ABSTRACT

Producing an Ovine Model of Cystic Fibrosis

by

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Cystic Fibrosis (CF) is an autosomal recessive disease that significantly affects quality of life and lifespan. There are currently no effective animal models of CF that mimic the human disease state. This prevents the development of pharmaceutical treatments for patients. Sheep have been considered for a useful animal model because of their size, life expectancy, and similarities in their anatomy and physiology. In order to generate a sheep transgenic model to study CF we have produced two Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene targeting DNA vectors containing large regions of homology to the CFTR gene in sheep. One of these targeting vectors (ΔF508Neo) contains sequences designed to delete the phenylalanine at amino acid position 508 of CFTR. Another targeting vector (G27XDTNeo) contains sequences designed to introduce an early stop codon into the CFTR gene such that no CFTR is produced. These two targeting vectors were used to transflect White Romney fibroblast cells. Donor sheep oocytes were collected for use in somatic cell nuclear transfer (scNT) with the two genetically modified cell lines. Embryos produced from scNT were
transferred to recipient ewes which resulted in the birth of 11 ΔF508 heterozygous lambs and 3 G27XDT heterozygous lambs.
Cystic Fibrosis (CF) is an autosomal recessive disease that significantly affects quality of life and lifespan. There are currently no effective animal models of CF that mimic the human disease state which prevents the development of pharmaceutical treatments for patients. Sheep have been predicted to serve as a useful animal model to use because of their size, life expectancy as well as anatomy and physiology. In order to generate a sheep model we will introduce DNA with the CF mutation into White Romney sheep cells. Two mutations will be used for this project, ∆F508 and G27XDT. These newly created cell lines will then be used with unfertilized donor sheep eggs in a process known as somatic cell nuclear transfer (scNT). During scNT, the nucleus is removed from the egg and the donor cell is inserted in its place. This newly formed embryo can be transferred into a recipient ewe resulting in an animal with a genetic profile that matches that of the cell with the CF mutation. The sheep models that will be produced should provide a viable option for studying CF treatments. This will lead to better treatments and improve quality of life for those diagnosed with CF.
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Cystic Fibrosis (CF) is the most common autosomal recessive disease among Caucasians with 1 out of every 2,500 Caucasians affected. Even with significant improvements in treatments for patients with CF, it remains a fatal disease (Drumm and Collins, 1993).

CF is caused by mutations in the Cystic Fibrosis Transmembrane Regulator (CFTR) gene (Ratjen and Doring, 2003). This gene encodes a glycoprotein that functions as a chloride channel located within the apical membranes of epithelial tissue (Engelhardt et al., 1994; Ratjen and Doring, 2003; Ballard et al., 2007; Bush and Davies, 2010). To date, more than 1,400 gene mutations have been characterized (Alibakhshi et al., 2008) with the most common mutation being a deletion of phenylalanine at position 508 (ΔF508) of the CFTR glycoprotein. This particular mutation is responsible for 70% of CF cases and is present in 90% of CF patients (Kerem et al., 1989; Shackleton et al., 1994; Bobadilla et al., 2002; Wang et al., 2000). G27X is a recently discovered and less common mutation that is caused by a G-T change at base 211 in exon 2 of the CFTR gene. The G27X mutation produces an early stop codon resulting in an absence of CFTR protein from epithelial tissue. There is some evidence that suggests that the absence of CFTR is less harmful than abnormal protein, making the G27X model useful for determining the effects of mutated CFTR (Shackleton and Harris, 1992). Animal models have been produced with differing mutations to provide further insight into the link between genotype and phenotype of CF (Tebbutt, 1995; Williams et al., 2003; Ostedgaard et al., 2007; Fisher et al., 2011; Keiser and Engelhardt, 2011).
Pathology resulting from defective CFTR is observed in respiratory, gastrointestinal and reproductive systems (Drumm and Collins, 1993; Donaldson and Boucher, 2006; Hodges, 2009). The airways and lungs generally have the most severe pathology (Quinton, 1990; Trezise et al., 1993; Engelhardt et al., 1994). Chronic lung infections from sustained bacterial infections lead to respiratory failure and cause more than 90% of the mortality in CF patients (Quinton 1990; Cho et al., 2011). The exact relationship between the lack of transepithelial transport of chloride and the development of typical CF lung pathology has not yet been established. This lack of information can be partially attributed to ineffective animal models.

A significant gastrointestinal symptom of CF is meconium ileus. Meconium ileus is an intestinal obstruction occurring at birth with a frequency of 13% to 17% of CF patients. Severity is variable among patients (Wilschanski and Durie, 1998; van der Doef et al., 2011).

In previous years, the murine model has been the most commonly used CF animal model. In 1989, the CFTR gene was first cloned. Three years later a CF mouse model was generated (Guilbault et al., 2007). At least 14 mouse models currently exist with differing genetic mutations and pathology (Fisher et al., 2011). Undoubtedly, the mouse model has contributed greatly to the advancement of CF research (Zeiher et al., 1995). However, due to important anatomical and physiological differences between humans and mice, this model is limited in its usefulness. The murine model lacks similar pathology in the lungs, pancreas and male reproductive tract (Grubb and Boucher, 1999; Guilbault et al., 2007; Rogers et al., 2008a; Egan, 2009; Fisher et al., 2011; Keiser and Engelhardt, 2011).
The high incidence of lung pathology in humans makes differences between mouse and human lung pathophysiology critically important. Submucosal glands are the primary location of CFTR. In combination with goblet cells, submucosal glands are the major producers of mucus within the airways (Engelhardt et al., 1992; Verkman et al., 2003; Wine and Joo, 2004). Humans have many more submucosal glands in a larger part of their airways as compared to mice, which have fewer submucosal glands in a much smaller portion of their airways (Liu et al., 2004). Normal mucous production aids in ciliary clearance of the lungs. CF patients suffer from an over production of mucous which builds up and provides an environment for bacterial growth (Wine and Joo, 2004).

