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Molecular origin of plasma membrane citrate transporter in human prostate epithelial cells

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The prostate is a highly specialized mammalian organ that produces and releases large amounts of citrate. However, the citrate release mechanism is not known. Here, we present the results of molecular cloning of a citrate transporter from human normal prostate epithelial PNT2-C2 cells shown previously to express such a mechanism. By using rapid amplification of cDNA ends PCR, we determined that the prostatic carrier is an isoform of the mitochondrial transporter SLC25A1 with a different first exon. We confirmed the functionality of the clone by expressing it in human embryonic kidney cells and performing radiotracer experiments and whole-cell patch-clamp recordings. By using short interfering RNAs targeting different parts of the sequence, we confirmed that the cloned protein is the main prostatic transporter responsible for citrate release. We also produced a specific antibody and localized the cloned transporter protein to the plasma membrane of the cells. By using the same antibody, we have shown that the cloned transporter is expressed in non-malignant human tissues.

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INTRODUCTION

The main function of the prostate gland is to produce and release large amounts (up to 180 mM) of citrate into prostatic fluid (Kavanagh, 1994). Prostatic citrate acts mainly as an energy substrate for sperm. It has been shown that sperm pre-incubated in citrate increase their ATP production (Medrano *et al*, 2006). Citrate is also important pathophysiologically, as in prostate cancer. In metastatic disease, citrate level decreases markedly. This phenomenon is already used in imaging techniques to detect cancerous sites in the prostate gland (Mueller-Lisse & Scherr, 2007).

In prostatic epithelial cells, an excess amount of citrate is produced in mitochondria, where mitochondrial aconitase regulated by hormones and Zn^{2+} is a rate-limiting enzyme, unlike in other cells (Costello & Franklin, 2002). Surprisingly, the molecular identity of the citrate release transporter crucial for prostatic physiology has not been defined. In our recent studies, we measured citrate release from normal human prostate epithelial PNT2-C2 cells (Mycielska & Djamgoz, 2004) and compared it with the strongly metastatic PC-3M cells (Mycielska *et al*, 2005). Release in both cases was K^+-dependent, whereas uptake of citrate remained K^+-dependent in normal prostatic cells but was largely Na^+-dependent in cancer cells. None of the known plasma membrane citrate transporters from the SLC13 gene family (NaCT, NaDC1 or NaDC3) was detected in either cell line by PCR (Mycielska *et al*, 2005). Importantly, some preliminary studies suggested similarity to SLC25A1, the mitochondrial citrate carrier (mCiC) encoded in the nucleus (Iacobazzi *et al*, 1997). In this study, we tested the hypothesis that the molecular nature of the prostatic release transporter is similar to the mitochondrial SLC25A1.

RESULTS

Identification of a new isoform of SLC25A1

By using RNA-ligase-mediated (RLM)-rapid amplification of cDNA ends (RLM-RACE) PCR, we obtained a variant of the SLC25A1 mCiC (Fig 1). The novel plasma membrane isoform (pmCiC) has its own transcription start site located 120 bp upstream from the beginning of exon 2 (inside the intron 1 sequence of SLC25A1). This would lead to formation of an alternative first exon (exon 1′) composed of the 3′-terminal part of intron 1 and subsequent exon 2 of SLC25A1. The splicing acceptor site at the intron 1/exon 2 junction of mCiC is not in use in pmCiC as it lacks an upstream splicing donor site. The unique 5′ part of the pmCiC sequence provides an alternative ATG translation start codon in frame with the rest of the mRNA sequence, which is common with mCiC. This results in the expression of a new protein isoform with the following unique 38-amino-acid amino-terminal sequence: MFPAALARRPR RPKSGTGEGPERQRPGGSLRSGFPVPA. We subcloned the complete open reading frame sequence of the new isoform into a pcDNA3.1-(+) vector and transfected the construct into human embryonic kidney (HEK) cells. To check the functionality of the clone, whole-cell patch-clamp measurements of citrate-induced currents were performed. Introducing 0.1 mM citrate through the patch pipette resulted in a significant outward current similar to the response generated in the native PNT2-C2 cells (Fig 2A). When the measurements were repeated on cells transfected with different amounts of vector (0.5–2 μg/ml), the citrate-induced outward current (180 ± 37 pA to 412 ± 45 pA) was dose-dependent (Fig 2B). No such current was observed in HEK cells transfected with empty vector alone (Fig 2B). A radiotracer technique was used to determine citrate uptake in transfected HEK cells. Compared with the control, HEK cells transfected with the cloned transporter showed a significant (around sevenfold) increase in citrate uptake (Fig 2C).

