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Involvement of CFTR in uterine bicarbonate secretion and the fertilizing capacity of sperm

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Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated chloride channel expressed in a wide variety of epithelial cells, mutations of which are responsible for the hallmark defective chloride secretion observed in cystic fibrosis (CF). Although CFTR has been implicated in bicarbonate secretion, its ability to directly mediate bicarbonate secretion of any physiological significance has not been shown. We demonstrate here that endometrial epithelial cells possess a CFTR-mediated bicarbonate transport mechanism. Co-culture of sperm with endometrial cells treated with antisense oligonucleotide against CFTR, or with bicarbonate secretion-defective CF epithelial cells, resulted in lower sperm capacitation and egg-fertilizing ability. These results are consistent with a critical role of CFTR in controlling uterine bicarbonate secretion and the fertilizing capacity of sperm, providing a link between defective CFTR and lower female fertility in CF.

The role of aberrant bicarbonate transport in CF pathogenesis has been overlooked until recently[1–3]. Although CFTR in several tissues, including the airway, intestine and pancreas, has been shown to conduct bicarbonate (refs 4, 5), the apparently low permeability of CFTR to bicarbonate raises doubt in its ability to secrete sufficient bicarbonate to be considered physiologically significant[6]. In fact, no previous study has shown a direct link between CFTR-mediated bicarbonate secretion and a pathological condition in CF.

Reduced fertility has long been observed in women with CF, but few studies have specifically assessed the contributing factors. The absence of any anatomical abnormality in the female reproductive tract, except for a thick and tenacious cervical mucus with altered water and electrolyte content[7,8], and the ability of a certain percentage of women with CF to achieve pregnancy successfully has led to the general belief that the genital factor contributing to lower fertility in women with CF is the thick cervical mucus acting as a barrier for sperm passage. The fact that CFTR mRNA has been detected throughout the female reproductive tract of rodents[9,10] and humans[11], including the uterus and oviduct in addition to the cervix, extends the possible pathophysiological basis beyond the cervix for reduced fertility in women with CF. It has long been recognized that uterine fluids contain substantial amount of bicarbonate, which could be more than twice that in the plasma[12], indicative of active bicarbonate transport across the endometrium, the functional uterine mucosal lining that is largely responsible for the formation of an optimal uterine fluid for various reproductive events. Accumulating evidence has also implicated bicarbonate as the component that is most responsible for sperm capacitation[13–15], a poorly understood molecular and physiological process that confers on the sperm the ability to fertilize during residence in the female reproductive tract. Taken together, these results make it plausible that CFTR might mediate or regulate bicarbonate secretion by the endometrium, defects in which might result in impaired sperm capacitation and therefore lower fertility in women with CF.

We undertook the present study first to demonstrate the involvement of CFTR in bicarbonate secretion across the endometrium. Although observations of high bicarbonate content in uterine fluids were made more than half a century ago, the cellular mechanisms underlying the formation of bicarbonate-rich uterine fluid remain largely unknown. Our previous studies[16] have demonstrated the involvement of a basolaterally located Na^+–HCO_3^- cotransporter (NBC) in the cellular accumulation of bicarbonate. The present study investigated the apical mechanisms for bicarbonate extrusion using our previously established mouse endometrial epithelial culture[17] in conjunction with fluorimetric intracellular pH (pH_i) measurements. Cellular alkalization was induced by removing bicarbonate/CO_2 from the perfusate, and the rate of pH_i recovery was taken as a measure of bicarbonate extrusion. The rate of pH_i recovery was greatly attenuated when chloride was absent from the apical solution in comparison with that observed in the Cl^--containing solution (Fig. 1a, b), indicating the involvement of the Cl^-/HCO_3^- exchanger (AE). In the absence of apical Cl^-, the normal bicarbonate extrusion process mediated by the AE was interrupted, slowing down the bicarbonate extrusion process and thus the rate of pH_i recovery. However, in the absence of apical Cl^-, the rate of pH_i recovery could be stimulated by an adenylyl cyclase

