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Lobe-Specific Carcinogenesis in the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) Mouse Model

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1. Introduction

Prostate cancer (PCA) is the most commonly diagnosed cancer affecting men in many countries and it has been estimated that there would be 33, 000 deaths due to PCA in the United States in 2011 alone [1]. The standard chemotherapy for metastatic castration-resistant prostate cancer (CRPC) is the taxane anticancer drug docetaxel in combination with the steroid prednisone. In the past two years, autologous immunotherapy sipuleucel-T (Provenge), the new taxane drug cabazitaxel (Jevtana) and a P450 C17 inhibitor drug abiraterone acetate (Zytiga) were also approved by the US FDA (Food and Drug Administration) [2]. Unfortunately, those drugs can only offer limited survival benefit but have significant side effects that negatively affect the quality of life of the patients.

In contrast to chemotherapy, cancer chemoprevention uses naturally occurring or synthetic chemicals to block, delay or reverse carcinogenesis, progression and metastasis and is increasingly being recognized as essential for winning the war on PCA. Animal models are essential for development of effective chemoprevention. Currently, the mouse models used for PCA research include human xenograft models in immunocompromised mice, mouse prostate reconstitution models, transgenic models and single stem cell-based prostate regeneration (see a comprehensive review by Jeet *et al.* [3]). Considering the facts that PCA is a complex disease of uncertain etiology and multifocal phenotypic heterogeneity, a desirable model should recapitulate some critical features of human PCA: initiation of PCA with PIN (prostatic intraepithelial neoplasia), followed by progression to invasive adenocarcinoma, and subsequent metastasis with defined kinetics. Although no single model is perfect, transgenic models are widely used to delineate the mechanisms of prostate carcinogenesis and evaluate the chemopreventive efficacy of candidate agents because the

lesions/cancers develop “naturally” *in situ* in the genetically engineered hosts and can be followed over a long time course [4].

2. TRAMP model represents at least two lineages of prostate carcinogenesis

The transgenic adenocarcinoma of mouse prostate (TRAMP) model was originally developed by Dr. Greenberg in 1995 [5]. It belongs to the first generation of models based on SV40 viral oncogenes [3]. In this TRAMP model, the rat probasin promoter (-426 to 28 bp) drives the expression of SV40 large T-antigen (T-Ag) and small t-antigen in the prostate. T-Ag abrogates P53 and Rb tumor suppressor proteins by direct binding. Simultaneously, small t-antigen interacts with protein phosphatase 2A [6] to regulate activity of the mitogen activated protein kinase activation pathway and the AP-1 transcription factor activity [7]. T-Ag and small t-antigen act spontaneously to propel the genesis of prostate epithelial lesions and malignant carcinomas and metastases. The TRAMP model is by far the most widely used PCA transgenic model for chemoprevention studies because of its simplicity in breeding compared to next generations of transgenic models based on deletions, insertions or mutations of mouse genes to mimic their changes in human PCA.

For more than one decade since its inception, the TRAMP model has been believed to represent a single lineage of epithelial lesion progression with well-defined kinetics and molecular marker alterations. The progression was thought to start from low and high grade PIN, to well-differentiated (WD) and moderately-differentiated (MD) adenocarcinoma in the dorsolateral prostate lobes (DLP) and finally by stochastic phenotypic conversion, to poorly differentiated adenocarcinomas (PD-Ca) with lymph node and other distant metastases [8]. Many subsequent publications interpreted the histology results and molecular characterizations using such a paradigm.

However, several recent studies have suggested that the poorly-differentiated neuroendocrine (NE)-like carcinomas (NECa) [9, 10] belonged to a distinct lineage from the epithelial lesions, which included low and high grade PIN, WD to MD “adenocarcinomas” of the original classification by Dr. Greenburg [8]. Those epithelial lesions were recently termed “atypical hyperplasias of T-Ag” (AHT) to distinguish from the human prostate cancer pathogenesis because they did not invade adjacent tissues [9]. In addition, the incidence of the NECa was found to be profoundly affected by the genetic background of the host mice [9]. In the C57BL/6 background, the lifetime incidence of NECa was estimated to be about 20% whereas in the FVB background, 87% NECa incidence was recorded by as early as 16 weeks of age and reached 100% by 20 weeks of age [9]. Furthermore, these NECa mostly arose in the ventral prostate (VP) lobes instead of the DLP in both strains [9, 10]. Tissue reconstitution experiments carried out by Chiaverotti *et al* indicated that NECa did not arise from the trans-differentiation of pre-existing AHT [9]. Tang *et al* reported that stress induced by anti-cancer treatments (castration and/or docetaxol) increased the incidence of NECa in TRAMP mice ((C57BL/6

x FVB) F1) [11]. However, the conclusion might be questionable since no mouse in the control group had NECa by the time of death, although they all developed adenocarcinoma in their prostates.

These findings significantly challenged the classical notion of single-lineage disease progression in the TRAMP DLP [8, 12]. Since the TRAMP model has been increasingly used for prostate cancer chemoprevention studies, it is very critical to further characterize the lobe-specificity of lesion lineages and NECa incidence in the prostate to consolidate the advantages of this model and minimize its limitations for both etiological and chemoprevention studies. To experimentally approach this, our group estimated the incidence of NECa based on 90 TRAMP mice in the C57BL/6 background spanning the age range of 16-50 weeks from several study cohorts [13-15]. We also characterized the histological features of different lineages of carcinogenesis in this model using archived tissue blocks from those studies [13-15]. In addition, by using state-of-the-art proteomics, we sought insights on mechanisms underlying carcinogenesis of different lineages and possible targets of chemopreventive reagents [15-17]. Here, we review our results as well as from other researchers' work to provide an objective analysis of the utility of this preclinical model for cancer chemoprevention studies.

3. Histological characteristics of prostate carcinogenesis in TRAMP model

In our studies, the female heterozygous C57BL/TGN TRAMP mice (line PB Tag 8247NG) were purchased from The Jackson Laboratory (Bar Harbor, ME), and were cross-bred with non-transgenic C57BL/6 males [13-15]. H&E and immuno-histological (IHC) staining were routinely performed in our lab and the results are summarized in Table 1 [15]. Figure 1a shows the H&E staining of representative morphological features of DLP and VP with micro-NECa in TRAMP mice [15]. As can be seen in Figure 1b-1e, the AR-expressing glandular prostate epithelial lesions, which are now termed atypical hyperplasias of T-Ag (AHT), express nuclear T-Ag, epithelial membrane-staining of E-cadherin and are negative for synaptophysin (SYP), which is a neuroendocrine cell marker [15]. Staining of Ki67 (Figure 1f), a proliferative index protein, showed a descending order of NE-Ca >> DLP AHT > VP epithelium. In the prostate from wild-type mice, single layer luminal epithelial cells were stained positive for nuclear AR, membrane E-cadherin with rare Ki-67 positive proliferating cells, while negative for T-Ag and SYP (Figure 2) [13-15]. In contrast, poorly-differentiated prostate carcinomas (PD) as classified by Greenberg [5] had distinct morphological features compared to AHT described above, regardless of the microscopic size found within the VP lobe (Figure 1, right panels) or those weighing over many grams [15]. Those lesions/tumors expressed strong T-Ag and SYP (except negative in trapped glandular epithelial islands), and were negative for AR and E-cadherin (except positive in the trapped glandular epithelial islands) [15].

