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Expression of 5-oxoETE receptor in prostate cancer cells: Critical role in survival ☆

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Abstract

Previously, we reported that metabolism of arachidonic acid through the 5-lipoxygenase (5-LOX) pathway plays an important role in the survival and growth of human prostate cancer cells. Inhibition of 5-LOX by pharmacological inhibitors triggers apoptosis in prostate cancer cells within hours of treatment, which is prevented by the metabolites of arachidonate 5-lipoxygenase, 5(*S*)-hydroxyeicosatetraenoic acid (5(*S*)-HETE), and its dehydrogenated derivative, 5-oxoeicosatetraenoic acid (5-oxoETE). These findings suggested that 5-lipoxygenase metabolites are critical survival factors of prostate cancer cells. However, molecular mechanisms by which 5(*S*)-HETE and its derivative 5-oxoETE exert their effects on prostate cancer cell survival are yet to be understood. Here, we report that human prostate cancer cells differentially express a G-protein-coupled 5-oxoETE receptor (5-oxoER) in them. Blocking expression of 5-oxoER by short-interfering RNA (siRNA) significantly reduced the viability of prostate cancer cells, suggesting that 5-oxoER is critical for prostate cancer cell survival, and that the 5-LOX metabolite, 5-oxoETE, controls survival of prostate cancer cells through its own G-protein-coupled receptor, 5-oxoER.

Keywords: Arachidonic acid; Prostate cancer; Eicosanoids; 5(*S*)-HETE; 5-oxoETE; 5-oxoER

Prostate cancer is the most common form of malignancy and second leading cause of cancer-related death in men in the United States [1]. Epidemiological studies and experiments with laboratory animals have repeatedly suggested a link between high-fat diets and clinical prostate cancer [2–6]. Biochemical and molecular studies have shown an association of fatty acid metabolism with prostate cancer cell proliferation. Specifically, metabolism of arachidonic acid has been shown to increase growth and promote survival of prostate cancer cells [7–13]. Arachidonic acid serves as substrate for several enzymes including lipoxygenases and cyclooxygenases. Metabolism of arachidonic acid by these enzymes generates a vast array of eicosanoids such as hydroxyeicosatetraenoids (HETEs), prostaglandins, and leukotrienes [14,15]. These metabolites have been shown to influence growth and survival of a variety of cancer cells including that of the prostate [7,8,16].

We have previously shown that inhibition of 5-LOX triggers apoptosis in prostate cancer cells. We have also shown that apoptosis triggered by 5-LOX inhibition could be prevented by exogenous 5(*S*)-HETE or its dehydrogenated derivative 5-oxoETE, and that 5-oxoETE was more effective in preventing apoptosis caused by 5-LOX inhibition [7,8,17]. Eicosanoids, such as the above arachidonic acid metabolites, have also been reported to play important roles in inflammatory and immune responses [18–24]. There are reports suggesting that these molecules act as signaling molecules in eosinophils and macrophages [18,19]. For instance, 5-oxoETE has been shown to be an eosinophil chemoattractant [20]. Though 5-oxoETE plays an essential role in the survival of prostate cancer cells, its

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mechanism of action in the regulation of survival of these cancer cells is still unknown.

Recently, Hosoi et al. and Jones et al. [25,26] have identified G-protein-coupled receptors (TG1019 and R527, respectively) for which 5-oxoETE, and to a lesser extent 5-HETE serve as ligands. Hosoi et al. [27], have also recently shown that the identified TG1019 receptor mediates 5-oxoETE-induced chemotaxis in CHO cells. Identification of 5-oxoER by Hosoi et al. [25] together with our earlier observations on apoptosis caused by 5-LOX inhibition and its prevention by 5-oxoETE led us to consider the possibility that a receptor of the 5-LOX metabolite, 5-oxoETE, is expressed in prostate cancer cells, and that this receptor plays a role in delivering survival signals of 5-oxoETE in these cells. We addressed these possibilities by RT-PCR using primer sets based on published sequences [25,26] and by gene knock-down experiments using siRNA. Here, we report the expression of 5-oxoETE receptor in prostate cancer cells and show that it plays an important role in the survival of these cells, suggesting that 5-oxoETE exerts its survival signal in prostate cancer cells through a receptor-mediated mechanism.

