

# TRPM8 in prostate cancer cells: a potential diagnostic and prognostic marker with a secretory function?

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## Abstract

During the past 5 years it has emerged that the transient receptor potential (TRP) family of Ca<sup>2+</sup>- and Na<sup>+</sup>-permeable channels plays a diverse and important role in cell biology and in pathology. One member of this family, TRPM8, is highly expressed in prostate cancer cells but the physiological and pathological functions of TRPM8 in these cells are not known. Here we address these questions, and the issue of whether or not TRPM8 is an effective diagnostic and prognostic marker in prostate cancer. TRPM8 is known to be activated by cool stimuli (17–25 °C) and cooling compounds such as menthol. The activation mechanism(s) involves voltage sensing of membrane potential, phosphatidylinositol 4,5-bisphosphate and Ca<sup>2+</sup>. In addition to prostate cancer cells, TRPM8 is expressed in sensory neurons where it acts as a sensor of cold. In prostate epithelial cells, expression of TRPM8 is regulated by androgen and is elevated in androgen-sensitive cancerous cells compared with normal cells. While there is some evidence that in prostate cancer cells Ca<sup>2+</sup> and Na<sup>+</sup> inflow through TRPM8 is necessary for survival and function, including secretion at the apical membrane, the function of TRPM8 in these cells is not really known. It may well differ from the role of TRPM8 as a cool sensor in sensory nerve cells. Androgen unresponsive prostate cancer is difficult to treat effectively and there are limited diagnostic and prognostic markers available. TRPM8 is a potential tissue marker in differential diagnosis and a potential prognostic marker for androgen-unresponsive and metastatic prostate cancer. As a consequence of its ability to convey Ca<sup>2+</sup> and Na<sup>+</sup> and its expression in only a limited number of cell types, TRPM8 is considered to be a promising target for pharmaceutical, immunological and genetic interventions for the treatment of prostate cancer.

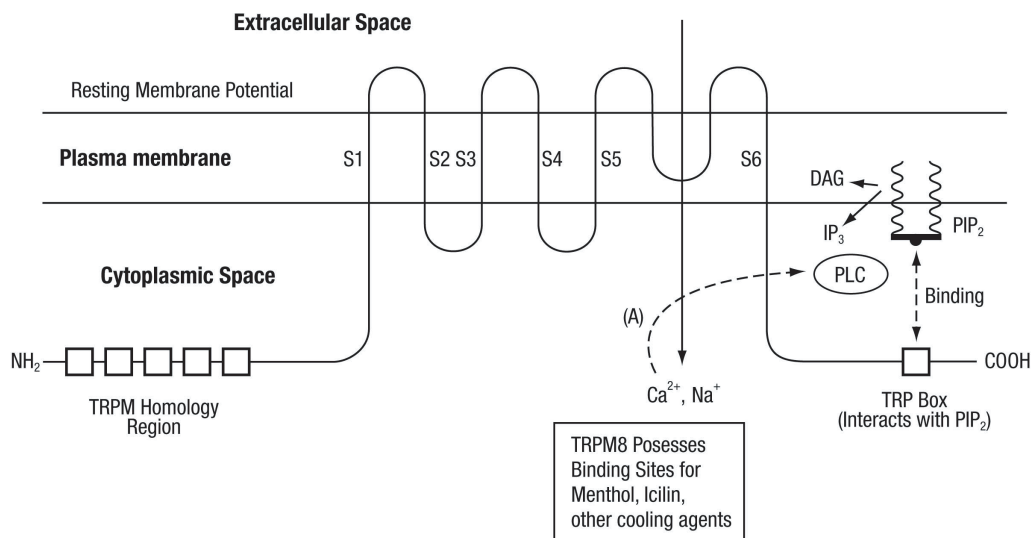
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## Introduction

TRPM8 is a member of the transient receptor potential (TRP) family of Ca<sup>2+</sup>-permeable non-selective cation channels, and belongs to the TRPM (melastatin) subfamily of TRP proteins. There are seven main subfamilies of TRP proteins. In addition to the TRPM subfamily, these are: TRPC (canonical or classical), TRPV (vanilloid), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin-like) and TRPN (no mechanoreceptor potential C, NOMPC) (reviewed in Clapham 2003, Montell 2005, Nilius *et al.* 2005). TRPM8 was discovered as a protein that is

upregulated in prostate and in some other types of cancer cells (Tsavaler *et al.* 2001). However, TRPM8 is also expressed in sensory neurons (McKemy *et al.* 2002, Peier *et al.* 2002, Zhang *et al.* 2004) and is one of two cold-sensing TRP channels, the other being TRPA1 (Story *et al.* 2003, McKemy 2005). By contrast, heat and heating compounds are sensed by several members of the TRPV subfamily, TRPV1, V2, V3, and V4 (reviewed in Clapham 2003).

Although there is evidence that TRPM8 is involved in thermo and pain sensation in sensory neurons, the knowledge of the functions of TRPM8 in other cell types, including prostate cancer cells, is very limited.



**Figure 1** A schematic representation of the structure of the TRPM8 polypeptide showing the main features of the membrane-spanning domains (S1-S6), cytoplasmic amino terminus, and cytoplasmic carboxy terminus. TRPM8 is activated by cold or menthol (or other cooling agents) in a process that involves a change in the activation curve (response) to membrane potential. Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) and, in some cases, the presence of Ca<sup>2+</sup> at the cytoplasmic mouth of the channel form part of the activation mechanism. PIP<sub>2</sub> is thought to interact with the TRP domain in the carboxy terminus. The activated channel is desensitised by the action of Ca<sup>2+</sup> at the cytoplasmic mouth of the channel which activates (A) a Ca<sup>2+</sup>-sensitive phospholipase C (PLC) (possibly PLC $\gamma$ 1) leading to the hydrolysis of PIP<sub>2</sub> to generate inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) and inactivation of the channel. The scheme is based on references cited in the text.