We analyzed all tumors and prostates in our cohorts with the above panel of biomarkers. The incidence of macroscopic tumors (>1 gram) in TRAMP mice of 16-18, 22-24 and 26

weeks of age (WOA) were 10%, 20% and 30%, respectively. They were all poorly differentiated NECa expressing SYP [13, 15]. In another study, 31 C57BL/6 TRAMP mice in the control group (no chemo-preventive agents administrated) were followed up to 50 WOA. Ten large tumors traceable to the prostate were found (32.3%) and they were all SYP positive poorly differentiated NECa [13]. Overall, we observed that there was a trend for increasing detection of visible macroscopic NECa in TRAMP mice and the life-time NECa incidence rate was 1 out of 3 TRAMP mice from our experiments. This value was slightly higher than that reported by Chiaverotti (20%) [9]. The possible reason might be a smaller number of and younger C57BL/6 TRAMP mice used in their study [9].

We attempted to identify the anatomical origin of the NECa. Out of 18 NECa's in a cohort of 90 C57BL/6 TRAMP mice of 22 to 24 WOA, 12 were traceable to the VP (66.7%), two tiny tumors were traced to DLP (11.1%), whereas 1 large tumor was found in the anterior prostate (AP) (5.6%). The other three tumors were not traceable to any lobe location due to their overwhelming large sizes. These data were consistent with two recent studies showing a preponderance of SYP positive NECa arising from VP in the TRAMP mice of both C57BL/6 and FVB backgrounds [9, 10]. In contrast, the weight of macroscopic tumor-free VP lobes was only slightly increased in TRAMP mice compared to their wild type littermates at 24 WOA (Table 2 [15]), whereas the DLP lobes underwent significant expansion (more than doubled) in the TRAMP mice in one study where these lobes were compared side by side [14, 15].

The Greenberg group had reported the prevalence of seminal vesicles (SV) problems such as papillary fibroadenoma in the TRAMP mice with C57BL/6 background shortly after the strain was established [12, 18]. Later on, several publications also described the histogenesis and pathology of SV neoplasms in the TRAMP mice [19, 20]. In one long-term survival experiment carried out by our group [13], 54.8% (17 out of 31) TRAMP mice in the control group developed tumors in their SV and SV tumor loads became significantly increased beyond 30 WOA [13, 15]. Tani *et al* reported that most of the tumors contained phyllode lesions, which were typically composed of single layer epithelial linings that were negative for T-Ag, but positive for E-cadherin and AR staining and stromal cells that were neoplastic. These stromal cells frequently exhibited mitotic figures (BrdU incorporation) and SV40-TAg protein expression in the nuclei and were positive for desmin immuno-histological staining [19]. Since they did not observe conclusive evidence of malignancy such as invasion or metastasis, they recommended diagnosis of the SV tumors as epithelial-stromal tumors. The descriptions matched phyllodes-tumors in the C57B/6 TRAMP mice reported by Hsu *et al* [18]. However, in our long term studies, tumors/lesions were found in pelvic lymph nodes, liver, lung or kidney of mice without significantly increased prostate size but much enlarged SV in very rare cases (<5%), suggesting metastatic lesions from the SV tumors [13, 15]. Tables 1&2 summarize the expression of biomarkers (by immunohisto staining) and tumor incidence in different lineages of carcinogenesis in TRAMP mice [15].

Protein Biomarker	Wild type Prostrate	TRAMP AHT*	TRAMP NECa/Metastasis	TRAMP Seminal vesicle Epithelial-stromal tumors/Metastasis
T-Antigen	-	+++	+++	+
Androgen Receptor	++	+++	-	+
Synaptophysin	-	-	+++	-
E-cadherin	+++	+++	-	-/+
Ki-67 (MIB-1)	<1%	++	+++	++

*AHT-atypical hyperplasia of T-antigen of glandular epithelia

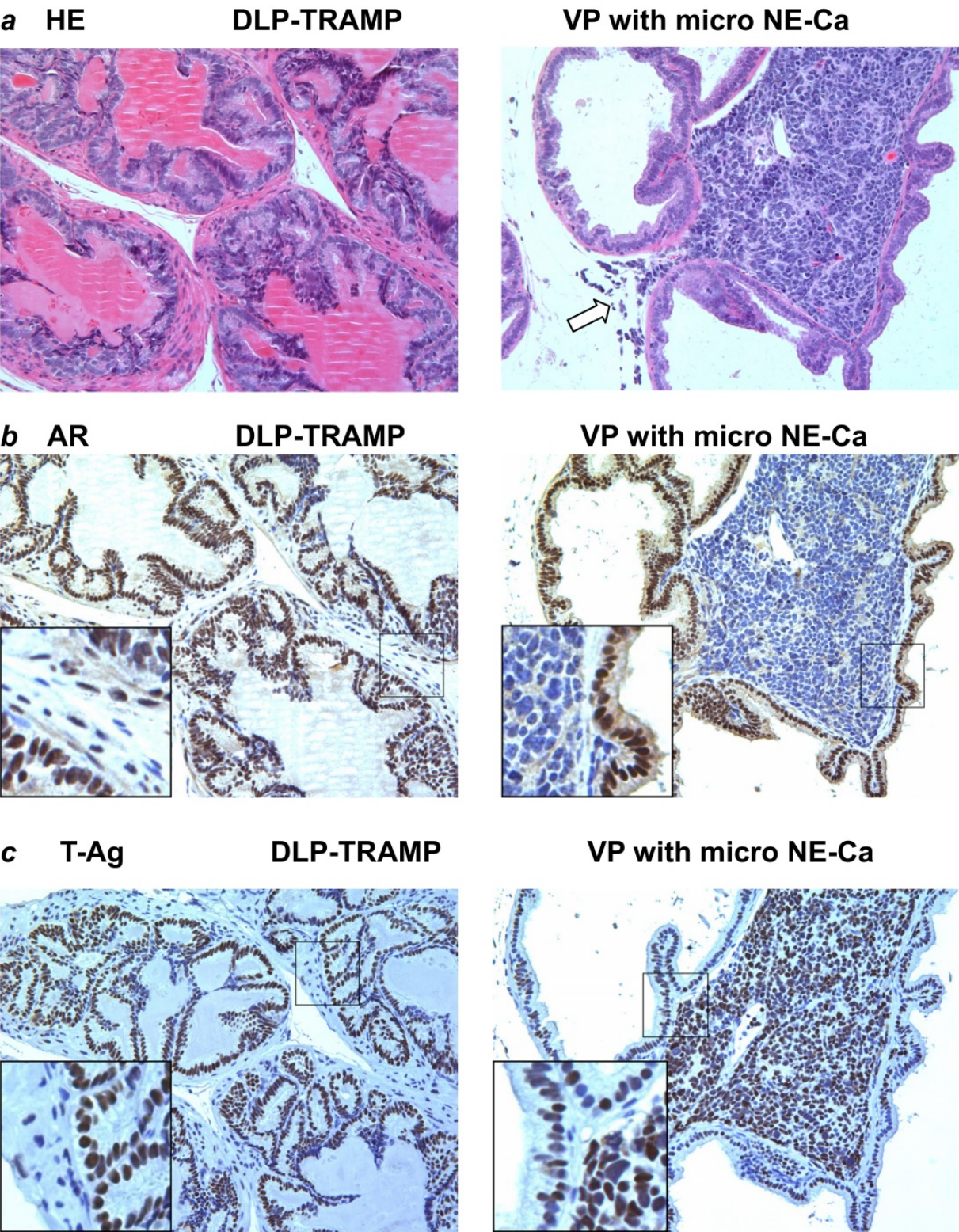
Table 1. Summary of Immunohistochemical Marker Expression in Prostate lesions and metastases in C57BL/6 TRAMP mice [15]