Materials and methods

Cell culture and reagents. Androgen-sensitive (LNCaP) and androgen-refractory (PC3) human prostate cancer cells were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) plus 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified CO_2-incubator. Cells were fed with fresh medium every third day and split at a confluence of ~80%. DNA primers for PCR were bought from Integrated DNA Technologies (Coralville, IA). SMARTpool siRNA against 5-oxoER was purchased from Dharmacon (Lafayette, CO). The lipid-based transfection reagent (TransIT-TKO) was purchased from Mirus (Madison, WI).

Expression and sequencing of 5-oxoETE receptor. Total RNA from LNCaP or PC3 cells was extracted using RNEasy mini kit (Qiagen, Valencia, CA). For RT-PCR, 25 ng of total RNA was used along with gene specific primers for 5-oxoETE receptor and GAPDH. First strand synthesis and amplification with gene specific primers were carried out with cMaster RT plus PCR system (Eppendorf, Westbury, NY). The following primer pairs were used to test the expression of 5-oxoETE receptor in LNCaP and PC3 cells: (upstream) 5′-TCT TCA TCT TCT GCA TCC ACA CG-3′, and (downstream) 5′-AGT GGC AGG AAG AAC TCC AGC AG-3′. The 5-oxoETE receptor fragment containing the entire coding region was amplified using primer set based on previously described method [25]. The coding region was then cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced using BigDye Terminator V3.1 cycle sequencing kit and ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA). Cloning and sequencing of the entire coding region of 5-oxoETE receptor was repeated (two independent experiments) to eliminate errors in sequencing.

Expression analysis. PCR products were resolved by 1% agarose gel electrophoresis. Bands were analyzed with Eagle Eye II Darkroom Cabinet still video imaging system using EagleSightv3.1 software (Stratagene, La Jolla, CA). Band intensities of the receptor resolved in agarose gel were normalized to that of GAPDH.

siRNA treatment and assay of cell viability. PC3 prostate cancer cells (~75,000 per well) were plated overnight onto 24-well plates in RPMI medium supplemented with 10% FBS. SMARTpool siRNA against 5-oxoER (with the following sense strand sequences: CAU GAG ACC UGG CGC UUU GUU, UCA CCU ACC UCA ACA GUG UUU, GGC GAG GUC UCU CUG GAA AUU, CAA AGU CAA CCU CUU CAU GUU) was complexed with TransIT-TKO transfection reagent (Mirus) as per manufacturer's instructions and added directly to the media at varying concentrations. A bioinformatically designed siRNA (not targeting any known human or mouse gene) was used as control under the same experimental conditions (Dharmacon). Plates were incubated further for 72 h at 37 °C in the CO_2 incubator. After isolation of total RNA, expression of 5-oxoER was detected and analyzed by RT-PCR using the primer set mentioned above. Expression of GAPDH was also measured in parallel as a control to verify the specific effect of siRNA treatment. For cell viability assays, PC3 cells were plated at ~3000 cells/well onto 96-well plates and treated with siRNA as mentioned above. Cell viability was measured by MTS/PES Cell Titer Aq assay (Promega, Madison, WI) as described previously [8].

Results

Prostate cancer cells express 5-oxoETE receptor

To understand the mechanism of action of 5-oxoETE in prostate cancer cell survival, first of all we wanted to know whether these cells express 5-oxoETE receptor (5-oxoER) by employing PCR-based analysis. We used gene specific primer sets for 5-oxoER according to published procedure [26] to perform RT-PCR. Electrophoretic analysis of the PCR product showed a band of expected size (453 bp) in both androgen-sensitive (LNCaP) and androgen-refractory (PC3) human prostate cancer cells (Fig. 1). DNA sequencing of the PCR products revealed exact match with the published sequence of 5-oxoER [25,26], confirming that human prostate cancer cells express 5-oxoETE receptor in them.