In patients with CF, chronic lung infections are caused by a small number of bacterial pathogens. The most commonly detected bacteria are \textit{P. aeruginosa}, \textit{Staphylococcus aureus}, \textit{Haemophilus influenzae}, and \textit{Stenotrophomonas maltophilia}. These bacterial infections are found in almost all patients with CF and are nearly impossible to eradicate (Wine, 1999; Ratjen and Doring, 2003). They lead to an inflammatory response that causes lung failure. Chronic infection induced lung failure is not observed in mice (Davidson et al., 1995; Liu et al., 2006; Guilbault et al., 2007; Keiser et al., 2011), highlighting a shortcoming of the mouse model and the need for a better CF model. Researchers have attempted to improve this aspect by exposing mice to \textit{P. aeruginosa} and \textit{Staphylococcus aureus}. However, such attempts have produced only minor improvements to the model (Heeckeren et al., 1997; Davidson et al., 2004).

Another failure of the mouse model is the lack of a pancreatic phenotype. Pancreatic insufficiency is a common ailment of CF patients that affects approximately 90% of patients (Sturgess, 1984; Quinton, 1999; Rowe et al., 2005). The number of
CFTR ion channels in the mouse pancreas is significantly less than in the human pancreas. Furthermore, an alternative, calcium activated chloride channel exists in the mouse pancreas that is not present in humans. This compensates for a lack of CFTR ion channels and results in much less severe pathology (Grubb and Boucher, 1999; Guilbalt et al., 2007; Egan, 2009). As a result, the mouse model is less than ideal for studying human CF pathophysiology.

In the case of male fertility, approximately 99% of male CF patients experience infertility due to congenital bilateral absence of vas deferens (CBAVD) (Jarzabeck et al., 2004). In contrast, most mouse models display no pathology in the male reproductive tract and have normal fertility rates (Grubb and Boucher, 1999; Bertog et al., 2000; Reynaert et al., 2000; Guilbault et al., 2007; Egan, 2009). Female CF mice take longer to become pregnant than their healthy counterparts, likely due to abnormal cervical mucus (Guilbault et al., 2007; Hodges et al., 2008), but the phenotypes observed in the mouse model deviate widely from those observed in humans. In humans, female fertility problems are usually mild and occur mostly in those with severe disease (Hodges et al., 2008; Lau et al., 2010).

In addition to the lack of comparable pathology in the mouse model, a large amount of CF mice die shortly after birth from intestinal blockage. The blockage develops after birth and is different than the meconium ileus that is seen in CF patients. This pathology is not seen in humans and is problematic in murine CF studies (Grubb and Boucher, 1999; Guilbault et al., 2007; Egan, 2009). Despite the great advancements provided by the murine model, clearly it is not the most advantageous way to study
pathophysiology of CF in humans. This has led researchers to seek for a more applicable model.

Pig and human organs demonstrate anatomical and physiological similarities and as a result, pigs have been used as animal models for numerous human diseases (Kühholzer et al., 2000; Rogers et al., 2008b; Welsh et al., 2009). Moreover, similarities between porcine and human organs are validated by the many studies dedicated to developing porcine organs for human xenotransplantation (Cooper et al., 2002). These studies have naturally led researchers to believe the porcine model of CF could be a viable option.

The human and porcine CFTR genes are 92% homologous, significantly greater than the percent similarity between the human and murine CFTR gene. With a few minor exceptions, the general anatomy and structure of porcine lungs parallels that which is seen in humans (Rogers et al., 2008a).

CFTR-null (-/-) and CFTR-ΔF508 piglets were created utilizing adeno-associated virus transfection in combination with somatic cell nuclear transfer (scNT). Studies conducted after birth show that CFTR<sup>-/-</sup> piglets lack any CFTR mRNA and expressed no protein. Piglets with the ΔF508 mutation (CFTR<sup>ΔF508/ΔF508</sup>), however, do maintain residual CFTR function but this residual function is not enough to prevent the disease phenotype in the piglets (Rogers et al., 2008b; Ostedgaard et al., 2011).

There are apparent similarities between human and porcine CF phenotypes. Both CFTR<sup>-/-</sup> and CFTR<sup>ΔF508/ΔF508</sup> piglets developed lung disease at a few months old, demonstrating that spontaneous lung infections do occur in the pig model (Rogers et al., 2008b; Chen et al., 2010; Stoltz et al., 2010; Ostedgaard et al., 2011). The porcine
model also demonstrates abnormalities in the male reproductive tract similar to those seen in human CF patients (Pierucci-Alves et al., 2011).

CFTR<sup>−/−</sup> piglets were born with severe pancreatic pathology that developed rapidly into pancreatic insufficiency while the CFTR<sup>ΔF508/ΔF508</sup> piglets had a slightly less severe phenotype. This same pattern is observed in the gallbladders of CFTR<sup>−/−</sup> and CFTR<sup>ΔF508/ΔF508</sup> piglets. CFTR<sup>−/−</sup> and CFTR<sup>ΔF508/ΔF508</sup> piglets also showed similar pathophysiology to humans in the liver and vas deferens (Rogers et al., 2008c).

The most pronounced difference between human and porcine CF lies within the gastrointestinal tract. Both CFTR<sup>−/−</sup> and the CFTR<sup>ΔF508/ΔF508</sup> porcine piglets are born with a 100% penetrance of meconium ileus which is a bowel blockage that is fatal unless operated upon. The meconium ileus is accompanied by an abnormally small colon, known as microcolon. In contrast, approximately 15% of human CF patients are born with this condition. It is interesting to note that even though there is residual CFTR in the CFTR<sup>ΔF508/ΔF508</sup> model, 100% of piglets still suffer from meconium ileus. This suggests that pigs require a higher level of CFTR than humans in order to maintain healthy intestines (Meyerholz et al., 2010; Ostedgaard et al., 2011).

