

## Role of Six Trans Membrane Protein of Prostate (STAMP) Proteins in Prostate Cancer- Relation with Survival Genes

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### Abstract

Prostate cancer studies focus on identification of androgen receptor (AR) regulated genes that are also highly expressed in the prostate. As a promising candidate, STAMP family genes STAMP1/STEAP2, STAMP2/STEAP4 and STEAP3 are involved in apoptosis and the cell cycle in metastatic prostate cancer. Vascular NADPH oxidase generates superoxide and other ROS, which stimulates IκB degradation and NF-κB activation by subunits of NADPH oxidases, namely p47phox and p67phox induced by different stimuli such as hydrogen peroxide. Hydrogen peroxide increased the expression levels of p67phox. They also have a role in redox-sensitive genes such as STAMP gene family. Flow cytometry analysis of LNCaP cells was performed using Annexin V staining and apoptotic index charts were drawn. STAMP1 and STAMP2 showed total anti-oxidant capacity versus control with hydrogen peroxide incubation. Using siRNA technology in LNCaP cells expressing mutant p53 silencing of p53 showed significant increase in MDM2 and decrease of caspase 9 mRNA levels at RT-PCR. Silencing of STAMP2, a significant decrease in p47phox was shown but STAMP1 silencing counteracted this effect on Cu/ZnSOD expression. As a conclusion, STAMP proteins have effects on oxidative stress-induced genes with significant and opposite changes.

**Keywords:** STAMP 1; STAMP2; STAMP3; Anti-apoptotic marker

### Abbreviations

Cu/Zn SOD: Cu/Zn Super Oxide Dismutase; eNOS: endothelial Nitric Oxide Synthase; HSP90: Heat Shock Protein 90; IKKβ: Inhibitor of NF-κB Kinase-β; MDM2: E3 Ubiquitin Ligase of P53 Tumor Suppressor Protein; NFκB: Nuclear Factor kappa B; STEAP: Six-Trans membrane Epithelial Antigen of Prostate; STEAP2/STAMP1: Six-Trans membrane Epithelial Antigen of Prostate 2/Six-Trans membrane Protein of Prostate 1; STEAP3/ TSAP6: Six-Trans membrane Epithelial Antigen of Prostate 3/ Tumor Suppressor-Activated Pathway 6; STEAP4/ STAMP2/TIARP : Six-Trans membrane Epithelial Antigen of Prostate 4/ Six-Trans membrane Protein of Prostate 2/Tumor Necrosis-Alpha Induced Adipose-Related Protein

### Introduction

Among in all cancer cases, prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer mortality in men in the Western World. STAMP genes that STEAP (six transmembrane epithelial antigen of prostate) [1] is the first characterized prostate enriched six transmembrane genes, expressed in metastatic prostate cancer samples. STAMP1/STEAP2 [2] and STAMP2/STEAP4 [3] are expressed in androgen receptor positive-prostate cancer cell line LNCaP, and androgen receptor-mediated regulation of STAMP2 was shown. Role in metabolic disease and essential position of STAMP2 for prevention of excessive inflammation and protection of adipocyte insulin sensitivity and systemic glucose homeostasis was reported in mice [4]. Other members of the STAMP

family include pHyde, a rat protein that has been implicated in apoptosis of prostate cancer cells [5], and its human homologue TSAP6 (also known as STEAP3), a p53-inducible gene involved in apoptosis and the cell cycle in prostate cancer and HeLa cells [6]. It is tempting to speculate that STAMP/STEAP family genes may be involved in similar functions with a role for both the normal biology and pathophysiology of prostate.

Activation of extracellular signal-regulated kinase (ERK), which has previously been implicated in prostate cancer progression, was reported with ectopic expression of STAMP1 in DU145 cells and, conversely, was strongly down regulated on STAMP1 knockdown in LNCaP cells [7]. Promoter region of the STAMP genes were analyzed and putative presence of tumor suppressor gene p53 and survival gene NFκB response elements were identified and confirmed at the promoter region of STAMP1 and STAMP2 genes [8].