Prostate lobes	Prostate lobes			
	Dorsolateral	Lobe uncertain*	Ventral	Anterior
Synaptophysin(+) NE carcinoma	2/18	3/18	12/18	1/18
# (%)	11.10%	16.70%	66.70%	5.60%
Average tumor weight (Weight range), g	0.09 (0.07, 0.11)	4.98 (3.44, 4.38, 7.11)	1.65 (0.02- 4.84)	1.1
TRAMP lobe weight at 24 weeks, mg	79.2±3.7 (n=17)**		15.3±1.1 (n=17)**	
Wild type lobe weight at 24 weeks, mg	31.7±2.9 (n=8)**		12.5±1.7 (n=8)**	
t-test	p<0.001		p>0.05	

*These 3 big tumors are hard to distinguish the exact originating sites.

**Presented as mean±SEM. t-test analysis between the TRAMP and wild type mice.

Table 2. Lobe specificity of NECa distribution in 90 TRAMP mice at ≤24 weeks of age (from different experiments) [15]



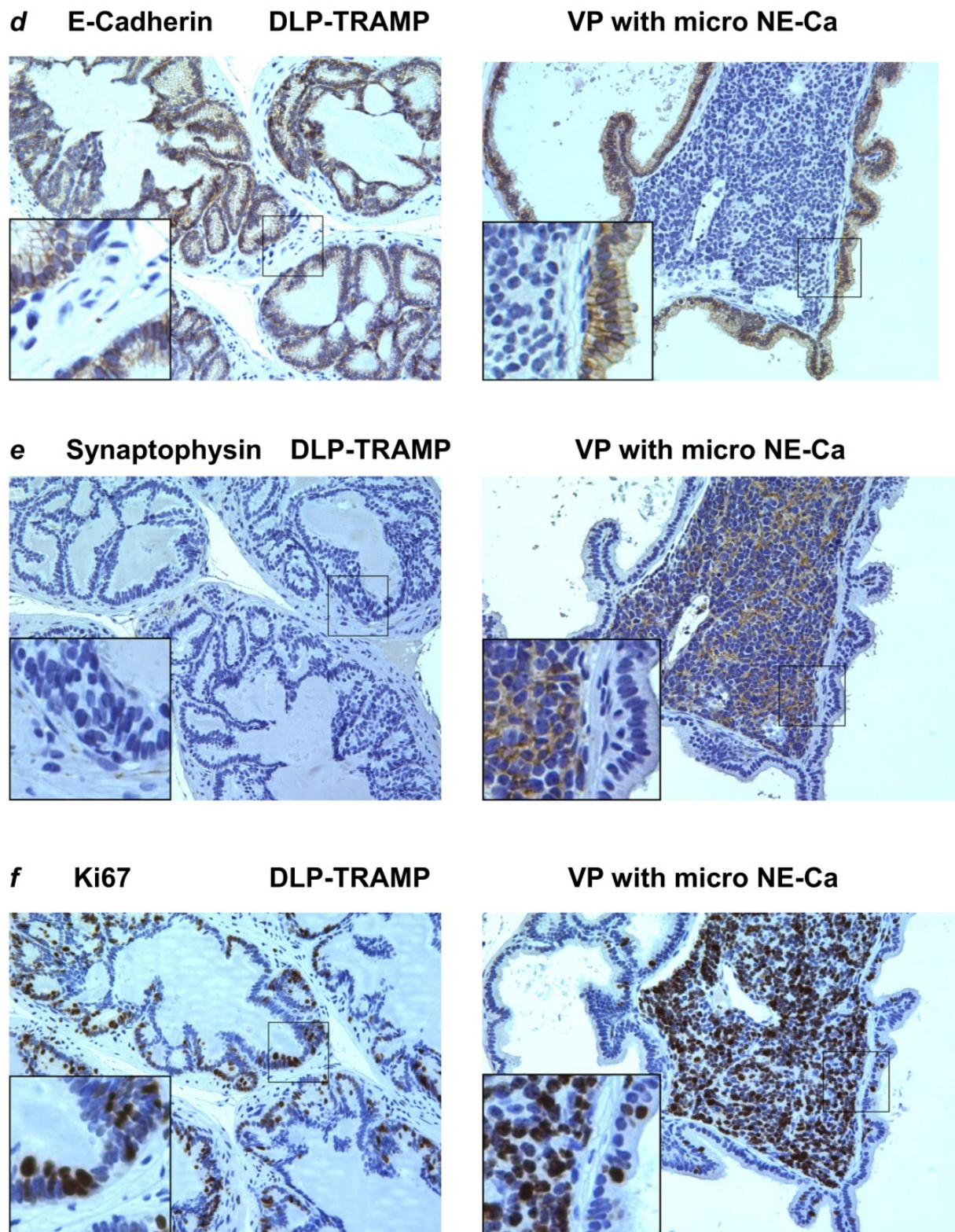


Figure 1. Immunohistochemical profiling of key protein markers in dorsal lateral (DLP) and ventral prostate (VP) with a microscopic neuroendocrine carcinoma (NECa) from a TRAMP B6 mouse. Serial sections were stained for hematoxylin and eosin (HE, a), androgen receptor (AR, b), SV40-T antigen (T-Ag, c), E-cadherin (d), synaptophysin (e) or Ki67 (Mib1) proliferation-associated protein (f). Magnification, 100x. Insets in panels show a higher magnification of staining patterns (400x) [15].

