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SHORT COMMUNICATION

# Functional expression of cystic fibrosis transmembrane conductance regulator in mouse chondrocytes

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## SUMMARY

**1. Cystic fibrosis transmembrane conductance regulator (CFTR) is well known for its role in the cystic fibrosis (CF). Recent studies have shown that CF patients and CFTR-deficient mice exhibit a severe abnormal skeletal phenotype, indicating that CFTR may play a role in bone development and pathophysiological processes. However, it is not known whether CFTR has a direct or indirect effect on bone formation. The aim of the present study was to detect the expression and function of CFTR in mouse chondrocytes.**

**2. Reverse transcription-polymerase chain reaction, western blotting and immunofluorescence were used to characterize the expression of CFTR in primary isolated mouse chondrocytes. Expression of CFTR mRNA and protein was detectable in mouse chondrocytes. Importantly, whole-cell patch-clamp analysis demonstrated that CFTR in mouse chondrocytes is functional as a cAMP-dependent $Cl^-$ channel that is inhibited by CFTRinh-172.**

**3. Thus, the results of the present study demonstrate that functional CFTR is expressed in mouse chondrocytes, which offers essential evidence for the potential direct role of CFTR in physiological and pathological processes of bone.**

**Key words: chondrocytes, cystic fibrosis, cystic fibrosis transmembrane conductance regulator (CFTR), mouse.**

## INTRODUCTION

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease with multi-organ disorders. It is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride channel in epithelial cell membranes. The CFTR mutation influences secretion and absorption by the epithelium in various organs, including the respiratory tract, pancreas, gastrointestinal tract and sweat glands.[1–4] Interestingly, recent studies report that an abnormal skeletal phenotype, including severe and persistent osteopenia, is present in $CFTR^{-/-}$ mice.[5–7] Importantly, it has been reported that osteoporosis is common in CF patients,[8–14] which implies involvement of CFTR in bone disease. However, it is not known whether the skeletal abnormalities of $CFTR^{-/-}$ mice are caused by a direct or indirect effect of CFTR.

As the only cell type in articular cartilage, chondrocytes are responsible for producing and maintaining the extracellular matrix and play a key role in endochondral bone formation.[15,16] During endochondral bone formation, chondrocytes exhibit a lifecycle of proliferation, differentiation, maturation and apoptosis to form the mineralized cartilaginous matrix. This process is followed by replacement of the cartilaginous matrix by trabecular bone.[17] Chondrocytes serve as a scaffold for the formation of trabecular bone and play an important role in bone development and pathophysiological function.[18] In $CFTR^{-/-}$ mice, abnormal skeletal phenotypes are seen, including abnormalities in bone mineral density, cortical bone thinning and altered trabecular architecture.[6] Thus, we speculated that CFTR expression in chondrocytes may have some role in bone formation. In the present study, we demonstrate that functional CFTR is highly expressed in mouse chondrocytes.

## METHODS

### Primary chondrocyte cultures

The isolation and culture of mouse chondrocytes was as reported previously.[19] Briefly, the cartilage from 5-day-old male CD1 mice was chopped into small pieces and then incubated with collagenase IA (Sigma, St Louis, MO, USA) for 2 h at 37°C with agitation. The cells obtained were plated in six-well plates, in which cover slips had been placed, and maintained at 37°C and 5% $CO_2$ for 4 h or 2–3 days. After the 4 h culture, mouse chondrocytes were used in patch-clamp experiments. After 2–3 days culture, mouse chondrocytes were used for immunofluorescence and reverse transcription-polymerase chain reaction (RT-PCR) studies.

### Reverse transcription-polymerase chain reaction

Total RNA from cultured chondrocytes was extracted with an RNeasy micro kit (Qiagen, Valencia, CA, USA) and cDNA was reverse transcribed from the total RNA using a SuperScript First-strand Synthesis System (Invitrogen,

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Carlsbad, CA, USA). The cDNA was used as a template for PCR amplification (94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s). Two pairs of specific primers were designed to amplify segments of mouse CFTR coding sequences, namely Primer A (5′-ATGCAGAAGTCGCCTTTGGAG-3′ (forward) and 5′-GAAGCAGTGTCCTGACAATGAAG-3′ (reverse)) and Primer B (5′-TCAGTAAGGCCAAGATCATAC-3′ (forward) and 5′-CTAGAGACGGGTTTCTTGAAC-3′ (reverse)). Using these two primer pairs yielded 420 and 360 bp fragments. The PCR products were analysed by agarose gel electrophoresis and DNA sequencing.