While the pig model of CF shows great potential, the 100% rate of meconium ileus and the immediate need for surgery postnatally, is a hindrance that prevents the pig model from being cost effective and readily maintained.

Ferret lung anatomy more closely resembles human anatomy than the murine lung and has previously been used to study human diseases (Maher and DeStefano, 2004). Ferret submucosal glands are found throughout the airways as is seen in humans. The expression of CFTR in these submucosal glands is also very similar to the expression
seen in human submucosal glands (Sehgal et al., 1996; Li and Engelhardt, 2003; Sun et al., 2010; Fisher et al., 2012).

Genetically, the human and ferret CFTR gene are 91% homologous. The protein amino acid identity between the two species is even more striking with a 97% homology (Li and Engelhardt, 2003).

With these commonalities in mind, research moved forward to create a ferret model of CF. Advancements in scNT techniques with ferret embryos have facilitated the necessary procedures for creating a ferret model of CF. Current knowledge of ferret CF models is derived from CFTR−/− kits. Creating a CFTRΔF508ΔF508 model will provide more insight and understanding of CF pathophysiology in ferrets (Li and Engelhardt, 2003).

CFTR null ferret kits display meconium ileus with a 75% penetrance. By 36 hours postnatal, they displayed significant morbidity and were euthanized 48 hours after birth. Microcolon was seen in 30% of the kits born with meconium ileus (Sun et al., 2010; Fisher et al., 2011; Keiser et al., 2011).

The kits that were born without meconium ileus failed to thrive and died within a few days of birth. These kits also suffered with early lung infections and elevated liver enzymes. Additionally, fat stores were extremely depleted throughout the body. With these observations in mind, it is clear that CFTR null kits display the most severe gastrointestinal pathology of the currently used CF model species. Ferrets differ from previously studied CF models in their carnivorous diet and as a result of their diet, ferrets do not have a cecum, an organ that aids in digestion of plant matter. They also have a much shorter digestive period than the other species. These differences could account for
the severity of gastrointestinal disease that is observed in the CFTR null ferrets (Sun et al., 2010).

In order to alleviate the malabsorption and failure to thrive, a proton-pump inhibitor was administered. This is believed to compensate for a diseased pancreas and aids in proper digestion. This resulted in slight weight gain but nonetheless the kits still died (Sun et al., 2010).

The CFTR null kits had pancreatic lesions as seen in human CF (Olivier et al., 2012). However, the gross anatomy and histopathology of the liver and gallbladder appeared normal, suggesting this model would not be ideal for studying human CF in these particular organs. In addition, the vas deferens was absent in 50% of the CFTR null kits. The remaining kits had significant degenerate pathology, with parts of the vas deferens missing or smaller than normal.

Despite the major problems observed in the CFTR null kits, abnormal Cl− permeability and secretions were detected in the submucosal glands of the trachea, similar to human CF (Sun et al., 2010; Fisher et al., 2011; Keiser et al., 2011).

The ferret model of CF has provided some insights into CF pathophysiology. This model is still in the early stages of use and further exploration could bring with it more information, however, this research will undoubtedly be hindered by the severe pathology of the gastrointestinal tract.

The previously mentioned animal models have the potential to provide advances in CF research but there are obvious disadvantages to each model (Ware, 2008). A sheep model of CF could provide a viable alternative to the existing CF animal models due to genetic and anatomical similarities. Moreover, the large size and docile nature of sheep
allow for easier surgical manipulation. The longevity of sheep (8-14 years) would ensure the ability to better understand long term effects of treatments (Harris, 1997; Scheerlinck et al., 2008).

The human and sheep CFTR genes are 90% homologous as compared with the 80% homology between the human and mouse CFTR genes. Furthermore, at the protein level there is a 95% similarity between human and sheep proteins, much greater than the 88% that is observed between human and mouse proteins (Tebbutt et al., 1995).

Extensive studies on sheep lung epithelium have shown there are striking anatomical, functional, and electrophysiological similarities between the human and ovine lung. Numerous studies have already used a sheep model to study human lung development and pathology and a large part of our understanding of human lung physiology has been derived from studies conducted with sheep (Joo et al., 2001; Davidson et al., 2006; Scheerlinck et al., 2008; Allen et al., 2009; Abeynaike et al., 2010; McLachlan et al., 2011).

Anatomical similarities include tracheobronchial branching patterns and epithelial cell populations (Plopper et al., 1983; Collie et al., 20011). Humans and sheep demonstrate dichotomous branching of the bronchi whereas monopodial branching is characteristic of mouse lungs (Meeusen et al., 2009). Cells found within the primary and secondary bronchioles of sheep include basal, intermediate, ciliated and Clara cells. The location and number of cells within the sheep airways resembles what is observed in other mammalian lungs, including that of humans (Emerson et al., 2003).

Sheep lungs experience inflammation and airway remodeling, a response to prolonged inflammation. This further demonstrates similarities between sheep and human
respiratory systems (Snibson et al., 2005; Matute-Bello et al., 2008). A sheep model should help alleviate a major drawback to studying CF lung treatments in mice. The size of their lungs and the pattern of breathing in mice greatly differs from the average human. Mice are obligate nose breathers, meaning, they are unable to breathe through their mouths. In contrast, sheep lung size and breathing patterns closely replicate human anatomy and physiology (Van der Velden and Snibson, 2011). Sheep are frequently used in studies related to breathing patterns in humans (Baier et al., 1985; Haouzi and Chennel, 2005). These similarities are particularly important when considering treatments for lung disease because drug delivery to the airways is affected by respiratory frequency and breathing volume. Respiratory rate is dependent on body size and sheep lungs are considerably more comparable in size to human lungs (Raeburn et al., 1992; Snibson et al., 2005; Meeusen et al., 2009).