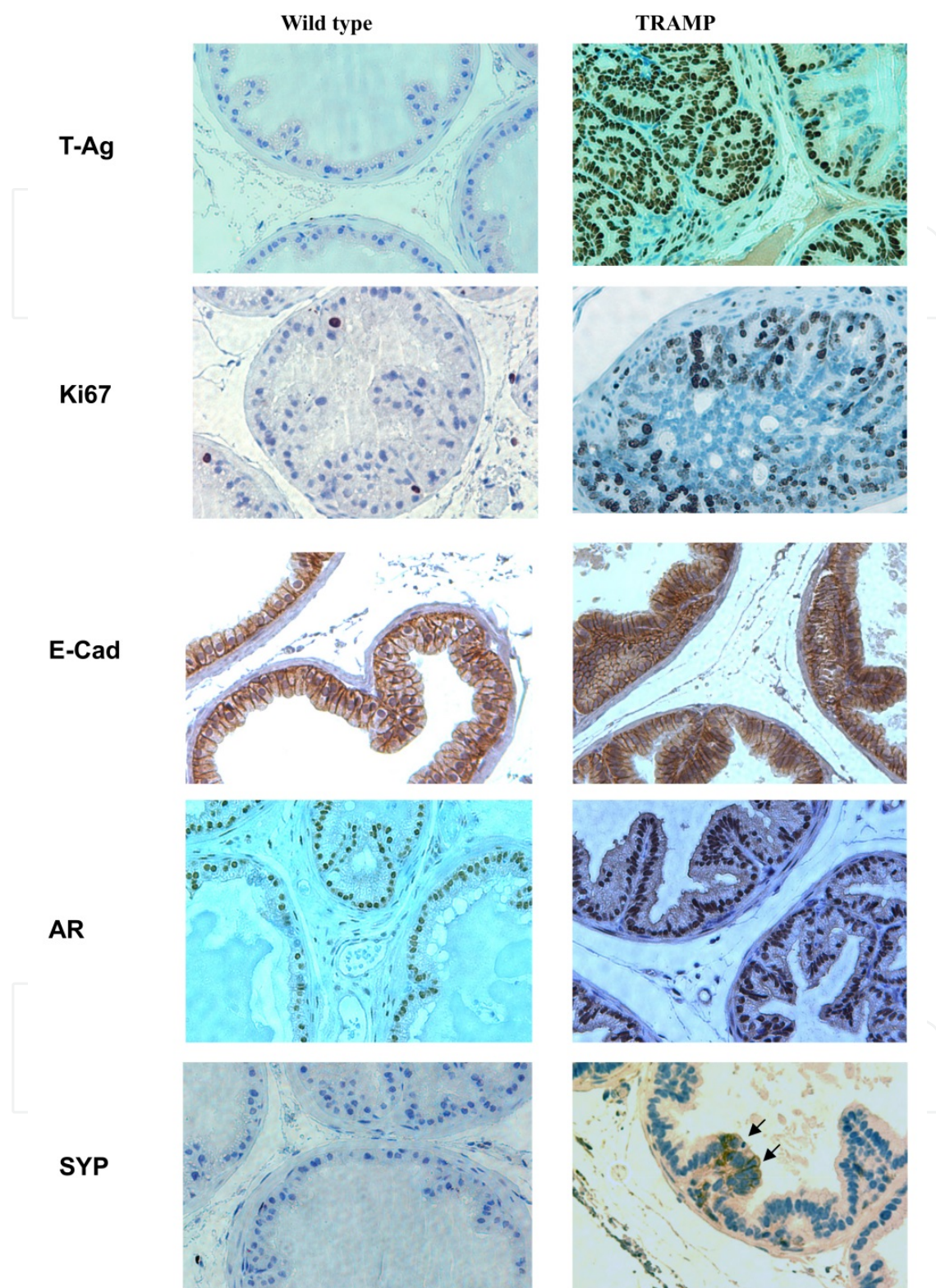


Figure 2. IHC staining patterns of wild type and TRAMP prostate for T-antigen (T-ag, nuclear), Ki67 proliferative antigen (nuclear), E-cadherin (E-Cad, cell membrane), and androgen receptor (AR, nuclear) and Synaptophysin (Syp, arrows point to representative positive NE-cells, cytosolic and cell membrane) [15].

4. Molecular changes in prostate carcinogenesis in TRAMP model

Classical methods detecting the steady levels of mRNAs and proteins, as well as “omics” approaches such as microarray, antibody array and two-dimension electrophoresis based proteomics have been used to study the molecular changes associated with TRAMP carcinogenesis using prostate or serum as starting materials. The Greenberg group reported increased expression of basic fibroblast growth factor (FGF2) in the prostates (whole prostate) of TRAMP mice ([C57BL/6 X FVB] F1) [21]. They found that the expression of the 25-kDa isoform of FGF2 was 2-fold higher in PIN and WD and MD tumors than in normal DLP and VP; the expression of the 22-kDa isoform of FGF2 was not elevated in PIN lesions, but was observed to be increased in WD, MD, PD and androgen-independent tumors. Interestingly, the 18-kDa isoform of FGF2 was only expressed in the samples representing PD and androgen-independent disease. These observations implicated specific changes in the FGF axis with the initiation and progression of PCA. The important role of FGF2 in PCA progression was further investigated in an *in vivo* study [22]. In that study, TRAMP mice (C57BL/6 background) were crossed with FGF2 knockout (*FGF2*^{-/-}, SV129 background) mice, and tumor progression in TRAMP mice that were either hemi- or homozygous for inactivation of the *FGF2* allele was compared with wild-type TRAMP mice. They found that even inactivation of one *FGF2* allele resulted in increased survival, less PD phenotype in primary tumors and a decrease in metastasis [22]. Later on, Agarwal’s group reported that the chemopreventive effect of dietary silibinin against TRAMP carcinogenesis (C57BL/6 background) was accompanied with a decreased plasma FGF2 level [23]. The observation further suggested FGF2 as a target for chemoprevention of PCA. Similarly, the roles of ERKs, EGFR, NF- κ B, clusterin, cyclins, insulin-like growth factor-I (IGF-I), DNMTs and other molecular pathways in the TRAMP carcinogenesis have also been reported by different groups [24-30].

Considering extracellular proteolysis as an important biochemical event in the invasion and metastasis of PCA, Bok *et al* studied the expression of matrix metalloproteases (MMP) and other related proteins in TRAMP mice (C57BL6/FVB mix) using gelatin zymography [31]. In their study, distinct lobes of the prostate (ventral, lateral, dorsal and anterior), seminal vesicles, as well as lymph nodes (LN) with metastases were isolated by micro-dissection if possible. They found increased expression of mainly pro-MMP 9 and pro-MMP2 in early TRAMP tumors, but substantial elevation of activated MMP9 and MMP2 only in late TRAMP tumors. In addition, they found a progressive increase in the activities of cysteine, serine and certain membrane-bound proteases (cathepsin B, MT-SP1, MT1-MMP) from normal to advanced prostate tumors. Interestingly, a gradual decrease in urokinase plasminogen activator (uPA) expression with tumor progression was also observed. Overall, the study suggested proteases as potential targets for the therapy and secondary prevention of PCA. Ruddat *et al* profiled the proteome of TRAMP ([C57BL/6TRAMP \times -FVB] F1) prostate by 2-D electrophoresis followed by peptide identification by MALDI-TOF [32]. In their study, the WD dorsal prostate (from mice approximately 14 WOA) was compared with normal dorsal prostate tissue. The prostates from mice approximately 25 WOA with PD CA were not further dissected since it is not practical to differentiate the lobes in an advanced

stage. The data revealed that there were few significant changes in the protein abundances in the WD dorsal prostates compared to wild-type dorsal prostates, with the exception of increases in proliferating cell nuclear antigen (PCNA) and beta tubulin, two proteins implicated in cell proliferation, and a more than 2-fold increase in an anti-apoptotic protein Hsp60. In contrast, there were substantial changes in protein abundance in the PD tumors compared to wild-type dorsal prostates. While some of those changes could be related to the disappearance of stromal tissue such as decreased expression of myosin light chain alkali, desmin and tropomyosin beta 2, the most notable was the overall decrease in calcium homeostasis proteins such as calreticulin and Hsp70 (10-fold decrease) and creatine kinase bb (40-fold decrease) in the cancerous tissue. The expression patterns of desmin, Hsp70 and calreticulin were further confirmed by Western blot. Although some of their results have been cross-validated by us (e.g. down-regulation of calreticulin and tropomyosin beta 2, to be discussed later) [16, 17], many of these changes could be due to the differences between the cells in the two different carcinogenesis lineages rather than the purported changes due to progression of diseases of the Greenberg paradigm.