Cloning and sequencing of 5-oxoER

Next we wanted to clone and know the complete sequence of the coding region of 5-oxoER in prostate cancer cells and compare it with published sequence in the database. We used primer sets flanking the coding region of the gene to isolate the full length functional 5-oxoER from androgen-sensitive (LNCaP) and androgen-refractory (PC3) prostate cancer cells. Agarose gel analysis of the PCR product showed a band of ~1.5 kb which matched with expected size of the 5-oxoER cDNA. We isolated the 1.5 kb PCR product and cloned onto pcDNA3.1/V5-His-TOPO vector for sequencing. Fig. 2 shows the

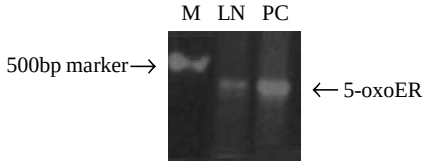

Fig. 1. Expression of 5-oxoETE receptor in prostate cancer cells. Total RNA (25 ng) from exponentially growing LNCaP (LN) or PC3 (PC) prostate cancer cells was used for RT-PCR with 5-oxoER gene specific primer set and the PCR products were resolved by agarose gel electrophoresis. Bands in lanes LN and PC correspond to an expected product size of 453 bp. Molecular weight standards were loaded in lane M.

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   1 ATGTTGTGTCACCGTGGTGGCCAGCTGATAGTGCCAATCATCCCA
   1 M  L  C  H  R  G  G  Q  L  I  V  P  I  I  P