Even without a current CF sheep model, researchers have utilized sheep to study CF treatments (Olver and Robinson, 1986; Ferrari et al., 2001; McLachlan et al., 2011). These studies validate the usefulness and demonstrate the potential efficiency of a sheep model of CF.

With the help of a sheep model of CF advancements in CF treatments can improve quality of life for CF patients, providing relief from an otherwise debilitating disease.
MATERIALS AND METHODS

Donor Cell Collection, Culture and Expansion

All media was purchased from Thermo Scientific (Logan, UT) unless otherwise stated. Ear notches were collected from an American Romney sheep. The biopsies were then transported back to the laboratory and used to initiate primary culture in a T-25 flask containing DMEM/F12 with 15% fetal bovine serum (FBS), 1X Penicillin Streptomycin (PenStrep). After reaching confluency, the fibroblast cells were treated with trypsin for 5 minutes. The trypsin was deactivated with 5 mLs DMEM/F12, centrifuged in a 15 mL tube at 400 X g and resuspended in DMEM/F12. The cell resuspension was expanded into more T-25 flasks. The subsequent flasks were then frozen in DMEM/F12 with 5% DMSO.

Bacterial Miniprep

LB stabs with E. Coli strain K-12 containing plasmids with a construct transformed with ΔF508 and the G27XDT mutations respectively, were received from Ann Harris at Northwestern University (Figures 1 and 2, shown later). Agar plates were streaked with the LB stabs and placed in a 37° C incubator overnight. Two milliliters of LB media with ampicillin was inoculated with a single colony from the streaked agar plates. The tubes with the inoculated broth were placed in a 37° C orbital water bath overnight. The Quicklyse Miniprep Kit from Qiagen was used to extract DNA from the inoculated broth. One and a half milliliters of cultured broth was placed in a 2 mL Quicklyse lysis tube. The broth was pelleted by centrifugation at 17,000 X g for 1 minute at room temperature. The media was removed by decanting and 374 μl of ice cold
complete lysis solution was added to the pelleted bacterial cells. The cells were vortexed for 30 seconds and incubated at room temperature for 3 minutes. The lysate was transferred to a quicklylyse spin column by pipetting. It was then centrifuged for 1 minute at 17,000 X g. The column was washed by adding 400 μl of diluted Buffer QLW and centrifuged for 1 minute at 17,000 X g. The flow through was discarded and the spin column was centrifuged for 1 minute at 17,000 X g to dry the column. The spin column was transferred to a new collection tube and 45 μl buffer QLE was added directly to the center of the quicklylyse spin column. The column was centrifuged for 1 minute at 17,000 X g. The DNA concentration was verified via a Nanodrop spectrophotometer and then stored at -20° C.

Fibroblast Cell Transfection

Transfection was performed by electroporation using Invitrogen’s Neon® Transfection System. Once ready to perform the transfection, cells were thawed and grown in 2 T-25 flasks, one for each construct. At 70% confluency, the cells were trypsinized for 5 minutes. The trypsin was neutralized with DMEM/F12 with 2% FBS in a 15 mL conical tube. An aliquot was taken from the neutralized trypsin cell suspension and cell density was determined using a hemocytometer. The cell suspension was centrifuged for 5 minutes at 400 X g. The media was decanted and the pelleted cells were washed with 1 mL of PBS (without Ca^{2+} and Mg^{2+}) and centrifuged for 5 minutes at 400 X g. The PBS was aspirated and the cell pellet resuspended in Buffer R to a final concentration of 1.0 X 10^{5} cells/μL. DNA was prepared by diluting to a concentration of 100 ng/μl. Six transfection reactions were done for each construct.
A 24-well plate was prepared by adding 5 mLs of DMEM/F12 with 15% FBS without antibiotic. The plate was prewarmed in a humidified 37°C/5% CO2 incubator.

DNA and the cells were combined in a 0.5 mL microfuge tube to a final concentration of 1 ug DNA and 1.0 X 10^5 cells. A neon tube was prepared by adding 3 mLs of Electrolytic Buffer. A pulse number of 1, pulse width of 20 and voltage of 1600 was programmed into the device. The combined DNA and cell mixture were pipetted using the 10 ul transfection pipette tips and electroporation was performed. Once electroporation was completed, cells were placed in the prewarmed culture dishes and cultured for 24 hours.

**Colony Selection**

Twenty-four hours post transfection, cell media was aspirated and replaced with DMEM/F12 with 15% FBS, 800 μg/mL of G418. The media was replaced every other day for a total of 2 weeks. At 2 weeks, non-transfected White Romney cells were completely eradicated from G418 treatment. The wells in the 24-well dish that had confluent cells were removed from the well with 0.25% trypsin. The trypsin was neutralized with DMEM/F12 with 5% FBS in a 15 mL centrifuge tube. The cells were centrifuged at 400 X g for 5 minutes. The resulting cell pellet was resuspended in 1 mL PBS (without Ca^{2+} and Mg^{2+}). The cells were then run through a BD Biosciences Special Order FACSaria™ II flow cytometer and sorted into groups of 10, 100, and 1,000. The cells were sorted into a 96-well, flat-bottomed cell culture dish. Upon reaching confluency the cells were trypsinized with 2 drops of 0.25% trypsin for 5 minutes. The cells were resuspended in 1.5 mLs of DMEM/F12 with 15% fetal bovine serum (FBS),
1X PenStrep. Cells from each well were grown to confluency and expanded further into a 6 well dish. Once again, at confluency cells were dissociated with 0.25% trypsin. Half of the cells were reseeded in a T-25 cell culture flask and the other half were saved for DNA extraction. DNA extraction was performed using the Qiagen DNeasy® Blood and Tissue Kit.

