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Gene expression profiling in R-flurbiprofen-treated prostate cancer: R-Flurbiprofen regulates prostate stem cell antigen through activation of AKT kinase

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ABSTRACT

We have used gene expression profiling to characterize genes regulated by the anti-tumor non-steroidal anti-inflammatory drug (NSAID)-like agent *R*-flurbiprofen (RFB) in murine TRAMP prostate cancer. Mice with spontaneous, palpable tumors were treated with RFB 25 mg/(kg d) × 7d orally, or vehicle only. RNA was then extracted from tumor tissue and used for microarray analysis with Affymetrix chips. Fifty-eight genes were reproducibly regulated by RFB treatment. One of the most highly up-regulated genes was prostate stem cell antigen (*psca*). We used TRAMP C1 murine prostate cancer cells to examine potential mechanisms through which RFB could regulate *psca*. RFB induced dose-dependent expression of PSCA protein, and activity of the *psca* promoter, in TRAMP C1 cells in culture. Increased *psca* promoter activity was also seen following treatment of cells with sulindac sulfone, another NSAID-like agent, but not with celecoxib treatment. RFB activation of the *psca* promoter could be attenuated by co-transfection of dominant-negative *akt* and *h-ras* constructs, but not by dominant-negative *mek1* plasmids. Immunoblotting revealed that RFB increased expression of phosphorylated AKT at concentrations that stimulated *psca* promoter activity, and that increased PSCA protein expression. In addition, RFB-dependent up-regulation of PSCA protein expression could be blocked by AKT inhibitors. These data demonstrate that RFB, and possibly other NSAID-like analogs, can increase expression of the *psca* gene both *in vivo* and in culture. They further suggest the utility of combining RFB with AKT inhibitors or with monoclonal antibodies targeting PSCA protein, for treatment or prevention of prostate cancer.

1. Introduction

New therapeutic approaches to the prevention and treatment of prostate cancer are of great interest, due to the high prevalence the disease and its poor response to conventional anti-tumor agents. Many data support the development of non-steroidal anti-inflammatory drugs (NSAIDs) as agents that could effect both the prevention and the treatment of

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prostate cancer [1–3]. Clinical development of NSAIDs has been limited, however, by the toxicities of the available drugs, such as aspirin, ibuprofen, and indomethacin. The recent development of less toxic NSAID-like drugs, sulindac sulfone (exisulind) and the cyclooxygenase (COX)-2 selective inhibitors such as celelcoxib, and exisulind, has renewed interest in these agents for chemoprevention and treatment of prostate cancer and other epithelial malignancies [4].

While the anti-tumor activity of such NSAID-like compounds is clear, the molecular mechanisms through which these drugs act are not. Inhibition of cyclooxygenase enzymes does not appear to be the primary mechanism [5–7]. Alternate molecular targets for NSAIDs have also been proposed. These include transcription factors such as NFκB [8], AP-1 [9], and PPARγ [10]; the AKT [11] and RSK2 [12] kinases; apoptosis-inducing proteins such as BAX [13] and PAR-4 [14]; and alternative arachidonic acid enzymes such as 15-LOX-1 [15]. Few studies compare various NSAIDs in the same assay system, preventing clear distinctions among the various agents. Furthermore, the observed effects are usually described from tissue culture systems, and occur at drug concentrations significantly higher than can be achieved in animals or patients. Not surprisingly, there are discrepancies between *in vitro* and *in vivo* anti-tumor effects of NSAIDs [16]. The rare studies that have identified NSAID-regulated targets in intact animals have focused on a small number of proteins. Thus, NS398, celecoxib, and rofecoxib have also been shown to decrease COX-2 protein in various tissues [17–19]. Rofecoxib can also decrease several proteins implicated in cancer development in a murine xenograft model, such as cyclin D1, β-catenin, and metalloproteinases [20]. However, there are no publications describing genome-wide searches for NSAID targets in animal models or patients.

Recently, we have investigated another NSAID-like compound, *R*-flurbiprofen (RFB, E-7869, MPC-7869), for potential use in the treatment or prevention of cancer. As with other drugs in the aryl propionic acid class, flurbiprofen can exist as *R*- and *S*-enantiomers that differ significantly in their biologic properties. The *S*-enantiomer is many times more active than RFB at inhibiting both COX-1 and COX-2. This may account for the near-complete lack of ulcerogenic activity of RFB, when tested in an animal model [21]. In spite of its lack of gastrointestinal toxicity, RFB demonstrates marked anti-proliferative activity [22]. In the APC^{min}/+ model of intestinal polyposis, RFB was able to both prevent the appearance of adenomas, and block the growth of established neoplasms, leading to a dramatic increase in lifespan of the treated animals [23,24]. RFB also strongly suppressed the development of both primary and metastatic adenocarcinomas in transgenic adenocarcinoma of murine prostate (TRAMP) mice [25].

Because of the lack of molecular data derived from clinically relevant models, we have performed gene expression profiling of TRAMP murine prostate cancers excised from mice treated for 7 days with RFB, to investigate early gene expression responses. We identified 58 genes that were reproducibly regulated by RFB. Several of these genes encoded tumor suppressors, differentiation markers and genes whose expression is lost during transformation, but some of up-regulated genes were associated with cell growth and survival, including the gene for prostate stem cell antigen, *psca*.

2. Materials and methods

2.1. Mice

TRAMP mice were derived from our breeding colony. The original breeding stock was kindly provided by Dr. Norm Greenberg (Fred Hutchinson Cancer Research Center) [26]. Breeding and use of the mice were under the auspices of an IACUC-approved protocol. Mice were used for study when they had palpable suprapubic tumor masses. RFB powder was suspended in 1% carboxymethylcellulose (CMC), then administered by gavage at 25 mg/(kg d) × 7d. Vehicle-treated mice received a similar volume of 1% CMC only.

2.2. Reagents

Pharmaceutical grade RFB (99% chirally pure) was synthesized as described [21].