The detection of TRPM8 in a number of normal urogenital tissues and the profile of up-regulated expression of TRPM8 in prostate cancer and other cancer cells suggest that this Ca<sup>2+</sup>-permeable channel may play diverse roles in different tissues.

## Structure of TRPM8

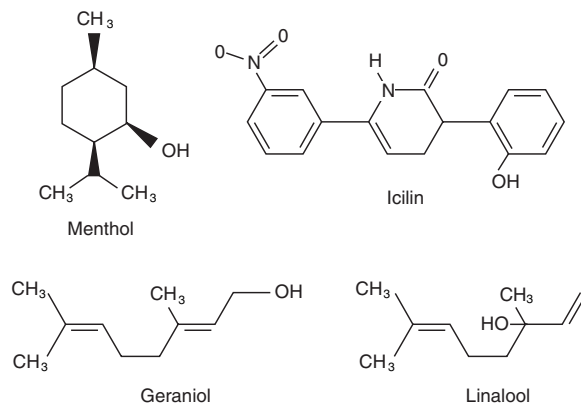
The human TRPM8 polypeptide consists of 1104 amino acids (Tsavaler *et al.* 2001, Peier *et al.* 2002). The predicted secondary structure includes six membrane-spanning domains and cytoplasmic amino and carboxy terminal domains (Fig. 1). The S1-S4 transmembrane domains exhibit weak voltage-sensing properties. It is proposed that the pore of the channel is composed of the region between the S5 and S6 membrane-spanning sequences in a tetramer comprising, most likely, four TRPM8 polypeptides (McKemy *et al.* 2002). The amino terminus contains several TRPM homology sequences, while the large carboxy terminus contains the TRP box (a sequence common to each member of the TRPV, TRPC and TRPM families) and eight potential N-linked glycosylation sites. Unlike many other members of the TRP family, no ankyrin repeat elements are present in the amino

terminus (Tsavaler *et al.* 2001, Peier *et al.* 2002, Clapham 2003). Electrophysiological studies conducted with TRPM8 heterologously expressed in mammalian cells indicate that the protein mediates an outwardly rectifying current with a selectivity for Ca<sup>2+</sup>:Na<sup>+</sup> of 3:1 (McKemy *et al.* 2002, Peier *et al.* 2002).

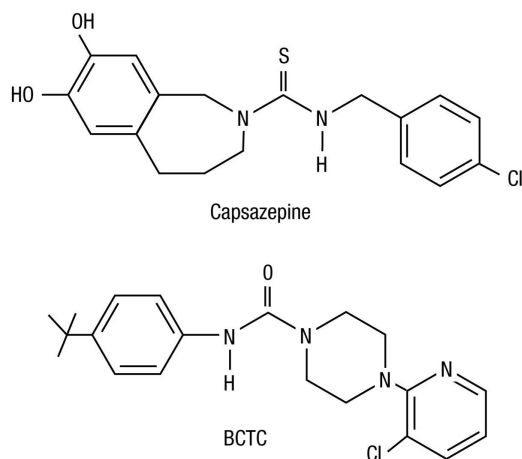
## Activation signals and mechanisms for TRPM8

TRPM8 can be experimentally activated by cooling (temperatures between 10 and 25°C), membrane depolarisation and by chemical agents. The voltage-dependent activation of TRPM8 is not strong and the voltage sensor is not known although it has been suggested that it is located in the carboxy terminus of TRPM8 (Nilius *et al.* 2005). Chemical activators of TRPM8 include menthol and icilin, and other cooling compounds, such as menthone, eucalyptol, geraniol and linalool (Fig. 2a) (McKemy *et al.* 2002, Peier *et al.* 2002, Behrendt *et al.* 2004, Brauchi *et al.* 2004, Voets *et al.* 2004). Capsazepine (shown in Fig. 2b) is an antagonist of menthol activation of TRPM8 (Behrendt *et al.* 2004). Other antagonists include

## (a) TRPM8 Chemical Activators



## (b) TRPM8 Chemical Antagonists



**Figure 2** The structures of some chemical activators (a) and inhibitors (b) of the TRPM8 channel.

*N*-(4-*tert*.butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H) carboxamide (BCTC) (Fig. 2b) and thiol BCTC (Behrendt *et al.* 2004). The activation of TRPM8 by cold and icilin, but not by menthol, is modulated by intracellular pH (Andersson *et al.* 2004).

The results of recent studies investigating the mechanism of activation of TRPM8 by cooling have provided evidence that cold or menthol change the weak voltage-dependent activation curves (i.e. change the activation energy) (Voets *et al.* 2004). It seems that menthol functions as a modifier of TRPM8 channel gating, shifting the activation curve to physiological membrane potentials. Thus membrane potential plays a critical role in the activation mechanism for TRPM8 (Brauchi *et al.* 2004, Voets *et al.* 2004, Hui *et al.* 2005).

Another agent that plays an important role in the activation of TRPM8 is phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). Liu & Qin (2005) showed that

the inactivation (desensitisation) of the TRPM8 channel is prevented by inhibitors of phospholipid phosphatases. Moreover, the application of exogenous PIP<sub>2</sub> was found to both activate the channel directly and to restore activity after desensitisation (Liu & Qin 2005). Using a slightly different experimental strategy, Rohacs *et al.* (2005) provided evidence that PIP<sub>2</sub> is necessary for the activation of TRPM8 by cold or cooling agents. Moreover, PIP<sub>2</sub> itself (in the absence of cold or a cooling agent) could also activate the channel. Cold or a cooling agent increases the apparent affinity of TRPM8 for PIP<sub>2</sub> and it is proposed that cooling activates the channel by increasing the sensitivity to PIP<sub>2</sub>. Evidence has been obtained to indicate that PIP<sub>2</sub> binds to positive changes in the TRP motif in the cytoplasmic carboxy terminus (shown schematically in Fig. 1). As pointed out by Rohacs *et al.* (2005), the activation mechanisms of TRPM8 are undoubtedly complex. It is likely that the role of PIP<sub>2</sub> is part of, or linked to, the effects of cold and cooling agents in changing the activation energy of the channel (Voets *et al.* 2004). Confirmation of these ideas will require further experiments.

