Decreased expression of cystic fibrosis transmembrane conductance regulator impairs sperm quality in aged men

Ruiying Diao^{1,2}, Kin Lam Fok³, Li Zhao⁴, Hao Chen^{1,2}, Hui Tang⁵, Jing Chen¹, Aiping Zheng⁶, Xiaohu Zhang³, Yaoting Gui¹, Hsiao Chang Chan³ and Zhiming Cai²

¹Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Shenzhen PKU-HKUST Medical Center, Peking University Shenzhen Hospital, Shenzhen 518036, China, ²Shenzhen Key Laboratory of Genitourinary Tumor, Shenzhen Second People's Hospital, First Affiliated Hospital of Shenzhen University, Shenzhen 518035, China, ³Faculty of Medicine, School of Biomedical Sciences, Epithelial Cell Biology Research Center, The Chinese University of Hong Kong, Hong Kong SAR, China, ⁴Department of Laboratory Medicine, the People's Hospital of Shilong, Southern Medical University, Dongguan 523321, China, Departments of ⁵Laboratory Medicine and ⁶Pathology, Peking University Shenzhen Hospital, Shenzhen 518036, China

Correspondence should be addressed to Z Cai; Email: caizhiming2000@163.com

Abstract

Sperm quality declines with aging; however, the underlying molecular mechanism remains elusive. The cystic fibrosis transmembrane conductance regulator (CFTR) has been shown to play an essential role in fertilizing capacity of sperm and male fertility. This study aimed to investigate the involvement of age-dependent CFTR downregulation in lowering sperm quality in old age. Two hundred and one healthy fertile men of three age groups (20–40 years, $n=64$; 40–60 years, $n=61$; and >60 years, $n=76$) were recruited. Expression of CFTR was determined by RT-PCR, western blot, and immunofluorescence staining. Collected sperm were treated with CFTR inhibitor or potentiator. Sperm quality was assessed by motility and bicarbonate-induced capacitation. The results showed that the expression of CFTR on the equatorial segment and neck region of sperm was significantly decreased in an age-dependent manner. Reduction of CFTR expression in sperm from old men was correlated with lowered forward motility and decreased HCO_3^- sensitivity required for sperm capacitation. Activation of CFTR by genistein partially rescued the decreased forward motility in sperm from old men. Decreased CFTR expression in sperm was also found to be associated with lowered sperm quality in aging mice. These results suggest that age-dependent downregulation of CFTR in sperm leads to lowered sperm quality in old age sperm. CFTR may be a potential target for rescuing sperm motility as well as a fertility indicator in old age men.

Reproduction (2013) **146** 637–645

Introduction

The effect of aging on fertility has become an important problem in public health due to the delayed marriage and parenthood in the modern world. Age-dependent decline in female fertility is well defined to be associated with the decrease in quality and number of oocytes produced and the increase in miscarriage (Menken *et al.* 1986, Munne *et al.* 1995). In men, fertility does not cease abruptly but continues during aging (Kuhnert & Nieschlag 2004). Although it is not uncommon for men to father children after 50 years of age, studies have shown a 23–38% decrease in pregnancy rate of male partners aged over 50 years compared with those under 30 years (Kidd *et al.* 2001). Furthermore, an increased risk of subfecundity was frequently associated with increased age in men (Kidd *et al.* 2001). These indicate that male fertility also exhibits an age-dependent decline. Whether sperm quality decreases during aging has been a matter

of debate. Early study revealed no difference in sperm function between older men and younger men, despite a subtle decrease in sperm motility in older men (Haidl *et al.* 1996). However, more recent studies on sperm parameters have demonstrated the decrease in seminal volume, sperm count, and sperm motility with aging (Eskenazi *et al.* 2003, Ng *et al.* 2004), suggesting a decline in both spermatogenesis and sperm maturation. However, the mechanism underlying the age-dependent fertility decline remains elusive.

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-modulated chloride/bicarbonate channel that is required for multifaceted functions in various organ systems (Wang *et al.* 2003). Defects of CFTR, as seen in cystic fibrosis (CF), are associated with chronic lung inflammation/infection, pancreatic insufficiency, intestinal obstruction, and infertility (Rowe *et al.* 2005, Chan *et al.* 2009, Chen *et al.* 2012). While previous studies have shown that around 95% of

the male infertility in CF is caused by congenital bilateral absence of the vas deferens (CBVD; [Chillon *et al.* 1995](#)), recent studies have demonstrated the presence of functional CFTR on sperm ([Figueiras-Fierro *et al.* 2013](#)) and revealed its essential roles in maintaining spermatogenesis, sperm motility, capacitation, and fertilizing capacity of sperm in mice and humans ([Hernandez-Gonzalez *et al.* 2007](#), [Xu *et al.* 2007, 2011](#), [Bai *et al.* 2010](#), [Chavez *et al.* 2012](#)). In the testis, CFTR expression on Sertoli cells has been shown to be involved in regulating the HCO_3^- -induced cAMP/PKA/CREB pathway ([Xu *et al.* 2011](#)), which may potentiate the effect of follicle-stimulating hormone on spermatogenesis. In sperm, CFTR mediates HCO_3^- entry, which in turn increases cAMP production and protein tyrosine phosphorylation, which are associated with sperm capacitation ([Wang *et al.* 2003](#)). CFTR-deficient mouse sperm have been demonstrated to have decreased sperm motility and fertilizing capacity ([Xu *et al.* 2007](#)). These results indicate an essential role of CFTR in sperm function and male fertility ([Chen *et al.* 2012](#)). We have previously observed age-dependent downregulation of CFTR in rat prostate ([Xie *et al.* 2012](#)). This prompted us to hypothesize that the age-dependent decrease in sperm quality may be due to downregulation of CFTR in sperm, which leads to poor sperm motility and fertilizing capacity in old age. We undertook this study to test this hypothesis.