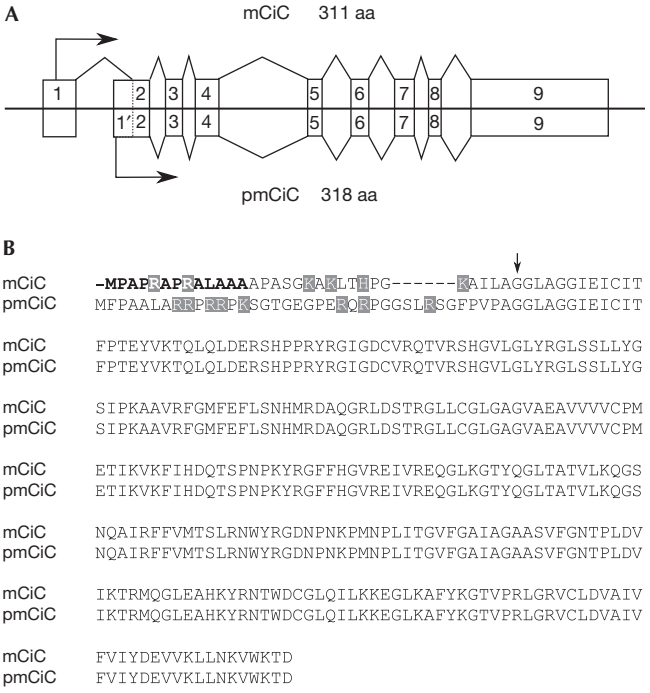

Fig 1 | Isoforms of human citrate-transporting proteins. (**A**) A schematic of the splicing patterns of mitochondrial (mCiC) and plasma membrane (pmCiC) citrate-transporting proteins. Exons are represented by squares. Translation initiation sites are depicted by arrows. Further details are in the text (see Results). (**B**) Alignment of the protein sequences of the isoforms. The 13-amino-acid (aa) pre-sequence in the mitochondrial isoform is shown in bold. Positively charged residues in the amino-terminal parts of the proteins are shaded. mCIC, mitochondrial citrate carrier; pmCiC, plasma membrane isoform of mCiC.

Silencing of pmCiC in native PNT2-C2 cells

We used several short interfering RNAs (siRNAs) targeting different parts of the pmCiC to silence the endogenous citrate transporter in PNT2-C2 cells. The siRNA specific for the pmCiC targeted the beginning (exon 1′) of its sequence. We also used two other siRNAs common for both mCiC and pmCiC, targeting exon 7/8 and exon 8. In all three cases, ^{14}C-citrate uptake was significantly reduced by 52–63% ($P = 0.02$–0.004, $n = 4$; Fig 3A). Similarly, all the siRNAs reduced the citrate-induced outward current in PNT2-C2 cells by 62–70% ($n = 4$, $P = 0.03$–0.025; Fig 3B).

We used siRNA specific for the mCiC (exon 1) to distinguish between the two isoforms of the citrate transporters. There was no effect on either citrate uptake or release from PNT2-C2 cells (Fig 3A,B, respectively). As a control for all of the tested siRNAs, mock (Lipofectamine) and scrambled siRNA-transfected cells were used (Fig 3). These produced no effect.

Immunocytochemistry and immunohistochemistry

Two polyclonal antibodies were used. One targeted pmCiC specifically and the other targeted the carboxyl-terminus common to both mCiC and pmCiC. Both antibodies allowed the determination of the localization pattern of either protein in native PNT2-C2 cells. As seen in Fig 4A, the antibody specific for pmCiC stained proteins in the plasma membrane only. By contrast, the antibody recognizing the C-terminus of both mCiC and pmCiC stained both mitochondria and the plasma membrane (Fig 4B). As shown previously, HEK cells do not express the citrate release mechanism in the plasma membrane (Mycielska & Djamgoz, 2004; also Fig 2A). Therefore, we used the N-terminus antibody against the pmCiC on non-transfected HEK cells as a negative control (Fig 4C). Conversely, the pmCiC antibody stained the plasma membrane of HEK cells transfected with pmCiC (Fig 4D). Cells had to be permeabilized to obtain any staining with either of the antibodies used, suggesting that both termini of the protein are located on the intracellular side of the plasma membrane.