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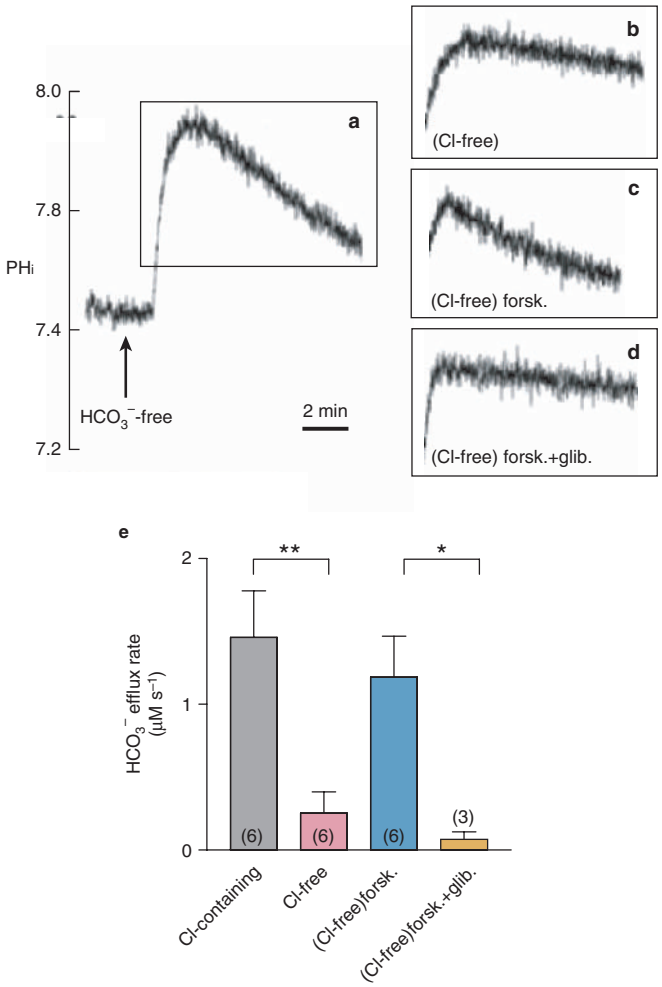

Figure 1 Involvement of CFTR in mediating cAMP-stimulated bicarbonate extrusion in mouse endometrial epithelial cells. (**a**, **b**) After cellular alkalinization induced by removing bicarbonate/CO_2 from perfusate, the pH_i recovered quickly owing to bicarbonate efflux in the presence of Cl^- (**a**) but slowly when Cl^- was absent from the apical perfusate (**b**). (**c**, **d**) In the absence of Cl^-, forskolin (forsk., 10 μM) stimulated pH_i recovery (**c**) which was blocked by glibenclamide (glib., 200 μM) (**d**). The scales in **a**–**d** are the same. (**e**) Summary of bicarbonate efflux rates under different conditions. Data are shown as means + s.e. for *n* experiments (*$P < 0.05$, **$P < 0.01$). Methods have been described previously[16].

activator, forskolin. The forskolin-induced pH_i recovery could be blocked by glibenclamide (Fig. 1c, d), a blocker known to inhibit CFTR[18]. Taking into account the changes in buffering capacity under different conditions, these data are expressed as a rate of bicarbonate efflux as shown in Fig. 1e, indicating the presence of an AE-independent but cAMP-dependent bicarbonate-transporting mechanism in the mouse endometrium, most probably involving CFTR. To test this further, we conducted experiments in Ussing chambers in which the transepithelial transport of bicarbonate was measured by the short-circuit current (I_{SC})[17]. We found (Fig. 2a, b) that forskolin or genistein, a plant-derived compound known to activate CFTR[19], stimulated a bicarbonate-dependent I_{SC} (with bicarbonate being the only permeable anion), which could be completely blocked by CFTR-sensitive diphenylamine-2-carboxylate (DPC) or glibenclamide but to a much smaller extent by CFTR-insensitive 4,4′-di-isothiocyanostilbene-2,2′-disulphonate (DIDS)[19]. Bicarbonate-dependent I_{SC} could also be suppressed by antisense oligonucleotide against CFTR (Fig. 2c), a treatment shown to substantially reduce CFTR expression in the same culture preparation (Fig. 2d). CFTR-mediated endometrial bicarbonate secretion was further demonstrated by measuring bicarbonate contents in the apical compartment (initially bicarbonate-free) 2 h after stimulation with forskolin. A substantial decrease in apical fluid bicarbonate contents was observed after treatment with blockers of CFTR or antisense oligonucleotides against CFTR (Fig. 2e) when compared with control. Taken together, these results are consistent with the CFTR's mediating uterine bicarbonate secretion, and indicate that defective CFTR might lead to impaired bicarbonate secretion in the uterus.