Materials and methods

Reagents and medium

CFTR inhibitor-172 (CFTRinh-172, C2992), genistein (G6649), and soluble adenylyl cyclase (sAC) inhibitor KH7 (sACinh-KH7, K3394) were purchased from Sigma-Aldrich Co. Rabbit anti-CFTR antibodies were from Santa Cruz Biotechnology (sc-10747) and Alomone Labs (ACL-006, Jerusalem, Israel). Mouse anti-phosphotyrosine antibody (05-321, clone 4G10) was purchased from EMD Millipore (Billerica, MA, USA). cAMP ELISA assay (KGE002B, Parameter, Minneapolis, MN, USA) was purchased from R&D Systems (Minneapolis, MN, USA). Quinn's Advantage Fertilization (HTF) Medium (ART-1020) was purchased from SAGE Media (Pasadena, CA, USA), which consisted of glucose, MgCl_2 , pyruvate, and lactic acid hemicalcium salt supplemented with glycylglutamine, taurine EDTA, and citric acid (pH 7.35). Percoll discontinuous density gradient (17-0891-01) was purchased from GE Healthcare Life Sciences (Pittsburg, PA, USA).

Samples and study design

This study was approved by the Human Ethics Committee of Peking University Shenzhen Hospital and informed consent was given by each subject. Subjects with a family history of endocrine or anatomical disorders were excluded from the study. Sperm samples were obtained from 20- to 65-year-old men in the Department of Physical Examination of Peking

University Shenzhen Hospital, according to World Health Organization Criteria (WHO 2010) ([Lu *et al.* 2010](#)). Every donor had a previous history of partner's achieving pregnancy. Samples were separated into three groups: young adult (20 to <40 years, $n=64$), middle age (40 to <60 years, $n=61$), and old age (>60 years, $n=76$). Semen collection was done by masturbation after 3–5 days of sexual abstinence. After liquefaction, semen volume, concentration, motility, viability, and morphology were analyzed by a computer-assisted semen analysis system (CASA, CASAS-QH-III, Qing Hua Tong Fang, Beijing, China) under 200 $\times$ magnification ([Lu *et al.* 2010](#)). A subset of samples, as indicated in each experiment, was randomly selected from the cohort of different age groups for expression profiling and functional studies.

Animals and sperm collection

Male C57/6BL mice (aged 3, 8, or 27 months, $n=4$ for each group) were obtained from Laboratory Animal Service Center of the Chinese University of Hong Kong. Ethics committee approval was obtained prior to the study (Approval ID: 01/031/MIS) and the animal experiment was conducted in accordance with the Laboratory Animals Service Center's Guidelines. Animals were killed by CO_2 asphyxiation. The epididymis was collected for sperm preparation as described by [Klinefelter *et al.* \(1991\)](#) and [Xu *et al.* \(2007\)](#). Briefly, cauda epididymides were dissected from C57 mice, trimmed free of fat, and then placed in 2 ml medium HTF (pH 7.4) at 37 °C to allow the dispersion of sperm. After 5–15 min, sperm were washed in 5 ml of the fresh medium by centrifugation of 800 g for 10 min. Sperm were then resuspended to a final concentration of 1×10^7 cells/ml for subsequent experiments.

Sperm motility analyses

After Percoll density gradient centrifugation ([Lambard *et al.* 2004](#)), human sperm samples were washed and adjusted to 1×10^6 cells/ml using the Neubauer cell chamber in HTF and then incubated at 37 °C under 5% CO_2 with or without 25 mM NaHCO_3 . Sperm was treated with CFTRinh-172 (25 μM), sACinh-KH7 (50 μM), and/or genistein (5 μM) for 1 h as indicated. At least 500 sperm were counted and the percentage of forward sperm motility was evaluated by CASA at 0, 15, 30, 45, and 60 min. DMSO was added as vehicle control.

For CASA analysis, 4 μl sperm suspension at a concentration of 1×10^6 cells/ml was placed in a counting chamber (0.01 mm^2 , 10 μm deep) and assessed for motility characteristics at 37 °C. For each sample, ten randomly selected fields containing more than 200 motile tracks were examined at 60 Hz. The parameters of sperm movement assessed were Grade a (the average velocity $\geq 25 \mu\text{m/s}$) and b (the average velocity between 15 and 25 $\mu\text{m/s}$). The setup parameters were as follows: imaging sampling frequency, 30 frames/s; number of frames, 20; minimum sampling for motility, two frames. Forward motility was defined by percentage of sperm showing Grade a+b motility pattern.

Immunofluorescence staining and confocal microscopy

Human sperm were treated by Percoll discontinuous density gradient, smeared onto superfrost slides, dried, and fixed in 4% paraformaldehyde for 20 min (Guo *et al.* 2007). After being permeabilized in 0.5% Triton X-100, sperm were blocked and incubated overnight with primary antibody specific to CFTR (1:100 dilution, sc-10747). Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) secondary antibody (1:500 dilution, Molecular Probes, Eugene, OR, USA) was added followed by washing in PBS for 15 min. Slides were counterstained with Hoechst 33258 (1 µg/ml, Invitrogen) and mounted with Prolong Gold Antifade Reagent (Invitrogen). Primary antibody pre-incubated with neutralizing peptide was used as a negative control. At least 200 sperm were visualized randomly under a confocal microscope (Zeiss, Germany) for statistical analysis using the software Image-Pro Plus 5.1.

Western blot

Sperm protein was extracted using Qproteome Mammalian Protein Preparation Kit (37901, Qiagen). Quantitative protein (40 µg in each panel) was boiled in 2× Laemmli sample buffer, separated by SDS-PAGE on 6% (v/v) polyacrylamide gel, and transferred onto PVDF membranes (Immobilon-P; Millipore). Membranes were blocked in 5% non-fat skim milk in TBST. Primary antibodies (rabbit anti-CFTR (ACL-006, 1:500) or rabbit anti-β-tubulin) were applied to the membranes overnight at 4 °C. Signals were detected using ECL kit according to the manufacturer's instructions (37071, Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Only one band was found at 165 kDa. No bands were detected when primary antibody was pre-incubated with neutralizing peptide as negative control. Band densitometry was analyzed as the ratio of CFTR/β-tubulin (%) using QuantityOne basic 4.6.3 (Bio-Rad Laboratories).