Sections of human benign prostatic hyperplasia (BPH) tissue biopsies treated with the pmCiC antibody showed staining mainly of the apical membranes of the epithelial cells facing the lumen of the ducts (Fig 5A). Conversely, BPH biopsies stained with the antibody for the C-terminus of the mCiC and pmCiC transporters showed more even distribution throughout the epithelial cells; however, apical membrane staining of the epithelial cells around the ducts was still prominent as this antibody also recognizes pmCiC (Fig 5B). Interestingly, epithelial cells around the ducts showed more prominent staining for mCiC as compared with cells in the surrounding stroma. Breast non-malignant tissue sections used as control showed no staining with the pmCiC antibody (data not shown).

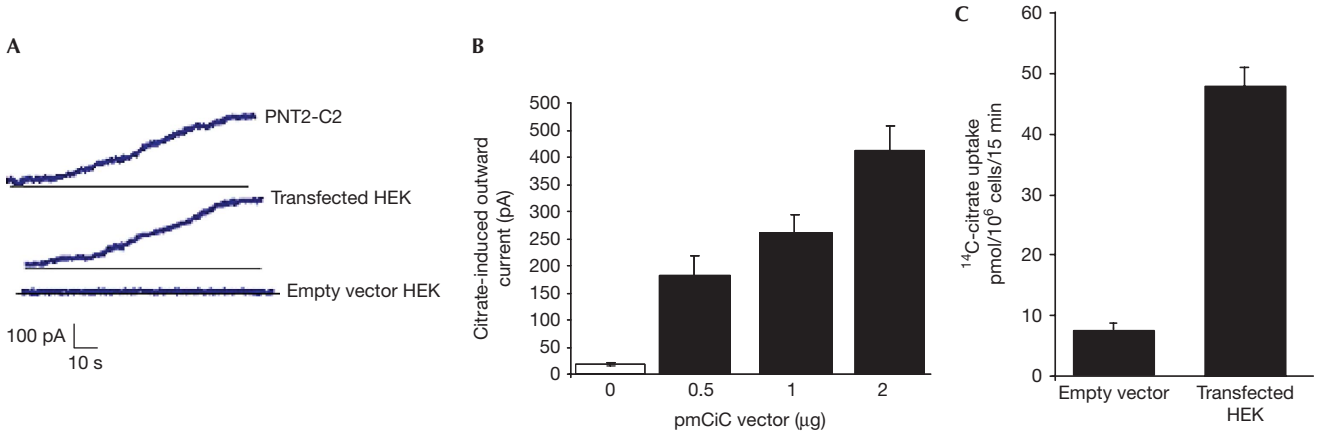

Fig 2 | Expression of pmCiC in human embryonic kidney cells. (**A**) Traces from electrophysiological recording of PNT2-C2 (upper trace) and HEK cells (lower traces) with 100 μM intracellular citrate. Citrate was introduced into the cells through the patch pipette. The upper trace shows a typical recording of the intracellular citrate-induced response of a normal prostate epithelial cell (PNT2-C2), whereas the lower traces are typical recordings for HEK cells transfected with the newly cloned transporter (middle) and transfected with empty vector cells (control; bottom). The zero current level is indicated by thin black lines. The slow rise for the currents is likely to be due to the diffusion of citrate in the cell. (**B**) Intracellular citrate-induced (as in panel **A**) response of control HEK cells (transfected with empty vector, white histobar) or cells transfected with the pmCiC (black histobars) with different amounts of the cloned transporter. The histobars show an average of 20–25 recordings. (**C**) Uptake of radiolabelled citrate (^{14}C) by HEK cells transfected with empty vector or with the newly cloned transporter. Cells were preincubated with 20 μM ^{14}C-citrate for 15 min, then washed and lysed. The readings show the values of ^{14}C per well (of 96) with confluent cells ($n=4$). HEK, human embryonic kidney; pmCiC, plasma membrane isoform of mitochondrial citrate carrier.