We then examined the effect of endometrial secretion on the fertilizing capacity of sperm by using a sperm–epithelium co-culture system. Computer-assisted sperm analysis (CASA) revealed that the sperm motility of the mouse was greatly enhanced by 40.5 ± 4.5% when sperm were co-cultured with the endometrial epithelia in comparison with that incubated with medium lacking both bicarbonate and cells. It should be noted that bicarbonate-free medium was first added to the apical compartment that was separated from the bicarbonate-containing (25 mM) basolateral compartment by the confluent epithelium (monitored by transepithelial resistance to exclude possible 'leaky' epithelia). Changes in bicarbonate content in the apical fluid therefore had to rely on endometrial secretion, over a period of 12–24 h before sperm were added to the apical compartment. The difference in sperm motility observed between co-cultured sperm and sperm incubated in bicarbonate-free medium reflected the capacity of the endometrial epithelium to secrete bicarbonate. We then examined the effect of bicarbonate on the hyperactivation of sperm motility because it is known to be associated with sperm capacitation. In fact, when bicarbonate was added to the incubation medium in the absence of endometrial cells, the hyperactivated motility of sperm was increased in a manner dependent on bicarbonate concentration, i.e. from 43 ± 1.2% in the absence of bicarbonate to 77.3 ± 1.2% at 15 mM and 86.3 ± 1.7% at 25 mM bicarbonate, confirming the influence of bicarbonate. In addition, the effect of CFTR-mediated endometrial secretion on sperm capacitation was also assessed by the chlortetracycline (CTC) fluorescence method[20]. As shown in Fig. 3a, the percentage of capacitated sperm was significantly decreased when CFTR expression in the endometrial epithelial cells was suppressed with antisense oligonucleotide against CFTR, compared with sense-treated controls ($P < 0.001$). *In vitro* fertilization (IVF) assays on zona-pellucida-intact mouse eggs were further performed to test the egg-fertilizing ability of sperm after co-culture, and the appearance of two-cell-stage embryos was taken to indicate successful fertilization. In these sets of experiments, sperm were capacitated in conditioned medium collected from the apical compartments of cell cultures instead of co-culturing with the endometrial cells directly, to avoid damage to the cells by the sperm. As shown in Fig. 3b, c, the number of two-cell embryos obtained with sperm capacitated in conditioned medium from CFTR antisense-treated endometrial cells (25 of 99) was significantly decreased compared with that obtained from sense-treated controls (54 of 74). The appearance of uneven blastomeres and fragmentation, indicating a deterioration of oocytes due to unsuccessful fertilization, was also observed in CFTR antisense-treated, but not sense-treated, samples. Taken together, these results indicate that sperm capacitation and egg-fertilizing ability might depend critically on CFTR and bicarbonate content.