2.3. RNA extraction and microarray analysis

Tumor tissue was excised and rinsed briefly with PBS. It was then cut into 3–4 mm cubes and immediately immersed in RNALater preservative solution (Ambion). Samples were stored in RNALater at −20 C for up to 1 week before RNA extraction. For RNA extraction, the RNALater was aspirated from the preserved tissues, and Trizol reagent was added. Tissues were homogenized in Trizol (Invitrogen) with a Tissue-Tearor instrument. RNA extraction then proceeded as per manufacturer's instruction, with the addition of a final, additional acid phenol–chloroform extraction, followed by ethanol precipitation. Satisfactory RNA preparations had an OD_{260}/OD_{280} ratio of 1.8–2.0, and showed intact 18S and 28S bands by agarose gel electrophoresis. Four biological replicates and two technical replicates were performed for each treatment condition. Two drug-treated animals and two vehicle-treated animals were used for each of two independent experiments, performed approximately 4 months apart. In each experiment, equal amounts of RNA from the two similarly treated animals were combined to create a drug-treated pool or vehicle-treated pool of RNAs.

First and second strand cDNA were synthesized from 5–15 μg of total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Gibco Life Technologies) and oligo-dT_{24}-T7 (5′-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-3′) primer according to the manufacturer's instructions. cRNA was synthesized labeled with biotinylated UTP and CTP by *in vitro* transcription using the T7 promoter-coupled, double-stranded cDNA as template and the T7 RNA Transcript Labeling Kit (ENZO Diagnostics Inc.). Briefly, double-stranded cDNA synthesized from the previous steps were washed twice with 80% ethanol and resuspended in 22 μL Rnase-free H_2O. The cDNA was incubated with 4 μL of 10× each Reaction Buffer, Biotin Labeled Ribonucleotides, DTT, Rnase Inhibitor Mix and 2 μL 20× T7 RNA Polymerase for 5 h at 37 °C. The labeled cRNA was separated from unincorporated ribonucleotides and precipitated at −20 °C for 1 h to overnight.

The cRNA pellet was resuspended in 40 μL Rnase-free H_2O and 10.0 μg was fragmented by heat and ion-mediated hydrolysis at 95 °C for 35 min in 200 mM Tris–acetate,

pH 8.1, 500 mM KOAc, 150 mM MgOAc. The fragmented cRNA was hybridized for 16 h at 45 °C to U74Av2 oligonucleotide arrays (Affymetrix; [27]). Arrays were washed at 25 °C with 6× SSPE (0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA + 0.01% Tween 20) followed by a stringent wash at 50 °C with 100 mM MES, 0.1 M [Na^+], 0.01% Tween 20. The arrays were then stained with phycoerythrein conjugated streptavidin (Molecular Probes) and the fluorescence intensities were determined using the GeneChip Scanner 3000 (Affymetrix). The scanned images were analyzed using GeneChip Operating Suite (Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to constant target intensity (250) for all arrays used. The expression data were analyzed as previously described [28]. The signal intensity for each gene was calculated as the average intensity difference, represented by [Δ(PM − MM)/(number of probe pairs)], where PM and MM denote perfect-match and mismatch probes. A number of housekeeping and spike in control transcripts were used to determine hybridization efficiency including Murine GAPDH, B-Actin, BioB, BioC, BioD, and Cre. Of the 12,489 probe sets contained in the U74Av2 chip, 58.2 ± 6.4% showed positive expression in the vehicle-treated samples and 61.3 ± 7.8% were positive in the RFB-treated samples. Genes whose expression was increased or decreased by more than two-fold in drug-treated animals (compared with vehicle-treated controls) in each of the two experiments were considered to be reproducibly regulated by the drug administration.

2.4. RT-PCR

Microarray results were validated by RT-PCR, using the original RNAs from the first gene expression profiling experiment as templates. Single-stranded cDNA was synthesized from RNA templates by a commercially available kit (Superscript III, Invitrogen). Primers used included the following—β-actin: sense 5′-GTGGGCCGCTCTAGGCACCA-3′, antisense 5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′; prostate stem cell antigen: sense 5′-CACAGCACAGATGACAACAGAGAC-3′, antisense 5′-TGAGCAGAACTTGGTAGGGCAC-3′; seminal vesicle autoantigen: sense 5′-AATGAACCCCGTAACTACACACTCACA-3′, antisense 5′-ACACACAATCCTCTCCAAGAGTACG-3′; cyp24: sense 5′-CGGAATCCCCAAGTGCAAC-3′, antisense 5′-TACCAG-GATGCCAAGATGCAG-3′; COX-2: sense 5′-GGACTACTATG-TACCTCCCCTTTGGA-3′, antisense 5′-TCCTATGCAGTCTGCT-TTATGCG-3′; SV40 TAg: sense 5′-TGTTTGGTTCTACAGGCTCT-GCTG-3′, antisense 5′-TCACTGCGTTCCAGGCAATG-3′. Amplification cycle number was varied to ensure that the results were derived from the linear portion of the amplification/product curve. Amplified fragments were visualized on agarose gels after ethidium bromide staining.

2.5. Cell lines

LNCaP cells were obtained from the ATCC, and grown in RPMI1640 medium with 10% iron-supplemented calf serum. TRAMP C1 cells were the kind gift of Dr. Norm Greenberg and were maintained in Dulbecco's medium supplemented with 10 pM dihydrotestosterone, 10% calf serum, and 5 μg/mL insulin.

