterization of two SV40-transformed rabbit tracheal epithelial cell lines. These cell lines, 5RTEo- and 9RTEo-, express the CF transmembrane conductance regulator (*CFTR*) gene, retain epithelial-specific differentiated morphology and show CFTR-based cAMP-dependent Cl^- ion transport across the apical membrane of a confluent monolayer. Immunocytochemical analysis indicates the presence of airway cytokeratins and tight-junction proteins in the 9RTEo- cell line after multiple generations. However, the tight junctions appear to diminish in their efficacy in both cell lines after at least 100 generations. Initial SCNT studies with

the 9RTEo- cells have revealed that SV40-transformed rabbit airway epithelial donor cells can be used to generate blastocysts. These cell systems provide valuable models for studying the developmental and metabolic modulation of *CFTR* gene expression and rabbit airway epithelial cell biology.

Keywords

Respiratory airway; Epithelial cell lines; Somatic cell nuclear transfer; SV40 transformation; Cystic fibrosis transmembrane conductance regulator; Blastocyst; Rabbit (New Zealand White)

Introduction

A limitation to achieving a better understanding of cystic fibrosis (CF) pathology and to the development of novel therapies for CF has been the ineffectiveness of the CF mouse model (Grubb and Boucher 1999). The recent development of the CF pig (Rogers et al. 2008b, 2008c) and ferret (Li et al. 2006; Sun et al. 2008) have provided additional animal models that can potentially be used to elucidate CF pathology further and to develop therapies for CF. The rabbit is also an excellent candidate for an animal model of CF, because of the anatomical, genetic and biochemical similarities between the rabbit and the human and because of their gestation time and reproductive ability (Bosze et al. 2003; Chen et al. 2001; Diamond et al. 1991; Fan and Watanabe 2003; Shiffman et al. 1983). Both the phylogenetic nature of the rabbit and its physiology and anatomy make the rabbit a relevant model for the biochemical, molecular and physiological characterization of CF pathology and for the development of CF therapies (Vuillaumier et al. 1997; Zeitlin et al. 1992). Since no viable embryonic stem (ES) cell systems exist that have been established for the development of transgenic animals and germline transfer outside of the mouse, the generation of large animal models is dependent on cloning technology and somatic cell nuclear transfer (SCNT; Challah-Jacques et al. 2003; Kasinathan et al. 2001; Matsuda et al. 2002).

Verification that the same or similar biochemical and molecular pathways are in place in the animal model to be developed for CF requires that in vitro cell systems relevant to CF are developed. Whereas primary airway epithelial cell systems have been a powerful tool in this regard (Gruenert et al. 1990; Widdicombe et al. 1985), their limited lifespan attributable to senescence or terminal differentiation (Hayflick 1974, 1998) has been an impediment to the analysis of biochemical pathways and the development of CF therapies. This limitation for airway epithelial cells has however been overcome through the transformation and/or immortalization of cultured primary cells (Cozens et al. 1992a; de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1988, 1995, 2004). The transformation and immortalization of airway epithelial cells has been integral in the study of airway disease and for expanding our understanding of metabolic and genetic mechanisms underlying airway epithelial cell functions associated with CF pathology.

Once established, the transformed cells exhibit an enhanced potential to proliferate. Ultimately, the transformed cultures generally but not always, go through a period of “crisis” during which the cells either die or become immortalized established cell lines (de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1995). Most immortalized airway epithelial cell lines have been established by using simian virus 40 (SV40)-based systems (Gruenert et al. 2004). One approach that has been proven to be particularly effective has been the transfection of the pSVori- plasmid that contains a replication defective SV40 genome (Gruenert et al. 1988; Small et al. 1982). Previous studies that have focused on the transformation, immortalization and characterization of human airway epithelial cells (Cozens et al. 1992a, 1994; de Semir et al. 2008; Ehrhardt et al. 2006; Goncz et al. 1999;

Gruenert 1987; Gruenert et al. 1988, 1995, 2004) clearly demonstrate that the development of large animal models for CF will benefit from in vitro cell systems with regard to the effective validation of the biochemical features of the animal model. The present study has evaluated the transformation/immortalization of rabbit airway epithelial cells with the pSVori- plasmid that contains an SV40 viral genome defective in the SV40 origin of replication (Small et al. 1982). The transformed/immortalized rabbit tracheal epithelial (RTE) cells have been characterized karyotypically, immunocytochemically and in terms of airway epithelial cell gene expression, including *CFTR*, keratin and zonula occludens factor 1 (ZO-1) and functional Cl^- ion transport. The analyses of these features suggest that the cells can be used as an in vitro model of the airway epithelium and are potentially useful for the development of therapies for CF. In addition to the generation of in vitro cell systems from primary cells, SCNT used in the development of transgenic animal models has provided another potential means for generating lineage-specific immortalized cell lines. Several studies have evaluated the potential of primary epithelial cells to act as donor cells for SCNT for the production of a viable embryo or blastocysts (Gong et al. 2004; Kishigami and Wakayama 2009). However, no studies have evaluated the potential of using transformed cells to generate viable blastocysts. This report demonstrates that transformed aneuploid rabbit airway epithelial cells will form blastocysts at frequencies higher than those observed by using primary cells (Du et al. 1995).

Materials and methods

Isolation of RTE cells

An excised trachea from a female New Zealand White rabbit (*Oryctolagus cuniculus*; Evergen Biotechnologies) was rinsed four times with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, Calif., USA) containing 5 mM dithiothreitol and twice in PBS alone. The tissue was incubated overnight at 4°C in PBS containing 1 mg/ml pronase (Roche, Mannheim, Germany). The following day, epithelial cells were collected by flushing the tracheal lumen with the 4°C PBS-pronase solution. The cells were then pelleted by centrifugation. The cell pellet was washed once with ice-cold PBS containing 10% fetal bovine serum (FBS) to neutralize the pronase. The cells were then resuspended in modified serum-free LHC-8e medium (MLHC8e) and plated on tissue culture plastic coated with a solution containing 10 mg/ml human fibronectin (BD laboratories, Franklin Lakes, N.J., USA), 30 mg/ml Vitrogen (Cohesion Technologies, Palo Alto, Calif., USA) and 100 mg/ml bovine serum albumin (BSA) Fraction V (FN/V/BSA) in LHC basal medium (Invitrogen; Gruenert et al. 1990). Cultures were routinely grown in a humidified atmosphere under 5% CO_2 at 37°C.