As a further demonstration of a link between defective CFTR-mediated bicarbonate secretion and a decreased fertilizing capacity of sperm as a possible cause of female infertility in CF, we conducted co-culture experiments with normal and CF pancreatic duct cell lines,

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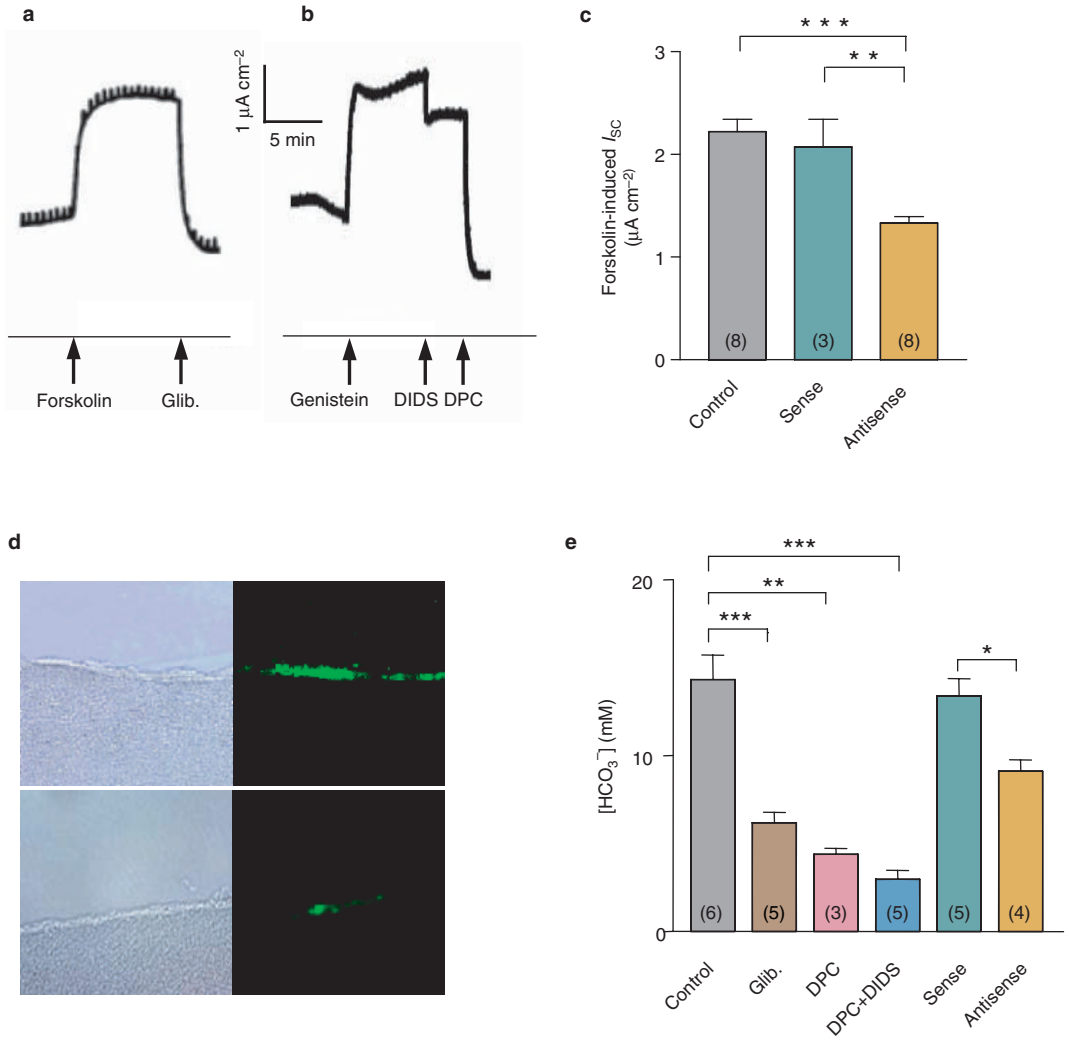