Mammalian cells do not survive in the absence of redox reactions; oxidation and reduction [9]. STAMP2 expression is deregulated in prostate cancer and significantly increases ROS through its iron reductase activity [10]. Reactive oxygen species (ROS) generation is linked to dynamic actin cytoskeleton reorganization, which is involved in tumor cell motility and metastasis [11]. Neutrophil cytosolic factor-1 (NCF1), also known as p47-phox (for phagocyte oxidase) [11-13] and neutrophil cytosolic factor-2 (NCF2), also known as p67-phox (for phagocyte oxidase), is a component of the NADPH oxidase complex [14-16]. It is confirmed that the p67 protein functions in the respiratory burst mediated by NADPH oxidase and that p67 may be complexed to p47-phox (NCF1) while it participates in oxidase activation. It is concluded that, the lack of reactive oxygen species contributes to the hyper inflammatory phenotype associated with

NADPH oxidase deficiencies, through a dysfunctional kynurenine pathway of tryptophan catabolism [17-19]. The SOD1 gene encodes superoxide dismutase-1, a major cytoplasmic antioxidant enzyme that metabolizes superoxide radicals to molecular oxygen and hydrogen peroxide, thus providing a defense against oxygen toxicity [20].

## Materials and Methods

### Cell culture

LNCaP cells were cultured in RPMI 1640 (GIBCO) with 10% fetal bovine serum, 1% L-glutamine and 1 U/ml of each penicillin/streptomycin. Cells were incubated at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere.

### Primer design, plasmid constructs and transfections

Full-length open reading frame of STAMP1, 2 and 3 were amplified using (10 pmol of each) primers which were designed using Light Cycler Probe Design Software 2 (Roche, Germany). The PCR product was cloned into pcDNA4-HisMax-TOPO (Invitrogen) vector according to the manufacturer's recommendations. The inserts were verified by PCR amplifications. All transfections including siRNA were performed using FuGENE HD (Roche, Germany) transfection reagent according to the manufacturer's recommendations. Briefly, cells were seeded in 6-well plates one day prior to transfection. Following day, the transfection solution was prepared in a 1.5 ml tube by 100 µl of pre-warmed RPMI without antibiotics and 1 µg of pcDNA4-HisMax-gene plasmid DNA were added incubated for 5 min. 3 µl of FuGENE HD transfection reagent into the mixture was added on DNA drop-wise and tapping to mix, after incubating for 15 min at room temperature and transfection mix was added on cells drop-wise.

### siRNA mediated knockdown of STAMP1, STAMP2 and p53

LNCaP cells were transfected with scrambled-control or STAMP1 specific siRNA, purchased from Santa Cruz Biotechnology Inc.

(Bergheimer, Germany). The sequences were provided by the manufacturer, labeled as below sc-37007 for control, sc-76587 for STEAP2/STAMP1 (h,) and sc-89820 for STAMP2 siRNA(h) and sc-29435 for p53siRNA(h) 100 pmol of siRNA (final conc. 50 nM) was used to transfect cells with 10 µl Fugene HD (Roche, Germany) and incubated for 1,2 and 5 days for STAMP1 and STAMP2, and 4 days for p53, respectively according to the manufacturer's recommendations.

### Apoptosis-panel primer couples for RT-PCR (Table 1)

Real-time PCR was performed using a Light Cycler® 480 (Roche Diagnostics) instrument and Light Cycler 480 SYBR Green 1 Master (Roche Diagnostics) kit. Briefly, reactions were performed in a 20 µl volume with 5 pmol of each primer and 1 µl of cDNA template derived from reverse-transcribed RNA of untreated ethanol (control) and siSTAMP1 transfected cells. The Homo sapiens glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an endogenous control and reference gene for relative quantifications. The same thermal profile was optimized for all primers: a pre-incubation for 5 min at 95°C for 1 cycle, followed by 40 amplification cycles of denaturation at 95°C for 10 sec, a primer annealing at 64°C for 20 sec, and a primer extension at 72°C for 10 sec. Water was included as a no-template control. Melting curves were derived after 40 cycles by a denaturation step at 95°C for 10 s, followed by annealing at 65°C for 15 sec, and a temperature rise to 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement. A final cooling was performed at 37°C for 30 sec. Melting curve analyses of each sample were performed using LightCycler 480 Software version LCS480 (Roche Diagnostics). The analysis step of relative quantification was a fully automated process accomplished by the software, with the efficiency set at 2 and the cDNA of untreated cells defined as the calibrator.

