Molecular profiling of hormone-resistant prostate cancer cells with TRPV6 oncochannel knockout/knockdown

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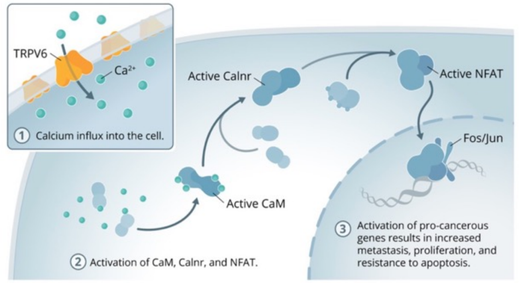
ABSTRACT: TRPV6 calcium channel is a recognized oncochannel over-expressed in a number of epithelial cancers (e.g. breast, ovarian, prostate). TRPV6, a member of one of seven subfamilies of TRP channels, plays an important role in Ca2+ absorption from the intestinal lumen. TRPV6 over-expression is associated with a poor prognosis particularly with breast and prostate cancers. Although some TRPV6 mechanistic work is available and indicates that NFAT is involved, there is a need to better understand pathways involved in its mechanism of action (MOA). These data could allow the identification of clinically relevant biomarkers and identify potential for synergy of TRPV6-targeting therapies with other anti-cancer treatments. To determine which key pathways TRPV6 influences in prostate cancer, two TRPV6 knockouts (KO) were produced in a castration-resistant prostate cancer cell line (PC-3) using the CRISPR-Cas9 approach with two different gRNAs. In addition, knockdowns (KD) were produced by transfecting PC-3 cells with two TRPV6 siRNA targeting different regions of TRPV6 mRNA. The mRNA expression of 184 genes was analyzed using an RT-qPCR TaqMan array. The panel consisted of genes involved in cancer calcium signalling, genes that have been associated with the MOA of TRPV6 and genes directly or indirectly involved in NFAT signalling. Gene profiling results were generally consistent between the TRPV6 KD and KO cells. Results showed a dramatic reduction in expression of TRPV6 mRNA in the KO/KD cells (14 to 166-fold). TRPV6 KO/KD affected multiple genes involved in cancer cell proliferation (e.g. ESR1, CDH1), metastasis (e.g. SNAI1, MUC1/16), resistance to apoptosis (e.g. Bcl-2-type proteins, Fos) and angiogenesis (e.g. VEGFA/B, FLT4), as well as genes involved in the M2 immune tumor microenvironment (e.g. IL6 and VEGFA). The TRPV6 KO/KD data confirm the basic MOA of TRPV6, activating NFAT by increasing cytosolic calcium and provide information on the downstream pathways involved (e.g. NF- kB, estrogen and MAPK). The data allow for the identification of clinical efficacy biomarkers for TRPV6 inhibitors (e.g. a panel of CXCL12, FLT4, MUC16 and IL- 6). Furthermore, results indicate TRPV6 inhibitors have the potential to modify the immune composition of the tumor microenvironment from a tumor promoting to a tumor control response, and thus may help trigger an anti-cancer immune defence. This study demonstrates a central role of TRPV6 and calcium signalling in cancer development.

Introduction

TRPV6 is a membrane bound channel that is highly selective for Ca^{2+}. TRPV6, a recognized oncochannel over-expressed in a number of epithelial cancers has been found up-regulated in breast, prostate, ovarian, thyroid and colon cancers with respect to normal controls. Increased expression of the constitutively active TRPV6 channels in the plasma membrane of cancer cells have cancer promoting effects by enhancing Ca^{2+}-dependent proliferative response, metastasis as well as resistance to apoptotic-induced cell death. The figure below describes the role of TRPV6 in cancer development through the calcineurin-NFAT pathway. This poster describes the genes that are differentially expressed due to disruption of the TRPV6 pathway by either CRISPR-Cas9 knockout or siRNA knockdown of TRPV6.



An increase in TRPV6 expression in the membrane of normal cells activates precancerous pathways by facilitating calcium influx into the cell (1). These calcium ions bind to the calcium binding protein calmodulin (CaM). This complex activates calcineurin (Calnr): a CaM/Ca2+ activated phosphatase that de-phosphorylates and activates Nuclear Factor of Activated T-cells (NFAT), a gene transcription factor (2). Active NFAT moves to the nucleus of the cell where, with Jun/Fos (two auxiliary transcription proteins from "cancer causing genes"), activates a number of intracellular pro-cancerous agents (3)

Materials & Methods

Cell Culture and TRPV6 knockdown by siRNA

PC-3 cells were obtained from ATCC and cultured as recommended. Cells were transfected with either TRPV6 siRNA 1156936 or 1156939 from BioNEER using Lipofectamine® RNAiMax. Cells were harvested at 80-100% confluence 72 hours after transfection.