  46 CTTTGCCCTGAGCACTCCTGCAGGGGTAGAAGACTCCAGAACCTT
  16 L  C  P  E  H  S  C  R  G  R  R  L  Q  N  L

  91 CTCTCAGGCCCATGGCCCAAGCAGCCCATGGAACTTCATAACCTG
  31 L  S  G  P  W  P  K  Q  P  M  E  L  H  N  L

 136 AGCTCTCCATCTCCCTCTCTCTCCTCCTCTGTTCTCCCTCCCTCC
  46 S  S  P  S  P  S  L  S  S  S  V  L  P  P  S

 181 TTCTCTCCCTCACCCTCCTCTGCTCCCTCTGCCTTTACCACTGTG
  61 F  S  P  S  P  S  S  A  P  S  A  F  T  T  V

 226 GGGGGGTCCTCTGGAGGGCCCTGCCACCCCACCTCTTCCTCGCTG
  76 G  G  S  S  G  G  P  C  H  P  T  S  S  S  L

 271 GTGTCTGCCTTCCTGGCACCAATCCTGGCCCTGGAGTTTGTCCTG
  91 V  S  A  F  L  A  P  I  L  A  L  E  F  V  L

 316 GGCCTGGTGGGGAACAGTTTGGCCCTCTTCATCTTCTGCATCCAC
 106 G  L  V  G  N  S  L  A  L  F  I  F  C  I  H

 361 ACGCGGCCCTGGACCTCCAACACGGTGTTCCTGGTCAGCCTGGTG
 121 T  R  P  W  T  S  N  T  V  F  L  V  S  L  V

 406 GCCGCTGACTTCCTCCTGATCAGCAACCTGCCCCTCCGCGTGGAC
 136 A  A  D  F  L  L  I  S  N  L  P  L  R  V  D

 451 TACTACCTCCTCCATGAGACCTGGCGCTTTGGGGCTGCTGCCTGC
 151 Y  Y  L  L  H  E  T  W  R  F  G  A  A  A  C

 496 AAAGTCAACCTCTTCATGCTGTCCACCAACCGCACGGCCAGCGTT
 166 K  V  N  L  F  M  L  S  T  N  R  T  A  S  V

 541 GTCTTCCTCACAGCCATCGCACTCAACCGCTACCTGAAGGTGGTG
 181 V  F  L  T  A  I  A  L  N  R  Y  L  K  V  V

 586 CAGCCCCACCACGTGCTGAGCCGTGCTTCCGTGGGGGCAGCTGCC
 196 Q  P  H  H  V  L  S  R  A  S  V  G  A  A  A

 631 CGGGTGGCCGGGGGACTCTGGGTGGGCATCCTGCTCCTCAACGGG
 211 R  V  A  G  G  L  W  V  G  I  L  L  L  N  G

 676 CACCTGCTCCTGAGCACCTTCTCCGGCCCCTCCTGCCTCAGCTAC
 226 H  L  L  L  S  T  F  S  G  P  S  C  L  S  Y

 721 AGGGTGGGCACGAAGCCCTCGGCCTCGCTCCGCTGGCACCAGGCA
 241 R  V  G  T  K  P  S  A  S  L  R  W  H  Q  A

 766 CTGTACCTGCTGGAGTTCTTCCTGCCACTGGCGCTCATCCTCTTT
 256 L  Y  L  L  E  F  F  L  P  L  A  L  I  L  F

 811 GCTATTGTGAGCATTGGGCTCACCATCCGGAACCGTGGTCTGGGC
 271 A  I  V  S  I  G  L  T  I  R  N  R  G  L  G

 856 GGGCAGGCAGGCCCGCAGAGGGCCATGCGTGTGCTGGCCATGGTG
 286 G  Q  A  G  P  Q  R  A  M  R  V  L  A  M  V

 901 GTGGCCGTCTACACCATCTGCTTCTTGCCCAGCATCATCTTTGGC
 301 V  A  V  Y  T  I  C  F  L  P  S  I  I  F  G

 946 ATGGCTTCCATGGTGGCTTTCTGGCTGTCCGCCTGCCGATCCCTG
 316 M  A  S  M  V  A  F  W  L  S  A  C  R  S  L

 991 GACCTCTGCACACAGCTCTTCCATGGCTCCCTGGCCTTCACCTAC
 331 D  L  C  T  Q  L  F  H  G  S  L  A  F  T  Y

1036 CTCAACAGTGTCCTGGACCCCGTGCTCTACTGCTTCTCTAGCCCC
 346 L  N  S  V  L  D  P  V  L  Y  C  F  S  S  P

1081 AACTTCCTCCACCAGAGCCGGGCCTTGCTGGGCCTCACGCGGGGC
 361 N  F  L  H  Q  S  R  A  L  L  G  L  T  R  G

1126 CGGCAGGGCCCAGTGAGCGACGAGAGCTCCTACCAACCCTCCAGG
 376 R  Q  G  P  V  S  D  E  S  S  Y  Q  P  S  R

1171 CAGTGGCGCTACCGGGAGGCCTCTAGGAAGGCGGAGGCCATAGGG
 391 Q  W  R  Y  R  E  A  S  R  K  A  E  A  I  G

1216 AAGGTGAAAGTGCAGGGCGAGGTCTCTCTGGAAAAGGAAGGCTCC
 406 K  V  K  V  Q  G  E  V  S  L  E  K  E  G  S

1261 TCCCAGGGCTGA 1272
 421 S  Q  G
```

Fig. 2. Nucleotide and deduced amino acid sequence of 5-oxoER isolated from PC3 cells. The codon at position 1219 corresponding to amino acid valine at position 407 is shown in boldface and underlined.

complete sequence of the coding region of 5-oxoER from PC3 prostate cancer cells. The sequence of the coding region of 5-oxoER from PC3 cells is essentially identical to that of TG1019 [25], except for a single nucleotide change (C to G) which corresponds to a single amino acid change from leucine to valine at position 407. Interestingly, this single amino acid difference was also observed in the 5-oxoETE receptor (R527) isolated by Jones et al. [26].

Androgen-sensitive (LNCaP) and androgen-refractory (PC3) prostate cancer cells differentially express 5-oxoETE receptor