Total RNA extraction and RT-PCR

To obtain somatic cell-free sperm for expression profiling, the samples were subjected to two sequential centrifugations through 40:80 Percoll discontinuous gradients as described by Ostermeier *et al.* (2002). Residual somatic contaminants were removed by washing in 0.5% Triton X-100 (Sigma) as described by Miller *et al.* (1999). The purity of sperm was verified by histology.

mRNA was isolated by use of AllPrep DNA/RNA Mini Extraction kit (80204, Qiagen). The first-strand cDNA was synthesized using oligo-dT primers (K1622, Fermentas, Waltham, MA, USA). PCR was carried out with Takara EmeraldAmp PCR Master Mix (DRR300A, Takara, Dalian, Japan). The primers used for human CFTR (NM_000492.3) are as follows: forward, 5'-AAACTTGGAT CCCTATGAAC-3';

reverse, 5'-GTGGGGAAAGAGCTTCA C-3'; the primers used for human GAPDH (NM_001256799) are as follows: forward, 5'-AGAA GGCTGGGGCTCATTTG-3'; reverse, 5'-AGGGGC-CATCCACAGTCTTC-3'. Band densitometry was analyzed as the ratio of CFTR to GAPDH (%) using QuantityOne basic 4.6.3 software.

Protein tyrosine phosphorylation of spermatozoa

Washed sperm from young adult and aged men were incubated under capacitation condition (25 mM NaHCO₃) with CFTRinh-172 or sACinh-KH7 for 1 h at 37 °C. Sperm protein was extracted using lysis cocktails with phosphatase inhibitors for western blot analysis. The monoclonal mouse antibody against phosphotyrosine (1:2000 dilution, 05-321, clone 4G10) was used.

Intracellular cAMP measurement

After incubation in HCO₃⁻-contained HTF medium, the reaction was terminated by the addition of five volumes of ice-cold 100 mM HCl in 100% ethanol. Sperm samples were kept on ice for 30 min, lyophilized, and assayed for competitive cAMP ELISA assay (KGE002B, Parameter) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was calculated using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, USA). Values are expressed as mean ± S.E.M. Comparisons were subjected to one-way ANOVA (for multi-group comparisons), two-way ANOVA with Bonferroni posttests, and Pearson correlation test. Statistical significance was set at $P < 0.05$.

Results

Decreased sperm quality in sperm from old men

We first collected sperm from donors of young age (20 to <40 years; $n = 64$), middle age (40 to <60 years; $n = 61$), and old age (>60 years; $n = 76$) and examined sperm quality in terms of concentration, motility, and forward motility (Grade a + b). Consistent with previous reports (Eskenazi *et al.* 2003, Ng *et al.* 2004), sperm from old age donors demonstrated significant decreases in sperm count (10.4 ± 11.2 million/ml compared with 95.3 ± 57.9 million/ml in young age; $P < 0.001$), sperm motility ($21.1 \pm 9.8\%$ compared with $61.1 \pm 8.4\%$ in young age; $P < 0.001$), and forward motility ($17.4 \pm 8.8\%$ compared with $56.2 \pm 4.5\%$ in young age; $P < 0.001$) (Table 1).

Table 1 Clinical information for semen in different age groups.

Group (years old)	Number	Age	Intensity (million/ml)	Motility (%)	Grade a + b (%)
20–40	64	29.5 ± 4.9	95.3 ± 57.9	61.1 ± 8.4	56.2 ± 4.5
40–60	61	48.0 ± 5.0	39.8 ± 24.6*	35.9 ± 9.0*	30.3 ± 8.5*
> 60	76	64.3 ± 3.2	10.4 ± 11.2* [†]	21.1 ± 9.8* [†]	17.4 ± 8.8* [†]

* $P < 0.001$ compared with young age; [†] $P < 0.001$ compared with middle age.

Furthermore, these sperm parameters exhibited an age-dependent decrease in the three groups, indicating decreased sperm quality during aging.

Abnormal CFTR expression in sperm from old men

To investigate the relationship between age and CFTR, we first compared the expression of CFTR in sperm obtained from men of different age groups. Western blot analysis detected a single band at ~ 160 kDa, which represents CFTR on human sperm lysate as previously reported (Xu *et al.* 2007). Notably, CFTR expression was significantly downregulated by 53.60 ($P < 0.001$) and 44.03% ($P < 0.05$) in old age (> 60 years) compared with young adult (20 to < 40 years) and middle age (40 to < 60 years) (Fig. 1A and B) respectively. Furthermore, age-dependent decrease in CFTR mRNA levels was also observed in old age ($P < 0.01$, Fig. 1C and D) compared with young adult, confirming the downregulation of CFTR in sperm from old men. Downregulation of CFTR was also observed in sperm obtained from 27-month-old mice compared with 3- and 8-month-old mice ($P < 0.05$) (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Next, we examined the localization of CFTR by immunofluorescence staining. A previous report has demonstrated the localization of CFTR at the equatorial segment of human sperm (Xu *et al.* 2007). Consistent with the previous report, CFTR was detected at the equatorial plate (arrow, Fig. 2A). Interestingly, CFTR was also detected at the neck of sperm. Of note, compared with the old group, a relatively small number of sperm from the young adult group expressed CFTR in the neck alone or CFTR was absent in both equatorial segment and neck region, suggesting that the downregulation of CFTR expression during aging may result from the loss of CFTR in the equatorial segment and neck region. By quantifying sperm percentage showing different CFTR expression patterns, we found a significant decrease in the percentage of sperm showing both the equatorial and neck expression of CFTR in middle age ($49.88 \pm 1.92\%$; $P < 0.05$) and old age ($42.95 \pm 1.20\%$; $P < 0.001$) compared with young adult ($56.73 \pm 2.60\%$) (Fig. 2B and Supplementary Table 1, see section on supplementary data given at the end of this article). On the other hand, significant increase in downregulation of CFTR was observed in middle age (50.12 ± 1.92 vs $43.27 \pm 2.60\%$; $P < 0.05$) and old age individuals (58.05 ± 1.33 vs $43.27 \pm 2.60\%$; $P < 0.001$) (Fig. 2B and Supplementary Table 1) respectively.