### Immunofluorescence

Cultured chondrocytes were fixed in 4% paraformaldehyde. After blocking and permeabilizing, cells were exposed to anti-CFTR monoclonal antibody (1 : 300 dilution, M3A7; Chemicon, Temecula, CA, USA) or an anti-collagen II antibody (1 : 200 dilution; Calbiochem, La Jolla, CA, USA) for 1 h at room temperature. After washing in phosphate-buffered saline (PBS), a Cy-3-conjugated secondary antibody (1 : 200 dilution; Sigma) was applied for 30 min at room temperature. As a positive control for CFTR immunostaining, COS7 cells transiently transfected with human CFTR in a pcDNA3.1 mammalian expression vector were used. Cells were washed in PBS four times for 5 min each time and examined and photographed under a fluorescence microscope (Olympus, Tokyo, Japan).

### Immunoblotting

Proteins extracted from mouse chondrocytes and CFTR-transfected COS7 cells were resolved by 7% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with primary CFTR antibody at 4°C overnight, washed three times for 10 min each time in Tris-buffered saline Tween-20 and then incubated with secondary antibody conjugated to horseradish peroxidase (1 : 5000 dilution; Sigma) before being detected with an enhanced chemoluminescence kit (Amersham, Buckinghamshire, UK).

### Whole-cell patch-clamp studies

Before patch-clamp recordings, the chondrocytes were treated with a cocktail containing 10 μmol/L forskolin and IBMX 5 min for CFTR activation, and then 20 μmol/L CFTRinh-172 was used for CFTR suppression. The final concentration of DMSO (about 0.1/100) has no effect on membrane currents.

#### *With a chloride gradient*

The pipette resistance in the bath solution was approximately 10 MΩ. Ionic currents were measured using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA). Currents traces were filtered at 1 kHz with a built-in four-pole Bessel filter and then digitized to the computer at a sampling rate of 2 kHz. Currents were recorded at room temperature (~ 25°C). In the gap-free recording mode, cells were held at 0 mV throughout the period of recording. The pipette potential and liquid junction potential were autocompensated for by the Multiclamp 700A amplifier before the electrode touched the cell. The composition of the pipette solution was (in mmol/L): aspartic acid 85; EGTA 10; tetraethylammonium-chloride (TEA) 20; MgATP 10; $MgCl_2$ 2; glucose 5.5; HEPES 10, pH 7.4 adjusted with CsOH. The composition of the bath solution was (in mmol/L): NaCl 119; $MgCl_2$ 2; $CaCl_2$ 1; glucose 5; HEPES 5, pH 7.4 adjusted with NaOH. Sucrose (20 mmol/L) and was added to the bath solution to prevent activation of swelling-induced currents.

#### *Without a chloride gradient*

Current/voltage (I–V) relationships were constructed by clamping the membrane potential to 0 mV and by applying pulses from −100 to +100 mV in 20 mV increments. The composition of the pipette solution in these studies was (in μmol/L): EGTA 10; TEA 121; MgATP 10; $MgCl_2$, 2; glucose 5.5; HEPES 10, pH 7.4 adjusted with CsOH. All other conditions were the same as described above.

## RESULTS

Cystic fibrosis transmembrane conductance regulator mRNA expression was detected in freshly isolated chondrocytes by RT-PCR (Fig. 1a). DNA sequencing of the PCR products showed 100% identity with segments of the mouse CFTR mRNA sequence (NM_021050). Western blot analysis showed a core glycosylated band (Band B) and a maturely glycosylated band (Band C) of CFTR protein at 160 kDa in chondrocyte lysates, which was identical to the expression pattern of human CFTR in transfected COS7 cells (Fig. 1b). Immunofluorescence further confirmed the presence of CFTR in cultured primary chondrocytes with transiently transfected COS7 cells used as a positive control (Fig. 1c,d).