Ca<sup>2+</sup> plays an important role in regulating the activity of TRPM8. Recent studies by Chuang *et al.* (2004) have shown that the activation of TRPM8 by icilin requires an increase in the cytoplasmic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>cyt</sub>) at the cytoplasmic mouth of the channel i.e. the flow of Ca<sup>2+</sup> through the channel (Fig. 1). They suggested that this requirement for Ca<sup>2+</sup> might represent the coincidence detection of signals by TRPM8 (Chuang *et al.* 2004). These authors also provided evidence that indicates that the region of the TRPM8 polypeptide that appears to interact with icilin is similar in structure to the region of hot-sensing TRPV1 that interacts with capsaicin.

Ca<sup>2+</sup> inflow also plays a role in modulating (desensitising) TRPM8. Thus it has been found that Ca<sup>2+</sup> inflow through TRPM8 activates phospholipase C (PLC) (possibly PLCγ1) which hydrolyses PIP<sub>2</sub>, leading to a decrease in PIP<sub>2</sub> and inhibition (desensitisation) of channel activity (Fig. 1) (Rohacs *et al.* 2005). This may be one of numerous examples (for many types of Ca<sup>2+</sup> permeable channel) of the physiological importance of limiting the amount of Ca<sup>2+</sup> which enters the cell via the open channel, and hence the necessity of there being effective mechanisms for achieving this.

Several research groups have compared the properties of the cold-sensing TRPM8 channels with those of the hot-sensing TRPV channels. It was found that TRPM8 is inhibited by 2-aminoethoxydiphenyl borate (2-APB) (Hu *et al.* 2004). 2-APB inhibits several types

**Table 1** Distribution of TRPM8 in normal and cancerous tissues and cells

Normal tissues	Signals detected	Reference	Cancerous tissues and/or cells	Signals detected	Reference
Small-diameter neurons in trigeminal ganglia, dose root ganglia, nodose ganglia	mRNA	McKemy <i>et al.</i> (2002), Peier <i>et al.</i> (2002), Zhang <i>et al.</i> (2004), Story <i>et al.</i> (2003), Nealen <i>et al.</i> (2003), Okazawa <i>et al.</i> (2004)	Melanoma	mRNA	Tsavalier <i>et al.</i> (2001)
	Protein	Abe <i>et al.</i> (2005), Stein <i>et al.</i> (2004), Tsukimi <i>et al.</i> (2005)			
Prostate epithelium	mRNA	Tsavalier <i>et al.</i> (2001), Henshall <i>et al.</i> (2003), Fuessel <i>et al.</i> (2003), Kiessling <i>et al.</i> (2003), Bidaux <i>et al.</i> (2005)	Prostate cancer	mRNA	Tsavalier <i>et al.</i> (2001), Henshall <i>et al.</i> (2003), Fuessel <i>et al.</i> (2003), Kiessling <i>et al.</i> (2003)
	Protein	Stein <i>et al.</i> (2004) Bidaux <i>et al.</i> (2005)			
Taste papillae	Protein	Abe <i>et al.</i> (2005)	Prostate LNCaP cells	mRNA	Tsavalier <i>et al.</i> (2001), Fuessel <i>et al.</i> (2003), Kiessling <i>et al.</i> (2003)
				Protein	Zhang & Barritt (2004)
Testis	mRNA	Tsavalier <i>et al.</i> (2001), Stein <i>et al.</i> (2004)	Prostate PC-3 cells	mRNA	Fuessel <i>et al.</i> (2003)
				Protein	Zhang & Barritt (2004)
Seminiferous tubules	mRNA	Stein <i>et al.</i> (2004)	Breast cancer	mRNA	Tsavalier <i>et al.</i> (2001)
Scrotal skin	mRNA	Stein <i>et al.</i> (2004)	Colorectal cancer	mRNA	Tsavalier <i>et al.</i> (2001)
Bladder urothelium	mRNA	Stein <i>et al.</i> (2004)			
	Protein	Stein <i>et al.</i> (2004)			
Urothelial cells in culture	Protein	Stein <i>et al.</i> (2004)			
Thymus	mRNA	Tsavalier <i>et al.</i> (2001)			
Breast	mRNA	Tsavalier <i>et al.</i> (2001)			
Small intestine mucosa	mRNA	Zhang <i>et al.</i> (2004)			

of plasma membrane  $\text{Ca}^{2+}$ -permeable channels and, under some conditions, it also inhibits inositol 1,4,5-trisphosphate receptors (Bootman *et al.* 2002). 2-APB can be used to distinguish TRPM8 from TRPV1, V2 and V3, since it activates TRPV1, V2 and V3 but inhibits TRPM8 (Hu *et al.* 2004). These observations most likely reflect differences in either the pores of the channels or in the activation mechanisms.

Other similarities and differences between TRPM8 and TRPV1 have been described by Weil *et al.* (2005) using the whole cell patch-clamp technique to study channel function. They showed that TRPM8 activity is modified by ethanol, TRPM8 is not affected by extracellular  $\text{H}^+$  whereas TRPV1 does respond to extracellular  $\text{H}^+$ , and many of the TRPV1 antagonists, including capsazepine, BCTC, (2R)-4-(3-chloro-2-pyridinyl)-2-methyl-N-[4-(trifluoromethyl)phenyl]-1-piperazonecarboxamide (CTPC) and SB-452533, are also antagonists of TRPM8. These results have

implications for both the structure and mechanism of activation of TRPM8, and also highlight potential difficulties in developing pharmaceutical interventions selective for TRPM8.