Using Affymetrix GeneChip Mouse Genome 430 2.0 micorarrays, Haram *et al* profiled the mRNAs from eight TRAMP (C57BL/6 background, 30±4 WOA)) tumors and DLP or VP of nine wild-type mice [33]. Statistical analysis indicated that 3,870 transcripts such as cyclin A2, cyclin B1, cyclin B2, cyclin E1, cyclin E2, cyclin F, Forkhead box M1, Aurora Kinase A and B were up-regulated in TRAMP tumors and 2,945 transcripts such as cyclin D2 and probasin were down-regulated in TRAMP tumors. Real-time RT-PCR confirmed the expression of the 11 genes mentioned above. In each real-time PCR experiment, three to four normal samples (typically one dorsolateral and three ventral) and four tumors (the same four tumor samples in every experiment) were used. Moreover, cross-referencing differentially expressed TRAMP genes to public human prostate array datasets revealed 66 genes with concordant expression in mouse and human PCA. Among the 66 genes, Sox4 and Tubb2a, which were reported to be up-regulated in primary PCA compared to normal prostates, were further validated by real-time RT-PCR. In addition to providing information regarding the mechanisms of TRAMP carcinogenesis, the concordance analysis between TRAMP and human PCA associated genes also supported the utility of the model. In another study, Morgenbesser *et al* studied the dynamic changes of mRNA profiles in TRAMP ([C57BL/6 TRAMP × FVB] F1) mice, especially the genes involved in the acquisition of androgen-independent and metastatic tumor growth, using both microarray and SAGE approaches [34]. The following cohorts were used in their study: C18 (prostates from wild-type mice at 18 WOA); C24 (prostates from wild-type mice at 24 WOA); T12 and T18 were prostates from TRAMP mice at 12 and 18 WOA representing PIN and MD stages of disease, respectively; T24 were PD, androgen-dependent (AD) primary tumors from TRAMP mice at 24 WOA whereas T(12)24 were PD, androgen-independent (AI) primary tumors from 24-week-old TRAMP mice that were castrated at 12 WOA. In their paper, differentially expressed genes between AD tumors and normal prostates, AI tumors and normal prostates, as well as AD and AI tumors were presented. The dynamic changes of ten transcripts (e.g. tissue inhibitor of metalloproteinase 4, GATA-binding protein 2, ER-resident protein ERdj5, paralemmmin, MYC-associated zinc finger protein) over the progress of

TRAMP carcinogenesis were further validated by real-time RT-PCR. Their analyses uncovered many transcripts that had not previously been implicated in PCA progression, especially in the transition from androgen-dependent to androgen-independent status. Again, these studies were conducted under the Greenberg paradigm of single lineage disease progression. Many of these changes could be due to the differences between the cells in the two different carcinogenesis lineages rather than the purported changes due to progression of the disease.

Therefore, it will be prudent to carefully read the descriptions of the tissue samples used in the published papers. For example, many older publications prepared lysate from the whole prostate whereas very few focused on particular lobes [24, 31, 32]. Recently, researchers started to carefully look into the molecular pathways involved in two lineages of carcinogenesis (different prostate lobes, mainly DLP and VP) in TRAMP mice after the lineage differences of the prostate carcinogenesis had been recognized [9, 13]. For instance, Slack-Davis *et al* studied the requirement for focal adhesion kinase (FAK) signaling in cancer progression in TRAMP mice with C57BL/6 background [35]. They found that loss of FAK or its inhibition with PF-562271 (small molecular inhibitor for FAK) did not alter the progression to AHT. However, continued FAK expression (and activity) is essential for the androgen-independent formation of NECa.

Our group [15] dissected prostate lobes and visible tumors separately in most of our experiments in order to assess the lobe-specific biochemical changes. Using these dissected lobes/tumors as starting materials, we studied the expression of classical biomarkers by immuno-blot. The expression patterns of proliferative cell nuclear antigen (PCNA) (Figure 3a) confirmed the Ki67 staining patterns by IHC staining described above [15]. Consistent with the lack of proliferation of normal prostate epithelium, PCNA was non-detectable in the DLP and VP in the wild type mice at 16-18 WOA, whereas significantly higher levels of PCNA were detected in the DLP and VP and NECa of TRAMP mice. Since the Ki67 proliferation index in the VP epithelium appeared to be lower than in TRAMP DLP as evidenced by IHC (Figure 1f), the higher level of PCNA in TRAMP VP (Figure 3b) than in DLP might be due to the presence of micro NECa foci in the TRAMP VP lobes (NECa has much higher Ki67 index, Figure 1f) [15]. We also confirmed the contrasting patterns of AR, SYP and E-cadherin expression patterns in the DLP AHT vs. NECa. Although the expression of AR and E-cadherin were increased in both DLP and VP in TRAMP mice at 16-18 WOA, there were more AR and E-cadherin in VP than in DLP of littermate wild type mice (Figure 3b) [15]. These patterns are in agreement with that published by the Greenberg group [8, 12] and others [9]. In addition, we found that the level of cleaved PARP (endogenous substrate for caspase 3) was higher in the NECa than in the DLPs of TRAMP and wild type mice (Figure 3a). The STAT3 level in TRAMP DLP was higher than in the wild type DLP and the NECa. However, there might be an isoform of STAT3 with higher molecular weight in the NECa, indicated by a mobility-retarded band (Figure 3a, arrow) [15]. Our observation was consistent with phosphorylative modification and activation of STAT3 in NECa, as reported by Aziz *et al* [36]. The changes of these biomarkers further support molecular differences between NECa and DLP AHT.