Generation of TRPV6 knockout Cell lines

Two TRPV6 knockout PC-3 cell lines (PC-3 TRPV6-1A and PC-3 TRPV6-2B) were generated using GeneArt™ CRISPR Nuclease Vectors with CD4 Enrichment and two different CRISPR crRNA (ThermoFisher Scientific). Two CRISPR nuclease vectors were created TRPV6-1 and TRPV6-2. PC-3 cells were transfected with either CRISPR-Cas9 TRPV6-1 or TRPV6-2 vector using Lipofectamine® 3000. Two days after transfection cells were harvested and CD4 positive PC-3 cells isolated using Dynabeads CD4 positive isolation kit. The isolated CD4 positive cells were cloned to isolate single cell colonies. The cloned colonies were screened for TRPV6 double allele knockouts using GeneArt™ Genomic Cleavage Detection Kit. Colonies positive for pure TRPV6 gene mutation were sent for sequencing for confirmation.

RNA isolation and RT-qPCR Analysis

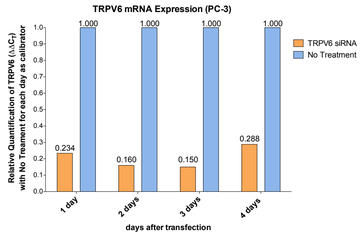
Cells were harvested by lysing directly in 6-well culture plates with lysis buffer from the PureLink RNA Mini Kit and then RNA isolation carried out as per manufacturers instructions (ThermoFisher Scientific). RNA was quantified using the Qubit Fluorometer. Reverse Transcription was performed and cDNA libraries created using SuperScript™ IV VILO Master Mix with ezDNase Enzyme. Each port of the Custom TaqMan® Array Card was loaded with 500 ng of cDNA and 1X TaqMan Fast Advanced Master Mix. RT-qPCR was performed using the Quantstudio™ 7Flex and analysis performed by ThermoFisher cloud software using GUSB and HRPT1 as endogenous controls and calibrated to PC-3 control cells.

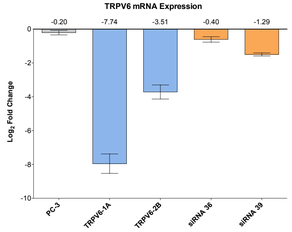
Results

PC-3 TRPV6 Knockout and Knockdown

TRPV6 mRNA expression was knocked-down 77% 1 day after TRPV6 siRNA 1156939 treatment and the TRPV6 mRNA suppression lasted 4 days. The lowest levels of TRPV6 mRNA were observed after 3 days of treatment, having an 85% reduction in TRPV6 expression.

Sequencing confirmed the TRPV6-1 CRISPR-Cas9 vector resulted in an insertion of 122 bp at the CRISPR-Cas9 cut-site at bp 293 of TRPV6 (exon 1) in the PC-3 TRPV6-1A cell colony, leading to a frameshift mutation that results in an nonfunctional TRPV6 protein. The 122 bp insert is also a dinucleotide GA repeat sequence, which can lead to DNA polymerase pausing and dissociation, down-regulating gene as seen in the mRNA reduction in TRPV6-1A. The second vector TRPV6-2 CRISPR-Cas9 resulted in a deletion of one G at the CRISPR-Cas9 cute site at bp 441 of TRPV6 (exon 3), causing a frameshift mutation. TRPV6 expression wasn't significantly decreased by siRNA 36, therefore was removed from analysis.

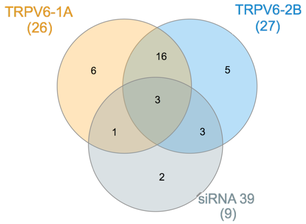




Analysis of 187 genes by RT-qPCR TaqMan Array

The Venn-Diagrams depicts the number of differentially expressed genes in each TRPV6 treatment; PC-3 TRPV6 knockout TRPV6-1A (n = 3), PC-3 TRPV6 knockout TRPV6-2B (n = 3) and TRPV6 siRNA 39 knockdown (n = 2), as well as how many differentially expressed genes are shared amongst TRPV6 treatments. The TaqMan Array consisted of 187 genes involved cell proliferation, metastasis, apoptosis, angiogenesis, immuno-oncology, and intracellular calcium regulation. Genes significantly different from PC-3 control (n = 3) (corrected p <0.10) were deemed as up or down-regulated.

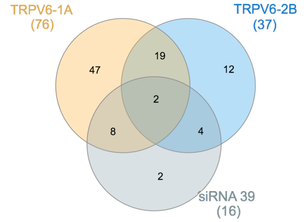
There were 26 genes down-regulated in TRPV6-1A, 27 in TRPV6-2B knockouts, 9 in siRNA 39 knockdown. There were significantly more genes up-regulated in both knockout and knockdown treatments than down-regulated, TRPV6-1A (76), TRPV6-2B (37) and siRNA 39 (16). A total of 23 genes were down-regulated in at least 2 TRPV6 treatments (Table 1) and 33 genes up-regulated in at least 2 TRPV6 treatments (Table 2).