### Expression and functions of TRPM8 in neuronal cells

The most clearly defined physiological function of TRPM8 is in sensory neuronal cells. It is proposed that TRPM8 channels constitute the cold sensors that detect ambient temperature. TRPM8 is found in pain- and temperature-sensing neurons (including small-diameter dorsal root ganglion neurons, trigeminal ganglia neurons and nodose ganglion neurons (McKemy *et al.* 2002, Peier *et al.* 2002, Thut *et al.* 2003, Babes *et al.* 2004, Tominaga & Caterina 2004, Zhang *et al.* 2004) and taste papillae (Abe *et al.* 2005)), and also in many other tissues including testis, scrotal

skin, bladder urothelium (Stein *et al.* 2004), thymus, breast, ileum and in melanoma, colorectal cancer and breast cancer cells (Tsavaler *et al.* 2001, Zhang *et al.* 2004). The known tissue distribution of the TRPM8 protein is summarised in Table 1. Further evidence for the function of TRPM8 as a sensor for cold in sensory neuronal cells has come from the transient transfection of cultured hippocampal neurones with TRPM8. This conferred sensitivity to cold and menthol that is not exhibited by non-transfected hippocampal neurones (de la Pena *et al.* 2005).

TRPM8 facilitates  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  inflow across the plasma membrane and may mediate the release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (ER) since some TRPM8 is found expressed in the ER as well as in the plasma membrane (Chuang *et al.* 2004). The activation of TRPM8 and subsequent inflow of  $\text{Na}^{+}$  and  $\text{Ca}^{2+}$  leads to depolarisation of the plasma membrane. A cold stimulus is most likely the physiological activator of TRPM8 in sensory neurones. However, it has also been suggested that an (unidentified) endogenous menthol-like ligand may act as a physiological activator of TRPM8 (McKemy *et al.* 2002).

### Expression of TRPM8 in prostate cancer cells

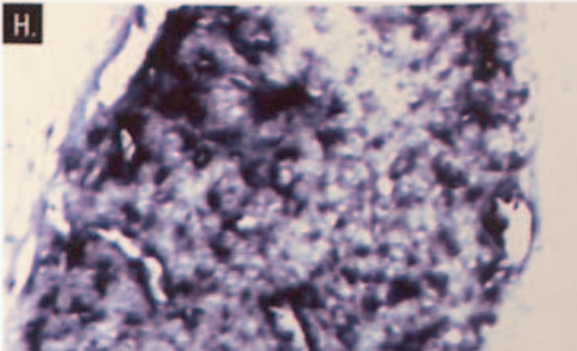
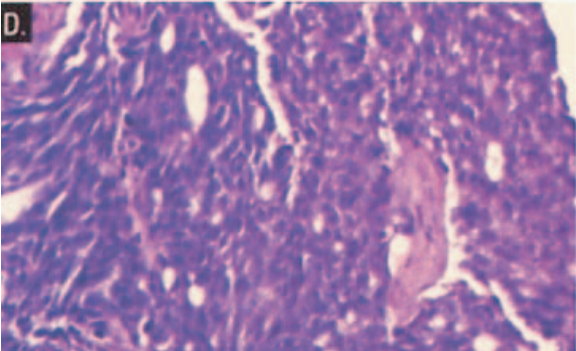
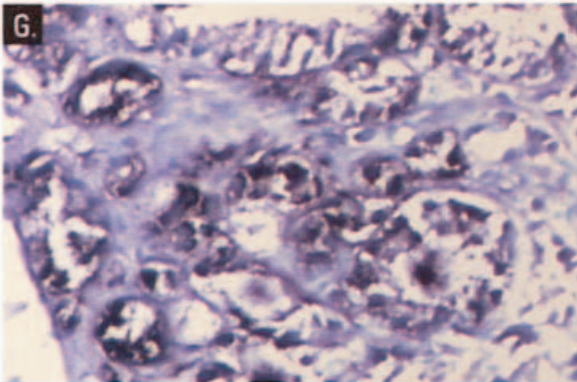
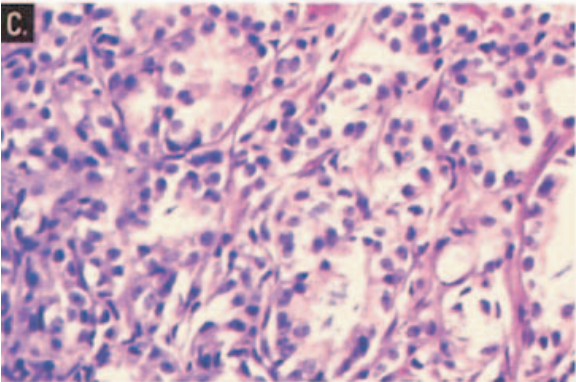
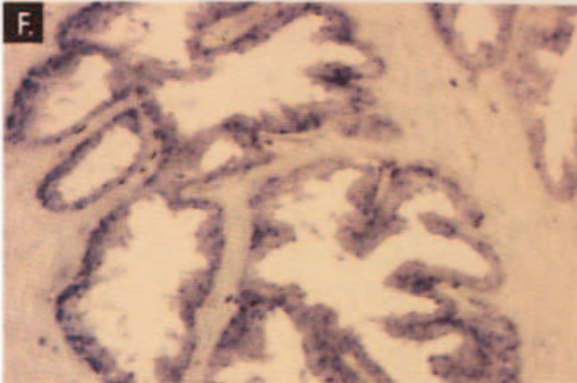
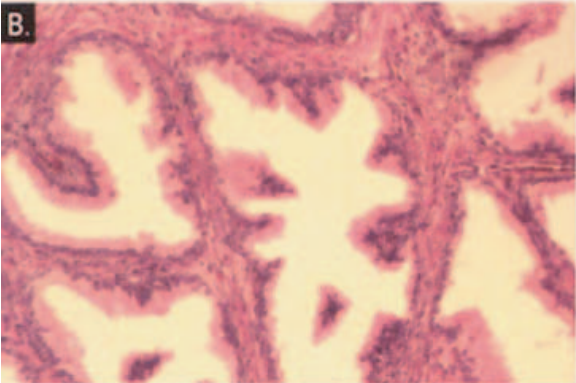
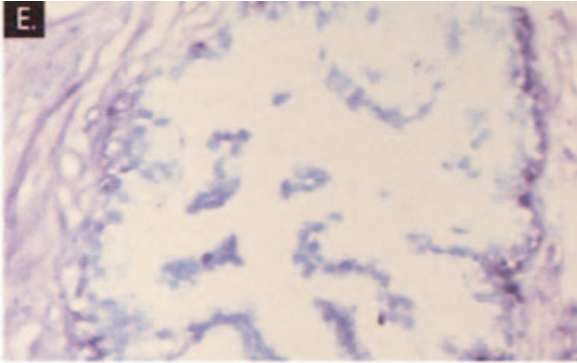
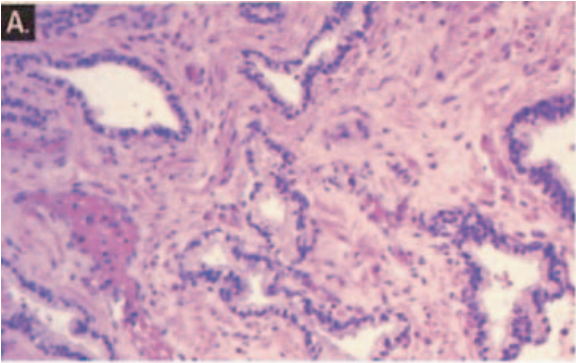
Tsavaler *et al.* (2001) found high levels of TRPM8 in both benign prostate hyperplasia and in prostate carcinoma cells, and low levels in normal (non-carcinoma) prostate epithelial cells (Fig. 3). Henshall *et al.* (2003) observed that with anti-androgen therapy, the expression of TRPM8 was greatly reduced. They also showed that TRPM8 expression is decreased when prostate cancer cells become androgen-independent, suggesting that TRPM8 is regulated by androgens (Henshall *et al.* 2003). The degree of expression of TRPM8 in prostate cancer tissue appears to be quite variable (Tsavaler *et al.* 2001, Henshall *et al.* 2003, Kiessling *et al.* 2003). Kiessling *et al.* (2003) examined prostate cancer tissue from 33 prostate cancer patients and found high but variable levels of TRPM8 mRNA expression in both tumorigenic and normal prostate tissue. However, a statistically significant greater expression of TRPM8 was observed in tumorigenic compared with normal tissue. No clear correlation of TRPM8 expression with the severity of prostate cancer was, however, observed.