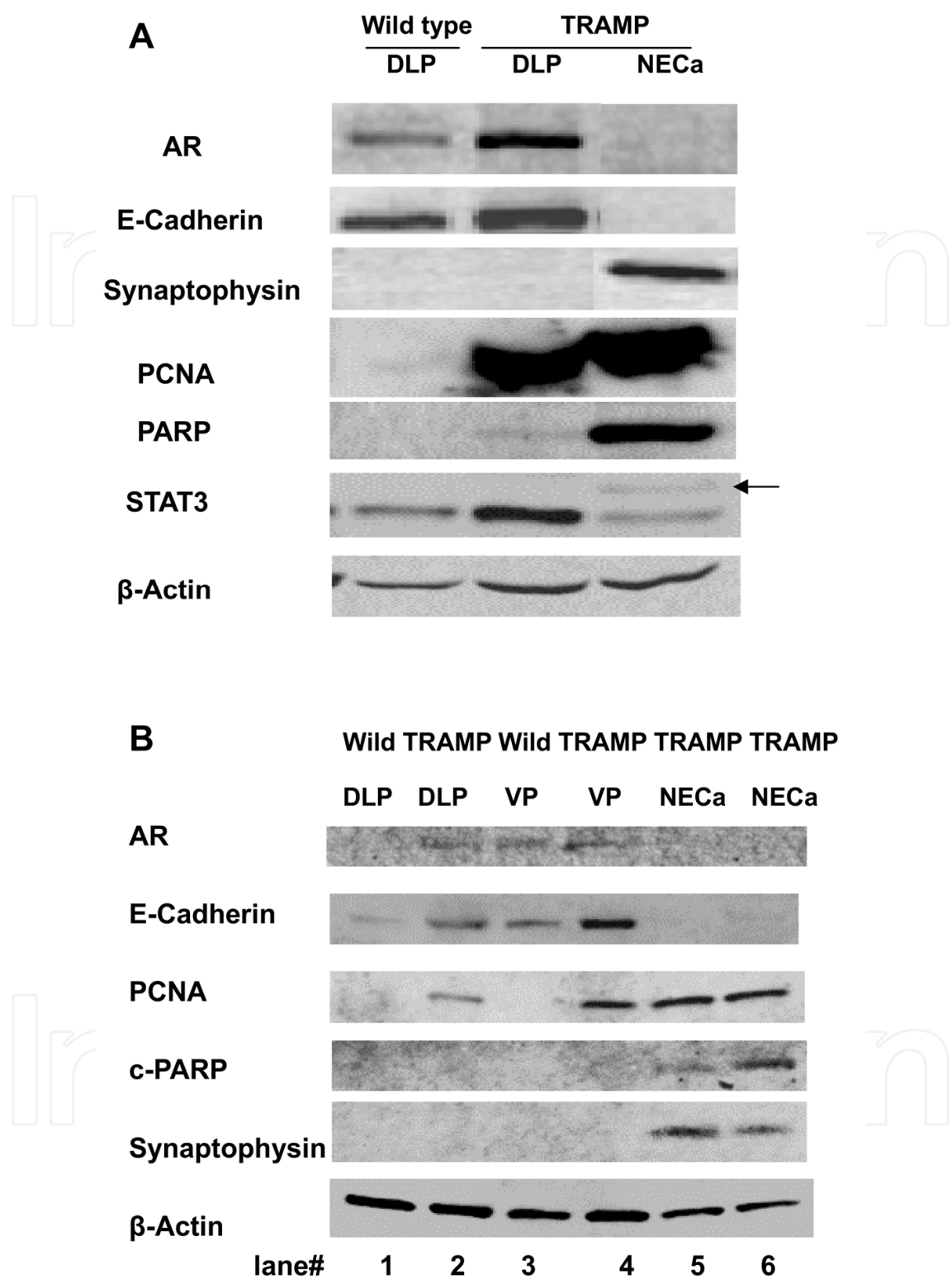


Figure 3. Western blot analyses of protein biomarkers in (A) wild type mouse prostate and in TRAMP DLP (DLP) and in NE-Carcinomas (NECa); in (B) DLP and VP of wild type and TRAMP mice at 16-18 WOA. AR, androgen receptor; PCNA, proliferating cell nuclear antigen; PARP, polyADPribose polymerase; STAT3, signal transduction and activator of transcription-3. Arrow marks mobility retarded STAT3 that is likely phosphorylated [15].

To provide more information on potential molecular correlates of the differences in DLP and VP for epithelial lesions and NE-carcinogenesis, we compared the expression level of proteins in respective lobes of wild type vs. TRAMP mice of 18 WOA by the iTRAQ proteomic approach, as we recently reported [16, 17]. We employed two-dimensional liquid chromatography coupled with tandem mass spectrometry (2D-LC-MS/MS) with iTRAQ labeling, which enables the concurrent identification and quantification of proteins through peptides generated upon tryptic digestion. The principle and advantages of the platform have been described in our previous publications [16, 17, 37]. In total, we identified 1068 proteins expressed in the DLP and VP of TRAMP mice and wild type mice. Among them, 483 and 748 proteins were identified at FDRs of 1% and 5% respectively. We found that the expression levels of 84 proteins were different between DLP and VP in wild-type mice [17]. For example, heat shock protein 5/glucose-regulated protein78 (GRP78), transglutaminase 4 (TGM4), experimental autoimmune prostatitis antigen 2 (EAPA2), probasin, beta-tropomyosin, calponin-1, as well as high mobility group box 1 & 2 were preferentially expressed in DLP, whereas there were higher levels of prostatic spermine-binding protein (SBP), serine peptidase inhibitor Kazal type 3 (SPINK3), polymeric immunoglobulin receptor (PIGR), solute carrier family 12 member 2, epidermal growth factor (EGF) and clusterin in the VP lobe. The expression pattern of GRP78 and clusterin were validated by immuno-blots [17]. The mRNA abundances of prostatic proteins in each lobe of mice or rats have been investigated by Fujimoto *et al* and Berquin *et al* using PCR and microarray, respectively [38-40]. Our results not only agreed with theirs but also extended to more lobe specific proteins. Importantly, we found that different sets of proteins were involved in lobe specific carcinogenesis in TRAMP mice. The expression levels of 118 proteins were significantly altered in DLP and/or VP during TRAMP carcinogenesis. 55 and 36 proteins were uniquely changed in DLP or VP respectively and only 27 proteins were found to be significantly modulated in both lobes during TRAMP carcinogenesis [17]. The majority (24 out of 27 proteins) shared the same trend, albeit the extent of change was not exactly the same. Shared changes in DLP and VP might indicate possible common molecular events associated with carcinogenesis such as elevated proliferation (e.g. up-regulation of HMGB1&2 and nuclear proteins such as histone(s)), oxidative stress (e.g. decreased antioxidant enzyme PRDX6) and disruption of stroma (e.g. down-regulation of SMMHC (myosin-11) and calponin-1) [17]. The three proteins, namely clusterin, polymeric immunoglobulin receptor and aldose reductase, which were differentially expressed in DLP versus VP, were more likely related to lobe-specific mechanisms of carcinogenesis. Interestingly, although some of prostatic proteins had been reported to be regulated by androgen signaling [38], EAPA2 and calreticulin were down-regulated in DLP whereas zinc-alpha-2-glycoprotein was only down-regulated in VP during carcinogenesis. These observations suggested that AR signaling could play different roles in carcinogenesis of different lobes of TRAMP mice.