2.6. Promoter studies

A 9 kb fragment of the human PSCA promoter was isolated, characterized, and inserted into a firefly luciferase-based reporter plasmid as previously described [29]. Transient transfections were performed as follows: target cells (80% confluent), grown in 6 cm plates, were transfected with Superfect reagent (Qiagen) and various plasmids per manufacturer's directions. Two micrograms of *psca* reporter plasmid and 2 μg of other plasmids (dominant negative signaling molecules or pCDNA3) were used per plate. Twenty hours after transfection the cells were trypsinized and replated into 24-well plates. Six hours later, test drugs were added, and incubation was continued for an additional 16 h. Cells were then assayed for firefly luciferase activity using Promega reagents. Briefly, lysates were prepared with Passive Lysis Buffer, and dispensed into 96-well plates, such that each treatment/plasmid combination was assayed in triplicate. Luciferase activity was then recorded using a plate luminometer (Microlumat PLUS [Berthold Technologies]). Luciferase activity was normalized to total protein concentrations, as measured by the Bradford method. Plasmids used included dominant-negative *akt* (Upstate Biological), dominant-negative *h-ras* (courtesy of A. Kraft, Medical University of South Carolina), and dominant-negative *mek1* (courtesy of E. Krebs, UWashington) constructs.

2.7. Immunoblotting

Lysates of TRAMP C1 cells treated, or not, with RFB, were prepared by extracting the cells with a non-denaturing lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and 5 mM sodium glycerophosphate). Lysate protein was quantitated by the BCA method (Pierce Chemical Co.). Proteins were resolved on SDS:PAGE gels, transferred to polyvinylidene difluoride membranes, and probed with various antibodies. Rabbit polyclonal antibodies to phosphoAKT (S473), and to total AKT, were purchased from Active Motif or Cell Signaling. An anti-PSCA antibody was obtained from Santa Cruz. A monoclonal antibody to β-actin was from Sigma. Immune complexes were identified by chemiluminescence after labeling with a horseradish peroxidase-coupled second antibody.

3. Results

3.1. Identification of differentially regulated genes in TRAMP prostate cancers treated with RFB

Two separate gene expression profiling experiments were performed, approximately 4 months apart. In the first experiment, 264 differentially expressed genes were identified in tumor from drug-treated animals. These genes showed two-fold or more increased or decreased expression compared with tumor from vehicle-treated animals. In the second experiment, 346 differentially expressed genes were identified. When the data sets were merged to find genes whose

Table 1 – Genes whose expression is reproducibly decreased following RFB treatment of TRAMP mice

Gene name	GenBank #	Fold-decrease mean (±range)	GenMAPP/KEGG classification
Cyp24a1	D89669	13.5 ± 3.6	
Acta1	M12347	8.95 ± 0.95	
Enpp2	AW122933	8.15 ± 3.25	Nicotinate/nicotinamide metabolism; pantothenate/CoA biosynthesis; purine metabolism; riboflavin metabolism; starch/sucrose metabolism
Crisp1	M92849	6.35 ± 4.25	
Defb1	AF003525	5.25 ± 1.75	
Saa3	X03505	5.20 ± 0.9	
Unknown	AW045808	5.05 ± 1.05	
Ptgs2	M88242	4.45 ± 0.15	Arachidonic acid metabolism
Eln	AA919594	3.85 ± 1.05	
Gpx3	U13705	3.45 ± 0.85	Aracidonic acid metabolism; glutathione metabolism
Folr1	M64782	3.35 ± 0.35	
Arg1	U51805	3.25 ± 0.25	Arginine/proline metabolism; urea cycle
Akr1b7	J05663	3.15 ± 0.55	Fructose/mannose metabolism; galactose metabolism; glycerolipid metabolism; pentose/glucuronate interconversion; pyruvate metabolism
Ccnd1	AI849928	3.00 ± 0.00	Cell cycle; focal adhesion; JAK-STAT pathway; Wnt signaling pathway
Ace	AV258262	2.90 ± 0.10	Ganglioside biosynthesis; glycan structures-biosynthesis
Aldh7a1	AI835461	2.75 ± 0.75	Arginine/proline metabolism; ascorbate/aldarate metabolism; bile acid biosynthesis; fatty acid metabolism; glycerolipid metabolism; glycolysis/gluconeogenesis; histidine metabolism; limonene/pinene degradation; lysine degradation; propanoate metabolism; pyruvate metabolism; tryptophan metabolism; Val/Leu/Isoleucine degradation; ala metabolism
MMTV env	Z22552	2.70 ± 0.60	
Ces3	AW226939	2.65 ± 0.35	Alkaloid biosynthesis
Twist1	M63649	2.65 ± 0.55	
Kcnn4	AF042487	2.55 ± 0.05	
Pcp4	X17320	2.50 ± 0.00	
Penk1	M55181	2.35 ± 0.25	
Smoc2	AA980164	2.20 ± 0.10	
Lgals9	AV049898	2.05 ± 0.05	

expression was increased or decreased by two-fold or more in both experiments, we identified 58 genes (Tables 1 and 2). Genes whose expression changed by only two- to three-fold in one experiment were often not detected in the other. However, the most highly regulated genes were repeatedly detected in the expression profiles, suggesting that they may truly be regulated *in vivo* by RFB treatment.

To determine if RFB regulated genes through drug modulation of the expression of the transforming SV40 TAg, we examined similarly treated TRAMP tumors by immunohistochemistry. Tumors derived from vehicle- and drug-treated mice expressed similar levels of SV40 TAg protein, suggesting that gene regulation was not a byproduct of changes in oncogene expression (data not shown).

Confirmation of differential gene expression was sought by RT-PCR, using RNA from TRAMP tumors of drug- or vehicle-treated mice (Fig. 1). We used the β-actin gene and SV40 TAg as control targets to demonstrate that there were equal amounts of total RNA and tumor-derived RNA in each sample. Primers were prepared for several of the most highly regulated genes. All (5/5) of the tested down-regulated genes were confirmed to be suppressed by RFB. In addition, 50% of the tested up-regulated genes (2/4) were seen by RT-PCR to be up-regulated in tumors from RFB-treated mice. Interestingly, one of the most highly up-regulated genes was *psca*, encoding prostate stem cell antigen.