Down-Regulated Genes

Table 1- Down-regulated genes in at least 2 of 4 TRPV6 treatments

Treatments	Total	Down-Regulated Genes
TRPV6-1A, TRPV6-2B, siRNA 39	3	IL6, TRPV6, TXNIP
TRPV6-1A, TRPV6-2B	19	BIRC5, CCL2, CDH1, CEACAM5, CEACAM6, CSF2, CXCL8, E2F1, EGF, ESR1, ESRP1, IL1RN, IL6, MUC1, NOS3, TGFB2, TRPV4, TRPV6, TXNIP
TRPV6-1A, siRNA 39	4	IL6, MYC, TRPV6, TXNIP
TRPV6-2B, siRNA 39	6	IL6, NFKBIA, PDCD1LG2, SERPINE1, TRPV6, TXNIP



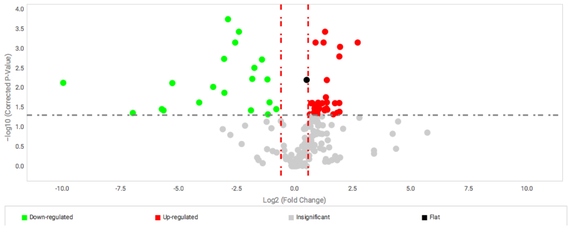
Up-Regulated Genes

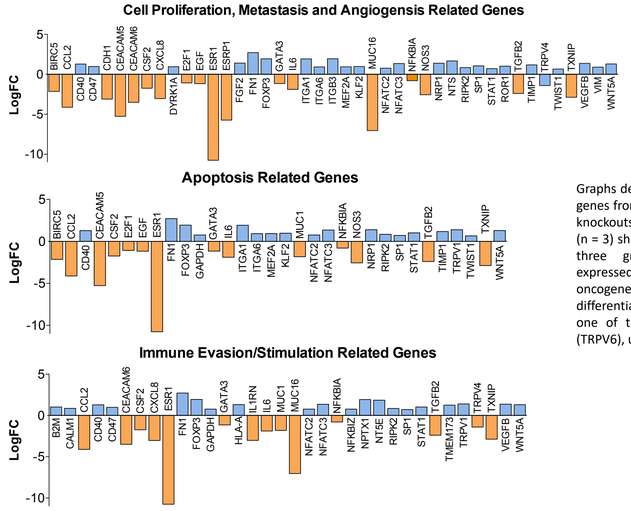
Table 2- Up-regulated genes in at least 2 of 4 TRPV6 treatments

Treatments	Total	Up-Regulated Genes
TRPV6-1A, TRPV6-2B, siRNA 39	2	NTS, ORAI3
TRPV6-1A, TRPV6-2B	21	FGF2, FN1, HLA-A, HRAS, ITGA6, ITGB3, KLF2, NFATC3, NPTX1, NRP1, NT5E, NTS, ORAI3, RIPK2, TIMP1, TMEM173, TRPC1, TRPV1, VEGFB, VIM, WNT5A
TRPV6-1A, siRNA 39	10	BMP1, CSF1, DYRK1A, KDR, MICB, NTS, ORAI3, PRKACA, ROR1, TGFB3
TRPV6-2B, siRNA 39	6	IL7, NFATC4, NTS, ORAI3, STAT1, TAP1

PC-3 TRPV6 Knockouts

The volcano plot shows the 57 differentially expressed genes (> 0.6 LogFC and corrected p < 0.05) when the TaqMan Array data from the two PC-3 TRPV6 knockout cell lines (TRPV6-1A and TRPV6-2B) are pooled (n = 6) and compared to the PC-3 control (n = 3).





Graphs depict the 57 differentially expressed genes from the analysis of the pooled TRPV6 knockouts (n = 6) compared to PC-3 Control (n = 3) shown in the volcano plot above. The three graphs group the differentially expressed genes by functional mechanism of oncogenesis. There were 3 genes differentially expressed but not grouped into one of the three graphs; Down-regulated (TRPV6), up-regulated (TRPC1 and ORAI3).

Summary

- Inhibiting/ablating TRPV6 expression leads to a cascade of dysregulated genes including transcription factors (e.g. ERSP1).
- Knocking out TRPV6 resulted in dysregulation of a high percentage of the genes in the panel (30%), that are involved in proliferation, metastasis, apoptosis, angiogenesis as expected but interestingly genes involved in immune evasion/stimulation.
- The mechanism of action of TRPV6 was confirmed in both CRISPR TRPV6 knockout and siRNA TRPV6 knockdown in castrate-resistant prostate cancer cells (apoptosis, proliferation, metastasis and angiogenesis).
- The TRPV6 knockout and knockdown data points to a central role TRPV6 in oncogenesis of prostate cancer and the opportunity of TRPV6 inhibitors in that field.