Ionic dependence of pmCiC

Similarly to PNT2-C2 cells (Mycielska & Djamgoz, 2004), the citrate release current in HEK cells transfected with the cloned pmCiC was K^+-dependent and Na^+-independent. Thus, in the presence of intracellular citrate introduced through the patch pipette, high extracellular K^+ (NaCl replaced with KCl) significantly reduced the outward current by 32 ± 8% ($P=0.01$; $n=10$–13; supplementary Fig S1A online). Low Na^+ (NaCl replaced with choline chloride) produced no effect on the citrate-induced outward current in pmCiC-transfected HEK cells ($P=0.07$; $n=12$; supplementary Fig S1A online). Surprisingly, however, uptake of the ^{14}C-citrate in these cells was Na^+-dependent (supplementary Fig S1B online). High extracellular K^+ decreased ^{14}C-citrate uptake by 30 ± 12% ($P=0.019$, $n=4$), whereas for low Na^+, the reduction was 51 ± 11% ($P=0.014$, $n=4$). Slightly elevated extracellular K^+ levels (10–20 mM) did not produce any significant effect on ^{14}C-citrate uptake. Comparison between outward and inward currents showed that, at the same concentrations of applied citrate, extracellular citrate induced 25 ± 3% of the outward current induced by the same amount of citrate introduced through the patch pipette. This was similar to that observed for PNT2-C2 cells (Mycielska & Djamgoz, 2004).

The mCiC was found previously to be electroneutral and to work as an anti-porter exchanging citrate for malate or another citrate molecule (Bisaccia *et al*, 1993). However, studies of the influence of extracellular malate on citrate release and uptake for pmCiC using both patch-clamp recording and radiotracer techniques showed no effect (data not shown).

DISCUSSION

The mechanism of citrate release is one of the main issues and unsolved problems of prostate gland physiology and pathophysiology. The manner in which citrate is released and its regulation are essential for the understanding of male infertility and could be useful for the treatment of prostate cancer. This study is the first, to our knowledge, to show the molecular nature of a prostatic transporter expressed in the plasma membrane.

New isoform of SLC25A1

The transporter we have cloned is an isoform of a protein encoded in the nucleus and expressed in the mitochondrial inner membrane (Bisaccia *et al*, 1989). The only difference between the two is the N-terminal region. Although both N-termini are positively charged, this is greater for pmCiC (Fig 1). Whether the extra positive charges have a role in guiding pmCiC to the plasma membrane and in modulating its structure and activity would require further studies. Interestingly, the pre-sequence of the mCiC has some signalling competence (Zara *et al*, 2009). It is known, however, that post-translational import of proteins of the SLC25 family into organelles is dependent on clusters of hydrophobic and hydrophilic residues within the protein.

In addition, WoLF PSORT software analysis predicted that the most probable localization of pmCiC would indeed be the plasma membrane (with a probability of 75%), with a 0% probability of this protein targeting mitochondria. Conversely, mCiC showed 17% probability of localizing to the mitochondria and lower than pmCiC probability (53%) of localizing to the plasma membrane.

Prostatic plasma membrane transporter

Several independent experimental techniques confirmed that the newly cloned protein (pmCiC) is a major citrate transporter expressed in the plasma membrane of normal human prostate PNT2-C2 cells and non-malignant prostate tissues. First, we used several siRNAs targeting different parts of the membrane transporter. Silencing pmCiC was effective in reducing up to