RTE cell transformation

The passage-2 primary RTE cells were grown in pre-coated 100-mm dishes to 70%-80% confluence, trypsinized and then electroporated/nucleofected with the pSVori- plasmid by using the T-07 program and the Basic Nucleofector Kit for primary mammalian epithelial cells (Amaxa, Gaithersburg, Md., USA) according to the manufacturer's instructions as described previously (Maurisse et al. 2010). After electroporation, the transfected cells were immediately plated onto coated 100-mm culture dishes and grown to confluence in MLHC8e, with routine medium changes every other day. The isolation of the viable proliferating transformants was straightforward, because the nontransformed airway epithelial cells undergo terminal squamous differentiation and senescence (de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1988, 1995). Colonies of transformed cells were isolated by using cloning rings and expanded on coated 100-mm dishes in Eagle's Minimum Essential Medium (MEM) with Earle's salts and supplemented with penicillin/streptomycin,

glutamine (200 mM) and 10% FBS for further characterization. Although, multiple clones were isolated, clones 5RTEo- and 9RTEo- were initially selected for further analysis.

Immortalized human bronchial epithelial cells

The clones were compared with a well-characterized immortalized human airway epithelial cell line, 16HBE14o- (Cozens et al. 1994) in terms of their airway-epithelial-specific features. As above, these cells were routinely grown on the same pre-coated dishes and/or flasks in MEM supplemented with antibiotics, glutamine and 10% FBS under 5% CO₂.

Densitometric analysis of amplification of CFTR mRNA by reverse transcription with the polymerase chain reaction

Cytoplasmic RNA was extracted from the primary and immortalized cells by using the RNeasy mini kit (Qiagen, Valencia, Calif., USA) and then treated with DNase I (8.3 U/μg RNA, Roche, Mannheim, Germany) for 1 h at 37°C. RNA (3 μg) was heated to 70°C for 10 min and then reverse-transcribed by using random hexamers (1 μg), oligo dT (1 μg) and Superscript II RNase H⁻ reverse transcriptase (Invitrogen). *CFTR* mRNA from this reverse transcription was analyzed by standard polymerase chain reaction (RT-PCR) with rabbit-specific primers r1278F: 5'-ggctggatc tactggtgctg-3' (exon 9) and r1830R: 5'-aaatccggccgtagacttt-3' (exon 11). PCR amplification was carried out in a 50-μl solution containing 5 μl 10× *Taq Platinum* polymerase buffer, 1.5 μl MgCl₂ (10 mM), 1 μl dNTPs (10 mM), 0.5 μl *Taq Platinum* polymerase (Invitrogen), 1 μl primers of r1278F and r1830R (15 μM) and 2 μl cDNA. The conditions for the amplification were as follows: an initial denaturation for 3 min at 94°C, followed by 32 cycles of: denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 50 s, with a final extension of 7 min at 72°C. The 552-bp amplicon was sized on an E-Gel pre-cast 2% agarose gel (Invitrogen). Bands were analyzed densitometrically by using ImageJ software.

Immunocytochemical analysis

Passage-2 (P2) primary and transformed cells (P1.3) were grown on well slides (Lab-Tek, Thermo Fisher Scientific, Rochester, N.Y., USA) coated with the FN/V/BSA solution. After being washed, fixed and dried, the cells on the slides were rehydrated in PBS and stained for immunofluorescence analysis. The primary antibodies used were: mouse monoclonal antibody Pab 101 (anti-SV40 T Ag; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 1:50 dilution; AE1/AE3 (anti-cytokeratin) mouse monoclonal anti-human cytokeratin 1,2,3,4,5,6,7,10,13,14,15,16,19 antibody (DakoCytomation, Carpinteria, Calif., USA) at 1:10 dilution; DC-10 mouse monoclonal anti-cytokeratin 18 antibody (Santa Cruz Biotechnology) at 1:200 dilution; H-300 rabbit polyclonal anti-zonula occludens-1 (ZO-1) antibody (Santa Cruz Biotechnology) at 1:5 dilution. The secondary antibody, namely bovine anti-rabbit IgG conjugated to fluorescein isothiocyanate (Santa Cruz Biotechnology), was used at a 1:100 dilution. All the antibodies listed above were incubated with the cells for 30 min at 37°C and the cells were then washed three times with PBS.

The presence of the SV40 large T antigen indicated that the cells were transformed/immortalized by the pSVori-plasmid. Staining with the cytokeratin 18 (K-18) or the AE1/AE3 antibodies indicated the presence of an airway epithelial cytoskeletal structure. ZO-1 as a component of the *zonula occludens* was an indicator of intercellular tight junctions.

Cytogenetic analysis

Primary and immortalized RTE cells were grown to 70%-80% confluence in MLHC8e medium or MEM, respectively, on coated 100-mm dishes. The cultures were incubated overnight at 37°C in the presence of 0.04 mg/ml colcemid (Invitrogen). After a 25-min

exposure to hypotonic 75 mM KCl (Sigma, St Louis, Mo., USA), the cells were removed from the dish by using a cell scraper, centrifuged and fixed in Carnoy's solution (3:1 methanol: acetic acid). The cells were resuspended and spread dropwise on frozen glass slides. They were then air-dried and stained with Giemsa (4% in 10× PBS; Sigma) for 7 min. The 5RTEo- and 9RTEo- cells were treated with 0.025% trypsin in 10× PBS for 2 s to obtain a G-banding pattern. Stained slides were viewed without a coverslip under a Nikon microscope at 100× magnification. The average chromosome number was determined by counting chromosomes on microphotographs by using 3, 9 and 20 metaphase spreads for the 5RTEo-, RTE and the 9RTEo- cell lines, respectively.