3.2. *RFB increases expression of psca promoter activity and protein in prostate cancer cells*

To examine potential mechanisms through which RFB could regulate *psca*, we studied TRAMP C1 cells, derived from a spontaneous TRAMP prostate cancer. RFB treatment produced a dose-dependent decrease in the proliferation of TRAMP C1 cells, consistent with its known anti-proliferative effects in animals (Fig. 2A; [22,24,25]). Over the same concentration range (25–200 μg/mL), we saw a dose-dependent increase in PSCA protein expression (Fig. 2B). We also examined RFB effects on the activity of the *psca* promoter. TRAMP C1 cells were transiently transfected with a *psca* promoter construct which controlled a firefly luciferase gene (Fig. 2C). RFB treatment of the transfected cells produced a biphasic response curve, with maximal reporter gene expression at a RFB extracellular concentration of 50–100 μg/mL. These maximally active concentrations of RFB mimic peak serum levels in drug-treated humans and mice [25]. Higher RFB concentrations had little or no effect on *psca* promoter activity. Another prostate cancer cell line, LNCaP, also showed a statistically significant increase in *psca* promoter activity after RFB treatment (Fig. 2D), though to a much lower degree than that seen in TRAMP C1 cells.

To determine if regulation of *psca* promoter activity was induced by another NSAID-like agents or by a COX-2 selective

Table 2 – Genes whose expression is reproducibly increased following RFB

Gene name	GenBank #	Fold-increase mean (±range)	GenMAPP/KEGG classification
Sva	L44117	8.75 ± 6.15	
Psca	AW209486	7.85 ± 4.35	
Hsd11b2	X90647	7.75 ± 2.85	Androgen/estrogen metabolism; C21-steroid hormone metabolism; prostaglandin synthesis
Msmb	U89840	6.75 ± 1.85	
Pdzk1	AW260404	6.10 ± 3.10	
Tgoln2	D50032	5.75 ± 1.75	
Tyki	L32973	5.55 ± 0.95	
Rhou	AV246963	5.25 ± 1.25	
Usp18	AW047653	4.75 ± 2.25	
Anp32a	U73478	4.20 ± 0.70	
Apof	AI528149	4.00 ± 0.00	
Ceacam1	M77196	3.90 ± 1.40	
Egf	V00741	3.65 ± 0.65	Cytokine-receptor interactions; focal adhesion; GAP junction; MAPK signaling pathway; regulation of actin cytoskeleton
Nfkbiz	AA614971	3.45 ± 0.25	
Xlr3b	L22977	3.30 ± 1.30	
Bnip3	AF041054	3.15 ± 0.35	Apoptosis
Ifit2	U43085	3.00 ± 1.00	
Gsto1	AI843119	2.85 ± 0.85	Glutathione metabolism; xenobiotic metabolism by CYP450
Lgals3	X16834	2.65 ± 0.55	
Cyp2f2	M77497	2.55 ± 0.05	Xenobiotic metabolism by CYP450
Pik3c3	AI847699	2.55 ± 0.45	Apoptosis; B cell receptor signaling; inositol phosphate metabolism; JAK-STAT signaling pathway; phosphatidylinositol signaling; regulation of actin cytoskeleton; regulation of autophage; T cell receptor signaling pathway; toll-like receptor signaling pathway
Sprr2a	AJ005559	2.55 ± 1.15	
Apod	X82648	2.50 ± 0.00	
Lmo7	AW047919	2.45 ± 0.35	
Anxa1	M69260	2.45 ± 0.35	
Hells	U25691	2.40 ± 0.10	
Anxa3	AJ001633	2.40 ± 0.40	
Cmas	AJ006215	2.35 ± 0.25	Aminosugars metabolism
Birc5	AB013819	2.30 ± 0.30	
Cited2	Y15163	2.30 ± 0.00	
Sox2	X94127	2.30 ± 0.30	
Unknown	AI849676	2.20 ± 0.10	
Tyms	M13352	2.10 ± 0.00	One carbon pool/folates; pyrimidine metabolism
Papss2	AF052453	2.05 ± 0.05	Purine metabolism; sulfur metabolism; selenoaminoacid metabolism

inhibitor, we treated TRAMP C1 cells with sulindac sulfone or celecoxib, as well as RFB (Fig. 3). The dose of celecoxib was lower, on a molar basis, than that of sulindac sulfone or RFB, due to its higher toxicity. Both RFB and sulindac sulfone increased *psca* promoter activity, although the effect of sulindac sulfone was not statistically significant. In contrast, celecoxib did not increase luciferase activity in the transfected cells, showing that it was unable to activate the *psca* promoter.

3.3. RFB activates psca promoter activity by an akt-dependent mechanism

To identify potential signaling pathways that might be regulated by RFB, we co-transfected the *psca* promoter construct into TRAMP C1 cells with a variety of plasmids encoding dominant negative kinases and other signaling molecules. No inhibition of the RFB-induced activation of *psca* promoter activity was seen following co-transfection of inhibitory *pak1*, *jak2*, or *pkc-theta* constructs (data not shown). A dominant-negative form of the *h-ras* gene inhibited the activation of the *psca* promoter, though the magnitude of the effect was not statistically significant (Fig. 4). The *h-ras* gene activates several downstream pathways, especially MEK-MAPK cascades and the AKT pathway. TRAMP cells transfected with a dominant-negative *akt* plasmid still showed an increase in *psca* promoter activity when treated with RFB. However, the magnitude of increase was significantly less than that seen in cells transfected with a control plasmid. A construct encoding a dominant-negative form of MEK1 protein had no inhibitory effect.