To determine whether CFTR expressed in mouse chondrocytes functions as a cAMP-regulated $Cl^-$ channel, we analysed $Cl^-$

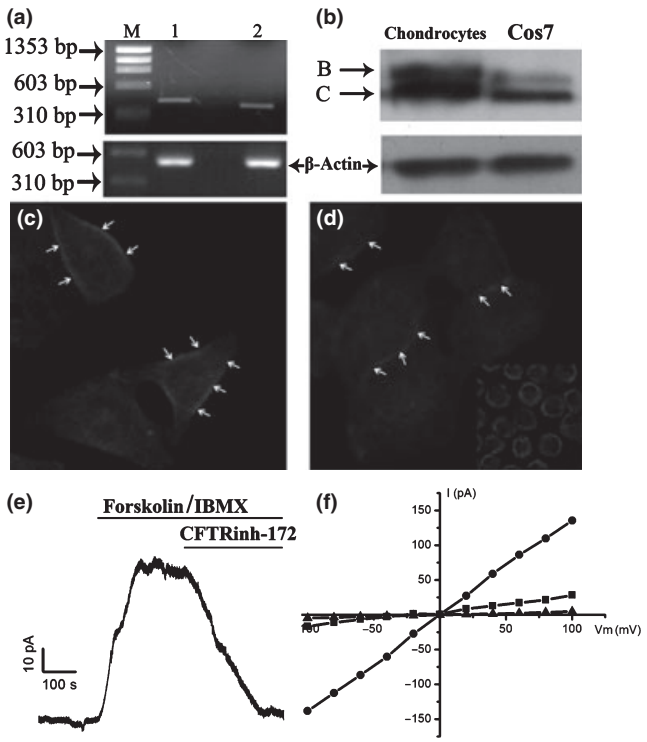


**Fig. 1** Expression and functional analysis of the cystic fibrosis transmembrane conductance regulator (CFTR) in mouse chondrocytes. (a) Reverse transcription-polymerase chain reaction. Lane 1, 420 bp PCR products; lane 2, 360 bp PCR products; M, marker. (b) Western blot analysis of CFTR in mouse chondrocytes and COS7 cells, with β-actin used as an internal control. Band B, core glycosylated band; Band C, maturely glycosylated band. (c,d) Immunofluorescence showing CFTR expression in plasma membranes of transfected COS7 cells (c) and mouse chondrocytes (d). (d, insert) Immunostaining of collagen II in chondrocytes shows high purity (> 98%) of isolated articular chondrocytes. (e,f) Representative whole-cell membrane currents (e) and current–voltage (I–V) relationship (f) evoked by voltages from −100 to 100 mV (in 20 mV increment) in chondrocytes ($n$ = 6) after stimulation with 10 μmol/L forskolin and 100 μmol/L IBMX (●) and in the presence of 20 μmol/L CFTRinh-172 (■). (▲), control. The holding potential was 0 mV and the interpulse duration was 4 s.

transport in mouse chondrocytes using whole-cell patch-clamp analyses after stimulation with the cAMP agonsist forskolin. The whole-cell membrane current was significantly activated after stimulation with 10 μmol/L forskolin and then suppressed by subsequent application of 20 μmol/L CFTRinh-172, a specific CFTR channel blocker (Fig. 1e).[20] Furthermore, the I–V relationship of the cyclic nucleotide-stimulated current was linear and independent of time or voltage (Fig. 1f), which is typical of the CFTR.

## DISCUSSION

Chondrocytes play an important role during endochondral ossification and serve as a scaffold for the formation of trabecular bone.[17,18] Moreover, chondrocyte differentiation requires the activation or upregulation of the membrane transporter and significant intracellular accumulation of inorganic osmolytes, such as $Cl^-$, which is important in cell metabolism.[21] Recently, Bonvin *et al.*[22] demonstrated that tracheal abnormalities and marked cartilaginous defects were present in $CFTR^{-/-}$ mice. Moreover, the abnormalities in cartilage rings seen in newborn $CFTR^{-/-}$ mice evolved during development. Thus, $Cl^-$ transport mediated by CFTR in mouse chondrocytes may play an important role in chondrocyte metabolism and participate in bone development. Dysfunction of CFTR in chondrocytes may be the reason underlying the bone disorders in $CFTR^{-/-}$ mice.[5] The findings of the present study prompt further investigation into the mechanisms underlying the occurrence of cartilage abnormalities in CFTR-deficient mice. However, no CFTR-deficient mice are available at present, so we cannot pursue this line of enquiry. Furthermore, studies using human cells and tissues will be required in the near future to determine the clinical significance of the expression of CFTR in chondrocytes.

In summary, the present study indicates that CFTR is highly expressed in chondrocytes and mediates cAMP-activated $Cl^-$ currents. The identification of CFTR expression in chondrocytes provides new insights into the potential role of CFTR in bone physiology and various pathological processes.

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