We further analyzed the correlation between CFTR expression pattern, age factor, and sperm forward motility. Intriguingly, there was a significantly negative correlation between normal CFTR expression pattern and age factor (Fig. 2C, $r = -0.56$; $P < 0.001$). Importantly, the increase in percentage of sperm showing normal distribution of CFTR (Fig. 2D, $r = 0.53$; $P < 0.001$)

Figure 1 Age-dependent downregulation of CFTR in human sperm.

(A) Representative western blot showing age-related decrease in CFTR in sperm from young adult (lanes 1–3), middle age (lanes 4–6), and old age (lanes 7–9) groups. β -tubulin was used as a loading control. (B) Statistical summary of CFTR protein expression levels by western blot ($n = 10$ for each group). (C) Representative RT-PCR expression analysis of CFTR mRNA levels from different age groups, including young adult (lanes 1–4), middle age (lanes 5–8), and old age (lanes 9–11). Template was omitted in blank control. GAPDH was used as an internal control. M, standard molecular weight marker. (D) Statistical summary of CFTR mRNA expression levels by RT-PCR ($n = 14$ for young adult, $n = 16$ for middle age and old age). *Compared with middle age, $P < 0.05$; **compared with young adult, $P < 0.01$; ***compared with young adult, $P < 0.001$.

was correlated with forward motility, indicating that age-dependent loss of CFTR in the equatorial segment and neck region may lead to decreased sperm forward motility.

Abnormal CFTR expression underlies the decreased HCO_3^- response in sperm from old men

As CFTR mediates HCO_3^- entry that increases cAMP production required for sperm motility, hyperactivation,

and capacitation (Xu *et al.* 2007, Bai *et al.* 2010), we reason that abnormal CFTR expression in aged individuals may lead to a decrease or abnormality in HCO_3^- sensitivity. As shown in Fig. 3A, cAMP levels in sperm from different ages were similar before capacitation (at baseline level, below 5 pmol/ml). Notably, compared with the old age group (increase from 2.20 ± 0.28 to 6.43 ± 0.88 pmol/ml), exposure in

HCO_3^- -containing capacitation buffer induced a time-dependent increase in cAMP production in sperm from young adults (increase by fourfold from 3.35 ± 0.65 to 16.46 ± 2.34 pmol/ml; $P < 0.01$), followed by middle age (increase from 2.45 ± 0.68 to 8.83 ± 0.69 pmol/ml; $P < 0.05$) (Fig. 3A). Subsequent analysis on sperm forward motility after HCO_3^- exposure further showed that significant time-related increase in sperm forward motility was observed, in the young and middle age adult groups but not the old age group (Fig. 3B). Specifically, sperm from old age had minimal increase (37%) in forward motility compared with those observed in middle age (58%, $P < 0.05$) and young adults (69%, $P < 0.01$) (Fig. 3B), suggesting that the decreased HCO_3^- responsiveness and reduced sperm motility may be attributed, at least in part, by abnormal CFTR expression in sperm during aging.

Previous studies have shown that CFTR-mediated HCO_3^- entry activates intracellular sAC and subsequently leads to increases in cAMP production in sperm (Xu *et al.* 2007). To further confirm that the decreased HCO_3^- sensitivity in sperm from old men was caused by the downregulation of CFTR and thus its downstream sAC signaling, we examined the effect of two inhibitors, CFTRinh-172 and sACinh-KH7, that specifically inhibit CFTR and sAC respectively on the HCO_3^- sensitivity in sperm obtained from different age groups. As shown in Fig. 3C and D, HCO_3^- induced a remarkable increase in both cAMP production and forward motility in sperm from young adults. The effect can be inhibited by either CFTRinh-172 or sACinh-KH7, confirming the involvement of the CFTR/sAC pathway in HCO_3^- -induced response. Consistently, sperm from middle age and old age individuals demonstrated significantly lowered HCO_3^- -induced cAMP production and forward motility compared with young adults ($P < 0.001$). Importantly, despite the statistical insignificance, the minor HCO_3^- response in sperm from old age can be inhibited by CFTRinh-172 and sACinh-KH7

Figure 2 Localization of CFTR in human sperm and its correlation with sperm motility. (A) Representative confocal images showing the localization of CFTR in equatorial plate and neck of sperms (arrow) and downregulation expression (arrow head) in human sperm. Negative control was treated by omitting primary antibody. Nuclei were counterstained with Hoechst 33258. Enlarged images are shown in the inset; at least three independent experiments were done. Scale bar = 50 μm . (B) Quantification of different CFTR expression patterns in different age groups (20 to <40, 40 to <60, and >60 years, $n = 20$ in each group). Data are presented as mean $\pm$ S.E.M. of averaged data from three independent experiments. (C) Graph showing the correlation between percentage of sperm with normal expression of CFTR and age factor with 20 individual men included in each age group. (D) Graph showing the correlation between percentage of sperm with normal distributed CFTR expression and sperm forward motility in different age groups ($n = 20$ for each cohort). *Compared with young adult, $P < 0.05$; ***compared with young adult, $P < 0.001$. Pearson r correlation: $P < 0.001$.