3.4. RFB increases activation of AKT protein

Since a dominant-negative *akt* construct attenuated the activation of the *psca* promoter by RFB, we examined TRAMP C1 cells directly for evidence that the drug activated AKT activity. TRAMP C1 cells were treated with RFB for intervals up to 24 h. Lysates were then used for immunoblotting with a variety of antibodies (Fig. 5). The drug increased PSCA expression by 3 h, with elevated levels of PSCA protein persisting for at least 12 h. Changes in the amount of phosphoAKT (S473) paralleled those of PSCA, while levels of

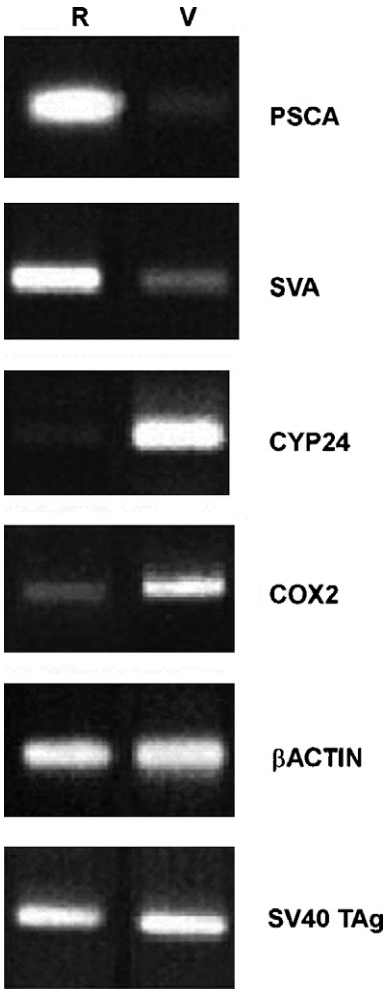

Fig. 1 – Verification of differential gene expression by RT-PCR. R, RFB by gavage, 25 mg/(kg d) × 7d; V, 1% carboxymethylcellulose by gavage, 0.1 mL/d × 7d. Amplified DNA was examined by agarose gel electrophoresis. Amplification of the cDNAs with primers for the β-actin and SV40 TAg genes ensured that there were equal amounts of total and tumor-derived cDNA in each amplification reaction. PCR results using primers for two genes whose expression was increased by RFB (*psca*, *sva*) and for two whose expression was decreased by RFB (*Cyp24*, *Ptgs2* [COX-2]) are presented here.

total AKT and β-ACTIN were stable over the experiment. The induction of AKT phosphorylation and PSCA expression by RFB was transient, returning to pretreatment levels by 24 h of drug exposure.

3.5. *RFB increases PSCA expression through an AKT-dependent mechanism*

To establish a direct relationship between activation of AKT and expression of *psca*, we used a variety of small-molecule inhibitors to perturb the function of AKT and other kinases (Fig. 6). TRAMP C1 cells treated with RFB showed the expected increase in PSCA and phosphoAKT (S473) proteins. Pretreatment of the cells with AKT1/2, an inhibitor of AKT kinases [30], substantially blocked the drug-induced increase in phosphoAKT (S473) protein as well as PSCA protein. In contrast, rapamycin (an inhibitor of the mTOR kinase [31]), quercetagetin (an inhibitor of the PIM1 kinase [32]), and BPIQ-II (an inhibitor of the EGFR kinase [33]) did not inhibit AKT activity and had minimal effects on the levels of PSCA protein.

4. Discussion

Gene expression profiling has become an extremely useful method for identifying differentially expressed genes without prior knowledge of drug effects. This methodology has previously been utilized to identify genes regulated by anti-tumor NSAIDs in tissue culture studies [34–37]. However, our report is the first to document gene expression profiles of spontaneous murine prostate cancers treated *in vivo* with clinically relevant doses of an anti-tumor NSAID-like agent—in this case, RFB.

Our studies identified 58 genes as being reproducibly up-regulated or down-regulated in tumor tissue following 7 days of treatment of tumor-bearing mice. We used a RFB dose that has been shown to have anti-proliferative and anti-tumor effects in transgenic animal models [24,25]. The choice of treatment duration was based on previous studies that showed biologic effects of RFB or various NSAIDs in tumor xenografts and normal tissues within a few days of treatment initiation [16,19,21,22].

The down-regulated genes include several known to be overexpressed, or otherwise implicated, in cancer development, such as *Cyp24a1* (vitamin D3 24-hydroxylase; [38]), *Enpp2* (autotaxin; [39]); *Saa3* (serum amyloid protein A3; [40]), *Ptgs2* (COX-2; [41]), *Gpx3* (glutathione peroxidase 3; [42]), *Folr1* (folate receptor 1; [43]), *Arg1* (liver arginase; [43]), *Ccnd1* (cyclin D1; [44]), and *Twist 1* (twist 1 homolog; [45]). Decreased expression of these genes would not be surprising following treatment with an anti-proliferative agent. Two of these genes, those for cyclin D1 and COX-2, have also been reported to be down-regulated in tissues or tumors from animals treated with the COX-2 inhibitors celecoxib and rofecoxib [19,20]. Signaling pathways that could contribute to the down-regulation of these genes remain to be identified.

The set of up-regulated genes in tumors from RFB-treated animals contains tumor suppressor genes, differentiation markers, and genes whose expression is lost during transformation. These include *Sprr2a* [46], *Lgals3* (galectin 3; [47]), *Papss2* [48], *Usp18* [49], *Ceacam1* [50], *Bnip3* [51], *Anp32a* (PHAP; [52]), *Msmb* (PSP94; [53]), *Wrch1* (Wnt-responsive CDC42 homolog 1; [54]), and *Pdzk1* [55]. These observations suggest that RFB can act as a differentiation-inducing agent. Interestingly, several additional up-regulated genes are typically associated with cell growth and survival. We decided therefore to examine some of the mechanisms through which RFB can increase gene expression.