**Cell DNA Extraction and Sequencing**

DNA extraction was performed using the Qiagen DNeasy® Blood and Tissue Kit. Cells were pelleted in a 1.5 mL microfuge tube at 400 X g. Cell culture media was decanted and the pellet was resuspended in 200 μl PBS (without Ca$^{2+}$ and Mg$^{2+}$). Twenty microliters of Proteinase K was added. Two hundred μl of Buffer AL was added, the tube was vortexed and then incubated at 56° C for 10 minutes. Two hundred microliters of Ethanol was added to the tube and it was vortexed and the contents of the tube were pipetted into a DNeasy mini spin column placed in a 2 mL collection tube. It was centrifuged at 8000 X g for 1 minute. The collection tube and flow-through were discarded. The spin column was placed in a new collection tube and 500 μl of Buffer AW1 was added. It was centrifuged at 8000 X g for 1 minute. The collection tube and flow-through were discarded. The spin column was placed in a new collection tube. Five hundred microliters of Buffer AW2 was added and the tube centrifuged at 20,000 X g for 3 minutes. The collection tube and flow-through were discarded. The spin column was placed in a new 1.5 mL microfuge tube. One hundred microliters of Buffer AE was pipetted directly on the DNeasy membrane. It was incubated for 1 minute and
centrifuged for 1 minute at 8000 X g. This DNA was submitted for sequencing and cells that had the correct mutation were frozen in anticipation of scNT.

**Somatic Cell Nuclear Transfer**

Animal care and use procedures were conducted by approval of the Utah State University Institute of Animal Care and Use Committee. Somatic cell nuclear transfer (scNT) cloning procedures were performed following our standard laboratory procedures (Meng et al., 2011). Domestic sheep ovaries were collected from an abattoir, Blue Mountain Meats in Monticello, Utah. Cumulus-oocyte complexes (COC’s) were collected by aspirating 2-8 mm follicles. Collected oocytes were incubated in maturation medium (TCM-199 supplemented with 20% FBS, 25 μg/mL gentamycin, 0.01 U/mL FSH, 0.01 U/mL LH) which was pre-equilibrated in a humidified 37°/5% CO₂ incubator. Oocytes were released from COC’s after 23-24 hours of culture in the formulated maturation media. The transfected fibroblast cells were thawed from cryopreservation and cultured in DMEM/F12 with 10% FBS in a humidified 37° C/5% CO₂. The cells were cultured in the same media with 0.5% FBS for 24 hours before scNT. Just prior to scNT, cells were disassociated with 0.25% trypsin and then resuspended in HEPES-buffered SOF (HSOF). Oocytes were enucleated by aspirating the first polar body and surrounding cytoplasm in HSOF supplemented with 10 μg/mL cytochalasin B (CB). Then, a single Romney cell was inserted into the perivitelline space of an oocyte that has been enucleated. Fusion of the cell and oocyte was induced by double 40 sec 1.8 kV/cm DC pulses in electrofusion medium (0.28M sorbitol in water, supplemented with 0.5mM HEPES, 0.1mM Ca(CH3COO)2, 0.5mM Mg(CH3COO)2 and
1mg/mL bovine serum albumin). After fusion, the embryos were incubated in SOFaa medium with 5 μg/mL CB for 40-90 minutes and then activated with 5 uM ionomycin for 5 minutes, followed by treatment with 1 mM 6-dimethylaminopurine (6-DMAP) and 5 μg/mL cycloheximide (CHX) in SOFaa for 4.5 hours at 38.5° C in 5% CO₂ for 1 day and transferred to recipients at the 1-cell stage.

**Embryo Transfer**

Estrus was synchronized in recipient ewes using vaginal pessaries (Chronogest® 20 Mg). They were inserted intravaginally on day 0 and removed on day 14. Twelve hours after the removal of the pessaries, the ewes were briefly exposed to a vasectomized ram at 2-3 hour intervals and observed for standing heat detection. If a ewe was seen to be in heat, the ewe number and time was noted and she was pulled from the group being exposed to the vasectomized ram.

Embryo transfers were performed by exteriorizing the oviduct via midline laparotomy. Insertion into the fallopian tube ipsalateral to the side of ovulation was accomplished with a Tom Cat catheter (Kendall, 3.5 french, 5.5 open end) attached to a 1 cc syringe.

ΔF508 cloned embryos were transferred into two groups of recipients on two consecutive days. Western White face ewes were used as recipients on both days. On October 11, 2012 one hundred seventy-five embryos were transferred to 10 recipients, 17 embryos per transfer. The next day, October 12, 2012, one hundred fifty embryos were transferred to 10 recipients, 15 embryos per transfer.
G27 embryo transfers also took place on two consecutive days. Suffolk sheep were used as recipients for these embryos. Eighty G27 cloned embryos were transferred into 4 recipients, 20 embryos per transfer on October 25, 2012. The following day, October 26, 2012, one hundred thirty-nine embryos were transferred into 7 recipients.

A ram with a marking harness was placed with the recipient ewes 30 days after embryo transfer to determine if estrus returns. All recipient ewes, including those marked by a ram, had an ultrasound scan at 57-60 post transfer. These scans performed via an Aloka portable scanner, allowed for assessment of pregnancies. It was determined that 7 ΔF508 recipient ewes and 1 G27 recipient ewe were pregnant. Four of the ΔF508 recipient ewes appeared to be pregnant with twins. A single G27 recipient ewe also appeared to be pregnant with a set of twins.