To shed light on the key expression signatures or “master switches” that may not be virtually identified due to technical limitation, we analyzed the lists of differentially expressed proteins in each lobe by IPA software for pathway connections based on gene ontology and functionality [17]. Proteins with altered expression in DLP during

carcinogenesis were preferentially clustered into Immunological Disease/Cancer/Antigen Presentation and Post-Translational Modification/Protein Folding/Cancer networks. 14-3-3 zeta/delta (YWHAZ, up-regulated only in DLP), NF- κ B, epidermal growth factor (EGF, up-regulated only in DLP), ERK1/2, PKCs were identified as distinct inferred network nodes for those networks. On the other hand, proteins with altered expression in VP were mapped to Connective Tissue Disorders/Developmental Disorder/Genetic Disorder and Cancer/Cell Cycle/Cellular Development pathways with ERK1/2 and FGF2 identified as distinct network nodes in the two networks. We further used immunoblot to study the expression pattern of FGF2 proteins, which were not identified by LC-MS/MS but unraveled by IPA analysis as important proteins in the TRAMP carcinogenesis of the VP lobe. The data indicated that different isoforms of FGF2 were expressed in each lobe and FGF2 was only up-regulated in the VP lobe of TRAMP mice [17]. To our knowledge, we are the first to systematically compare the different protein profile changes between DLP and VP lobes during TRAMP carcinogenesis. Our results further support the concept that the C57BL/6 TRAMP mouse represents two lineages of prostate carcinogenesis in DLP and VP lobes. Further efforts to narrow down possible target proteins and investigation on the functional significance of those proteins will not only help understand the mechanisms of TRAMP carcinogenesis, but also facilitate the understanding and use of TRAMP model in the field of prostate cancer chemoprevention.

5. Effects of chemopreventive agents on prostate carcinogenesis in TRAMP model and possible mechanisms

Because the breeding strategy of TRAMP mice is straightforward, TRAMP mice have been widely used in evaluating potential preventive modalities by many groups in the past decade. Besides the effect of androgen deprivation therapy (ADT) on the progression of PCA by surgical castration of TRAMP mice [41, 42], the effects of many agents with chemopreventive potential including green tea [26, 43], NSAIDs [44, 45], flutamide [46], retinoic acid [47], vitamin E analog [48], genistein [24, 49], epigallocatechin-3-gallate (EGCG) [50], silibinin [23, 51], dietary restriction [52] and immunotherapy [53, 54], have been studied using this model. While most of the publications reported anti-cancer effects of their test compounds, El Touny *et al* showed that feeding TRAMP mice (TRAMP-FVB) with a diet containing 0.25% genistein from 12 to 20 WOA induced an aggressive progression of PCA, as evidenced by a 16% increase in the number of WD and PD prostates, coinciding with a 70% incidence of pelvic lymph node metastases as opposed to 0% in the control group [49].

The recognition of different lineages of carcinogenesis in the TRAMP prostate has important implications on the interpretation of chemoprevention data. Due to the distinct characteristics of AHT in DLP and NECa in VP, their sensitivity to different chemopreventive agents might not be the same. Knowledge of lineage-specific effects of each agent will be essential for selecting additional models to confirm the efficacy and to ultimately benefit clinical translation studies. In addition, it can provide valuable insights into mechanism studies for molecular pathway(s) and targets specific to the particular

lineage of carcinogenesis. Using this paradigm, our group evaluated the chemopreventive efficacy of next-generation selenium compounds methylseleninic acid (MSeA) and methylselenocysteine (MSeC) in TRAMP mice with C57BL/6 background [13]. In a short-term experiment, TRAMP mice of 8 WOA were given an oral dose of MSeA or MSeC at 3 mg Se/kg daily and were euthanized at either 18 or 26 WOA. By 18 WOA, the genitourinary tract and DLP weights for both treatment groups were lower than for the control ($p < 0.01$). At 26 weeks, 4 of 10 control mice had genitourinary weight > 2 g whereas only 1 of 10 in each of the treatment groups did. The efficacy was accompanied by delayed lesion progression, increased apoptosis, and decreased proliferation without appreciable changes of T-antigen expression in the DLP. In another experiment, giving MSeA to TRAMP mice from 10 or 16 WOA increased their survival to 50 weeks of age and delayed the death due to SYP-positive NECa, SYP-negative prostate lesions and seminal vesicle hypertrophy [13]. Interestingly, although MSeA and MSeC were considered as precursors of methylselenol, the proteins they modulated in the DLP of TRAMP mice were quite different as indicated by proteomic profiling. The data suggest that MSeA and MSeC should be developed as separate agents rather than as equal precursors of methylselenol [16]. Very recently, our group also demonstrated that oral administration of the alcoholic extract of *Angelica gigas* (AGN), a traditional Korean herb, had strong inhibitory effect on two lineages of carcinogenesis in TRAMP mice, especially the NECa [55, 56]. In contrast to the examples of MSeA, MSeC and AGN described above, we also published a lack of efficacy of a novel sulindac derivative sulindac sulfide amide (SSA) against NECa, whereas it exerted a significant protection against the DLP epithelial lesions [14]. Consistent with the fact that NECa originated from VP independently of AR, suppressing AR signaling in DLP was one of the important mechanisms underlying the chemopreventive effect of SSA, both *in vivo* and *in vitro* [14]. Similar to our strategy, Harper *et al* gave TRAMP mice (C57BL/6) water containing 0.06% EGCG starting at 5 WOA and dissected DLP and VP carefully when mice were euthanized at 12 WOA [50]. They found that EGCG significantly reduced the incidence of HG-PIN (Grade 3) from 100% to 17% in the VP of TRAMP mice but did not have a protective effect in DLP. At 28 WOA, it was difficult to dissect DLP and VP separately and EGCG did not have any inhibitory effect against TRAMP carcinogenesis in general.

As mentioned above, the life-time NECa incidence has been estimated to be approximately 30% in TRAMP mice with C57BL/6 background. Rest of the mice will be free of NECa and may therefore model the epithelial lineage lesions that are more relevant to the human prostate epithelial adeno-carcinogenesis, since the majority of human PCA are adenocarcinomas.

On the other hand, NECa occurs in nearly 100% of the TRAMP mice in the FVB background and the DLP undergoes more epithelial lesion growth than does the VP [9, 12]. This might have contributed to the assumption that a single lineage of carcinogenesis progressed from DLP epithelial lesions to poorly differentiated Ca (i.e., NECa) by the Greenberg group. Based on the current information, the C57BL/6 background will be a preferred choice over

the FVB background for chemoprevention studies since the former allows a clear separation of estimation of the impact of the test agents on both lineages of lesions in the prostate, provided that the studies are terminated before SV tumors in the C57BL/6 TRAMP mice become a serious complication to survival.