Figure 2 Involvement of CFTR in uterine bicarbonate secretion. (**a**, **b**) The bicarbonate-dependent short-circuit current (I_{SC}) stimulated by forskolin (10 μM) (**a**) and genistein (50 μM) (**b**), which were completely blocked by DPC (1 mM) or glibenclamide (glib., 1 mM) but not by DIDS (200 μM). (**c**) The forskolin-induced I_{SC} was suppressed by antisense oligonucleotides against CFTR (10 μg ml^{-1}), but not with sense control oligonucleotides. (**d**) Immunohistochemical demonstration of CFTR expression in sense (top right; average fluorescence intensity 60.3 ± 4.7, n = 12) and antisense (bottom right; average fluorescence intensity 33.7 ± 2.2, n = 10) treated cultured endometrial monolayers[17], with corresponding phase-contrast micrographs (top left and bottom left, respectively) showing cultured endometrial monolayers grown on permeable supports (original magnification ×200). (**e**) Summary of measurement of bicarbonate contents in the apical fluids (initially bicarbonate-free) under various conditions obtained 2 h after stimulation with forskolin (*P < 0.05; **P < 0.01; ***P < 0.001).

CAPAN-1 and CFPAC-1, respectively. CAPAN-1 retains characteristic bicarbonate secretion of human pancreatic duct *in vivo*[21,22], whereas CFPAC-1, which has the ΔF508 mutation most frequently associated with CF, is defective in bicarbonate secretion[23]. We confirmed a decrease in bicarbonate content in CFPAC-1-derived apical fluid similar to that observed in endometrial cells treated with CFTR blockers or CFTR antisense oligonucleotides (data not shown). CTC analysis revealed that the percentage of capacitated sperm in bicarbonate-free or CFPAC-1-conditioned medium was significantly decreased in comparison with that in 15 mM bicarbonate or CAPAN-1-conditioned medium (Fig. 4a). Sperm capacitation was restored when 20 mM bicarbonate was added to CFPAC-1-derived medium, or when CFPAC-1 cells were transfected with wild-type CFTR (Fig. 4a). The egg-binding and egg-penetrating ability of sperm after co-culture was also tested. Mouse sperm capacitated in CFPAC-1-conditioned medium showed decreased sperm binding and penetration of zona-pellucida-free mouse eggs compared with that in CAPAN-1 medium (Fig. 4b). However, the impaired egg-binding and penetrating ability was restored either by the addition of bicarbonate or the transfection of wild-type CFTR into CFPAC cells (Fig. 4b). Further IVF experiments on zona-pellucida-intact mouse eggs showed that 86.2% of eggs (73 of 83) were fertilized by sperm co-cultured with CAPAN-1-conditioned medium but only 49.7% (43 of 85) by sperm with CFPAC-1-conditioned medium (Fig. 4c). Percentages of fertilization also varied with different concentrations of bicarbonate (Fig. 4c). A closer examination of live sperm under a light microscope after incubation with eggs revealed a significant difference between sperm incubated with CAPAN-1-conditioned medium and those incubated with CFPAC-1-conditioned medium. Sperm in CAPAN-1-conditioned medium had a higher percentage of motile sperm and displayed vigorous beating and

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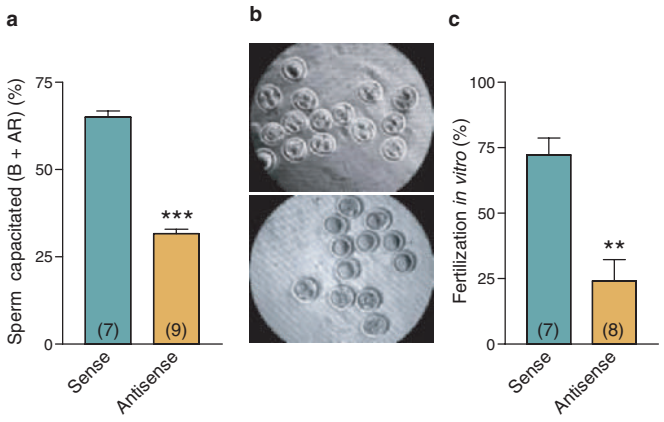

Figure 3 CFTR-suppressed endometrial epithelial cells decreases sperm capacitation and egg-fertilizing ability. The percentage of capacitated sperm (B + AR pattern) detected by chlortetracycline (CTC) fluorescence staining (**a**) and the rate of IVF of zona-pellucida-intact eggs indicated by the percentage of two-cell embryos (**b**, **c**) were significantly attenuated when sperm were capacitated in conditioned medium from endometrial epithelia treated with CFTR antisense (10 μg ml^{-1}), in comparison with that in sense-treated control cells. Representative IVF results are shown in the upper and lower panels of **b** for sense and antisense, respectively. The total number of eggs tested was 173.