**Embryo Lysis and Sequencing**

On each day of embryo transfer 4-5 embryos were placed in a 0.5 microfuge tube in 10 μl of molecular grade water. Using forceps, the tube was immersed in a canister of liquid nitrogen for 1 minute, thawed and immersed again to ensure lysis of the embryos. The tube was spun briefly for 30 seconds and 1 μl was used in nested polymerase chain reaction resulting in enough DNA to be submitted for sequencing.

**Genomic DNA Extraction**

Tissue biopsies were taken from the tails of lambs at birth and transported to the laboratory. In the laboratory they were cleaned with 70% ethanol and rinsed with Hank’s Balanced Salt Solution (HBSS). With a scalpel they were dissected into small pieces and added to a 15 mL conical tube containing 5 mLs of 0.25% trypsin. The tube was placed in
a shaking water bath that was prewarmed to 37° C. The tissue sample was incubated
in the water bath for 15 minutes with vigorous shaking every 3 minutes. After 15
minutes 5 mLs of DMEM/F12 with 5% FBS was added and the tube was centrifuged at
400 X g for 5 minutes. The media was decanted and the tissue was resuspended in
DMEM/F12 15% FBS with 1X Amphotericin B (amp-B). This was transferred to a T-25
flask where cells were grown until confluency and were then cryopreserved.

Small tissue samples (25 mg) were also taken and DNA was extracted using the
Qiagen DNeasy® Blood and Tissue Kit. The tissue was cut into small pieces and placed
in a 1.5 mL microfuge tube. 180 ul Buffer ATL and 20 ul proteinase K was added. The
tube was vortexed and incubated at 56° C overnight. The next day, the tube was vortexed
for 15 seconds and 200 μl of Buffer AL was added and it was vortexed again. Then 200
μl of 100% ethanol was added and it was vortexed again. The mixture was pipetted into
the DNeasy Mini spin column and placed in a 2 ml collection tube. It was centrifuged at
6,000 X g for 1 minute. The flow-through and collection tube were discarded. The
DNeasy Mini spin column was placed in a new 2 mL collection tube and 500 μl of Buffer
AW1 was added. The tube was centrifuged at 6,000 X g for 1 minute and the flow-
through and collection tube was discarded. The DNeasy Mini spin column in a new 2 ml
collection tube and 500 μl Buffer AW2 was added. It was centrifuged for 3 min at
20,000 x g. The DNeasy Mini spin column was placed in a clean 1.5 ml microfuge tube
and 100 μl of Buffer AE was pipetted directly onto the spin column membrane. It was
incubated for 1 minute at room temperature and then centrifuge at 20,000 X g for 1
minute. The DNA that was extracted was submitted for sequencing at Utah State
University’s Genomics Laboratory.
Homologous Recombination Screening

Long-range PCR amplification of the targeting constructs was performed with DNA from each cloned lamb. For the ΔF508 lambs, the primers FLONGF (5’-TACCTCTGATACATTATTAAGTTGACT-3’) and FLONGR (5’-CTCATATAATAATCATACGCAAGCCT-3’) amplified a 2.2 kb fragment of intron 8 of the CFTR gene. Each reaction contained 65 ng genomic DNA, a final concentration of .5 uM of each primer, 12.5 µl of GoTaq® Green Master Mix and Nuclease-free water for a final volume of 25 ul. The reaction conditions were 95°C for 5 minutes, then 40 cycles of 95°C for 30 seconds, 60°C for 2 minutes and 72°C for 2 minutes followed by a final step of 72°C for 5 minutes.

The end-point PCR product should be 2.2 kb. If homologous recombination (HR) has occurred in at least one of the CFTR alleles, 1.75 kb of this PCR band will be derived from the ΔF508 targeting construct. The ΔF508 targeting construct has a 16 bp deletion of intron 8 resulting in the loss of a BglII site. Thus, PCR products from alleles that have undergone HR will not be digested by BglII. Amplified wild-type DNA will be digested by BglII resulting in 1.4 kb and 0.78 kb products (Fig. 1).

Restriction enzyme digests contained 100 ng of PCR product, 2 µl of 10X FastDigest buffer, 1 µl of BglII and H2O to 20 µl. Reactions were incubated at 37°C for 5 minutes. Inactivation was performed by adding 1 µl of 0.5 M EDTA.

To analyze DNA from the G27 lambs, long-range PCR was performed with the primers GLONGF (5’-AGAGGTGAGGGGAAGGATGG-3’) and GLONGR (5’-
TGACATCAAACCTGCTTTACCC-3’). This primer pair amplifies the intron 1/exon 2 region of the CFTR gene and will be 7 kb if the 6.39 kb targeting construct has been inserted correctly. The targeting construct contains an engineered Eco RI restriction enzyme site. Wild-type DNA would be 5 kb and lack the Eco RI site (Fig. 1).

PCR utilized Qiagen LongRange PCR kit. Each reaction contained 5 μl of LongRange PCR Buffer, 2.5 μl dNTP mix, 1 μl of each primer, 100 ng of genomic DNA and water to reach a final volume of 50 μl.

The reaction conditions were 93°C for 3 minutes followed by 35 cycles of 93°C for 15 seconds, 60°C for 30 seconds and 68°C for 10 minutes.

After PCR restriction enzyme digests were performed with Eco RI. Each reaction contained 2 μl of FastDigest Buffer, 1 μl Eco RI, 2 μl of PCR product and H₂O for a final volume of 20 μl. Reactions were incubated at 37°C for 5 minutes and inactivated at 80°C for 5 minutes.
RESULTS

Embryo Sequencing

Embryos not transferred to recipient ewes were lysed two days after the scNT procedure and were found to contain the correct sequence for the respective desired mutations (Figures 2 and 3).