To detect the relative levels of expression of 5-oxoER in androgen-sensitive and androgen-refractory prostate cancer cells, we used the same primer set (as in Fig. 2) covering the entire coding region of 5-oxoER for semi-quantitative RT-PCR experiments. Densitometric analysis of the PCR-generated product showed ~3.4-fold higher levels of the receptor in the androgen-refractory (and more aggressive) PC3 cells compared to the androgen-sensitive LNCaP cells (Fig. 3). The normal prostate epithelial cells (PrEC) did not show any detectable expression of 5-oxoER under the same experimental conditions (not shown).

5-OxoER plays an important role in prostate cancer cell survival

Since we previously observed that 5-oxoETE plays a critical role in the survival of prostate cancer cells, we

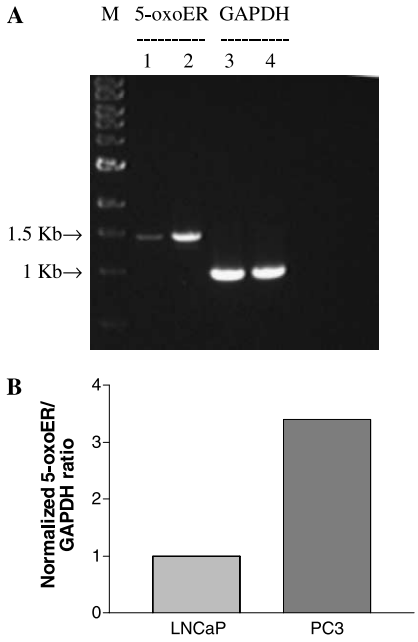

Fig. 3. Differential expression of 5-oxoER in prostate cancer cells. (A) Expression levels of 5-oxoER in androgen-sensitive (LNCaP) and androgen-refractory (PC3) prostate cancer cells were measured by RT-PCR using gene specific primers flanking the coding region. Lanes 1 and 3, LNCaP; lanes 2 and 4, PC3. Molecular weight standards are shown in lane M. (B) Shows results from densitometric analysis of 5-oxoER expression in prostate cancer cells. Band densities of 5-oxoER and GAPDH were measured and the ratios were calculated for each cell type. 5-oxoER/GAPDH ratio for LNCaP was considered to be one unit of measurement.

wanted to know whether its receptor (5-oxoER) also plays a similar role in the survival of these cells. In order to investigate the role of 5-oxoER in prostate cancer cells, we studied the effect of its inhibition by gene silencing using short-interfering RNA (siRNA) to make these cells functional knockout of this receptor. We used androgen-refractory PC3 prostate cancer cells for siRNA treatment because of their higher level of expression of 5-oxoER. Results showed a dose-dependent decrease in the expression of 5-oxoER in PC3 cells (Fig. 4A) and their viability (Fig. 4B) upon treatment with 5-oxoER siRNA. These findings support our hypothesis that the growth-promoting and apoptosis-preventing effects of 5-oxoETE in prostate cancer cells are mediated through its receptor, 5-oxoER. In order to ensure that the observed effects were not a generic response to RNA interference, we used a bioinformatically designed control siRNA (not targeting any known human or mouse gene) in parallel under the same experimental conditions, which showed no significant effect on the expression of 5-oxoER, or the viability of cells.

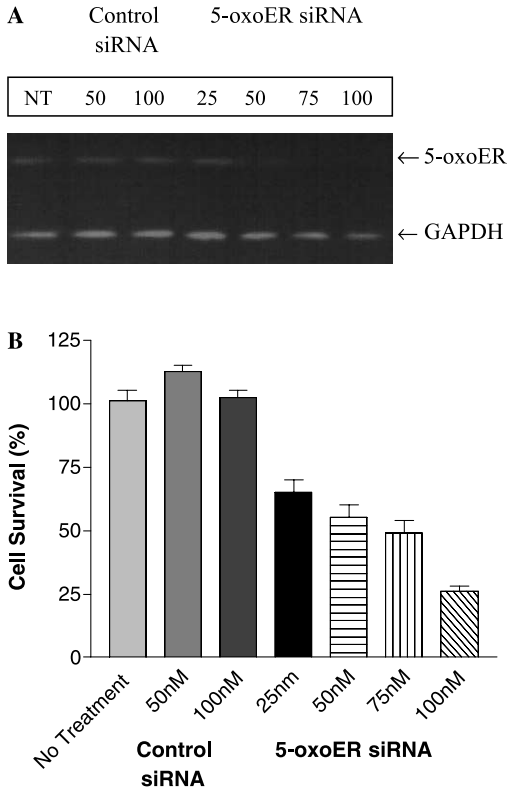

Fig. 4. Effects of siRNA treatment on 5-oxoER gene expression and viability of prostate cancer cells. PC3 prostate cancer cells (75,000 per well) were plated onto 24-well plates. Indicated concentrations of control (50, 100 nM) or 5-oxoER (25, 50, 75, and 100 nM) siRNA were added 24 h after seeding and the plates were incubated further for 72 h. NT represents "no treatment." (A) Shows expression levels of 5-oxoER detected by RT-PCR as in Fig. 1. Expression level of GAPDH was used as reference. (B) Shows viability of PC3 cells 72 h after siRNA treatment. Absorbance values of untreated cells (no treatment) were designated to be 100% cell viability. Results are shown as mean values of each data point ± standard error ($n = 3$).

Discussion

Our data, for the first time, document the expression of 5-oxoETE receptor (5-oxoER) in human prostate cancer cells. Based on our previous findings about the essential role of 5-oxoETE in the survival and regulation of growth of prostate cancer cells [7,8], and published reports about the possible existence of typical seven transmembrane G-protein-coupled receptors for this eicosanoid [28,29] we hypothesized that 5-oxoETE may signal through its own G-protein-coupled receptor in regulating survival and growth of prostate cancer cells. Recent work by Hosoi et al. [25] and Jones et al. [26] using genome-mining strategy for identification of GPCR made it possible to detect and characterize 5-oxoETE receptor gene expression through adopting a PCR based approach. G-protein-coupled receptors of related eicosanoids, such as LTB4 and cysteinyl leukotrienes, have already been identified and characterized [30–36]. Using primer sets based on published sequences of 5-oxoER [25,26] we were able to detect, clone, and sequence 5-oxoETE receptor in human prostate cancer cells.