Figure 3 Abnormal CFTR expression underlies the decreased bicarbonate sensitivity in sperm from old men. (A) Intracellular cAMP concentration in sperm after HCO₃⁻ incubation at different age groups ($n=5$ for each cohort) assessed by ELISA. (B) Time-dependent inductions of capacitation medium on sperm forward motility in different age groups ($n=5$ for each cohort). Effect of CFTRinh-172 or sACinh-KH7 on HCO₃⁻-induced cAMP production (C) and sperm forward motility (D) ($n=5$ in each group). (E) Western blot for protein tyrosine phosphorylation in young adults and old men stimulated by HCO₃⁻-free or HCO₃⁻-containing buffer with CFTRinh-172 or sACinh-KH7 respectively. Shown is a representative plot from five independent experiments. *Compared with old, $P<0.05$. **Compared with old, $P<0.01$. #Compared with young adult, $P<0.05$; ### compared with young adult, $P<0.001$; ^acompared with HCO₃⁻-treated sperm, $P<0.001$.

as well, suggesting that the remaining CFTR in sperm from old age was functional and capable of mediating the minimal HCO₃⁻ response through sAC signaling.

We further examined HCO₃⁻-induced tyrosine phosphorylation, which is the main downstream target of cAMP that leads to capacitation (Visconti *et al.* 1995). The result showed that HCO₃⁻ could increase protein tyrosine phosphorylation in sperm both from young adults and old age, and the HCO₃⁻-induced effect could be reversed by CFTRinh-172 and sACinh-KH7. However, reduced or lost phosphorylated sperm proteins (especially those above 150 kDa and below 50 kDa) are observed in old age during *in vitro* sperm capacitation (Fig. 3E). Taken together, our results suggested that

age-dependent downregulation of sperm CFTR decreases the sperm response to HCO₃⁻ by suppressing the sAC/cAMP pathway, which is required for sperm motility and capacitation.

CFTR potentiator or increasing HCO₃⁻ exposure partially rescues the decreased motility in old age sperm

The decrease in sperm motility and capacitation that was associated with downregulation of CFTR is suggestive of a causal relationship, while the residual CFTR expression is functional and capable of mediating minimal HCO₃⁻ response. Therefore, we next asked whether enhancing CFTR or increasing HCO₃⁻ could rescue the sperm motility defects. First, we treated sperm from different age groups with genistein, a CFTR potentiator that acts as a tyrosine kinase and in a protein phosphatase-independent manner (French *et al.* 1997), and examined the effect of CFTR activation on sperm motility. As shown in Fig. 4A, genistein treatment caused a subtle increase (5.42%) in forward motility of sperm from young adults, suggesting that HCO₃⁻ response already reached a maximum in young adults prior to genistein treatment. Intriguingly, activating CFTR by genistein led to a 14.17 (Fig. 4B) and 42.60% (Fig. 4C, $P<0.05$) increase in forward motility of sperm from middle age and old age.

Figure 4 CFTR potentiator (A, B, and C) or increasing HCO₃⁻ exposure (D) can partially rescue the decreased motility in old age sperm. Genistein (5 μ M) significantly promotes forward motility induced by HCO₃⁻ in the old age group (C) but not in the young adult (A) or middle age groups (B) ($n=5$ in each group). CFTR inhibitor (CFTRinh-172, 25 μ M) can abolish genistein-induced forward motility. Increasing HCO₃⁻ concentration (25–80 mM) exposure would improve the sperm forward motility in old age (D). 1×10^6 sperm in each group was incubated with HCO₃⁻ (25 mM). Forward motility was detected by CASA. Data are present as mean $\pm$ s.e.m. of five patients. *Compared with HCO₃⁻ treatment, $P<0.05$; ***compared with HCO₃⁻ treatment, $P<0.001$; **compared with HCO₃⁻ plus genistein treatment, $P<0.001$; ^acompared with HCO₃⁻ (25 mM) treatment, $P<0.05$.

Furthermore, inhibiting CFTR by CFTRinh-172 can abolish genistein-induced forward motility in the old age group during sperm capacitation ($P < 0.01$, Fig. 4C), suggesting that activating residual CFTR in sperm from old men may partially rescue the sperm motility and other HCO_3^- -induced events. In addition, increasing HCO_3^- exposure would also improve the sperm forward motility in old age ($P < 0.05$) (Fig. 4D), further indicating a causative link between decreased HCO_3^- responsiveness and abnormal CFTR expression in sperm from old age.

Discussion

This study has demonstrated that age-dependent downregulation of CFTR contributes significantly to the impairment of sperm quality in aged men, providing a molecular mechanism underlying the decline of sperm quality during aging. We have previously demonstrated an important role of CFTR in sperm capacitation and male fertility in the mouse and guinea pig (Wang *et al.* 2003, Xu *et al.* 2007, Chen *et al.* 2009). Bai *et al.* (2010) have further analyzed the expression levels of CFTR in teratospermic, asthenoteratospermic, asthenospermic, and oligospermic sperm and found a correlation between CFTR expression and sperm quality, supporting the notion that CFTR is essential for sperm-fertilizing capacity and determinant of sperm quality. In this study, we found age-dependent downregulation of CFTR, which is correlated with age-dependent reduction in sperm forward motility, suggesting that abnormal CFTR function in sperm underlies the age-dependent decline in sperm quality in old men. We have previously found an age-dependent decline of CFTR expression in rat prostate, which is due to the decline of testosterone (Xie *et al.* 2012). In castrated rats, CFTR expression was significantly reduced compared with the sham control, and replacement of testosterone could significantly increase CFTR expression in castrated rat prostate, indicating a role of testosterone in regulating CFTR expression (Xie *et al.* 2012). Age-dependent decline of testosterone has been observed in rats (Wang *et al.* 1993), mice (Reed *et al.* 2007), and humans (Leifke *et al.* 2000, Snyder 2008), which has been suggested to be responsible for the impaired sperm quality in aged men (Takahara *et al.* 1987, Jung *et al.* 2002). These suggested that CFTR may be an effector of the testosterone-dependent effect on the quality of sperm in old age.