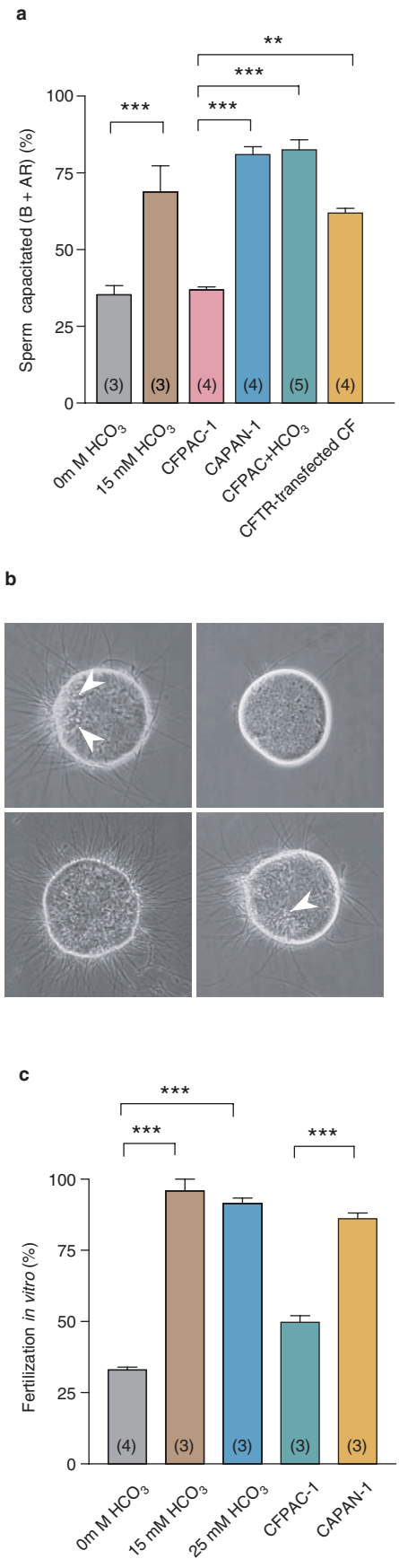

Figure 4 Defective bicarbonate-secreting CF cells decreases sperm capacitation and egg-fertilizing ability. (**a**) Comparison of sperm capacitation obtained 50 min after incubation in different medium. (**b**) Phase-contrast microscopy (original magnification × 200) obtained after sperm–egg incubation for 4 h and vigorous washing, showing a zona-pellucida-free mouse oocyte bound or penetrated by sperm capacitated in CAPAN-1-conditioned medium (top left), decreased sperm binding and fewer penetrations by sperm capacitated in CFPAC-1-conditioned medium (top right), and restored egg-binding and egg-penetrating ability with the addition of bicarbonate (bottom left) or the transfection of wild-type CFTR into CFPAC-1 cells (bottom right). Arrowheads indicate the presence of penetrated and decondensed sperm heads in the ooplasm. (**c**) Comparison of IVF of zona-pellucida-intact oocytes, showing a significantly greater percentage of mouse eggs fertilized by sperm capacitated in CAPAN-1-conditioned medium or medium containing 15 or 25 mM bicarbonate than that in CFPAC-1-conditioned medium or bicarbonate-free medium. The total number of eggs assessed was 263.

progressive directed movement characterized as hyperactivation, an indication of sperm capacitation. However, sperm in CFPAC-1-conditioned medium had a lower percentage of motile sperm, with sluggish and less directed movement characteristics. These results demonstrated that aberrant bicarbonate secretion, due to defective CFTR, could lead to impaired sperm capacitation and decreased fertilizing capacity.