Ultrasounds

Of the 20 recipient ewes that received ΔF508 scNT embryos, 10 were marked open by the vasectomized teaser ram, which indicated they were not pregnant following embryo transfer and had returned to estrus. 5 of the 11 G27 recipients were also marked open by the ram.

Seven ΔF508 recipient ewes and 1 G27 recipient ewe were pregnant following ultrasound. Four of the ΔF508 recipient ewes were diagnosed as having twins. A single G27 recipient ewe was pregnant with twins.

Birth of Lambs

There were 11 ΔF508 lambs born. All of the lambs cloned from this cell line exhibited multiple congenital abnormalities that were incompatible with life (Table 1). Most notably, 10 of the 11 lambs had severe palatoschisis (cleft palate) of both the hard and soft palate. The lamb without a cleft palate was stillborn.

Three G27X lambs were born. None of the G27X lambs had a cleft palate. The phenotype of the G27X lambs was much less severe than was seen in the ΔF508 lambs.
(Table 2). One was stillborn, the second G27X lamb born died from a lung infection, and the third lamb born remains alive.

DNA extracted from lamb tissues was sequenced with an ABI PRISM™ 3730 DNA Analyzer using BigDye terminator chemistry. This genetic analysis showed that nine ΔF508 lambs were heterozygous for the ΔF508 mutation and two were homozygous.

Both ΔF508 homozygous lambs were stillborn and too necrotic for a complete necropsy of internal tissues. One of the ΔF508 homozygous lambs did not have a cleft palate, the other one did.

DNA sequencing of the G27X lambs showed they were all heterozygous for the G27X mutation.

**Homologous Recombination Screening**

PCR product from ΔF508 cloned lambs digested with BglII resulted in 2 bands sized at 1.4 kb and 0.78 kb (Fig. ). These results demonstrate that the BglII was still present and homologous recombination did not occur.

PCR product from G27 digested with EcoRI had only one band sized at 5 kb. This demonstrates that HR did not occur as there was no EcoRI site present. It can also be concluded that the 6.39 kb targeting construct was not inserted correctly as the band size was not large enough for the targeting construct to be present.
Figure 1. Diagrams of the ΔF508 and G27 targeting vectors. The neomycin cassette and long range PCR primers are shown. The Diptheria Toxin A gene is not shown because it should be removed after homologous recombination. Restriction Enzyme sites are as follows; A, Bam HI; C, Sac I; D, Dra III; E, Eco RI; G, Bgl II; H, Bsp HI; K, Kpn I; L, Bsp I; P, Sap I; S, Sal I; T, Sru I; U, Bsu 36I.
DISCUSSION

There is extensive evidence that sheep can provide a viable alternative to current animal models of CF (Abraham, 2008; Matute-Bello et al., 2008). Results from this study do not provide insight into the usefulness of sheep as a CF model as was hoped.

The purpose of this project was to create a founder CF sheep for breeding and eventually provide a model for the study of CF treatments. The results show us that none of the animals produced were viable and could not be used for breeding. It is apparent that further measures, including negative selection, long arms of homology, and intensive screening of cell colonies must be taken to ensure homologous recombination (HR) occurs resulting in a healthy and viable heterozygous phenotype (Barzel and Kupiec, 2008).

HR is a method used to replace an allele with new gene of interest without disrupting other regions within the genome. A major setback to creating animal models is the inefficiency of targeted HR. An HR targeting construct should provide a means to insert an engineered mutation into a specific location within the animal’s genome. The mechanisms of HR are poorly understood but the engineered construct will find the gene of interest and recombination will occur in a process similar to mechanisms involved with meiosis and mitosis. HR occurs at a low frequency in mammalian cells, approximately one event per $10^5$ to $10^7$ cells. Non-homologous recombination (non-HR) events occur more than 1,000 times more frequently than HR. Random insertion occurs in about one cell per $10^2$ to $10^4$ transfected cells. With such a high probability of non-HR
occurring, targeting constructs must be designed with methods of selection in mind (Hanson and Sedivy, 1995; Vasquez et al., 2001).

New genetic engineering technologies exist that have improved on the rates of targeted HR. Transcription activator-like effector nucleases (TALENs) are novel method for genetic engineering. TALENs are composed of a DNA binding domain and a Fok I domain. The DNA binding domain provides specificity to a targeted DNA sequence (Cermak et al., 2011). It is derived from transcription activator-like effectors (TALEs). These proteins derive from a type of bacteria, *Xanthomonas*, which affects plants and functions by directly modifying the host’s genome (Lei et al., 2013). The Fok I domain is a restriction endonuclease which introduces a double strand break. In transfected fibroblast cell colonies 54% and 17% resulted in mono- or biallelic modifications respectively (Carlson et al., 2012). This new technology provides a valid option for alternative methods of genetically engineering a model of CF.

Negative selection is a way to determine which cells have correctly incorporated the new DNA. The Diptheria Toxin A (DTA) gene will prevent protein synthesis and was used for negative selection in this project. The DTA cassette was placed outside of areas of homology within the vector and is not incorporated into the genome when HR occurs. Random insertion results in the incorporation of the DTA gene and causes cell death (Williams et al., 2003).

Another important aspect of the gene targeting vector are the long arms of homology. These large areas of homology promote the occurrence of HR. The longer these arms are the higher the chance of HR. The minimum recommended combined length for the two arms is 6-8kb (Hall et al., 2009).
The occurrence of cleft palate in the ΔF508 lambs show evidence of a genetic or epigenetic disruption of an important developmental gene (Mendoza-Londono et al., 2013). Understanding the genetic and environmental factors affecting palate formation is still a work in progress (Roy et al., 2012) Rapid amplification of cDNA ends (RACE) would show where in the genome the construct inserted and could provide insight into midline development (Yeku et al., 2011).