GeneBank/ Symbol	Description	Gen Name	Primers	
NM_004322 BAD	BCL2-antagonist of cell death	BBC2/BCL2L8	Forward Reverse	AGGATCCGTGCTGTCTCCTTTG CAAACCTCCGATGGGACCAAG
NM_001188 BAK1	BCL2-antagonist /killer 1	BAK/BCL2L7	Forward Reverse	GGGTGTAGATGGGGAACTGTG AAGACCCTAGGCTGTGCCCAAT
NM_138578 BCL2L1	BCL2-like 1	BCL-X/BCL-XL	Forward Reverse	GTGTGAGGAGCTGCTGGCTTG AGCATCAGGCCGTCCAATCTC
NM_001205 BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	NIP1/TRG-8	Forward Reverse	CAGGTTGGATGGAACACAGTGC ATCCCAATGCCAGACCTTCTC
NM_032982 CASP2	Caspase 2, apoptosis-related cysteine protease	CASP-2/ICH-1L	Forward Reverse	TCTCCCATGGTCCCTAGCAAAA AAGGCTCACAACCAACCAAAA
NM_001227 CASP7	Caspase 7, apoptosis-related cysteine protease	CMH-1/ICE-LAP3	Forward Reverse	AAGTGAGGAAGAGTTTATGGCAAA CCATCTTGAAAAAAGTGCCAAA
NM_001229 CASP9	Caspase 9, apoptosis-related cysteine protease	APAF-3/APAF3	Forward Reverse	TCCTGAGTGGTGCCAAACAAAA AGTGGTTGTCAGGCGAGGAAAG

NM_005157 ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1	ABL/C-ABL	Forward Reverse	GGCCTTGAAGACAGAGCAAAGC GGAAGGGACCAGTACCTCATGG
NM_005163 AKT1	V-akt murine thymoma viral oncogene homolog 1	PKB/PRKBA	Forward Reverse	TCCCCCTCAGATGATCTCTCCA CGGAAAGGTTAAGCGTCGAAAA
NM_005427 TP73	Tumor protein p73	P73	Forward Reverse	AGCAGCCCATCAAGGAGGAGTT TCCTGAGGCAGTTTTGGACACA
NM_000546 TP53	Tumor protein p53 (Li-Fraumeni syndrome)	CYS51STOP/P53	Forward Reverse	AGATGGGGTCTCACAGTGTTCG GCGGCCAGGGTATGTACATGA
NM_078467 P21	Homo sapiens cyclin-dependent kinase inhibitor 1A	CDKN1A	Forward Reverse	GGCAGACCAGCATGACAGATT GCGGCCAGGGTATGTACATGA
NM_002392 MDM2	Homo sapiens Mdm2, transformed 3T3 cell double minute 2	HDMX/MGC71221	Forward Reverse	GGGTTTCGACCATTCTCCTG GGCAGATGACTGTAGGCCAAGC
NM_016335 PRODH	Homo sapiens proline dehydrogenase (oxidase) 1	PIG6/HSPOX2	Forward Reverse	TTTTTCACCCACACTTGCGAGA TGTCCCAGGCAGGTATCAGGTT
NM_001101 ACTB	Homo sapiens actin, beta	PS1TP5BP1, beta-actin	Forward Reverse	CAATGTGGCCGAGGACTTTGAT AGTGGGGTGGCTTTTAGGATGG
NM_002046 GAPDH	Homo sapiens glyceraldehyde 3-phosphate dehydrogenase	G3PD, GAPD	Forward Reverse	CATTGCCCTCAACGACCCTTT GGTGGTCCAGGGGTCTTACTCC
NM_152999.3 STAMP1	Homo sapiens STEAP family member 2, metalloreductase (STEAP2), transcript variant 1	STEAP2/ STAMP1	Forward Reverse	ATAGGAAGTGGGGATTTTGC AGATGTCTCAGGTCCACAA
NM_024636.3 STAMP2	Homo sapiens STEAP family member 4 (STEAP4), transcript variant 1	STEAP4/ STAMP2	Forward Reverse	GCACTTACACTGCTTGC CAGTGGTCAAGCCAGTC

**Table 1:** Gene List and Primers of Apoptosis Panel Taken to Real-Time PCR (LC480, Roche) The table lists the names of genes with GenBank reference sequences from National Center for Biotechnology Information (RefSeq) accession numbers and forward and reverse primers used in the quantitative RT-PCR assay.