One of the most highly up-regulated gene in tumors from the RFB-treated animals was *psca*—prostate stem cell antigen. This gene encodes a 123-amino acid, GPI-anchored cell surface protein that is a member of the Thy-1/Ly-6 superfamily. Expression of the PSCA protein is largely restricted to the genitourinary and gastrointestinal tracts [56]. In the prostate

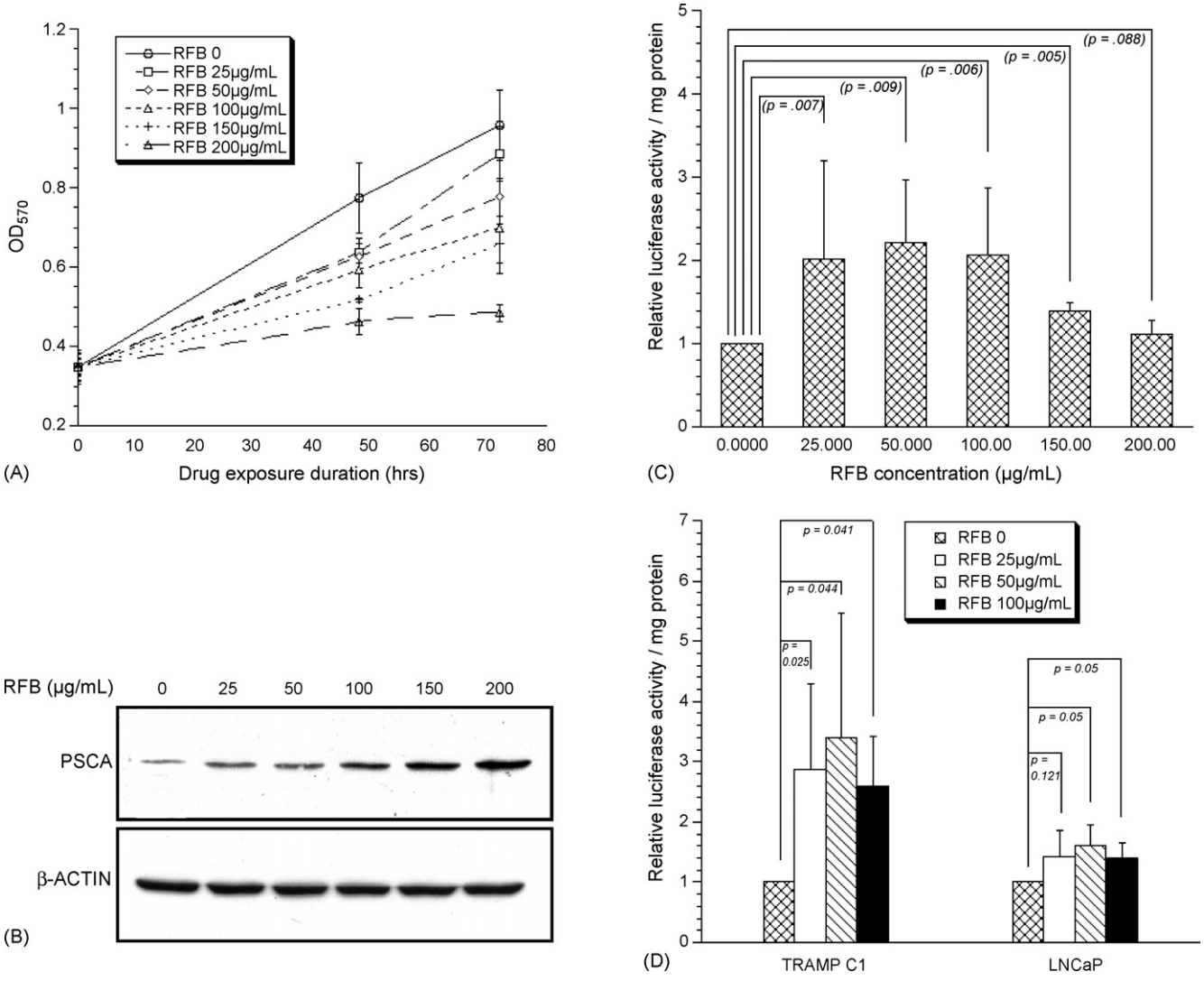

Fig. 2 – (A) Inhibition of TRAMP C1 cell growth by RFB. Cells were plated in 12-well plates at 8×10^4 cells per well, and allowed to grow for 12 h. RFB or vehicle were then added, and culture continued for up to 72 h. Cell number was quantitated at intervals by crystal violet staining. Each point represents the mean of the means (±S.E.M.) from three independent experiments. Growth inhibition was statistically significant at both 48 and 72 h for cells treated with RFB 50 μg/mL ($p = 0.041$, 0.027), 100 μg/mL ($p = 0.05$, 0.013), 150 μg/mL ($p = 0.038$, 0.004), and 200 μg/mL ($p = 0.006$, 0.008). *p*-Values were calculated by use of T tests. (B) Dose-dependent increase in PSCA protein by RFB in TRAMP C1 cells. Cells were treated with the indicated dose of RFB for 4.5 h, then lysates were prepared for immunoblotting. (C) Dose-dependent enhancement of *psca* promoter activity by RFB in TRAMP C1 cells. TRAMP C1 cells were transfected with the *psca* promoter plasmid. Twenty hours later, the cells were trypsinized and plated into 24-well plates. After 6 h incubation, cells were exposed to RFB or vehicle for an additional 16 h. Luciferase activity was then measured with Promega reagents and a fluorescent plate reader. Each bar represents the mean (±S.E.M.) from three independent experiments. *p*-Values were calculated by paired T tests. (D) Enhancement of *psca* promoter activity by RFB in other prostate cell lines. Cells were treated as described under (C). Each point represents the mean (±S.E.M.) of measurements from three to four independent experiments. *p*-Values were calculated by paired T tests.

psca is regulated by both androgen-dependent and androgen-independent mechanisms [28] and is a marker of late intermediate prostate epithelial cells [57]. Expression increases during the development of carcinomas, and correlates with adverse prognostic indicators such as high Gleason score, seminal vesicle invasion, or capsular penetration [58]. Monoclonal antibodies to PSCA have been shown to retard the growth of prostate cancer xenografts in immunosuppressed mice [59].