Furthermore, the possibility of multiple insertions must be studied through a Southern Blot assay using a probe for the positive selection neomycin cassette.

New genetic engineering technologies exist that can move this work forward. Transcription activator-like effector nucleases (TALENs) are novel method for genetic engineering. TALENs are composed of a DNA binding domain and a Fok I domain. The DNA binding domain provides specificity to a targeted DNA sequence. It is derived from transcription activator-like effectors (TALEs). These proteins are from a bacteria, Xanthomonas, which affects plants and functions by directly modifying the host’s genome. The Fok I domain is a restriction endonuclease which introduces a double strand break. In transfected fibroblast cell colonies 54% and 17% resulted in mono- or biallelic modifications respectively. This new technology provides a valid option for alternative methods of genetically engineering a model of CF.

Due to fertility issues that may present themselves in sheep homozygous for the CF mutation, cloning with a female cell line alongside the male cell line would improve the outcome of this study. For future use we have prepared tissue biopsies from female Romney ewes. They will be transfected using the same techniques for the male cell line.
This will provide a female heterozygote to be used in breeding and should result in homozygous CF sheep.

While this particular project did not yield the desired results future measures can ensure a successful outcome. These include a new, efficient genetic modification method and extensive cell screening after transfection which should include PCR, restriction enzyme digests and Southern Blot assays. With this in mind it is reasonable to continue further research efforts towards creating a sheep model of CF.
Figure 2. Lysed ΔF508 embryo chromatogram. The left arrow designates an adenine to thymine transition. The right arrow designates the deletion of three thymines.

Figure 3. Lysed G27X embryo chromatogram. The left arrow designates a guanine to thymine transition. The right arrow designates a single base pair insertion of cytosine.
Figure 4. ΔF508 Lamb DNA chromatogram. The left arrow designates an adenine to thymine transition. The right arrow designates the deletion of three thymines.

Figure 5. G27X lamb DNA chromatogram. This animal was heterozygous and the chromatogram from sequencing results shows a mixture of genotypes. The left arrow designates a red peak which represents a guanine to thymine transition. The right arrow designates a blue peak which represents a single base pair insertion of cytosine.
<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Birth</th>
<th>Death</th>
<th>Details</th>
<th>Necropsy Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>9089</td>
<td>3/6/13</td>
<td>3/6/13</td>
<td>Euthanized</td>
<td>Severe palatoschisis, ventricular septal defect, hydronephrosis, myopathy, dilated central veins, premature lung, slight cortical atrophy</td>
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<tr>
<td>1002</td>
<td>3/12/13</td>
<td>3/12/13</td>
<td>Stillborn</td>
<td>Brachygnathia inferior, palatoschisis, hydronephrosis</td>
</tr>
<tr>
<td>1002</td>
<td>3/12/13</td>
<td>3/12/13</td>
<td>Euthanized</td>
<td>Brachygnathia inferior, palatoschisis, hydronephrosis</td>
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<tr>
<td>1052</td>
<td>3/12/13</td>
<td>3/12/13</td>
<td>Euthanized</td>
<td>Severe palatoschisis, hydronephrosis, myopathy, pulmonary atelectasis, lymphoid depletion</td>
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<tr>
<td>1148</td>
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<td>3/12/13</td>
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</tr>
<tr>
<td>1128</td>
<td>3/14/13</td>
<td>3/14/13</td>
<td>Euthanized</td>
<td>Brachygnathism, palatoschisis, high ventricular septal defect, hydronephrosis, myopathy, pulmonary atelectasis, hepatic fibrosis, tunica media hypertrophy, lymphoid depletion</td>
</tr>
<tr>
<td>9085</td>
<td>3/15/13</td>
<td>3/15/13</td>
<td>Euthanized</td>
<td>Severe palatoschisis, brachygnathism, hydronephrosis, hemorrhage, myopathy, pulmonary atelectasis, hepatic fibrosis, tunica media hypertrophy, lymphoid depletion</td>
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<tr>
<td>1128</td>
<td>3/15/13</td>
<td>3/15/13</td>
<td>Stillborn</td>
<td>Advanced autolysis precludes meaningful interpretation of internal tissues</td>
</tr>
<tr>
<td>1128</td>
<td>3/15/13</td>
<td>3/15/13</td>
<td>Stillborn</td>
<td>Advanced autolysis precludes meaningful interpretation of internal tissues</td>
</tr>
<tr>
<td>9083</td>
<td>3/18/13</td>
<td>3/18/13</td>
<td>Stillborn</td>
<td>Anasarca, brachygnathia inferior, palatoschisis, hydronephrosis, ventricular septal defect</td>
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<tr>
<td>Y1057</td>
<td>3/19/13</td>
<td>3/19/13</td>
<td>C-section, euthanized</td>
<td>Hydronephrosis, palatoschisis, brachygnathism, high ventricular septal defect, tracheal submucosal dysplasia, central vein and sinusoid dilation</td>
</tr>
</tbody>
</table>

**Table 1.** Synopsis of ΔF508 cloned lamb births.
<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Birth</th>
<th>Death</th>
<th>Details</th>
<th>Necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>377</td>
<td>3/14/13</td>
<td>3/14/13</td>
<td>Stillborn</td>
<td>prognathism, high ventricular septal defect, hydronephrosis, myopathy, pulmonary atelectasis, hepatic fibrosis, tunica media hypertrophy, lymphoid depletion</td>
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<tr>
<td>363</td>
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<td>tracheal mucosal hyperplasia, hydronephrosis, intrabronchiolar and intraalveolar rod-shaped bacteria</td>
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<td>648</td>
<td>3/24/13</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
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</table>

**Table 2.** Synopsis of G27X cloned lamb births.
REFERENCES