Transepithelial electrophysiology

Stock cultures of confluent transformed/immortalized RTE cells (5RTEo-P1.12 and 9RTEo-P1.5) were trypsinized, resuspended in fresh MEM and plated on coated (as above) 12-mm clear polyester Snapwell inserts (Corning, Lowell, Mass., USA). Approximately 10^5 cells were plated onto each insert and grown for 9–11 days. Snapwell inserts were mounted via sliders into Easy Mount Ussing chambers (Physiologic Instruments, San Diego, Calif., USA) and short circuit current (I_{SC}) measurements were performed as previously described for CFBE41o- monolayers (Illek et al. 2010). Briefly, a typical four-electrode voltage clamp (VCC MC6, Physiologic Instruments, San Diego, Calif., USA) was connected via Ag/AgCl electrodes (World Precision Instruments, Sarasota, Fla., USA) to the solutions through 3% Agar bridges containing 1 M KCl. I_{SC} was continuously recorded at 5 Hz by an analog-to-digital board (DATAQ Instruments, Akron, Ohio, USA) and at 60 s intervals, the transepithelial voltage was clamped for 1 s from 0 to 1 mV to monitor transepithelial resistance (R_t) by using Ohm's law. A basolateral-to-apical Cl^- gradient was established to increase the electrochemical driving force for Cl^- secretion across the apical membrane and the transepithelial I_{SC} measured under these conditions was termed I_{Cl} . The basolateral solution was composed of 120 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM NaH_2PO_4 , 5.6 mM glucose, 1.0 mM $CaCl_2$ and 1.2 mM $MgCl_2$, whereas the apical Cl^- -free solution was composed of 120 mM Na-gluconate, 20 mM $NaHCO_3$, 5 mM $KHCO_3$, 1.2 mM NaH_2PO_4 , 5.6 mM glucose, 2.5 mM $Ca(gluconate)_2$ and 1.2 mM $MgSO_4$. Both chamber compartments were separately perfused with 5 ml of each solution at 37°C, respectively and gassed with 5% CO_2 in air to give a pH of 7.4. Positive currents were defined as the movement of anions in the basolateral-to-apical direction. To determine the magnitude of transcellular Cl^- ion movement, CFTR blockers (CFTR-inh172, GlyH-101) were used at the end of each experiment to block Cl^- exit across apical CFTR Cl channels. I_{Cl} and the change in forskolin-stimulated Cl currents (ΔI_{Cl}) were compared between clones 5RTEo- and 9RTEo-.

Oocyte collection and SCNT

Rabbit oocytes were collected and prepared as described previously (Du et al. 2009). All micromanipulations were carried out by using a standard protocol (Du et al. 2006). Individual tracheal epithelial donor cell nuclei (9RTEo- P1.6) were transferred into the perivitelline space of an enucleated oocyte. Cell fusion and monitoring of the success of SCNT was performed as reported previously (Du et al. 2009).

Results

Clonal isolation

Two cell lines were generated, namely 9RTEo- and 5RTEo-, after transfection of primary RTE cells with the pSVori- plasmid (de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1988, 1995). Individual colonies were isolated and grown until confluent.

Immunocytochemical characterization

Although transformation and enhanced growth potential is readily discernable in epithelial cell cultures, verification of epithelial origin requires a more comprehensive immunocytochemical analysis of key epithelial-specific features to avoid culture contamination with other cell types, e.g., fibroblasts (de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1988, 1995). The 5RTEo- and 9RTEo- clones were screened with an SV40 large T-antigen-specific antibody (Fig. 1) in order to determine whether the transformed cells with enhanced growth properties expressed the SV40 large T antigen and therefore had the potential to become immortalized (Cozens et al. 1992a, 1992b, 1994; de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1988, 1995). Like the immortalized 16HBE14o- cell line (see Cozens et al. 1994), both the 5RTEo- and the 9RTEo- clones showed the expression of the SV40 large T antigen in 100% of cell nuclei, whereas primary RTE cells were devoid of any signal. All the cell systems (RTE, 5RTEo-, 9RTEo- and 16HBE14o-cells) showed characteristic staining of cytokeratin filaments and tight-junction proteins, indicating epithelial origin, when immunocytochemically assayed for these epithelial-specific features (Fig. 1).

Karyotype analysis

Any gross genetic changes affecting *CFTR* gene expression and/or clonal stability had to be assessed; therefore, the 5RTEo- and 9RTEo- clones were cytogenetically evaluated and compared with the primary RTE cells. Primary diploid rabbit cells have 44 chromosomes, as was observed in nine RTE cell metaphases (Fig. 2a). However, when three 5RTEo- and twenty 9RTEo- cell metaphases were analyzed, they ranged from 55 to 81 chromosomes per cell and were aneuploid, with an average of 65 (5RTEo-) and 70 (9RTEo-) chromosomes per cell (Fig. 2b). The aneuploidy observed was consistent with that seen in numerous cell systems transformed with SV40 (Cozens et al. 1994; de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1988, 1995, 2004).

RT-PCR analysis

Since the transformed cells will eventually be used as in vitro models to evaluate airway disease including CF, they were characterized in terms of their *CFTR* mRNA expression (Pilewski and Frizzell 1999). RT-PCR analysis of the two clones (5RTEo- and 9RTEo-) and of the RTE cells showed robust *CFTR* mRNA expression (Fig. 3). Specifically, clone 9RTEo- showed a higher *CFTR* gene expression (25%) when compared with 5RTEo- or RTE cells most probably because of the selection of a higher-expressing clone during the process of clonal isolation. This finding further substantiated the epithelial origin of the transformed cells and showed that the expression of *CFTR* was not disrupted during transformation.

Transepithelial ion transport

Transepithelial chloride current (I_{Cl}) and resistance (R_t) measurements were carried out in Ussing chambers. Original I_{Cl} traces and corresponding current deflections caused by 1 mV pulses are shown in Fig. 4. Average values for I_{Cl} and R_t are summarized in Table 1. The mean R_t for the 9RTEo- cell monolayers was $960 \pm 510 \Omega \text{cm}^2$ ($n=11$) and $31 \pm 10 \Omega \text{cm}^2$ ($n=12$) for the 5RTEo- cell monolayers indicating that 9RTEo- cells formed tighter epithelial monolayers that were more suitable for transepithelial measurements. Whereas the 5RTEo- cells had limited use in Ussing chambers because they did not form adequately tight monolayers at passage P 1.12, they did show the presence of ZO-1 staining at earlier passages (Fig. 1). Because of the high R_t of the 9RTEo- epithelial cell monolayers, the gradient-driven baseline I_{Cl} was low ($6.3 \pm 2.5 \mu\text{A}/\text{cm}^2$, $n=11$, Table 1). Conversely, the low R_t for the 5RTEo- cells resulted in a high baseline I_{Cl} ($211.3 \pm 86.0 \mu\text{A}/\text{cm}^2$, $n=12$). Both