Since many studies had followed the Greenberg paradigm for interpretation of their results, it will be prudent to carefully look into the genetic background of TRAMP mice, the manner with which prostate/tumors were collected and the end point(s) chosen to evaluate the efficacy. Some of the studies as shown below could be questionable in term of the conclusions. In the study by Adhami *et al* [43], TRAMP mice (C57BL/6 background) were given water containing 0.1% green tea polyphenols (GTP) starting at ages representing different stage of the disease: 6 WOA (normal prostate), 12 WOA (PIN), 18 WOA (WD) and 28 WOA (MD). Follow-up monitoring showed that the earlier the treatment started, the greater was tumor-free survival extended. The mean genitourinary weights showed the same trends. IGF-I and its downstream targets including phosphatidylinositol 3-kinase and pAkt were significantly inhibited only when GTP treatment was initiated no later than 18WOA. In another study, TRAMP mice (C57BL/6 background) starting at 4, 12, 20, and 30 WOA were fed with control or 1% silibinin supplemented diet for 8 to 15 weeks [51]. In general, silibinin feeding inhibited neoplastic progression of the prostate in TRAMP mice at various stages. For the most part, the tumor stage at onset time of treatment determined the mechanisms for silibinin's efficacy. Silibinin treatment during the early stages of prostate tumor development in TRAMP mice inhibited the progression at PIN stage *via* anti-proliferation. When the mice were burdened with higher stage lesions, silibinin significantly delayed the progression of the disease *via* both anti-proliferative and anti-angiogenesis mechanisms. Anti-angiogenesis, along with inhibition of epithelial-mesenchymal transition (EMT) *via* decreasing the expression of MMPs, snail-1, and fibronectin, as well as increasing E-cadherin expression levels might also contribute to the anti-metastatic effect of silibinin. Studies aiming to define the "stage-specific" efficacy and mechanisms of chemoprevention are very crucial for the identification of *in vivo* targets mediating the chemopreventive effect of corresponding agents. In addition, the information will be very important to select the right indications for clinical trials.

6. The concept of cancer stem cells in TRAMP model

The concept of cancer stem cells (CSC) or tumor-initiating cells assumes that cancers are mainly sustained by a small pool of neoplastic cells, which are responsible for cancer initiation and/or progression. Although currently no single protein is widely accepted as a definitive stem cell marker in the prostate, investigations using *in vitro* and *in vivo* models, especially tissue recombination strategies, reveal a multifaceted nature of stem cells for prostate cancer and highlight the importance of targeting cancer stem cells in the therapy and prevention of prostate cancer [57-59]. The role of "cancer stem cells" in TRAMP carcinogenesis has received some attention. Chiaverotti *et al* observed an increased frequency of neuroendocrine precursor lesions in TRAMP mice with FVB background as

early as 4 WOA [9]. Some of these lesions exhibited properties of bi-potential stem cells as evidenced by co-expressing the transcriptional factors *Foxa1* and *Foxa2*, and markers of epithelium (E-cadherin) and neuroendocrine (SYP). In their proposed model of two lineages of carcinogenesis [9], a pluripotent stem cell of the normal prostate is transformed by SV40 Tag expression and starts to proliferate. Initially these proliferative foci maintain a transitional epithelial/NE phenotype co-expressing E-cadherin, SYP, as well as *Foxa1* and *Foxa2*. These bipotential cells continue to proliferate, undergo full NE differentiation with loss of E-cadherin and then progress to an overt NECa. On the other hand, luminal cells of the normal prostate proliferate and form focal areas of hyperplasia after transformed by SV40 Tag expression. These foci expand to the entire glandular lumen and end up with AHT but do not invade the surrounding stroma and do not metastasize.

Huss *et al* reported the expression and function of a breast cancer resistance protein (BCRP/ABCG2) in putative prostate stem cells and prostate tumor stem cells in several models including TRAMP mice (C57BL/6 TRAMP X FVB F1) [60]. BCRP is a member of the ATP-binding cassette (ABC) transporter family and expressed consistently by adult stem cell populations that possess pluripotentiality and long-term repopulation capability. IHC staining indicated that the BCRP⁺ putative tumor stem cells were localized to foci of AR-cells in glands of VP, where the greatest number of NECa would arise after castration. The AR- foci that contained the BCRP⁺ cells were preexisting and not induced by androgen deprivation since the frequency of BCRP⁺ cells was similar in intact (2.0%) and castrated (1.8%) TRAMP mice (between 1 and 14 days post-castration). In TRAMP mice, BCRP⁺/AR-cells behaved as label-retaining cells, a stem cell characteristic. The role of BCRP⁺ putative tumor stem cells as the nidus of NECa was further supported by the fact that NECa harvested from castrated TRAMP mice contained large focal areas of proliferating, BCRP⁺/AR-/SYP⁺ cells. BCRP-mediated efflux of androgen might be one of the mechanisms for maintenance of the prostate stem cell phenotype since it might be associated with their insensitivity to androgen-mediated differentiation and androgen deprivation-induced apoptotic cell death.

ADT was reported to result in a state of androgen independence with more malignant behavior in TRAMP mice [41]. Whether certain types of “cancer stem cells” were activated during or after castration awaits further investigation. Recently, Tang *et al* studied the effect of surgical castration on the expression of stem cell markers in the prostates of TRAMP mice [61]. They castrated TRAMP mice (genetic background not specified) at 12 WOA and dissected the DLP lobes at two time-points: 10 weeks (Cas-10; n=12) and 20 weeks (Cas-20; n=9) after castration. They found that stem cell markers Sca-1 (stem cell antigen-1), CD133 and c-Kit (CD117) were overexpressed in the luminal cells of the Cas-10 group, but not in the Cas-20 group. Immuno-blots showed that the expression of bcl-2 and GRP78 were significantly higher in the DLP from the Cas-10 compared to that from the Cas-20 group. Using anti-Sca-1 antibody conjugated magnetic beads, they estimated the abundance of Sca-1 positive cells in the cell suspension prepared by digesting the DLP lobes with collagenase/hyaluronidase/DNase I. They found that the abundance of Sca-1

cells was more than 3 times higher in the TRAMP Cas-10 group than in wild-type mice at 12WOA. However, it was dramatically lower in the TRAMP Cas-20 group, which was consistent with the IHC data. Although their work described the dynamic expression of certain stem cell markers during TRAMP carcinogenesis in DLP, the information is too preliminary to draw any conclusion on the effect of castration on “cancer stem cells” in TRAMP mice.

In one recently published paper [15], our group compared different treatment regimens with MSeA on TRAMP mice (C57BL/6) to investigate whether MSeA could irreversibly inhibit early events in TRAMP carcinogenesis (e.g. activation of “cancer stem cells”). MSeA exposure to TRAMP mice (C57BL/6) from 5 to 15 WOA was sufficient to elicit protective effects against the two lineages of carcinogenesis to the same extent as continuous MSeA exposure from 5 to 23 WOA [15]. Similar findings in a chemically-induced mammary carcinogenesis model was reported by Ip *et al* [62], in which selenium-enriched garlic given for 1-month duration right after a single carcinogen exposure was as effective as when it was provided throughout the 6-month post-initiation period of the study. Those data suggested a possibility that MSeA treatment during the initiation stage of carcinogenesis permanently inactivated early critical initiated (“cancer stem”) cells in both lineages. The potential mechanisms of inactivation of “cancer stem cells” by MSeA might be apoptosis, which is consistent with the pro-apoptotic effect of MSeA and MSeC in TRAMP DLP reported previously [13], or another lasting epigenetic modification.

7. Conclusion

The paradigm of distinct lineages of carcinogenesis in the TRAMP model and our data with MSeA, a sulindac derivative compound and AGN extract shed new light on the utility of this preclinical model for chemoprevention studies in spite of much concern regarding of the NE nature of the resultant carcinomas and metastasis. Future data collection and analyses should incorporate this new knowledge for efficacy assessment and for molecular target validations, avoiding “apple versus orange” comparisons.

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