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

Ruiying Diao1,2, Kin Lam Fok3, Li Zhao4, Hao Chen1,2, 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 HCO3- 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.

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 subfertility 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 (CBVAD; 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 glycyglycine, 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=16). 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× 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 Klínefelter 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$^6$ 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 ≥25 μm/s) and b (the average velocity between 15 and 25 μm/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. To 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 2X Laemmli sample buffer, separated by SDS–PAGE on 6% (v/w) 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'-GTTGGGAAAGAGCTTCA C-3'; reverse, 5'-GTGGGGAAAGAGCTTCA C-3'; the primers used for human GAPDH (NM_001256799) are as follows: forward, 5'-AGAA GCCGGGGCTCATTTG-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. 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.

**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±SE. 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±9.8% compared with 61.1±8.4% in young age; P<0.001), and forward motility (17.4±8.8% compared with 56.2±4.5% in young age; P<0.001) (Table 1).

<table>
<thead>
<tr>
<th>Group (years old)</th>
<th>Number</th>
<th>Age (median±IQR)</th>
<th>Intensity (million/ml)</th>
<th>Motility (%)</th>
<th>Grade a+b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-40</td>
<td>64</td>
<td>29.5±4.9</td>
<td>95.3±57.9</td>
<td>61.1±8.4</td>
<td>56.2±4.5</td>
</tr>
<tr>
<td>40-60</td>
<td>61</td>
<td>48.0±5.0</td>
<td>39.8±24.6*</td>
<td>35.9±9.0*</td>
<td>30.3±8.5*</td>
</tr>
<tr>
<td>&gt;60</td>
<td>76</td>
<td>64.3±3.2</td>
<td>10.4±11.2*</td>
<td>21.1±9.8*</td>
<td>17.4±8.8*</td>
</tr>
</tbody>
</table>

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

---

Table 1: Clinical information for semen in different age groups.
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±1.92%; P<0.05) and old age (42.95±1.20%; P<0.001) compared with young adult (56.73±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±2.60%; P<0.05) and old age individuals (58.05±1.33 vs 43.27±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) 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 HCO3⁻ response in sperm from old men**

As CFTR mediates HCO3⁻ 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 $2.20 \pm 0.28$ to $6.43 \pm 0.88$ 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 $3.35 \pm 0.65$ to $16.46 \pm 2.34$ pmol/ml; $P<0.01$), followed by middle age (increase from $2.45 \pm 0.68$ to $8.83 \pm 0.69$ 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 insensitivity, 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 \(\mu\)m. (B) Quantification of different CFTR expression patterns in different age groups (20 to 40, 40 to 60, and > 60 years, \(n=20\) for each cohort). Data are presented as mean $\pm$ S.D. of averaged data from three independent experiments. (C) 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: $P<0.001$. 

www.reproduction-online.org
tyrosine phosphorylation in young adults and old men stimulated by forward motility (D) (different age groups (inductions of capacitation medium on sperm forward motility in (with old, independent experiments. Compared with old, 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; †††Compared 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.

![Image](https://via.placeholder.com/150)

**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; †††Compared with HCO₃⁻– treated sperm, P<0.001.

**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 μM) 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 μM) can abolish genistein-induced forward motility. Increasing HCO₃⁻– concentration (25–80 mM) exposure would improve the sperm forward motility in old age (D). 1×10⁶ sperm in each group was incubated with HCO₃⁻– (25 mM). Forward motility was detected by CASA. Data are present as mean±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; ††Compared 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 (Leikie et al. 2000, Snyder 2008), which has been suggested to be responsible for the impaired sperm quality in aged men (Takihara 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 ecticopic 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 (Tu 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 (tyrophostin 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 (CXR201104220045A), 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


Chan HC, Ko WH, Zhao W, Fu WO & Wong PY 1996 Evidence for independent Cl− and HCO3− secretion and involvement of an apical Na+ + HCO3− cotransporter in cultured rat epididymis epithelium. Experimental Physiology 81 515–524


Xu WM, Chen J, Chen H, Diao CY, 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