Our data indicate that RFB can increase *psca* expression through a mechanism involving *akt* (and possibly *ras*), but not *mek1*. This conclusion is demonstrated by correlative studies of protein expression, as well as by the use of genetic and pharmacologic inhibitors of AKT kinase activity. We therefore have established a linear relationship between AKT kinase activity and *psca* promoter activity and protein levels. Our observations are in agreement with previous studies that

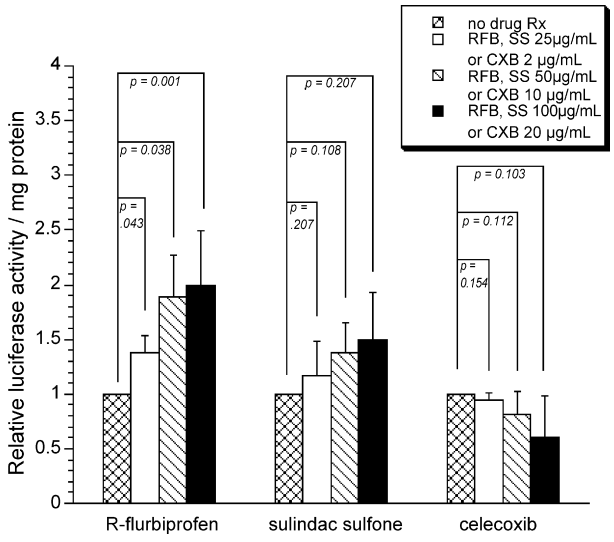

Fig. 3 – Regulation of *psca* promoter activity in TRAMP C1 cells by various anti-tumor NSAIDs. TRAMP C1 cells were transfected with *psca* promoter plasmid and exposed to drugs as described under Fig. 2C. Celecoxib was used at a different dose range because of its higher cytotoxicity compared with sulindac sulfone and RFB. Each point is the mean (±S.E.M.) from three independent experiments. *p*-Values were calculated by paired T tests.

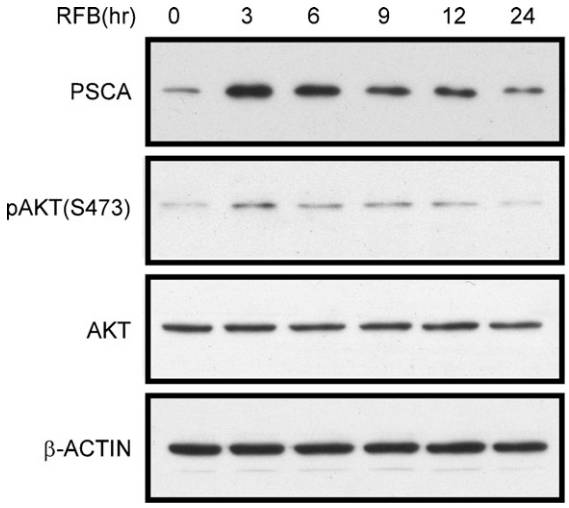

Fig. 5 – Time course of regulation of AKT activation and PSCA expression by RFB. TRAMP C1 cells were treated with RFB 100 μg/mL for up to 24 h. Lysates were prepared at intervals and then used for immunoblotting with the indicated antibodies.

show an increase in *psca* expression in prostate tissue from mice with targeted PTEN alleles [60]. Such mice have constitutive activation of the *akt* kinase. A similar increase in *psca* expression is seen in mice with enforced expression of an activated AKT allele [61]. Our gene expression studies also support the activation of the AKT kinase by RFB treatment of TRAMP mice. Gene expression profiles of prostate tissue from mice with enhanced AKT signaling (as a result of targeted disruption of the PTEN gene) have identified multiple genes whose increased or decreased expression may be due to the kinase [62]. *Psca* was again one of the most highly up-regulated genes in PTEN-deficient prostate tissue. Our microarray studies detected additional genes described from this database, whose expression appeared to be modified by AKT. Of the 34 up-regulated genes in RFB-treated prostate tumors, 9 have been identified as being increased in PTEN-deficient prostates (*psca*, *ceacam1*, *bnip3*, *ifit2*, *sprr2a*, *anxa3*, *cmas*, *birc5*, and *tyms*). Furthermore, among the 24 RFB-inhibited genes, 6 have shown to be decreased in the prostates of PTEN-targeted mice (*gpx3*, *ccnd1*, *kcnn4*, *pcp4*, *penk1*, and *smoc2*). These data are consistent with the view that RFB can activate AKT in the tumors of treated mice, and regulate expression of AKT-dependent genes such as *psca*.

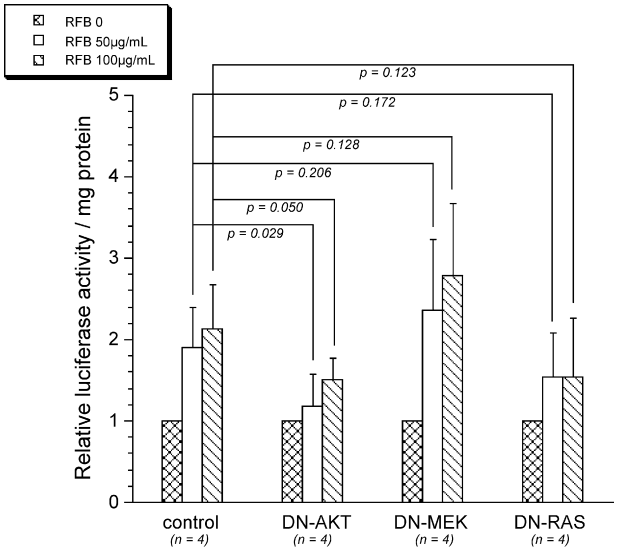

Fig. 4 – Activation of *psca* promoter activity by RFB, through a *ras/akt*-dependent pathway. TRAMP C1 cells were transfected with *psca* promoter plasmid, along with plasmid encoding dominant-negative signaling molecules or empty plasmid, and exposed to RFB as described in Fig. 2C. Total amount of plasmid DNA was the same for each transfection. Each value is the mean (±S.E.M.) of three independent experiments. *p*-Values were calculated by paired T tests.