Here we have demonstrated a CFTR-mediated bicarbonate-transporting mechanism in mouse endometrium, a defect in which might result in impaired fertilizing capacity of sperm. Although no report is available on the bicarbonate content in CF female reproductive tract, the content is likely to be decreased because of defective CFTR in the uterus and oviduct. It therefore seems that, as well as the thick cervical mucus long thought to be the problem in CF, the newly established link between defective CFTR-mediated bicarbonate secretion and lower fertilizing capacity of sperm might also account for the lower female fertility in CF. Interestingly, it has been reported that two women with CF failed to achieve pregnancy after four repeated

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intrauterine inseminations, a procedure for sperm to circumvent the cervical problem, but that pregnancy occurred after IVF[24]. This is consistent with a defect beyond the cervix causing female infertility in CF. The present finding provides further evidence supporting the role of aberrant bicarbonate transport in CF pathogenesis. The CFTR-mediated bicarbonate secretion might therefore be of critical importance not only in sperm physiology and fertility but also in CF pathogenesis in the gastrointestinal tract and airways. □

METHODS

Co-culture of sperm and epithelial cells. Sperm from ICR mice aged 20–24 weeks were selected by two-step Percoll density gradient (35–70%) centrifugation and resuspended in modified Tyrode's medium (mT) without bicarbonate. Endometrial cells, CAPAN-1and CFPAC-1 cells were grown in their respective normal bicarbonate containing culture medium[17,22] until confluence was reached. The apical medium was replaced with 60 μl mT (bicarbonate-free) medium and basolateral medium with the same bicarbonate-containing (25 mM) DMEM for all cell cultures 12 h before co-culturing. Adrenaline or secretin (1 μM) was added to the basolateral culture medium 1 h before collection of apical secretions. Variations in pH before co-culture were titrated back to pH 7.4 to avoid any possible effect of pH.

Assessment of sperm capacitation and fertilization. Sperm capacitation in mice was detected by CTC staining as described previously[20]. A total of 200 sperm were counted to assess the different CTC staining patterns recognized previously. B and AR patterns were taken to indicate capacitated sperm: the B pattern was capacitated and acrosome-intact sperm; the AR pattern was capacitated and acrosome-reacted sperm. The methods for IVF in mice followed those described[25], with the exception that the incubation was conducted in a heated water bath (37 °C). After incubation with conditioned medium from apical compartments of endometrial, CAPAN-1 or CFPAC-1 cultures for 1 h, sperm suspension was added to an oocyte-containing insemination drop of human tubular fluid (HTF) medium with 30 mg ml^{-1} BSA under mineral oil (sperm density 2×10^6 ml^{-1}). After incubation for 4 h at 37 °C in air, the medium was replaced by mHTF with 14 mM bicarbonate and followed by incubation for a further 20 h before assessment of fertilization.

Transfection with antisense oligonucleotide against CFTR. Antisense oligonucleotide was complementary to the translation initiation of region of mRNA specific for the mouse CFTR (5′-CATGATGTCTCCGTGA-3′). Sense oligonucleotide to CFTR (5′-TCACGGAGACATCATG-3′) was used as control. Transfection was performed with Lipofectin transfection reagent (Gibco-BRL). In brief, oligonucleotides (10 μg ml^{-1}) and Lipofectin transfection reagent were incubated together for 45 min at 25°C room temperature and then added to apical medium of confluent cell cultures in serum-free and antibiotic-free DMEM/F12 culture medium for 24 h. The expression of CFTR after antisense treatment was checked by fluorescent immunostaining, and the fluorescent intensity was measured by SPOT RT image analysis software (version 3.5; Diagnostic Instruments, Inc.). The fluorescent intensity over total area under a microscope objective (200 ×) was measured for all samples examined.

Bicarbonate concentration measurement. Bicarbonate concentrations were obtained by measuring the pH of the fluid in the apical compartment of the cell culture (200 μl) with a micro-pH electrode (part number 511083; Beckman). Cell cultures were kept at 5% CO_2 and 37 °C during the measurement. The bicarbonate concentration was then calculated with the Henderson–Hasselbalch equation.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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