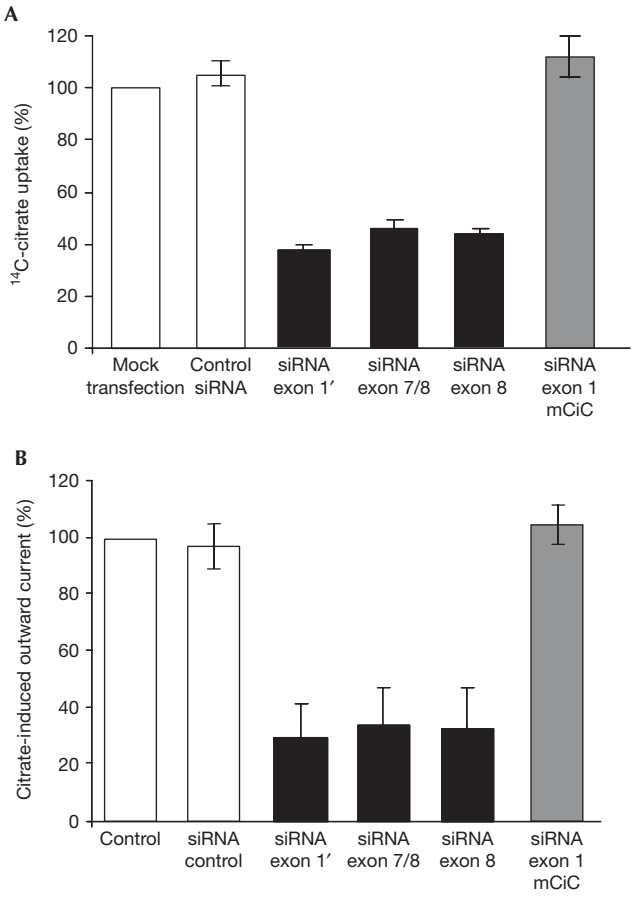

Fig 3 | pmCiC-mediated citrate transport in PNT2-C2 cells. Percentage decrease of (**A**) ^{14}C citrate uptake or (**B**) outward current induced by intracellular 100 μM citrate in PNT2-C2 cells silenced with different siRNAs. siRNA exon 1′ was specific for the pmCiC whereas siRNAs for exons 7/8 and 8 were common for mCiC and pmCiC. siRNA for exon 1 of mCiC was specific for the mitochondrial isoform. For citrate uptake, $n = 4$ repeats; for patch clamp experiments, $n = 20$–25 cells. mCIC, mitochondrial citrate carrier; pmCiC, plasma membrane isoform of mCiC; siRNA, short interfering RNA.

70% of total citrate transport as determined from both patch-clamp and tracer experiments. By contrast, there was no effect on citrate transport by silencing mCiC, suggesting that pmCiC and mCiC are separate proteins and localized to different membranes. Second, specific antibodies targeting either pmCiC or mCiC/pmCiC showed different expression patterns in BPH tissue biopsies consistent with the former being confined to the plasma membrane. Overall, both cell and tissue studies would strongly suggest that pmCiC is the main plasma membrane citrate transporter expressed in prostatic cells. However, we cannot exclude the possibility that there might be other transporters present that are of minor importance.

Ionic characteristics

We have previously characterized citrate transport in prostate epithelial cells by whole-cell patch-clamp recordings. Citrate release and uptake were electrogenic and K^+-dependent with the proposed stoichiometry of 1 cit^{3-}: 4 K^+ (Mycielska & Djamgoz, 2004). Citrate release, which is the normal activity of pmCiC, from HEK cells transfected with pmCiC was also electrogenic and K^+-dependent as measured electrophysiologically. Surprisingly, however, citrate uptake, which occurs against the normal working mode of the transporter, in pmCiC-transfected HEK cells was Na^+-dependent. At present, the reasons for this are unclear, but it could be due to any of several factors. First, it was observed that HEK cells had a high basal Na^+ conductance, which was negligible in PNT2-C2 cells. Second, although pmCiC by itself can transport citrate in an electrogenic manner, there might be another auxiliary protein enhancing transporter activity and/or its K^+ dependence. As observed earlier (Mycielska & Djamgoz, 2004), citrate current in PNT2-C2 cells was reduced by 4-AP (a K^+ channel blocker) so functional association with another protein regulating K^+ levels might be possible. In fact, studies of citrate release in plants also favour this possibility, as the same inhibitor inhibited citrate release in the roots of *Arabidopsis* (Murphy *et al*, 1999). In addition, we have preliminary data suggesting an association of pmCiC with a secondary component (M.P. Mazurek, M.B.A. Djamgoz and M.E. Mycielska, unpublished observations), but further work is required to determine its nature.