Fuessel *et al.* (2003) have studied the expression of TRPM8 in relation to other established and potential prostate cancer markers. They found that in both malignant and non-malignant tissue samples, prostate

specific antigen (PSA) is expressed at the highest level (in terms of absolute amounts of mRNA), with lower levels of human kallikrein 2 (hK2) and TRPM8, and very low levels of prostate stem cell antigen (PSCA). However, on the basis of relative amounts of mRNA, TRPM8 expression (but not PSA expression) in malignant tissue was significantly greater than that in non-malignant tissue. When only organ-confined prostate cancer tissue samples were considered, this difference for TRPM8 was greater, and a difference between malignant and non-malignant tissue was also observed for PSA.

In malignant tissue specimens from patients with a total serum PSA concentration in the 'grey-zone' of 4–10 ng/ml, TRPM8 mRNA, but not PSA mRNA was significantly elevated. Thus the detection of TRPM8 expression could be useful in the differential diagnosis of prostate cancer in biopsy specimens to facilitate therapeutic decisions particularly in ambiguous cases often found in this total PSA 'grey-zone'. More interestingly, in identified groups of low- and high-grade prostate cancer, significant differences in expression between malignant and non-malignant tissue samples were found for TRPM8 but not for other prostate cancer markers, including PSA. It was concluded that, compared with the other markers (PSA, hK2 and PSCA), TRPM8 is a more specific indicator of prostate cancer and a potential candidate for targeted gene therapy or other interventions (Fuessel *et al.* 2003). However, as discussed below, expression of TRPM8 in prostate cancer cells is closely linked to the presence of a functional androgen receptor. Bidaux *et al.* (2005) have drawn attention to the complexities caused by this link and hence in using TRPM8 as a prognostic marker for prostate cancer.

A significant proportion of TRPM8 is observed in the plasma membrane of prostate cancer cells, although the protein has also been observed in intracellular membranes (Zhang & Barritt 2004). Extracellular domains of the protein can potentially activate cytotoxic T-lymphocytes. One such region, the GLMKYIGEV, has been identified (Kiessling *et al.* 2003). This may provide an endogenous mechanism that enhances the destruction of prostate cancer cells expressing TRPM8 — part of the normal immunological defence mechanisms. Loss of TRPM8 expression in metastatic prostate cancer cells may, therefore, be associated with a reduction in prostate cancer cell destruction. On the other hand, the activation of cytotoxic T-lymphocytes by the GLMKYIGEV region may offer the possibility of an immunotherapeutic approach to killing prostate cancer cells based on this



or another TRPM8 peptide as target (Kiessling *et al.* 2003).

### Regulation of TRPM8 by androgens in prostate cancer cells

The early studies of Tsavaler *et al.* (2001) and Henshall *et al.* (2003) suggested that expression of TRPM8 in prostate cancer cells is regulated by androgen. This has been confirmed in recent more direct experiments. Zhang & Barritt (2004) reported androgen-dependent TRPM8 expression in the androgen-responsive LNCaP cell line, but not in the androgen-unresponsive PC-3 cell line. Bidaux *et al.* (2005) have shown that expression of TRPM8 in prostate epithelial cells requires a functional androgen receptor. They used an anti-androgen antagonist and also transfected cDNA encoding the androgen receptor into PNT1A cells (which have lost androgen receptor). The response of TRPM8 to androgens was demonstrated in prostate cancer cell lines, and in primary cultures of normal, hyperplastic and cancerous prostate epithelial cells (Bidaux *et al.* 2005). The extremely low level of androgen receptor expression in androgen refractory prostate cancer cells such as the PC-3 cell line (Culig *et al.* 1993) probably contributes to the much lower level of TRPM8 gene expression and the lack of response to androgen regulation (Zhang & Barritt 2004).