The importance of CFTR in sperm capacitation and initiation of sperm motility lies in its ability to transport HCO_3^- , either directly or indirectly, which acts on its sensor, sAC (Okamura *et al.* 1985, Litvin *et al.* 2003), leading to the activation of cAMP and phosphorylation of tyrosine kinases required for sperm motility (Esposito *et al.* 2004) or capacitation (Hess *et al.* 2005). In this study, the downregulation of CFTR expression in sperm from old men is found to be associated with reduced levels of cAMP and tyrosine kinase phosphorylation in

response to HCO_3^- , consistent with a role of CFTR downregulation in the decline of sperm quality. The observed inhibition of the HCO_3^- -induced cAMP production and tyrosine kinase phosphorylation as well as sperm motility by inhibitors of either CFTR or sAC confirmed the involvement of CFTR and sAC in mediating the effect of HCO_3^- on sperm functions. This is further supported by the observation that genistein, a CFTR potentiator (Hernandez-Gonzalez *et al.* 2007, Al-Nakkash *et al.* 2008, Billet *et al.* 2010), was able to improve sperm quality in sperm from old men (Fig. 4), indicating a partial functionality of residual CFTR in sperm from old men after ectopic potentiation or activation. As the activator of CFTR ion channel, genistein had a relatively high selectivity for CFTR HCO_3^- conductance in a cAMP-independent pattern by interacting with CFTR directly (Tuo *et al.* 2009). Previous studies have shown that genistein can influence CFTR function directly or indirectly through its tyrosine kinase inhibitor activity (Gadsby & Nairn 1999). However, the Cl^- influx generated by genistein in noncapacitated sperm was inhibited only by the CFTR antagonist DPC but not by inhibitors of tyrosine kinases (tyrphostin 47), phosphatases (NaVO_4), or PKA (H-89). These suggest that genistein is directly activating CFTR (Hernandez-Gonzalez *et al.* 2007). Taken together, our results suggest that the impaired sperm quality in aged men is indeed associated with the downregulation of CFTR. As CFTR is also reported to affect spermatogenesis (Xu *et al.* 2011) and epididymal secretion (Chan *et al.* 1996, Pierucci-Alves *et al.* 2011, Ruan *et al.* 2012), which may affect sperm maturation, the age-dependent downregulation of CFTR in the testis and epididymis may also affect sperm quality, apart from its direct effect on sperm functions.

In this study, we found that CFTR expression was decreased in an age-dependent manner, particularly the decrease or loss of CFTR at the equatorial plate and neck region. By correlation analysis, we found that the percentage of CFTR in a downregulation pattern, especially those with decreases or loss in equatorial plate and neck region, was correlated with a reduced rate of sperm forward motility. Therefore, decreased CFTR expression in sperm may indicate impaired sperm quality. It should be noted that compared with Caucasians, relatively low incidence of CF or CFTR gene mutations is found in Asian populations (Wong *et al.* 2003, Wu *et al.* 2005). Therefore, it is unlikely that CFTR mutations would play a significant role in this study. Alternatively, apart from changes in hormonal levels, DNA methylation and histone deacetylation may contribute to CFTR transcriptional regulation (Rochwerger *et al.* 1994, Bartoszewski *et al.* 2008), which may be altered during aging.

In conclusion, this study has provided a causative correlation between downregulation of CFTR and age-dependent impairment of sperm quality. The present

findings further support an essential role of CFTR in fertilizing capacity of sperm and male fertility, indicating that CFTR may be a useful marker in the assessment of semen quality and sperm function, which would have practical applications in assisted reproduction technologies and diagnosis of male infertility.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-13-0146>.

Declaration of interest

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

Funding

This work was supported by grants from the National Key Scientific Program of China (No. 2011CB944303), the National Natural Science Foundation of China (No. 81070529, 31271244, 31100841 and 81270740), the Promotion Program for Shenzhen Key Laboratory (CXB201104220045A), Shenzhen Projects of Science and Technology (200901015 and 201202035), and the Biobank of Complex Diseases in Shenzhen (CXC201005260001A).

Acknowledgements

The authors are grateful to Dr Yong Zhang (The Department of Laboratory Medicine, Peking University Shenzhen Hospital, China) for intracellular cAMP measurement.