### Flow cytometry and analysis

Apoptotic response of hydrogen peroxide induction at LNCaP cells was studied by flow cytometry using FACSCanto (BD Biosciences, USA) and analysis software FACS Diva 5.0.

Briefly, cells in growth medium subsequently stained with Annexin V-FITC Apoptosis Dedection kit 1 containing propidium iodide (10 µg/ml). Analysis was performed, and apoptotic index of cells were plotted (mean values).

### Total anti-oxidant capacity

Trolox is used to standardize antioxidants, with all other antioxidants being measured in Trolox equivalents. Measurement of the combined non enzymatic antioxidant capacity of biological fluids and other samples provides an indication of the overall capability to counteract reactive oxygen species (ROS), resist oxidative damage and combat oxidative stress-related diseases. In some cases, the antioxidant contribution of proteins is desired whereas in other cases only the contribution of the small molecule antioxidants is needed. TAC Assay

Kit (Catalog #K274-100), which can measure either the combination of both small molecule antioxidants and proteins or small molecules alone in the presence of our proprietary Protein Mask. Cu<sup>2+</sup> ion is converted to Cu<sup>+</sup> by both small molecule and protein (BioVision Research Products, CA, USA). The Protein Mask prevents Cu<sup>2+</sup> reduction by protein, enabling the analysis of only the small molecule antioxidants. The reduced Cu<sup>+</sup> ion is chelated with a colorimetric probe giving a broad absorbance peak around 570 nm, proportional to the total antioxidant capacity.

### Cell Lysis, Protein Extraction and Western Blotting

For protein extraction, cells were grown on 60 mm culture dishes (Orange, Italy) and washed once with PBS prior to cell lysis. Cells were resuspended in 250 µl of modified RIPA as lysis buffer [10 mM Tris-Cl (pH:8.0), 1% Triton X-100, 0,1% SDS, 0,1% Na deoxycholate, 1 mM EDTA, 1 mM EGTA, 140 mM NaCl] containing protease and phosphatase inhibitors. Cells were collected from culture plates using a cell scraper and transferred to eppendorf tubes. Incubated on ice for 1 hour with pipeting up/down every 10 minutes, centrifuged at 13,000

rpm for 30 minutes and cleared supernatants were collected. Protein concentration was determined using Q-BIT quantitation assay (Invitrogen) where appropriate. SDS-PAGE and western blots were performed under standard conditions using 20 µg of lysate per lane. Proteins were separated on a 10% gel and transferred to PVDF membrane (Amersham, UK) by semi-dry transfer blotter (VWR). PVDF membrane was blocked with 10% dry milk in PBS-T (Phosphate Buffer Saline-sol'n containing 0.1% Tween 20) for 10 minutes. Primary and secondary antibody incubations were carried out using PBS-T containing 0.5% dry milk at 4°C degrees overnight. Membranes were developed using ECL plus reagent (Amersham, UK) for 5 min, and photographed at chemiluminescence FX7 dark room camera (Vilbert L11ourmat, France). The primary antibodies that had been used were purchased from Cell Signaling Technologies (Germany/Austria), Chemicon/ Milipore (CA/ USA) Sigma (Germany) and Santa Cruz Biotechnology Inc. (Bergheimer, Germany), respectively. Antibodies labeled as below beta-actin mouse Sigma A5316, for STEAP2/STAMP1 goat sc- 82367, for TP53 mouse sc-81168, for MDM2 mouse sc-813, for NFkappaB-p65 rabbit #3034, for IKKbeta monoclonal Ab cat# 2370, for eNOS mouse #9572, for HSP90 (E289) #4875, for p47-phox #07-001, for p67-phox #07-002 and for Cu/Zn-SOD #07-403.