The question of what mechanism underlies the androgen regulation of TRPM8 in androgen-responsive prostate cancer cells has been addressed, and several putative androgen response elements have been detected (Zhang & Barritt 2004, Bidaux *et al.* 2005). Analysis of 5' flank regions of the TRPM8 gene using GenoMatix reveals that a region which spans 1500 bp upstream of the transcription initiation code in the TRPM8 gene may be the core promoter for TRPM8 DNA transcription. This promoter region contains a variety of putative transcriptional factor binding sites including at least three TATA boxes, a transcription factor Sp1, a transcript enhancer factor Tef, three NKX3.1 binding sites and an androgen-responsive element (Table 2, Fig. 4). The last mentioned exhibits a high degree of homology to a

motif corresponding to the TRANSFAC androgen-responsive element consensus sequence (c.f. Zhang & Barritt 2004, Bidaux *et al.* 2005). Further analysis reveals another nine putative androgen-responsive elements in the introns of the TRPM8 gene (Fig. 4), and two in the 3' untranslated region. It is possible that in androgen-responsive prostate cancer cells (which have functional androgen receptors) the androgen-androgen receptor complex binds with androgen response elements in either the promoter region or introns in the TRPM8 gene. Together with other transcription co-factors, this may initiate TRPM8 gene transcription.

NKX3.1 is an androgen-regulated homeodomain protein that is predominantly localised in the adult prostate in men (He *et al.* 1997, Prescott *et al.* 1998) and is a candidate tumour-suppressor oncogene (Lundgren *et al.* 1988, Carter *et al.* 1990, Phillips *et al.* 1994, Voeller *et al.* 1997). Thus the three putative prostate-specific NKX3.1 binding sites in the TRPM8 promoter region are compatible with the androgen-dependence and prostate-predominant expression profile of TRPM8.

### Physiological functions of TRPM8 in prostate cancer cells

Evidence from immunofluorescence experiments indicates that, in the androgen-responsive LNCaP cell line, the TRPM8 protein is expressed in the plasma membrane and ER, and acts as a Ca<sup>2+</sup>-permeable channel (assessed using fura-2 to measure increases in the [Ca<sup>2+</sup>]<sub>cyt</sub>) in each of these membranes (Zhang & Barritt 2004). The results of experiments employing LNCaP cells, the TRPM8 antagonist capsazepine, and siRNA targeted to TRPM8 suggest that TRPM8 is required for cell survival. These results indicate that Ca<sup>2+</sup> and Na<sup>+</sup> inflow through TRPM8 plays an essential role in cellular Na<sup>+</sup> and Ca<sup>2+</sup> homeostasis in prostate epithelial cells.

It has been proposed that, in LNCaP cells, an increased growth rate correlates with an increase in the amount of Ca<sup>2+</sup> in intracellular stores, whereas a decreased rate of growth correlates with a reduced load

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**Figure 3** *In situ* hybridisation of a TRPM8 RNA probe to normal prostate, benign prostate hyperplasia, and prostate cancer tissue. Serial sections (4 µm) were stained with haematoxylin and eosin (A, B, C and D) or used in the *in situ* hybridisation with the TRPM8 RNA probe (E, F, G and H). (A and E) Normal prostate; (B and F) benign prostate hyperplasia; (C and G) prostate cancer tissue (Gleason score 7); (D and H) prostate cancer tissue (Gleason score 10). Magnifications: A, ×40; B and F, ×100; C, D, E, G and H, ×200. From Tsavaler *et al.* (2001) reprinted with permission of the American Association for Cancer Research from Cancer Research.

**Table 2** Putative transcriptional binding sites identified in TRPM8 promoter region. (References are cited in the text.)

Motif	Position (bp)	Strand	Sequence
Myf (myogenic regulatory factors)	–59 to –48	+	agagaccagcag
	–295 to –284	+	agctaagagcag
Tef (transcription enhancer factor)	–73 to –69	+	agcttctcct
	–218 to –207	+	cgtattccactg
TATA box (Bound by TBP (TATA binding protein), a component of TFIID)	–87 to –73	+	ttataaagctttcc
	–613 to –593	+	gtttaaaggggacag
	–1266 to –1253	+	ctatatatgtgcttg
Sp1 (GC box element/bound by transcription factors in the Sp/KLF (kruppel-like factor) family)	–113 to –101	+	agaaggcgggct
MyT1 (MyT1 zinc finger transcription factor involved in primary neurogenesis)	–153 to –141	+	ctaagtittttg
NKX3.1 (prostate-specific homeodomain protein NKX3.1.)	–154 to –142	+	gcttaagtitttt
	–337 to –325	+	aattaagtattag
	–689 to –677	+	athtaagtaaaat
Ets (c-Ets-2 binding site)	–603 to –593	+	gacaggaata
ARE (androgen responsive element)	–242 to –257	+	aaaataccatgttca
PSE.02 (proximal sequence element (PSE) of RNA polymerase III-transcribed genes)	–446 to –428	+	ctaacttaattaagagct
CEBP.02 (CCAAT/enhancer binding protein)	–519 to –505	+	gggatgaggaaagt
CEBPB.01 (CCAAT/enhancer binding protein beta)	–837 to –817	+	tgcttgtgcaatca
Myc (binding motif for Myc-Max dimers)	–697 to –688	+	ggcacgtgat
	–1500 to –1491	+	ggcacgtgcc
			tgacctgtctgaccttgaa
MOK2 (ribonucleoprotein associated zinc finger protein MOK-2 (human))	–1051 to –1031	+	
P53 (tumor suppressor p53 (3' half site))	–1052 to –1032	–	tccaaggctcagacaggtcat
ZBP-89 (zinc-finger binding protein-89. Transcriptional repressor, binds to elements found predominantly in genes that participate in lipid metabolism)	–1108 to –1086	+	acagcccccacccttccactat
Mef-2 (myocyte enhancer factor 2)	–1215 to –1204	+	acccatatttag
	–1472 to –1461	+	ttgatatttag
	–1481 to –1470	+	ggctaattttg
CRE (cAMP response element)	–1417 to –1406	+	cctgacatcaag