forskolin (20 μ M) and 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate (CPT-cAMP, 500 μ M) significantly stimulated CFTR Cl^- currents across 9RTEo- cell monolayers (by $6.6 \pm 2.4 \mu\text{A}/\text{cm}^2$). However, elevation of the cellular cAMP levels in 5RTEo- monolayers only slightly changed the I_{Cl} , because the baseline Cl^- currents drifted upward as a result of the applied serosal-to-mucosal Cl^- gradient. The cAMP-stimulated Cl^- secretory response across 9RTEo- monolayers was partially blocked by CFTR-inh172 (50 μ M) and fully blocked by GlyH-101 (50 μ M). The mean of the fully blocked currents was $-10.6 \pm 3.6 \mu\text{A}/\text{cm}^2$. Approximately 50% of the cAMP-dependent CFTR Cl^- currents were inhibited by CFTR-inh172, whereas GlyH-101 blocked currents to unstimulated baseline levels (Fig. 4a). The 5RTEo- monolayers showed a minor but not significant, decline of I_{Cl} in response to GlyH-101 indicating that the measured I_{Cl} was primarily attributable to paracellular and not transcellular Cl^- movement.

CFTR Cl^- currents in RTE cells were further characterized in 9RTEo- monolayers by testing the efficacy of the common CFTR potentiator compounds genistein (10 μ M; Illek et al. 1996) and VRT-532 (10 μ M; Van Goor et al. 2006). In the presence of forskolin, both genistein (Fig. 5a) and VRT-532 (Fig. 5b) further stimulated forskolin-activated I_{Cl} by ~1.4-fold suggesting that both genistein and VRT-532 were effective stimulators of CFTR Cl^- transport in the rabbit cell model. Additional Ussing chamber analyses of clones 5RTEo- and 9RTEo- were carried out at higher passages. Whereas both clones were responsive to cAMP stimulation consistent with CFTR-associated Cl^- ion transport, at the higher passages (>10), they lost their transepithelial resistance and the uniformity of their ZO-1 staining (unpublished). Some of the cells within the population appeared to exhibit ZO-1 staining; however, not all the cells showed ZO-1 staining. This suggested that the isolation of subclones of cells that maintain their polarity should be possible.

In vitro development of rabbit embryos by SCNT

Transformed 9RTEo- cells were cultured to confluence and then subjected to SCNT (Fig. 6a). Individual donor cell nuclei (Fig. 6b, arrow) were transferred into the perivitelline space of an oocyte and subsequently fused with an enucleated oocyte by an electrical pulse (Fig. 6b). Cloned embryos were expanded and developed into blastocysts after 5 days in culture (Fig. 6c). Of 103 SCNTs, 66 embryos (64%) were obtained. A significant portion (19 of 66; 28.8%) developed to the morula stage, 17 of which developed into blastocysts (17 of 66; 25.7%; Table 2).

Discussion

The present study describes the transformation of primary RTE cells with the pSVori-plasmid (Cozens et al. 1992a, 1992b, 1994; de Semir et al. 2008; Gruenert et al. 1988, 1995, 2004; Small et al. 1982). Clones of transformed cells have been characterized with respect to their epithelial-specific features and their karyotype. Immunocytochemical characterization has indicated that the transformed cells express the SV40 large T antigen and cytokeratin, a hallmark of epithelial cells. The primary and transformed cells also possess ZO-1 indicating that they have maintained their epithelial-specific junctional complexes (Cozens et al. 1992a, 1994; de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1988, 1995, 2004). Although the transformed cells are aneuploid, they also retain the epithelial-specific expression of *CFTR*. Furthermore, when nuclei from the transformed cells are used as donor nuclei for SCNT, they are effective in generating multicellular blastocysts.

Over the last two decades, a number of rabbit cell lines from diverse tissue origins have been generated after transfection of the SV40 early genes (Burns et al. 1996; Nachtigal et al. 1990; Taub et al. 2002; Thenet et al. 1992) and numerous airway epithelial cell lines have been developed to study various airway diseases such as CF (Gruenert et al. 2004). An

aneuploid karyotype is generally associated with cell transformation (de Semir et al. 2008; Gruenert 1987; Gruenert et al. 1995) and has been observed in transformed human airway epithelial cells and in SV40-transformed rabbit kidney or corneal epithelial cells (Kang et al. 2001; MacDonald et al. 1991). Kang et al. (2001) have observed a mean of 72.8 chromosomes per cell in their SV40 immortalized cells, a value that is consistent with the mean number of chromosomes per cell observed in this study. Another study involving the use of SV40 large T antigen transformation of an RTE cell line has evaluated squamous cell differentiation (Lotan et al. 1992); however, these cells have not been extensively assessed for tight junctions, cytokeratins, or karyotype. Extensive Ussing chamber analysis of ion transport has shown that the 9RTEo- and the 5RTEo- cells maintained cAMP-dependent Cl^- ion transport and indicates that the cells at early passages maintain their tight junctions and CFTR-dependent Cl^- transport. At later passages, the clones that we have assayed begin to lose transepithelial resistance, even though they retain their CFTR-dependent Cl^- ion transport. Apparently, some cells within the population lose their ability to retain tight junctions, whereas others do not, thus resulting a “porous” monolayer. To circumvent the loss of tight junctions, additional clones generated from the initial transformation can be analyzed. Alternatively, 9RTEo- and 5RTEo- cell lines can be subcloned to select for those cell colonies that retain their tight junctions.

The study of diseases such as CF has relied primarily on murine models (Grubb and Boucher 1999; Guilbault et al. 2007). However, the mouse is not an ideal model for the study of CF disease pathology, in particular, airway disease. Therefore, the development of (1) alternative animal models to study the progression of CF disease and (2) cell systems to elucidate the molecular and biochemical mechanisms underlying the cellular dysfunction associated with CF is crucial. The recent development of the CF pig (Rogers et al. 2008a, 2008b, 2008c) and CF ferret (Li et al. 2006; Sun et al. 2008) has added to the repertoire of animal model systems. However, long-term cell culture systems have not been developed for either of these animal models.