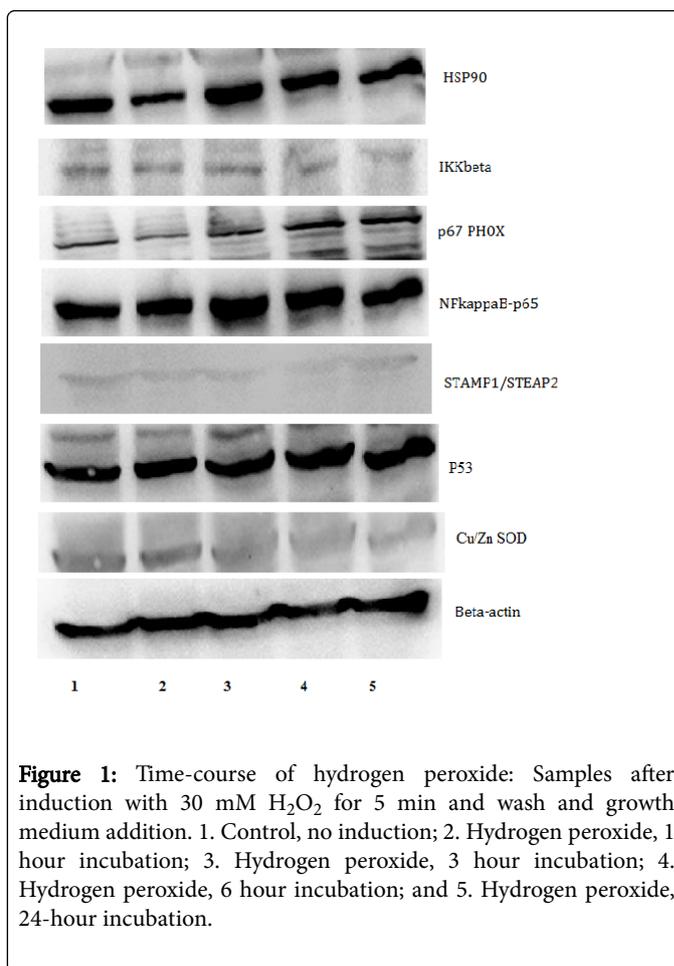
### Statistical analysis

All the illustrated results represent one of at least three independent experiments with similar outcomes.

### Results

Hydrogen peroxide time-course induced oxidant and apoptotic gene samples and repressed anti-oxidant and survival genes

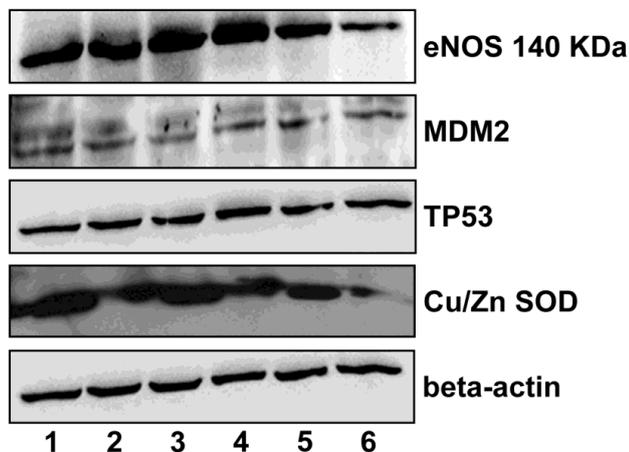
LNCaP cells after induction with 30 mM H<sub>2</sub>O<sub>2</sub> for 5 min, wash and growth medium addition, time-course protein samples from control, 1, 3, 6 and 24 hours were collected and western labeling were done with spesific antibodies. Heat shock protein (Hsp)-90, IKKbeta, p67 PHOX, NFkappa B, STEAP2/STAMP1, P53 and Cu/Zn SOD expression changes were shown at increasing exposure time points to oxidative stress at Figure 1.



**Figure 1:** Time-course of hydrogen peroxide: Samples after induction with 30 mM H<sub>2</sub>O<sub>2</sub> for 5 min and wash and growth medium addition. 1. Control, no induction; 2. Hydrogen peroxide, 1 hour incubation; 3. Hydrogen peroxide, 3 hour incubation; 4. Hydrogen peroxide, 6 hour incubation; and 5. Hydrogen peroxide, 24-hour incubation.