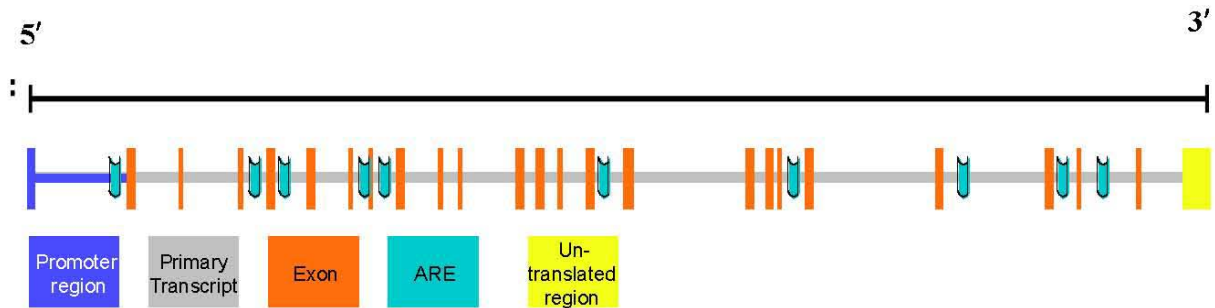
of Ca<sup>2+</sup> in intracellular stores (Legrand *et al.* 2001). TRPM8 may, with other ER-located Ca<sup>2+</sup> pool regulators (e.g. ER (Ca<sup>2+</sup> + Mg<sup>2+</sup>)ATPase), modulate the amount of Ca<sup>2+</sup> in intracellular stores and subsequently regulate cell growth and proliferation (Fig. 5A).

While a number of proposals have been made, the normal functions of Ca<sup>2+</sup> and Na<sup>+</sup> inflow through TRPM8 in prostate epithelial cells are not known, nor have the physiological activators of TRPM8 been described (Fig. 5A). It has been suggested that TRPM8 serves as a cold sensor in the prostate (Stein *et al.* 2004). Such a function has also recently been suggested for TRPM8 in the bladder (Tsukimi *et al.* 2005). TRPM8 may also be involved in other functions such as the regulation of proliferation and/or apoptosis (and hence the control of cell number) and in ion and protein secretion in prostate epithelial cells (Zhang &

Barritt 2004). One interesting possibility comes from the recent observation that geraniol activates TRPM8 (Behrendt *et al.* 2004). The pyrophosphate ester of geraniol is an intermediate in cholesterol synthesis (Voet & Voet 1990), and geraniol enhances cell proliferation in prostate epithelium (Paubert-Braquet *et al.* 1998). Thus it is possible that, in prostate epithelial cells, TRPM8 is involved in the regulation of cell proliferation and responds to geraniol as an intracellular messenger (Zhang & Barritt 2004).

A more detailed study of the tissue and intracellular locations of the TRPM8 protein in the prostate has shown that TRPM8 is principally expressed in prostate epithelial cells (Bidaux *et al.* 2005). A low level of TRPM8 expression was also found in smooth muscle cells. In prostate epithelial cells, the highest level of TRPM8 expression was seen in the apical region of secretory epithelial cells, with much lower amounts





**Figure 4** A schematic representation of the TRPM8 gene showing the locations of putative androgen response elements (ARE). The TRPM8 gene contains 24 exons and spans 95 kb. The 5' untranslated region (–1 to –1500 bp) is the predicted (GenoMatix) promoter region for driving TRPM8 transcription. Ten putative AREs, one in the promoter region and others in introns of the gene, can be identified (Zhang & Barritt 2004).

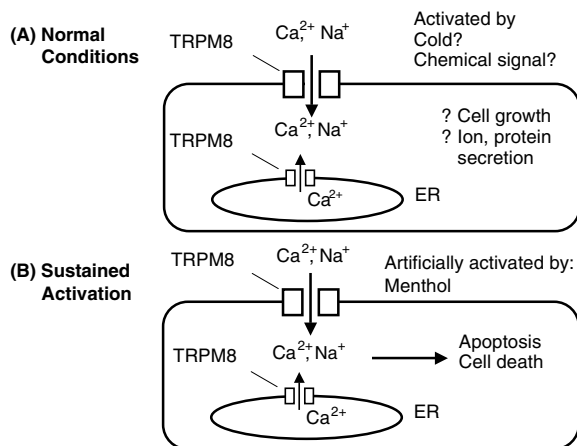
of TRPM8 found in the basal regions. Expression of TRPM8 in secretory epithelial cells of the prostate decreased with time as the cells were grown in primary culture and became dedifferentiated. Bidaux *et al.* (2005) have suggested that TRPM8 is involved in the secretory function of prostate epithelial cells. Secretory products include citric acid, fibrinolysin, acid phosphatase, several other enzymes and lipids. Noting the androgen-dependence of TRPM8 expression and a role for androgens in oocyte fertilisation, these authors have suggested that one of the functions of androgen-regulated TRPM8 is in fertilisation.

In addition to normal prostate epithelium, TRPM8 is also detected in a number of urogenital tissues including testis, seminiferous tubules, scrotal skin, and

bladder urothelium (Tsukimi *et al.* 2005) (Table 1). This systemic expression profile implies that TRPM8 may have yet to be identified roles associated with reproduction and/or sexuality.