References

- Al-Nakkash L, Springsteel MF, Kurth MJ & Nantz MH 2008 Activation of CFTR by UCCF-029 and genistein. *Bioorganic & Medicinal Chemistry Letters* **18** 3874–3877. (doi:10.1016/j.bmcl.2008.06.051)
- Bai Y, Li M & Hwang TC 2010 Dual roles of the sixth transmembrane segment of the CFTR chloride channel in gating and permeation. *Journal of General Physiology* **136** 293–309. (doi:10.1085/jgp.201010480)
- Bartoszewski R, Rab A, Twitty G, Stevenson L, Fortenberry J, Piotrowski A, Dumanski JP & Bebek Z 2008 The mechanism of cystic fibrosis transmembrane conductance regulator transcriptional repression during the unfolded protein response. *Journal of Biological Chemistry* **283** 12154–12165. (doi:10.1074/jbc.M707610200)
- Billet A, Melin P, Jollivet M, Morron JP, Callebaut I & Becq F 2010 C terminus of nucleotide binding domain 1 contains critical features for cystic fibrosis transmembrane conductance regulator trafficking and activation. *Journal of Biological Chemistry* **285** 22132–22140. (doi:10.1074/jbc.M110.120683)
- Chan HC, Ko WH, Zhao W, Fu WO & Wong PY 1996 Evidence for independent Cl^- and HCO_3^- secretion and involvement of an apical $\text{Na}^+(\text{+})\text{-HCO}_3^-$ cotransporter in cultured rat epididymal epithelia. *Experimental Physiology* **81** 515–524.
- Chan HC, Ruan YC, He Q, Chen MH, Chen H, Xu WM, Chen WY, Xie C, Zhang XH & Zhou Z 2009 The cystic fibrosis transmembrane conductance regulator in reproductive health and disease. *Journal of Physiology* **587** 2187–2195. (doi:10.1113/jphysiol.2008.164970)
- Chavez JC, Hernandez-Gonzalez EO, Wertheimer E, Visconti PE, Darszon A & Trevino CL 2012 Participation of the $\text{Cl}^-/\text{HCO}_3^-$ exchangers SLC26A3 and SLC26A6, the Cl^- channel CFTR, and the regulatory factor SLC9A3R1 in mouse sperm capacitation. *Biology of Reproduction* **86** 1–14. (doi:10.1095/biolreprod.111.094037)
- Chen WY, Xu WM, Chen ZH, Ni Y, Yuan YY, Zhou SC, Zhou WW, Tsang LL, Chung YW, Hoglund P *et al.* 2009 Cl^- is required for HCO_3^- entry necessary for sperm capacitation in guinea pig: involvement of a $\text{Cl}^-/\text{HCO}_3^-$ exchanger (SLC26A3) and CFTR. *Biology of Reproduction* **80** 115–123. (doi:10.1095/biolreprod.108.068528)
- Chen H, Ruan YC, Xu WM, Chen J & Chan HC 2012 Regulation of male fertility by CFTR and implications in male infertility. *Human Reproduction Update* **18** 703–713. (doi:10.1093/humupd/dms027)
- Chillon M, Casals T, Mercier B, Bassas L, Lissens W, Silber S, Romey MC, Ruiz-Romero J, Verlingue C, Claustres M *et al.* 1995 Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens. *New England Journal of Medicine* **332** 1475–1480. (doi:10.1056/NEJM199506013322204)
- Eskenazi B, Wyrobek AJ, Slotter E, Kidd SA, Moore L, Young S & Moore D 2003 The association of age and semen quality in healthy men. *Human Reproduction* **18** 447–454. (doi:10.1093/humrep/deg107)
- Esposito G, Jaiswal BS, Xie F, Krajnc-Franken MA, Robben TJ, Strik AM, Kuil C, Philipsen RL, van Duin M, Conti M *et al.* 2004 Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. *PNAS* **101** 2993–2998. (doi:10.1073/pnas.0400050101)
- Figueiras-Fierro D, Acevedo JJ, Martinez-Lopez P, Escoffier J, Sepulveda FV, Balderas E, Orta G, Visconti PE & Darszon A 2013 Electrophysiological evidence for the presence of cystic fibrosis transmembrane conductance regulator (CFTR) in mouse sperm. *Journal of Cellular Physiology* **228** 590–601. (doi:10.1002/jcp.24166)
- French PJ, Bijman J, Bot AG, Boomaars WE, Scholte BJ & de Jonge HR 1997 Genistein activates CFTR Cl^- channels via a tyrosine kinase- and protein phosphatase-independent mechanism. *American Journal of Physiology* **273** C747–C753.
- Gadsby DC & Nairn AC 1999 Regulation of CFTR Cl^- ion channels by phosphorylation and dephosphorylation. *Advances in Second Messenger and Phosphoprotein Research* **33** 79–106.
- Guo X, Gui YT, Tang AF, Lu LH, Gao X & Cai ZM 2007 Differential expression of VASA gene in ejaculated spermatozoa from normozoospermic men and patients with oligozoospermia. *Asian Journal of Andrology* **9** 339–344. (doi:10.1111/j.1745-7262.2007.00253.x)
- Haidl G, Jung A & Schill WB 1996 Ageing and sperm function. *Human Reproduction* **11** 558–560. (doi:10.1093/HUMREP/11.3.558)
- Hernandez-Gonzalez EO, Trevino CL, Castellano LE, de la Vega-Beltran JL, Ocampo AY, Wertheimer E, Visconti PE & Darszon A 2007 Involvement of cystic fibrosis transmembrane conductance regulator in mouse sperm capacitation. *Journal of Biological Chemistry* **282** 24397–24406. (doi:10.1074/jbc.M701603200)
- Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, Miyamoto C, Zippin JH, Kopf GS, Suarez SS *et al.* 2005 The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Developmental Cell* **9** 249–259. (doi:10.1016/j.devcel.2005.06.007)
- Jung A, Schuppe HC & Schill WB 2002 Comparison of semen quality in older and younger men attending an andrology clinic. *Andrologia* **34** 116–122. (doi:10.1046/j.0303-4569.2001.00487.x)
- Kidd SA, Eskenazi B & Wyrobek AJ 2001 Effects of male age on semen quality and fertility: a review of the literature. *Fertility and Sterility* **75** 237–248. (doi:10.1016/S0015-0282(00)01679-4)
- Klinefelter GR, Gray LE Jr & Suarez JD 1991 The method of sperm collection significantly influences sperm motion parameters following ethane dimethanesulphonate administration in the rat. *Reproductive Toxicology* **5** 39–44. (doi:10.1016/0890-6238(91)90108-R)
- Kuhnert B & Nieschlag E 2004 Reproductive functions of the ageing male. *Human Reproduction Update* **10** 327–339. (doi:10.1093/humupd/dmh030)
- Lambard S, Galeraud-Denis I, Martin G, Levy R, Chocat A & Carreau S 2004 Analysis and significance of mRNA in human ejaculated sperm from normozoospermic donors: relationship to sperm motility and capacitation. *Molecular Human Reproduction* **10** 535–541. (doi:10.1093/molehr/gah064)