Although, there is only a small change in the amino-acid sequence between pmCiC and mCiC, there seem to be significant differences in the way citrate is being transported. Whereas mCiC was found to work as an anti-porter (exchanging citrate for malate or another citrate), pmCiC was coupled mainly to K^+ and malate did not affect the efficiency of citrate transport. However, whether K^+ or Na^+ might be involved in the transport mechanism of mCiC is unknown. Differences in the way other homologous solute transporters work have previously been observed. For example, the plasma membrane citrate transporter from the SLC13 family, NaCT, which is an orthologue of the *Drosophila* Indy (I am Not Dead Yet) transporter is electrogenic and Na^+-dependent even though Indy is electroneutral and Na^+-independent (Inoue *et al*, 2002).

Conclusion

This study describes a novel citrate release transporter cloned from prostate epithelial cells that is an isoform of the mitochondrial mCiC. It was confirmed by several techniques that the cloned transporter is responsible for the majority of citrate release from prostatic cells. In addition, prostatic tissue staining confirms the *in vivo* relevance of this transporter.

METHODS

RNA isolation, RLM-RACE, cloning and real-time PCR. Total RNA was isolated from PNT2-C2 cells using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). Genomic DNA contamination was assessed by control PCR (-RT) using β-actin-specific primers (data not shown). The RNA was further processed using the GeneRacer kit (Invitrogen) and amplification-ready RACE cDNA was prepared using oligo-dT primer from the kit. Two rounds of 5′-RACE PCR amplification were subsequently performed. The sequences of the gene-specific primers used were as follows: first round, 5′-GCTTAGTCCGTCTTCCACACT TTGTTGA-3′; and second round, 5′- AGCAGCTTCACCACTTCAT CATAGATGA-3′. Forward primers were provided in the kit. The PCR product obtained was cloned into the pCR2.1-TOPO vector (Invitrogen) and then sequenced (Eurofins MWG). On the basis of the sequencing result, primers for two rounds of 3′-RACE PCR were