### Transfections of STAMP1 and STAMP3 increased anti-oxidant Cu/Zn SOD expression

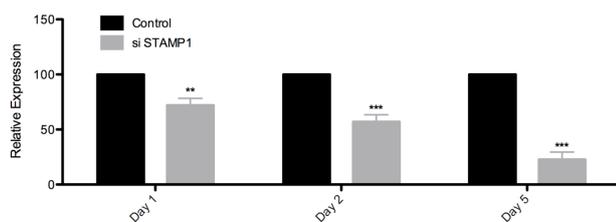
HisMax-STAMP1 and HM-STAMP3 transfections to LNCaP cells followed by hydrogen peroxide induction for 24 hours repressed anti-oxidant gene Cu/Zn SOD levels and significant inhibition of eNOS signalling was seen with STAMP3 transfected sample at Figure 2.



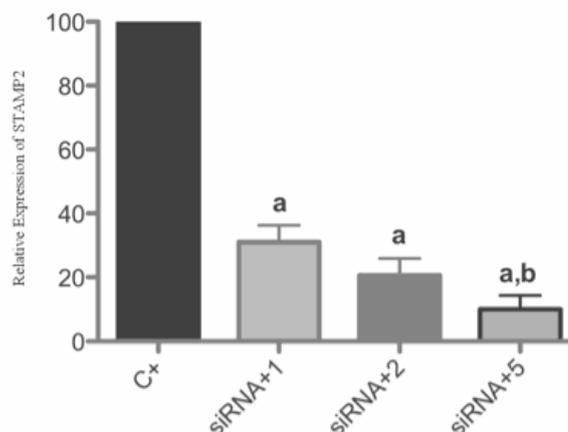
**Figure 2:** Western blot for inflammation-related proteins after transfection with the STAMP1 and STAMP3 constructs. 1. Control, no induction; 2. Control, H<sub>2</sub>O<sub>2</sub> induction; 3. HisMax-STAMP1 transfection, no induction; 4. HM-STAMP1 transfection, H<sub>2</sub>O<sub>2</sub> induction; 5. HM-STAMP3 transfection, no induction; and 6. HM-STAMP3 transfection, H<sub>2</sub>O<sub>2</sub> induction.

### Time-course silencing of STAMP1 and STAMP2 followed by changed responses of oxidant and anti-oxidant genes

A time course of gene silencing was performed with si-STAMP1 and si-STAMP2 for 1, 2 and 5 days post-transfection (Figures 3A and 3B), the strongest silencing occurred at day 5, and the protein extractions were done at the strongest silencing time points.



**Figure 3A:** Gene silencing of STAMP1 at LNCaP cells was performed with a time course for 1, 2 and 5 days post-transfection. Two-way ANOVA followed by Bonferroni Test, \*\*P<0.01, \*\*\*P<0.001, (n=3)

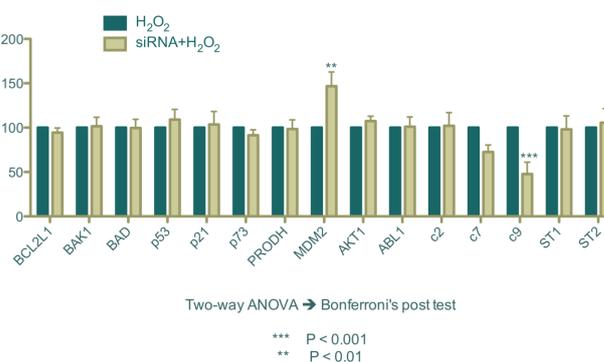


**Figure 3B:** Gene silencing of STAMP2 at LNCaP cells was done 1, 2 and 5 days of transfections. RT-PCR analysis were done with STAMP2 gene, C+ vs siRNA+1 a. P < 0.001, C+ vs siRNA+2; a. P < 0.001, C+ vs siRNA+5 ; a. P < 0.001, siRNA+1.day vs siRNA+5.day b.P < 0.05 ANOVA followed by Tukey's Multiple Comparison Test.