In addition to the pig and ferret, the rabbit is an attractive candidate as an animal model to gain a better understanding of the mechanisms that underlie CF pathophysiology. The rabbit not only is anatomically relevant to the human but also has a short gestation period (28–31 days), is relatively low maintenance and is easy to handle. Whereas SCNT and animal cloning has become routine (Li et al. 2009; Matsuda et al. 2002), rabbit cloning is considered difficult (Yang et al. 2007). To date, only four reported cases worldwide have achieved success in SCNT in the rabbit. However, the favorable outcome of these studies demonstrates the feasibility of rabbit cloning by using cumulus cells (Chesne et al. 2002; Du et al. 2009), stem cells (Challah-Jacques et al. 2003) and adult fibroblasts (Shi et al. 2008) as nuclei donors.

In a previous study, a comparison of primary cultures of cumulus cells with fetal fibroblasts for their efficiency in SCNT has revealed that the donor cell type influences the efficiency of rabbit cloning (Cervera and Garcia-Ximenez 2003). Whereas fetal fibroblasts are more effective at fusion than the cumulus cells (67% versus 45%), in vitro development to the morula (cumulus cells at 41% versus fetal fibroblasts at 14%) and the blastocyst (cumulus cells at 27% versus fetal fibroblasts at 3%) stages is different between cell types. The 66% fusion efficiency observed on using the pSVori- transformed RTE cells (Table 2) is comparable with that observed for the fetal fibroblasts. However, the 28.8% and 25.7% observed with regard to progress to the morula and blastocyst stages, respectively, are similar to the percentage observed when cumulus cells are used as donors (Cervera and Garcia-Ximenez 2003), i.e., almost all the embryos that reach the morula stage develop into blastocysts (17 of 19).

Few reports have assessed the feasibility of SCNT and cloning by using immortalized cell lines as nuclei donors (Cui et al. 2003; Zakhartchenko et al. 1999). In species other than rabbit, either a failure to generate blastocysts from a spontaneously immortalized bovine mammary gland epithelial cell line (Zakhartchenko et al. 1999) or an inability to generate embryos from a telomerase-immortalized sheep fibroblast (Cui et al. 2003) has been experienced. This is the first report demonstrating that blastocysts can be obtained from a donor cell line that has been transformed with an origin of replication-defective SV40 plasmid.

Two groups have shown that rabbit embryonic stem (rES) cell lines can be derived from preimplantational embryos (Graves and Moreadith 1993) or from non-immortalized SCNT blastocysts (Fang et al. 2006). These results and the finding that various cell types (fibroblasts, ES cells, or trophoblasts) can be generated from blastocysts of species other than rabbit (Gomez et al. 2006; Hatoya et al. 2006; Li et al. 2003; Shimada et al. 2001) provide the novel possibility of using the blastocysts generated from these immortalized cell nuclear donors to develop immortal in vitro cell systems with cell-type-specific properties that not only can give insights into human diseases but also facilitate the development of novel therapies.

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References

- Bosze Z, Hiripi L, Carnwath JW, Niemann H. The transgenic rabbit as model for human diseases and as a source of biologically active recombinant proteins. *Transgenic Res.* 2003; 12:541–553. [PubMed: 14601653]
- Burns KD, Regnier L, Rocznia A, Hebert RL. Immortalized rabbit cortical collecting duct cells express AT1 angiotensin II receptors. *Am J Physiol.* 1996; 271:F1147–F1157. [PubMed: 8997388]
- Cervera RP, Garcia-Ximenez F. Oocyte age and nuclear donor cell type affect the technical efficiency of somatic cloning in rabbits. *Zygote.* 2003; 11:151–158. [PubMed: 12828414]
- Challah-Jacques M, Chesne P, Renard JP. Production of cloned rabbits by somatic nuclear transfer. *Cloning Stem Cells.* 2003; 5:295–299. [PubMed: 14733748]
- Chen JM, Cutler C, Jacques C, Boeuf G, Denamur E, Lecointre G, Mercier B, Cramb G, Ferec C. A combined analysis of the cystic fibrosis transmembrane conductance regulator: implications for structure and disease models. *Mol Biol Evol.* 2001; 18:1771–1788. [PubMed: 11504857]
- Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP. Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat Biotechnol.* 2002; 20:366–369. [PubMed: 11923842]
- Cozens AL, Yezzi MJ, Chin L, Simon EM, Finkbeiner WE, Wagner JA, Gruenert DC. Characterization of immortal cystic fibrosis tracheobronchial gland epithelial cells. *Proc Natl Acad Sci USA.* 1992a; 89:5171–5175. [PubMed: 1375758]
- Cozens AL, Yezzi MJ, Yamaya M, Steiger D, Wagner JA, Garber SS, Chin L, Simon EM, Cutting GR, Gardner P, et al. A transformed human epithelial cell line that retains tight junctions post crisis. *In Vitro Cell Dev Biol.* 1992b; 28A:735–744. [PubMed: 1282914]