- Leifke E, Gorenoi V, Wichers C, Von Zur Muhlen A, Von Buren E & Brabant G 2000 Age-related changes of serum sex hormones, insulin-like growth factor-1 and sex-hormone binding globulin levels in men: cross-sectional data from a healthy male cohort. *Clinical Endocrinology* **53** 689–695. (doi:10.1046/j.1365-2265.2000.01159.x)
- Litvin TN, Kamenetsky M, Zarifyan A, Buck J & Levin LR 2003 Kinetic properties of "soluble" adenyl cyclase. Synergism between calcium and bicarbonate. *Journal of Biological Chemistry* **278** 15922–15926. (doi:10.1074/jbc.M212475200)
- Lu JC, Huang YF & Lu NQ 2010 WHO Laboratory Manual for the Examination and Processing of Human Semen: its applicability to andrology laboratories in China. *Zhonghua Nan Ke Xue* **16** 867–871.
- Menken J, Trussell J & Larsen U 1986 Age and infertility. *Science* **233** 1389–1394. (doi:10.1126/science.3755843)
- Miller D, Briggs D, Snowden H, Hamlington J, Rollinson S, Lilford R & Krawetz SA 1999 A complex population of RNAs exists in human ejaculate spermatozoa: implications for understanding molecular aspects of spermiogenesis. *Gene* **237** 385–392. (doi:10.1016/S0378-1119(99)00324-8)
- Munne S, Alikani M, Tomkin G, Grifo J & Cohen J 1995 Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertility and Sterility* **64** 382–391.
- Ng KK, Donat R, Chan L, Lalak A, Di Piero I & Handelsman DJ 2004 Sperm output of older men. *Human Reproduction* **19** 1811–1815. (doi:10.1093/humrep/deh315)
- Okamura N, Tajima Y, Soejima A, Masuda H & Sugita Y 1985 Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *Journal of Biological Chemistry* **260** 9699–9705.
- Ostermeier GC, Dix DJ, Miller D, Khatri P & Krawetz SA 2002 Spermatozoal RNA profiles of normal fertile men. *Lancet* **360** 772–777. (doi:10.1016/S0140-6736(02)09899-9)
- Pierucci-Alves F, Akoyev V, Stewart JC III, Wang LH, Janardhan KS & Schultz BD 2011 Swine models of cystic fibrosis reveal male reproductive tract phenotype at birth. *Biology of Reproduction* **85** 442–451. (doi:10.1095/biolreprod.111.090860)
- Reed MJ, Karres N, Eyman D, Cruz A, Brekken RA & Plymate S 2007 The effects of aging on tumor growth and angiogenesis are tumor-cell dependent. *International Journal of Cancer* **120** 753–760. (doi:10.1002/ijc.22351)
- Rochwerger L, Dho S, Parker L, Foksett JK & Buchwald M 1994 Estrogen-dependent expression of the cystic fibrosis transmembrane regulator gene in a novel uterine epithelial cell line. *Journal of Cell Science* **107** 2439–2448.
- Rowe SM, Miller S & Sorscher EJ 2005 Cystic fibrosis. *New England Journal of Medicine* **352** 1992–2001. (doi:10.1056/NEJMra043184)
- Ruan YC, Shum WW, Belleannee C, Da Silva N & Breton S 2012 ATP secretion in the male reproductive tract: essential role of CFTR. *Journal of Physiology* **590** 4209–4222. (doi:10.1113/jphysiol.2012.230581)
- Snyder PJ 2008 Decreasing testosterone with increasing age: more factors, more questions. *Journal of Clinical Endocrinology and Metabolism* **93** 2477–2478. (doi:10.1210/jc.2008-0922)
- Takahara H, Cosentino MJ, Sakatoku J & Cockett AT 1987 Significance of testicular size measurement in andrology: II. Correlation of testicular size with testicular function. *Journal of Urology* **137** 416–419.
- Tuo B, Wen G & Seidler U 2009 Differential activation of the HCO₃(–) conductance through the cystic fibrosis transmembrane conductance regulator anion channel by genistein and forskolin in murine duodenum. *British Journal of Pharmacology* **158** 1313–1321. (doi:10.1111/j.1476-5381.2009.00398.x)
- Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS 1995 Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121** 1129–1137.
- Wang C, Leung A & Sinha-Hikim AP 1993 Reproductive aging in the male brown-Norway rat: a model for the human. *Endocrinology* **133** 2773–2781. (doi:10.1210/en.133.6.2773)
- Wang XF, Zhou CX, Shi QX, Yuan YY, Yu MK, Ajonuma LC, Ho LS, Lo PS, Tsang LL, Liu Y *et al.* 2003 Involvement of CFTR in uterine bicarbonate secretion and the fertilizing capacity of sperm. *Nature Cell Biology* **5** 902–906. (doi:10.1038/ncb1047)
- Wong LJ, Alper OM, Wang BT, Lee MH & Lo SY 2003 Two novel null mutations in a Taiwanese cystic fibrosis patient and a survey of East Asian CFTR mutations. *American Journal of Medical Genetics. Part A* **120A** 296–298. (doi:10.1002/ajmg.a.20039)
- Wu CC, Alper OM, Lu JF, Wang SP, Guo L, Chiang HS & Wong LJ 2005 Mutation spectrum of the CFTR gene in Taiwanese patients with congenital bilateral absence of the vas deferens. *Human Reproduction* **20** 2470–2475. (doi:10.1093/humrep/dei077)
- Xie C, Jiang XH, Zhang JT, Sun TT, Dong JD, Sanders AJ, Diao RY, Wang Y, Fok KL, Tsang LL *et al.* 2012 CFTR suppresses tumor progression through miR-193b targeting urokinase plasminogen activator (uPA) in prostate cancer. *Oncogene* **32** 2282–2291, 2291.e1–7. (doi:10.1038/ncr.2012.251)
- Xu WM, Shi QX, Chen WY, Zhou CX, Ni Y, Rowlands DK, Liu GY, Zhu H, Ma ZG, Wang XF *et al.* 2007 Cystic fibrosis transmembrane conductance regulator is vital to sperm fertilizing capacity and male fertility. *PNAS* **104** 9816–9821. (doi:10.1073/pnas.0609253104)
- Xu WM, Chen J, Chen H, Diao RY, Fok KL, Dong JD, Sun TT, Chen WY, Yu MK, Zhang XH *et al.* 2011 Defective CFTR-dependent CREB activation results in impaired spermatogenesis and azoospermia. *PLoS ONE* **6** e19120. (doi:10.1371/journal.pone.0019120)

Received 8 April 2013

First decision 23 May 2013

Revised manuscript received 29 July 2013

Accepted 27 September 2013