Real-time PCR was performed using a Light Cycler® 480 (Roche Diagnostics) instrument and Light Cycler 480 SYBR Green 1 Master (Roche Diagnostics) kit. Briefly, reactions were performed in a 20 µl volume with 5 pmol of each primer and 1 µl of cDNA template derived from reverse-transcribed RNA of untreated ethanol (control) and siSTAMP1 transfected cells.

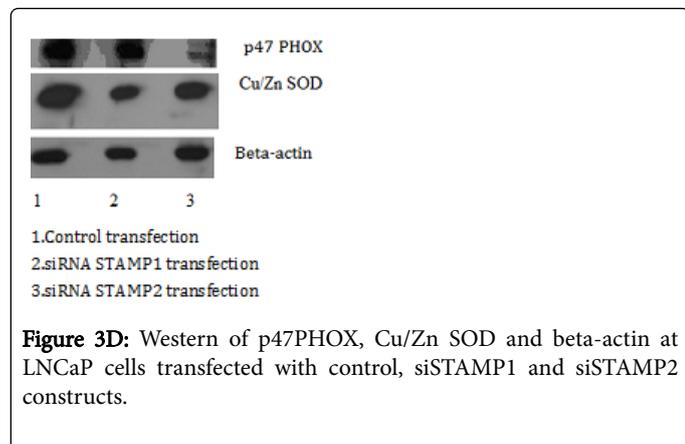
All experiments were performed in triplicate analyzed with apoptosis-panel primer couples at RT-PCR (Table 1).

When LNCaP cells were transfected with sip53 for 4 days, significant up-regulation of MDM2 and Caspase9 decrease with H<sub>2</sub>O<sub>2</sub> induction was shown at RT-PCR (Figure 3C).



**Figure 3C:** LNCaP cells were transfected with sip53, and apoptosis-panel amplifications were performed with samples from -/+ H<sub>2</sub>O<sub>2</sub> induced cells. Two-way ANOVA followed by Bonferroni Test, \*\*\*P<0.001, (n=3)

As a reported anti-apoptotic gene, STAMP1 silencing weakens the anti-oxidant Cu/Zn SOD expression. While STAMP2 silencing caused a significant decrease at oxidant p47PHOX level was taken that confirmed the opposite effect at Cu/Zn SOD at Figure 3D.

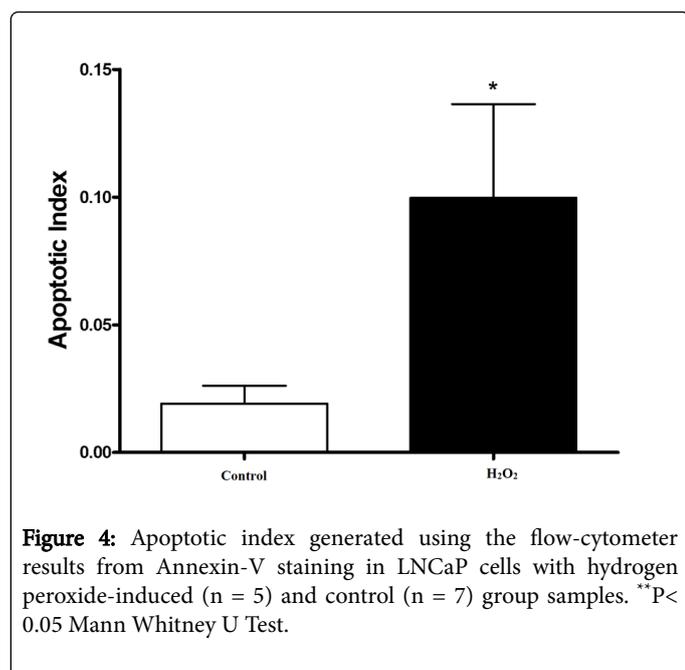


**Figure 3D:** Western of p47PHOX, Cu/Zn SOD and beta-actin at LNCaP cells transfected with control, siSTAMP1 and siSTAMP2 constructs.

### Flow cytometry and analysis

The apoptotic response of with siSTAMP1 in LNCaP cells was assessed by flow cytometry using FACSCanto and analysis software FacsDiva 5.0 (BD Biosciences, USA).