- Cozens AL, Yezzi MJ, Kunzelmann K, Ohnishi T, Chin L, Eng K, Finkbeiner WE, Widdicombe JH, Gruenert DC. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol*. 1994; 10:38–47. [PubMed: 7507342]
- Cui W, Wylie D, Aslam S, Dinnyes A, King T, Wilmut I, Clark AJ. Telomerase-immortalized sheep fibroblasts can be reprogrammed by nuclear transfer to undergo early development. *Biol Reprod*. 2003; 69:15–21. [PubMed: 12606403]
- de Semir, D.; Maurisse, R.; Vock, E.; Gruenert, DC. Immobilization strategies for epithelial cells in primary culture. In: Ehrhardt, C.; Kim, K-J., editors. *Biotechnology: pharmaceutical aspects; drug absorption studies—in situ, in vitro and in silico tools*. Springer; New York: 2008. p. 616-639.
- Diamond G, Scanlin TF, Zasloff MA, Bevins CL. A cross-species analysis of the cystic fibrosis transmembrane conductance regulator. Potential functional domains and regulatory sites. *J Biol Chem*. 1991; 266:22761–22769. [PubMed: 1719001]
- Du F, Giles JR, Foote RH, Graves KH, Yang X, Moreadith RW. Nuclear transfer of putative rabbit embryonic stem cells leads to normal blastocyst development. *J Reprod Fertil*. 1995; 104:219–223. [PubMed: 7473411]
- Du F, Shen PC, Xu J, Sung LY, Jeong BS, Lucky Nedambale T, Riesen J, Cindy Tian X, Cheng WT, Lee SN, Yang X. The cell agglutination agent, phytohemagglutinin-L, improves the efficiency of somatic nuclear transfer cloning in cattle (*Bos taurus*). *Theriogenology*. 2006; 65:642–657. [PubMed: 16045975]
- Du F, Xu J, Zhang J, Gao S, Carter MG, He C, Sung LY, Chaubal S, Fissore RA, Tian XC, Yang X, Chen YE. Beneficial effect of young oocytes for rabbit somatic cell nuclear transfer. *Cloning Stem Cells*. 2009; 11:131–140. [PubMed: 19090660]
- Ehrhardt C, Collnot EM, Baldes C, Becker U, Laue M, Kim KJ, Lehr CM. Towards an in vitro model of cystic fibrosis small airway epithelium: characterisation of the human bronchial epithelial cell line CFBE41o. *Cell Tissue Res*. 2006; 323:405–415. [PubMed: 16249874]
- Fan J, Watanabe T. Transgenic rabbits as therapeutic protein bioreactors and human disease models. *Pharmacol Ther*. 2003; 99:261–282. [PubMed: 12951161]
- Fang ZF, Gai H, Huang YZ, Li SG, Chen XJ, Shi JJ, Wu L, Liu A, Xu P, Sheng HZ. Rabbit embryonic stem cell lines derived from fertilized, parthenogenetic or somatic cell nuclear transfer embryos. *Exp Cell Res*. 2006; 312:3669–3682. [PubMed: 16996056]
- Gomez MC, Pope CE, Dresser BL. Nuclear transfer in cats and its application. *Theriogenology*. 2006; 66:72–81. [PubMed: 16620927]
- Goncz KK, Feeney L, Gruenert DC. Differential sensitivity of normal and cystic fibrosis airway epithelial cells to epinephrine. *Br J Pharmacol*. 1999; 128:227–233. [PubMed: 10498856]
- Gong G, Dai Y, Zhu H, Wang H, Wang L, Li R, Wan R, Liu Y, Li N. Generation of cloned calves from different types of somatic cells. *Sci China C Life Sci*. 2004; 47:470–476. [PubMed: 15623160]
- Graves KH, Moreadith RW. Derivation and characterization of putative pluripotent embryonic stem cells from preimplantation rabbit embryos. *Mol Reprod Dev*. 1993; 36:424–433. [PubMed: 8305204]
- Grubb BR, Boucher RC. Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiol Rev*. 1999; 79:S193–S214. [PubMed: 9922382]
- Gruenert DC. Differentiated properties of human epithelial cells transformed in vitro. *Biotechniques*. 1987; 5:740–749.
- Gruenert DC, Basbaum CB, Welsh MJ, Li M, Finkbeiner WE, Nadel JA. Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. *Proc Natl Acad Sci USA*. 1988; 85:5951–5955. [PubMed: 2457904]
- Gruenert DC, Basbaum CB, Widdicombe JH. Long-term culture of normal and cystic fibrosis epithelial cells grown under serum- free conditions. *In Vitro Cell Dev Biol*. 1990; 26:411–418. [PubMed: 1693142]
- Gruenert DC, Finkbeiner WE, Widdicombe JH. Culture and transformation of human airway epithelial cells. *Am J Physiol*. 1995; 268:L347–L360. [PubMed: 7900815]
- Gruenert DC, Willems M, Cassiman JJ, Frizzell RA. Established cell lines used in cystic fibrosis research. *J Cyst Fibros*. 2004; 3(Suppl 2):191–196. [PubMed: 15463957]

- Guilbault C, Saeed Z, Downey GP, Radzioch D. Cystic fibrosis mouse models. *Am J Respir Cell Mol Biol.* 2007; 36:1–7. [PubMed: 16888286]
- Hatoya S, Torii R, Kondo Y, Okuno T, Kobayashi K, Wijewardana V, Kawate N, Tamada H, Sawada T, Kumagai D, Sugiura K, Inaba T. Isolation and characterization of embryonic stem-like cells from canine blastocysts. *Mol Reprod Dev.* 2006; 73:298–305. [PubMed: 16292778]
- Hayflick L. The longevity of cultured human cells. *J Am Geriatr Soc.* 1974; 22:1–12. [PubMed: 4587223]
- Hayflick L. A brief history of the mortality and immortality of cultured cells. *Keio J Med.* 1998; 47:174–182. [PubMed: 9785764]
- Illek B, Fischer H, Machen TE. Alternate stimulation of apical CFTR by genistein in epithelia. *Am J Physiol.* 1996; 270:C265–C275. [PubMed: 8772453]
- Illek B, Lei D, Fischer H, Gruenert DC. Sensitivity of chloride efflux vs. transepithelial measurements in mixed CF and normal airway epithelial cell populations. *Cell Physiol Biochem.* 2010; 26:983–990. [PubMed: 21220929]
- Kang SS, Wang L, Kao WW, Reinach PS, Lu L. Control of SV-40 transformed RCE cell proliferation by growth-factor-induced cell cycle progression. *Curr Eye Res.* 2001; 23:397–405. [PubMed: 12045889]
- Kasinathan P, Knott JG, Wang Z, Jerry DJ, Robl JM. Production of calves from G1 fibroblasts. *Nat Biotechnol.* 2001; 19:1176–1178. [PubMed: 11731789]
- Kishigami S, Wakayama T. Somatic cell nuclear transfer in the mouse. *Methods Mol Biol.* 2009; 518:207–218. [PubMed: 19085136]
- Li J, Villemoes K, Zhang Y, Du Y, Kragh PM, Purup S, Xue Q, Pedersen AM, Jorgensen AL, Jakobsen JE, Bolund L, Yang H, Vajta G. Efficiency of two enucleation methods connected to handmade cloning to produce transgenic porcine embryos. *Reprod Domest Anim.* 2009; 44:122–127. [PubMed: 18564317]
- Li M, Zhang D, Hou Y, Jiao L, Zheng X, Wang WH. Isolation and culture of embryonic stem cells from porcine blastocysts. *Mol Reprod Dev.* 2003; 65:429–434. [PubMed: 12840816]
- Li Z, Sun X, Chen J, Liu X, Wisely SM, Zhou Q, Renard JP, Leno GH, Engelhardt JF. Cloned ferrets produced by somatic cell nuclear transfer. *Dev Biol.* 2006; 293:439–448. [PubMed: 16584722]
- Lotan R, Pieniazek J, George MD, Jetten AM. Identification of a new squamous cell differentiation marker and its suppression by retinoids. *J Cell Physiol.* 1992; 151:94–102. [PubMed: 1348511]
- MacDonald C, Watts P, Stuart B, Kreuzburg-Duffy U, Scott DM, Kinne RK. Studies on the phenotype and karyotype of immortalized rabbit kidney epithelial cell lines. *Exp Cell Res.* 1991; 195:458–461. [PubMed: 2070827]
- Matsuda J, Takahashi S, Ohkoshi K, Kaminaka K, Kaminaka S, Nozaki C, Maeda H, Tokunaga T. Production of transgenic chimera rabbit fetuses using somatic cell nuclear transfer. *Cloning Stem Cells.* 2002; 4:9–19. [PubMed: 12006152]
- Maurisse R, de Semir D, Enamekhoo H, Bedayat B, Abdolmohammadi A, Parsi H, Gruenert DC. Comparative transfection of DNA into primary and transformed mammalian cells from different lineages. *BMC Biotechnol.* 2010; 10:9. [PubMed: 20144189]
- Nachtigal M, Legrand A, Nagpal ML, Nachtigal SA, Greenspan P. Transformation of rabbit vascular smooth muscle cells by transfection with the early region of SV40 DNA. *Am J Pathol.* 1990; 136:297–306. [PubMed: 2154928]
- Pilewski JM, Frizzell RA. Role of CFTR in airway disease. *Physiol Rev.* 1999; 79:S215–S255. [PubMed: 9922383]
- Rogers CS, Abraham WM, Brogden KA, Engelhardt JF, Fisher JT, McCray PB Jr, McLennan G, Meyerholz DK, Namati E, Ostedgaard LS, Prather RS, Sabater JR, Stoltz DA, Zabner J, Welsh MJ. The porcine lung as a potential model for cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol.* 2008a; 295:L240–L263. [PubMed: 18487356]
- Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, Prather RS. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J Clin Invest.* 2008b; 118:1571–1577. [PubMed: 18324337]

- Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB Jr, Zabner J, Prather RS, Welsh MJ. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science*. 2008c; 321:1837–1841. [PubMed: 18818360]
- Shi LH, Ai JS, Ouyang YC, Huang JC, Lei ZL, Wang Q, Yin S, Han ZM, Sun QY, Chen DY. Trichostatin A and nuclear reprogramming of cloned rabbit embryos. *J Anim Sci*. 2008; 86:1106–1113. [PubMed: 18245503]
- Shiffman ML, Spitzer RE, Swender PT, Galey WR. Altered bicarbonate reabsorption in the pancreas of reserpine-treated rabbits—a model for cystic fibrosis. *Pediatr Res*. 1983; 17:486–490. [PubMed: 6877902]
- Shimada A, Nakano H, Takahashi T, Imai K, Hashizume K. Isolation and characterization of a bovine blastocyst-derived trophoblastic cell line, BT-1: development of a culture system in the absence of feeder cell. *Placenta*. 2001; 22:652–662. [PubMed: 11504534]
- Small MB, Gluzman Y, Ozer HL. Enhanced transformation of human fibroblasts by origin-defective simian virus 40. *Nature*. 1982; 296:671–672. [PubMed: 6280060]
- Sun X, Yan Z, Yi Y, Li Z, Lei D, Rogers CS, Chen J, Zhang Y, Welsh MJ, Leno GH, Engelhardt JF. Adeno-associated virus-targeted disruption of the CFTR gene in cloned ferrets. *J Clin Invest*. 2008; 118:1578–1583. [PubMed: 18324338]
- Taub M, Han HJ, Rajkhowa T, Allen C, Park JH. Clonal analysis of immortalized renal proximal tubule cells: Na(+)/glucose cotransport system levels are maintained despite a decline in transport function. *Exp Cell Res*. 2002; 281:205–212. [PubMed: 12460651]
- Thenet S, Benya PD, Demignot S, Feunteun J, Adolphe M. SV40-immortalization of rabbit articular chondrocytes: alteration of differentiated functions. *J Cell Physiol*. 1992; 150:158–167. [PubMed: 1309824]
- Van Goor F, Straley KS, Cao D, Gonzalez J, Hadida S, Hazlewood A, Joubbran J, Knapp T, Makings LR, Miller M, Neuberger T, Olson E, Panchenko V, Rader J, Singh A, Stack JH, Tung R, Grootenhuys PD, Negulescu P. Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. *Am J Physiol Lung Cell Mol Physiol*. 2006; 290:L1117–L1130. [PubMed: 16443646]
- Vuillaumier S, Kaltenboeck B, Lecointre G, Lehn P, Denamur E. Phylogenetic analysis of cystic fibrosis transmembrane conductance regulator gene in mammalian species argues for the development of a rabbit model for cystic fibrosis. *Mol Biol Evol*. 1997; 14:372–380. [PubMed: 9100367]
- Widdicombe JH, Welsh MJ, Finkbeiner WE. Cystic fibrosis decreases the apical membrane chloride permeability of monolayers cultured from cells of tracheal epithelium. *Proc Natl Acad Sci USA*. 1985; 82:6167–6171. [PubMed: 3862125]
- Yang F, Hao R, Kessler B, Brem G, Wolf E, Zakhartchenko V. Rabbit somatic cell cloning: effects of donor cell type, histone acetylation status and chimeric embryo complementation. *Reproduction*. 2007; 133:219–230. [PubMed: 17244748]
- Zakhartchenko V, Durcova-Hills G, Scherthaner W, Stojkovic M, Reichenbach HD, Mueller S, Steinborn R, Mueller M, Wenigerkind H, Prella K, Wolf E, Brem G. Potential of fetal germ cells for nuclear transfer in cattle. *Mol Reprod Dev*. 1999; 52:421–426. [PubMed: 10092122]
- Zeitlin PL, Crawford I, Lu L, Woel S, Cohen ME, Donowitz M, Montrose MH, Hamosh A, Cutting GR, Gruenert D, et al. CFTR protein expression in primary and cultured epithelia. *Proc Natl Acad Sci USA*. 1992; 89:344–347. [PubMed: 1370353]

Fig. 1.