Activation of AKT kinase by RFB, an anti-proliferative agent, may seem paradoxical. Enforced AKT activity in prostate tissue can lead to increased growth, diminished apoptosis, prostatic intraepithelial neoplasia, and progression to androgen independence in prostate cancer [61,63]. Alternate possibilities have been described however. Increased activity of AKT can be associated with decreased proliferation, but increased survival of cancer cells under some conditions. Aspirin, another NSAID, can simultaneously inhibit proliferation of colon cancer cells, and enhance their survival, through an AKT-dependent mechanism [64]. These studies have recently been expanded to show that aspirin treatment of mice for 7 days can increase AKT activity in murine tumors, leading to enhanced survival and cytotoxic drug resistance [65]. We feel that neither the activation of AKT kinase activity, nor the expression of the AKT-dependent gene *psca*, are involved in the known anti-tumor effects of RFB. Rather,

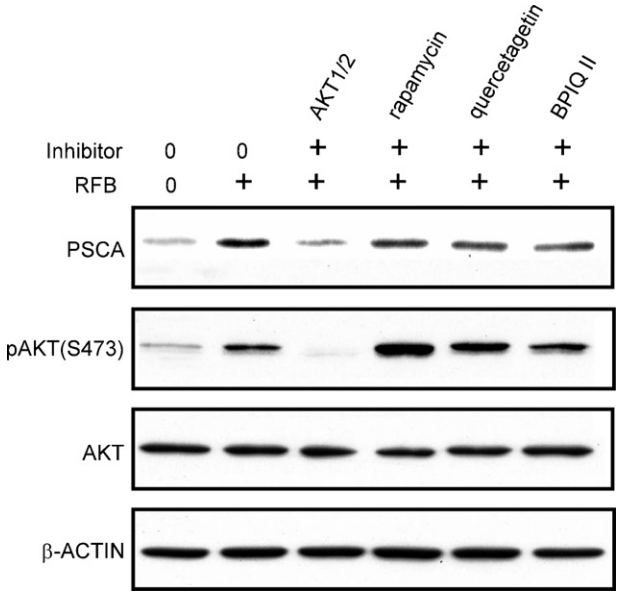

Fig. 6 – Effect of kinase inhibitors on RFB-dependent regulation of PSCA expression. TRAMP C1 cells were treated with the indicated inhibitors for 2 h, at the following concentrations: AKT1/2 = 10 μM; rapamycin = 100 nM; quercetagetin = 7 μM; BPIQ-II = 100 nM. RFB (100 μg/mL) was then added for 4.5 h. Lysates were then prepared and used for immunoblotting with the indicated antibodies.

activation of AKT signaling by RFB could be seen as a component of a stress response pathway that actually impairs the anti-proliferative effects of the drug. Other studies have shown that some cytotoxic and anti-proliferative drugs can induce activation of AKT, resulting in a blunting of the cytotoxic effect and increased cell survival [66,67]. Furthermore, the related anti-tumor NSAID-like compound *R*-etodolac has been shown to activate pro-survival signals mediated through the Her2 tyrosine kinase [68]. Blocking Her2 activity by a monoclonal antibody, during treatment with *R*-etodolac, resulted in synergistic anti-tumor effects. Thus, combinations of RFB and inhibitors of AKT-pathway activation may lead to enhanced anti-proliferative effects.

The dose-dependent increase in *psca* promoter activity and protein expression at RFB concentrations up to 100 μg/mL are likely due to activation of the AKT kinase, since the small molecule inhibitor AKT1/2 substantially blocks PSCA expression in this setting. The continued increase in PSCA protein levels at concentrations above 100 μg/mL is not necessarily inconsistent with the decreased *psca* promoter activity at these higher, supra pharmacologic drug concentrations, and does not automatically imply additional regulatory mechanisms. Because the *psca* promoter studies required transient transfection and subsequent protein synthesis to express the reporter gene, the total time course of these experiments was substantially longer than that of the immunoblotting studies. The time course immunoblot (Fig. 5) demonstrated a marked decrease in PSCA protein between 12 and 24 h after RFB addition. Inhibitory effects are likely to be even more prominent at higher drug concentrations.

The time course of RFB-induced activation of AKT likely will be significantly different between cultured cells and the tissues of treated mice. In TRAMP C1 cells, the increase in activated AKT kinase was a transient event. Activation was apparent by 3 h after drug addition, but the levels of phosphoAKT protein returned to baseline by 24 h. In TRAMP mice, however, PSCA expression was high at 7 days after the start of drug treatment. This increase in PSCA expression likely reflects activation of AKT in tumor tissue, since this mechanism is implicated in parallel studies of cultured cells. Our results are also in agreement with a recent report showing activation of AKT in tumor cells from mice treated for 7 days with the NSAID drug aspirin [65]. Unfortunately, no data exist to characterize gene expression profiles at earlier or later time points during RFB treatment. Thus, we cannot state whether AKT activation will be persistent, or whether, as with cultured cells, it will be a transient phenomenon.

PSCA protein is not invariably a marker for cancer. Increased PSCA expression was shown to be associated with human and murine prostate cancer progression [69]. However, expression of PSCA protein is also known to be *decreased* in cancers of the stomach and esophagus, compared with the corresponding normal tissues, and PSCA staining decreases with increasing grade and invasion of transitional cell carcinomas of the bladder [70,71]. Anti-PSCA antibodies have shown promise in the treatment of prostate cancer in animal models [59]. The efficacy of anti-tumor monoclonal antibodies is dependent on target antigen density. Our studies suggest that it may be possible to increase expression of the *psca* mRNA, and possibly protein, on prostate cancer cells in animals, through treatment with RFB. Studies of the combination of anti-PSCA antibodies and anti-tumor NSAID-like drugs, such as RFB, seem appropriate exercises to improve the treatment or prevention of prostate cancer.

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