The complete cDNA sequence of 5-oxoER in prostate cancer cells (Fig. 2) shows 99.9% identity to TG1019 [25], and suggests that it is a full length variant of TG1019 with a single nucleotide difference (C to G) which corresponds to a protein of 423 amino acids in length with a single amino acid change from leucine to valine at position 407. The functional significance of this change in 5-oxoER in prostate cancer cells is not known at this time and is an interesting area of future study. Interestingly, this single amino acid difference was also observed in the 5-oxoETE receptor (R527) isolated by Jones et al. [26]. However, the R527 receptor is only 384 amino acids in length (less 39 amino acids from the N-terminus) but functional (capable of inducing calcium mobilization and suppressing cAMP formation) when transfected into HEK293 cells. R527 and TG1019 5-oxoETE receptors have been characterized to show G-protein-coupled receptor activity by Jones et al. [26] and Hosoi et al. [27] respectively.

We observed different levels of 5-oxoER expression in the androgen-sensitive (LNCaP) and the androgen-refractory (PC3) prostate cancer cells (Fig. 3). PC3 prostate cancer cells showed ~3.4-fold higher levels than LNCaP cells. LNCaP and PC3 cell lines were originally isolated from metastatic prostate cancer lesions of lymph node and bone, respectively [37]. These two cell lines differ in a number of biological and molecular characteristics. Typically, LNCaP cells express androgen receptor, produce PSA (prostate-specific antigen), and have functional p53, whereas PC3 cells have none of these properties. Also, LNCaP cells have a longer generation time than PC3 cells and are much less tumorigenic in nude mice [37]. Considering the growth-promoting and apoptosis-preventing effects of 5-oxoETE, it is tempting to speculate that higher levels of expression of 5-oxoER in PC3 prostate cancer cells allow them to deliver stronger signals through 5-oxoER, and thus may

contribute to their more aggressive growth and tumorigenic characteristics.

Treatment of prostate cancer cells with siRNA against 5-oxoER indicates that the receptor plays an important role in the survival of these cells (Fig. 4). This is in direct correlation with our earlier observations that 5-oxoETE (the ligand of 5-oxoER) promotes prostate cancer cell growth and prevents apoptosis in these cells triggered by inhibition of arachidonate 5-lipoxygenase. Altogether, these findings suggest that 5-oxoETE exerts its pro-survival effects through 5-oxoER-mediated pathway. However, downstream signaling mechanisms of 5-oxoETE and 5-oxoER in the regulation of prostate cancer cell growth and survival are not known at this time. Role of 5-oxoER in mediating 5-oxoETE-induced chemotaxis has recently been characterized by Hosoi et al. transfecting CHO cells with 5-oxoER gene [27]. However, role of 5-oxoER in the survival of any cell type has not been documented so far. The expression of 5-oxoER in prostate cancer cells and its critical role in prostate cancer cell survival suggest that the growth-promoting and apoptosis-preventing effects of 5-oxoETE in prostate cancer cells are mediated through its cognate G-protein-coupled receptor, 5-oxoER, and that 5-oxoER comprises an appropriate molecular target for the treatment of prostate cancer.

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