On the basis of the observation that TRPM8 expression is upregulated in organ-confined prostate cancer cells and in a number of other cancerous cell types, TRPM8 could function as an oncogene. TRPM8 may contribute to the initiation, promotion and progression of carcinogenesis by disturbing  $\text{Ca}^{2+}$  homeostasis in pre-neoplastic cells. It has been reported that early pre-neoplastic cells are highly susceptible to apoptosis, whereas later pre-neoplastic cells are quite resistant (Preston *et al.* 1997). Moreover, increases in  $[\text{Ca}^{2+}]_{\text{cyt}}$  and intra-organelle  $\text{Ca}^{2+}$  play key roles in apoptosis (Mattson & Chan 2003). Although several hypotheses have been proposed to explain the resistance of cells (such as androgen-unresponsive prostate cancer cells) to apoptosis, a plausible mechanism involving  $\text{Ca}^{2+}$  signalling has been presented (reviewed in Prevarskaya *et al.* 2004) in which reduced  $\text{Ca}^{2+}$  levels in the ER were observed in early pre-neoplastic cells that undergo apoptosis compared with a higher level of stored  $\text{Ca}^{2+}$  in the ER in late pre-neoplastic cells. Over-expressed or upregulated TRPM8 channels could cause an increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  and an overfilling of ER  $\text{Ca}^{2+}$  store and this change in  $\text{Ca}^{2+}$  homeostasis would make the late pre-neoplastic cells more resistant to apoptotic stimuli and subsequently promote cell transformation into a neoplastic stage.

In addition, the detection of putative binding sites for tumour suppressor protein p53, prostate-specific homeodomain protein NKX3.1, and zinc-finger binding protein-89 (ZBP-89; Merchant *et al.* 2003) (Table 2) in the TRPM8 gene promoter region implies that TRPM8 may serve as a downstream target of



**Figure 5** A schematic representation of the hypothesised functions of  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  inflow mediated by TRPM8 in prostate cancer cells. (A) Prostate cancer cells under 'normal' conditions when TRPM8 is activated by physiological or pathophysiological activators. (B) Prostate cancer cells under conditions of sustained TRPM8 activation such as induced by menthol or other cooling chemical agents.

tumour-suppressor genes. (ZBP-89 is a butyrate-regulated co-activator of p53, and is able to induce p21(Waf1) gene expression through both p53-dependent and -independent mechanisms to inhibit cell growth (Merchant *et al.* 2003).) Thus TRPM8 may be involved in the regulation of cell growth and carcinogenesis. Taken together, the above results suggest that TRPM8 is a novel prostate cancer biomarker, is likely to play an important role in the pathophysiology of prostate epithelial cells, and is a potential target for the action of drugs in the treatment of prostate cancer.

### **The consequences of sustained activation of TRPM8 induced by pharmacological intervention in prostate cancer cells**

In contrast to the results that provide evidence that under 'normal' conditions TRPM8 is required for the survival of prostate epithelial cells, the non-physiological activation of TRPM8 by menthol can induce cell death in the androgen-sensitive LNCaP prostate cancer cell line (Zhang & Barritt 2004). Moreno *et al.* (2005) further showed that some selective TRPM8 agonists inhibit the growth of TRPM8-positive tumours in mice by 77% compared with control untreated tumours (Moreno *et al.* 2005). This is presumably by inducing a sustained increase in the cytoplasmic  $Ca^{2+}$  and  $Na^{+}$  concentrations (Fig. 5B). These effects most likely reflect a pathophysiological action of TRPM8, as it is unlikely that TRPM8 would normally be activated in a sustained manner. Interestingly, these findings may provide an explanation for the chemopreventive role of menthol on rat mammary tumours initiated by 7,12-dimethylbenz[a]anthracene (Russin *et al.* 1989) because TRPM8 is also expressed at significant levels in breast cancer cells (Table 1). Further experiments are needed to test the hypothesis and to evaluate whether menthol and its analogues could be promising agents for the chemoprevention of prostate and other cancers.

### **Conclusions**

TRPM8 has clearly defined roles as a sensor for cooling in sensory neurones. While it is expressed in prostate cancer cells and is associated with the pathophysiology of these cells, tumorigenic progression and metastasis, its physiological or pathophysiological roles (in terms of the functions of  $Ca^{2+}$  and  $Na^{+}$  which enter the cell via TRPM8) are not understood. They may be involved in sensing cold

and/or chemical activators, in regulating the secretion of prostate epithelial cells, and/or in conveying signals which control cell growth and death. It is possible that TRPM8-mediated cool sensation is part of a more complex sensation network in the reproductive tract. In terms of molecular interactions, the mechanisms of activation of TRPM8 are rapidly being understood. These involve membrane potential,  $PIP_2$  and intracellular  $Ca^{2+}$ . The TRPM8 over-expression profile in mainly organ-confined prostate cancers suggests a possible role of TRPM8 in the development of the cancer from the organ-confined stage to the metastatic stage.

TRPM8 is potentially a valuable diagnostic tissue marker and prognostic indicator for the progress of prostate cancer. Additional knowledge, which might establish this role for TRPM8 could include measurement of TRPM8 expression at the protein (as well as the mRNA) level. Further studies are needed to compare TRPM8 expression with other established prostate cancer markers in terms of accuracy and probability. The limitation of using TRPM8 as a marker is that prostate tissues are needed for the examination of TRPM8 levels. The regulation of TRPM8 expression by androgens is important in terms of both fundamental knowledge and understanding the role of TRPM8 in prostate cancer. Further experiments might be directed at identifying androgen response elements and perhaps other hormone response elements in the promoter and intron regions, and at understanding the mechanisms underlying up-regulation of TRPM8 in cancerous tissues. Finally, TRPM8 may be a potential target for pharmaceutical or genetic interventions for the treatment of prostate cancer and other cancers with over-expression of TRPM8. Further experiments might include screening for the specific and potent agonists for activation of the TRPM8 channel and exploring the strategy *in vivo*.

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