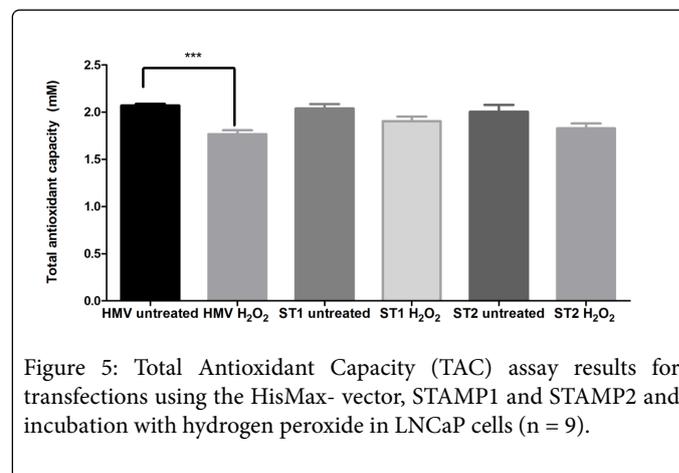
Briefly, cells in growth medium were subsequently stained with an Annexin V-FITC Apoptosis Dedection kit 1 containing propidium iodide (10 µg/ml). The analysis was performed and plotted as histograms (mean values) of live, early-apoptotic and apoptotic cell groups. Annexin-V staining provides to investigate the apoptosis ratio versus different stimuli. In our experiment a statistically significant increase with hydrogen peroxide 24 hours induction was shown at Figure 4.



**Figure 4:** Apoptotic index generated using the flow-cytometer results from Annexin-V staining in LNCaP cells with hydrogen peroxide-induced (n = 5) and control (n = 7) group samples. \*\*P< 0.05 Mann Whitney U Test.

### Total anti-oxidant capacity

STAMP1 and STAMP2 transfected cells versus control were compared after hydrogen peroxide induction and TAC assay kit determined the capacity Figure 5.



**Figure 5:** Total Antioxidant Capacity (TAC) assay results for transfections using the HisMax- vector, STAMP1 and STAMP2 and incubation with hydrogen peroxide in LNCaP cells (n = 9).

### Discussion

Soluble cytoplasmic SOD1 is a copper and zinc-containing enzyme; the SOD1 gene maps to chromosome 21q22 [21]. It is demonstrated that superoxide dismutase catalyzes the oxidation/reduction conversion of superoxide radicals to molecular oxygen and hydrogen peroxide. The name 'superoxide dismutase' comes from the fact that the reaction is a 'dismutation' of superoxide anions. The protein had been known for over 30 years as a copper-containing, low molecular weight cytoplasmic protein identified in erythrocytes, referred to as 'erythrocuprein' or 'hemocuprein.' See review [22]. Inhibition of SOD causes accumulation of cellular superoxide radical and leads to free radical-mediated damage to mitochondrial membranes, the release of cytochrome c from mitochondria, and apoptosis of the cancer cells. It is concluded that targeting SOD1 may be a promising approach to the selective killing of cancer cells and that mechanism-based combinations of SOD inhibitors with free radical-producing agents may have clinical applications [23-30].

Studies reported that STAMP family members have metalloredutase activities associated with iron and copper uptake into HEK-293T cells [31,32] though mentioned activities have not been shown for prostate cells yet.

Taken together, prostate-specific STAMP genes and their regulatory influences on the p53 and caspase-related pathways, oxidative stress relation were characterized. These results will guide us through our future work aiming the control of oxidation/reduction changes on proliferation in neoplastic transformation.

Although, total anti-oxidant capacity of STAMP1 and STAMP2 transfected cells versus control were shown after hydrogen peroxide induction, using siRNA technology in LNCaP cells, silencing of STAMP2, a significant decrease in p47 phox was shown but STAMP1 silencing decreased Cu/ZnSOD expression. STAMP proteins have effects on oxidative stress-induced genes.

The opposite effects of two STAMP genes on oxidation were shown for the first time at prostate cancer lymph node metastasis cell line-LNCaP.

## Author Contributions

Conceived and designed the experiments: CGK. Performed the experiments: CGK, GS, GG, MZA, GY, LV, BR, AK, MSA, DO analysed the data: MZA, GG. Contributed reagents/materials/analysis tools: CGK, GG, MZA, GY, LV, Wrote the paper: CGK, BR.

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