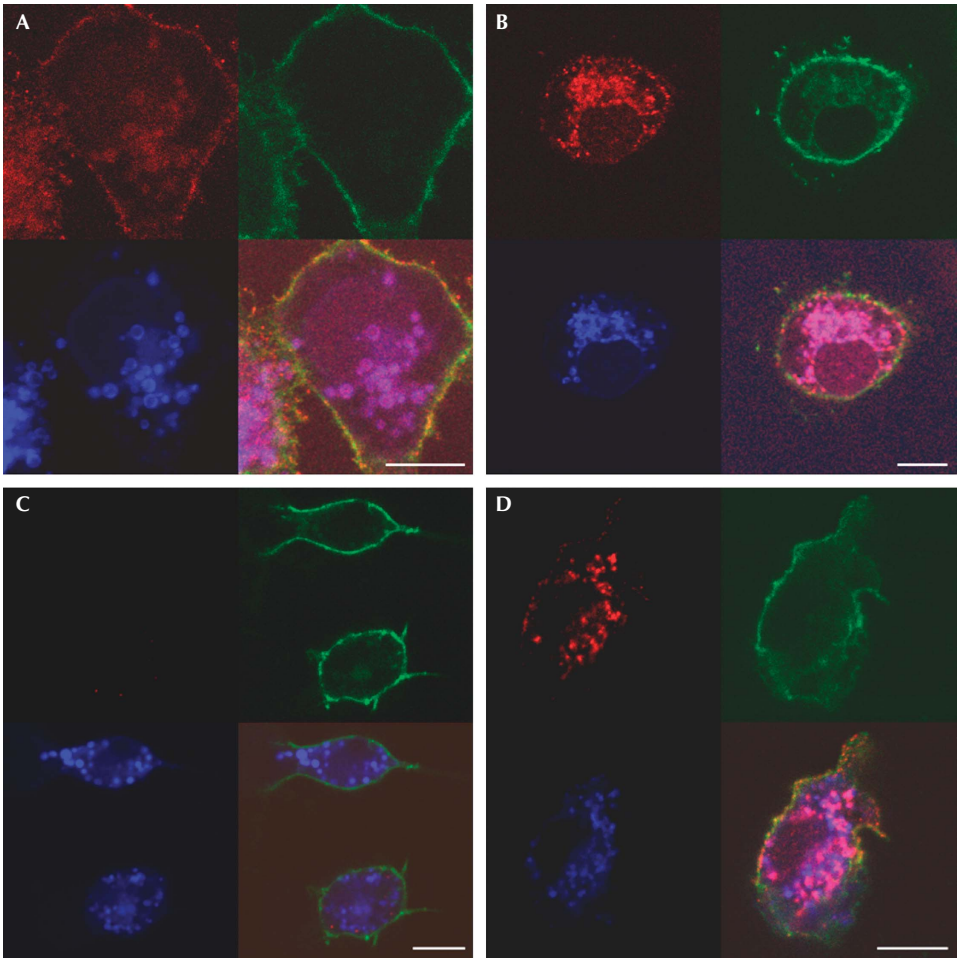

Fig 4 | Plasma membrane expression of pmCiC. Confocal images of single stacks of (**A**,**B**) PNT2-C2 cells stained with antibody (red) for (**A**) pmCiC or (**B**) the carboxyl-terminus of both mCiC and pmCiC, ConA (green) for plasma membrane and Mitotracker deep red (blue) for mitochondria. The lower right panels within (**A**) and (**B**) show colocalization of the staining for citrate transporter with plasma membrane (orange) or mitochondria (violet). (**C**,**D**) HEK cells transfected with empty vector (**C**) and with pmCiC (**D**), stained as above. For panels **C** and **D**, specific antibody for pmCiC only was used. Scale bars, 10 μm. HEK, human embryonic kidney; mCIC, mitochondrial citrate carrier; pmCiC, plasma membrane isoform of mCiC.

designed: first round, 5′-ATGTTCCCCGCGGCACT-3′; and second round, 5′-GGGAGTCTCAGGAGCGGGTT-3′. Reverse primers were provided in the kit. The product was cloned into the pCR2.1-TOPO vector and sequenced. The complete open reading frame sequence of the newly identified isoform was subsequently amplified from cDNA in the PNT2-C2 cells by using the following primers: forward, 5′-TAGGATCCATGTTCCCCGCGGCACT-3′; and reverse, 5′-TTTCT AGATTAGTCCGTCTTCCACA-3′, and cloned into pCR2.1-TOPO for sequence confirmation. It was further subcloned into *Bam*HI/*Xba*I-digested pcDNA3.1-(+) mammalian expression vector (Invitrogen) using a standard restriction/ligation protocol. The final construct was used for functional expression. Real-time PCR was performed as described previously (Brackenbury *et al*, 2007), with PUM1 as the normalizing gene.

Cell culture and transfections. Human normal prostate PNT2-C2 and HEK cells were grown as described previously (Mycielska & Djamgoz, 2004). Before transfection, cells were plated on the appropriate dishes. After 24 h, the cells were transfected according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen) and either siRNA or citrate transporter construct. The cells were incubated with the transfecting reagents for 48 h. In siRNA experiments, the amount of knockdown of the mRNAs was determined by real-time PCR. The reduction achieved was 70–75% for all siRNAs used ($P = 0.004$–0.006, $n = 4$–5). Scrambled siRNA had no effect on any of the mRNAs tested. siRNA knockdown at the protein level was measured by using confocal images. The stained particles were identified, counted and measured using ImageJ (1,2). Stacks were processed with the 3D Object Counter. For each siRNA, at least three repeats were performed and at least 100 cells were measured for each repeat. The protein level in siRNA-treated cells was 53–57% lower compared with that in the cells transfected with empty vector.

Electrophysiology. Whole-cell patch-clamp recordings were performed as described previously (Mycielska & Djamgoz, 2004; Mycielska *et al*, 2005).