Immunocytochemical characterization of primary and immortalized rabbit tracheal cells. Nuclear SV40 large T antigen, cytokeratins and tight-junction molecules were identified in primary and transfected rabbit tracheal epithelial (RTE) cells. Cells were grown on well slides, fixed and incubated with specific antibodies. Representative micrographs at 40 $\times$ magnification are shown (*16HBE* pSVori- immortalized human bronchial epithelial cells (*16HBE14o-*, control), *RTE* primary RTE cells, *9RTEo-* pSVori- transfected RTE clone 9, *5RTEo-* pSVori- transfected RTE clone 5, *AE1/3* anti-cytokeratin antibody, 1:10 dilution [**a**, **d**, **h**, **l**]), *K1-18* anti-cytokeratin 18 antibody, 1:200 dilution [**b**, **e**, **i**, **m**], *ZO-1* antibody against *Zonula occludens-1*, 1:5 dilution [**c**, **f**, **j**, **n**]), *SV40* anti-SV40 antibody, 1:50 dilution [**g**, **k**, **o**]). Secondary antibody: bovine anti-rabbit IgG conjugated to fluorescein isothiocyanate used at a dilution of 1:100. Bars 100 μ m

Fig. 2.

Representative micrographs of (a) RTE P1 (passage 1, P 1) diploid metaphase, (b) 9RTEo- P1.10 aneuploid metaphase and (c) 5RTEo- P1.10 aneuploid metaphase. **a** RTE cells were incubated with colcemid and stained with Giemsa. All nine metaphase spreads showed a 44 chromosome count (normal rabbit diploid karyotype: $2n=44$). **b, c** Passage 10 (post-transformation, P 1.10) 9RTEo- and 5RTEo- cells were incubated with colcemid, stained with Giemsa and treated with trypsin for 2 s. Whereas 71 chromosomes are shown, the mean chromosome numbers was 70 and 65 after the analysis of 20 metaphases spreads for 9RTEo- and three metaphases for 5RTEo-, respectively. Chromosomal spreads were viewed without a coverslip at a 100 $\times$ magnification

Fig. 3.

Assessment of mRNA expression of the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) by reverse transcription with the polymerase chain reaction (PCR). First-strand cDNA was prepared and amplified by PCR with primers r1278F/r1830R from exons 9 and 11. The 552-bp amplicons (*arrow right*) were banded on a pre-cast 2% agarose E-Gel and stained with ethidium bromide (*lane 1* 9RTEo-, *lane 2* 5RTEo-, *lane 3* RTE, *lane 4* distilled H₂O, *lane M* 100-bp marker)

Fig. 4.

Ussing chamber analysis of CFTR Cl⁻ transport and R_t in two clonal isolates of transformed/immortalized RTE cells (9RTEo⁻, 5RTEo⁻). Original I_{Cl} traces and corresponding current deflections in response to 1 mV pulses (as a measure of R_t) in (a) 9RTEo⁻ monolayers (P 1.5) and (b) 5RTEo⁻ monolayers (P1.12). CFTR-mediated I_{Cl} was stimulated by addition of the cAMP analog 8-(4-chlorophenylthio)-adenosine 3', 5'-cyclic monophosphate (cpt-cAMP, 500 μM). The CFTR blockers CFTR-inh172 (50 μM) and GlyH-101 (50 μM) were used to identify CFTR-mediated I_{Cl}. Note in a that CFTR I_{Cl} values were less effectively blocked by a maximal dose of CFTR-inh172, whereas a similar dose of GlyH-101 caused full inhibition. In these experiments, R_t was 710 Ωcm² in the 9RTEo⁻ monolayer and 23 Ωcm² in the 5RTEo⁻ monolayer

Fig. 5. Stimulation of CFTR Cl^- transport by genistein and the CFTR potentiator, VRT-532, in monolayer cultures of transformed/immortalized 9RTEo-cells (passage 5). Transepithelial Cl^- currents (I_{Cl}) were sequentially stimulated by the addition of the cAMP agonist forskolin (20 μM) and (a) genistein (10 μM) or (b) VRT-532 (10 μM). The stimulated I_{Cl} was blocked by GlyH-101 (50 μM)

Fig. 6.

Nuclear transfer with 9RTEo- as donor cell. **a** Immortalized 9RTEo- cells were cultured to confluence before being subjected to nuclear transfer. **b** Individual donor cell nucleus (*arrow*) was transferred into perivitelline space of oocyte. Subsequent fusion with enucleated oocyte was by an electrical pulse. **c** Cloned embryos expanded and developed into the hatching blastocyst stage after 5 days of culture in vitro. *Bar* 75 μm

Table 1

Transepithelial electrical parameters of the rabbit tracheal epithelial cell lines (*P* passage). All measurements were made in the presence of serosal-to-mucosal Cl^- gradients. Chloride currents (I_{Cl} in $\mu\text{A}/\text{cm}^2$) and transepithelial resistances (R_T in Ωcm^2) were measured before treatment (*Unstimulated*), after the addition of forskolin (*Forskolin*) and following the addition of the CFTR inhibitor (*GlyH-101*). $\Delta I_{\text{Cl}} = I_{\text{Cl}}$ (after) – I_{Cl} (before) treatment.

Cells	Unstimulated		Forskolin		GlyH-101		Forskolin	GlyH-101
	I_{Cl}	R_t	I_{Cl}	R_t	I_{Cl}	R_t	ΔI_{Cl}	ΔI_{Cl}
9RTEo- (P 1.5)	6.3±2.5 (n=11)	960±486 (n=11)	12.5±4.0* (n=11)	1017±352 (n=11)	6.3±2.6 (n=9)	1094±442 (n=9)	6.6±2.4 (n=14)	-10.6±3.6 (n=13)
5RTEo- (P 1.12)	211.3±86.0 (n=12)	31±10 (n=12)	232.2±91.1 (n=13)	34±12 (n=13)	237.2±86.8 (n=11)	23±10 (n=11)	15.6±10.7 (n=11)	-3.7±6.7 (n=10)

* Measurements significantly different from unstimulated values by one way analysis of variance. Values are the mean±SD (*n* number of experiments)

Table 2

In vitro development of rabbit cloned embryos from 9RTEo-cells. Somatic cell nuclear transfer (SCNT) of 9RTEo- nuclei into rabbit oocytes. The nuclei transferred (*NT*) were monitored in terms of their developmental progression to the blastocyst stage. The proportion of SCNT oocytes progressing to each stage is indicated as a percent of the total

Number of NT assays	Fused (%)	Cleavage (%)	Four-cell stage (%)	Morula (%)	Blastocyst day 5 (%)
103	66 (64.1)	34 (51.5)	19 (28.8)	19 (28.8)	17 (25.7)