Radiotracer uptake. The cells were plated in 96-well dishes, three wells for each experimental condition. After 24 h, the medium was

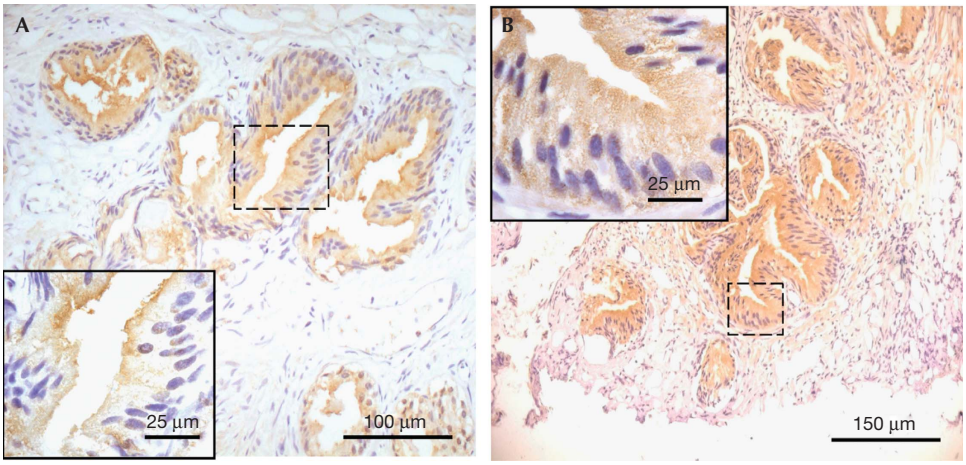

Fig 5 | pmCiC expression in human tissue. Immunohistochemical staining of BPH tissue with an antibody against the pmCiC (**A**) or mCiC (for both mCiC and pmCiC carboxyl-terminus) (**B**). (**A**) The strongest staining was evident in the apical membrane of epithelial cells, with little immunoreactivity in the surrounding stroma. (**B**) Staining was evident all over the epithelial cells, with significant immunoreactivity in the surrounding stroma. The highest immunoreactivity was observed at the apical side of the ductal cells as this antibody recognizes both isoforms (mCiC and pmCiC). Insets in both panels **A** and **B** show a higher magnification view of the area outlined in the dashed square to highlight the membrane staining pattern. Samples were counterstained with haematoxylin. Controls performed without the addition of the primary antibody did not yield evident staining. Three separate BPH biopsies showed similar results. BPH, benign prostate hyperplasia; mCIC, mitochondrial citrate carrier; pmCiC, plasma membrane isoform of mCiC.

enriched with Lipofectamine-2000 with addition of nucleic acids. The cells were washed with Ringer solution of appropriate ionic composition and incubated with 20 μM ^{14}C-citrate for 15 min. Then, the cells were five times put on ice and washed with ice-cold Ringer solution of the same composition. The cells were lysed with 50 μl per well of RIPA buffer (Sigma-Aldrich, St Louis, MO, USA) and transferred into optiplate wells with 100 μl of Microscint 40 solution. Radioactivity measurements were made using the lysates using a Top Count NXT Microplate Scintillation and Luminescence Counter (PerkinElmer, Waltham, MA, USA).

Immunocytochemistry and immunohistochemistry. Cells were plated onto glass coverslips. Staining of mitochondria (with Mitotracker deep red) was performed using live cells according to the manufacturer's instructions. The cells were then washed with phosphate-buffered saline and fixed with 2% paraformaldehyde for 4 min followed by 25 min of incubation with ConA (plasma membrane staining). A 0.1% solution of saponin was used to permeabilize the cells. A primary antibody against the C-terminus of mCiC and pmCiC was raised in rabbits by GenSscript (Piscataway, NJ, USA) as described previously (Capobianco *et al*, 1995). For the newly cloned isoform, the immunizing peptide was designed on the basis of the N-terminal amino-acid sequence specific only to the plasma membrane isoform: CGEGPERQRPGGSLR. Goat anti-rabbit IgG Alexa Fluor-488 conjugate was used as the secondary antibody (Invitrogen, Molecular Probes, Eugene, OR, USA). Staining of cells with primary or secondary antibody was used as a control and no fluorescence was observed. Tissue staining was performed as described previously (Fraser *et al*, 2005), with the following modifications: a 1:100-fold dilution of both antibodies was used and the primary antibodies were incubated for 1 h at 21 °C.

Statistical analyses. All data are presented as means ± standard errors (s.e.m.) Statistical analyses were performed using raw data by Student's *t*-tests, unless specified otherwise.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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