

Original Article

## Establishment of an Invasive Prostate Cancer Model in Transgenic Rats by Intermittent Testosterone Administration

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**Abstract:** We have established a transgenic rat for adenocarcinoma of the prostate (TRAP) model that features uniform adenocarcinoma development in prostatic lobes at high incidence within a short experimental period. However, no invasive carcinomas with reactive stroma characteristics similar to those in man were observed. We therefore have focused on a new model for invasive carcinoma of the prostate using TRAP rats. In experiment 1, male TRAP rats in groups 1 and 2 were treated with orchietomy at day 0 of the experiment. Rats in groups 1–3 underwent testosterone propionate (TP) implantation from weeks 1 to 4 and from weeks 6 to 16. Rats in groups 1 and 3 were given 3,2'-dimethyl-4-aminobiphenyl (DMAB) after TP implantation. The rats of group 4 served as controls. In experiment 2, the rats were divided into three groups, none of which received DMAB or orchietomy, treated with TP continuously or with the treatment withdrawn once or twice. In experiment 1, invasive adenocarcinomas with abundant collagenous stroma were found in the dorsolateral and anterior prostate, some of which showed perineural space invasion at week 16. The number of invasive carcinoma foci was most frequent in group 3. In experiment 2, invasive adenocarcinoma development in the lateral prostates was correlated with the number of TP administration/withdrawal cycles. In conclusion, our newly established rat model for invasive adenocarcinoma of the prostate could serve as a useful preclinical model for evaluating the *in vivo* efficacy of preventive and therapeutic agents targeting of the tumor microenvironment. (DOI: 10.1293/tox.2013-0052; J Toxicol Pathol 2014; 27: 43–49)

**Key words:** prostate cancer, animal model, cancer invasion, transgenic rat, testosterone propionate, intermittent administration

### Introduction

Prostate cancer is the most common cancer and the second leading cause of death from cancer among men in the US. It has been estimated there will be approximately 238,590 new cases of prostate cancer and 29,720 deaths from prostate cancer in the US in 2013<sup>1</sup>. In Japan, the prevalence and mortality of prostate cancer has also been increasing, along with in the so-called nutrition transition<sup>2,3</sup>. Androgen ablation therapy is generally applied for prostate cancer because of hormone-dependent growth. However, outgrowth of androgen-independent and metastatic cancer cells is a frequent outcome, eventually leading to death of the patient. Therefore, understanding of the mechanisms of the acqui-

sition of metastatic potential or the androgen-independent phenotype of cancer cells is urgently required.

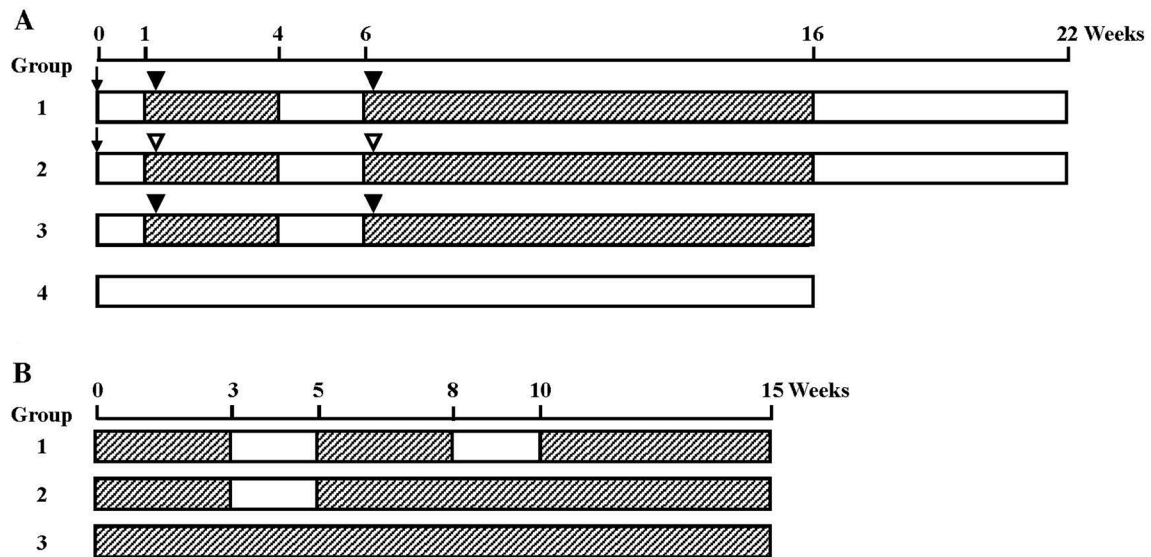
We have established a rat cancer model responding to the need for *in vivo* systems that adequately reproduce the spectrum of human prostate cancers. Administration of 3,2'-dimethyl-4-aminobiphenyl (DMAB) induces noninvasive and androgen-dependent adenocarcinomas in the ventral prostate, while additional long-term treatment with testosterone propionate (TP) causes development of invasive and metastasizing androgen-independent adenocarcinomas arising from the dorsolateral and anterior prostate and seminal vesicles<sup>4,5</sup>. However, a long period of about 60 weeks is required to induce prostate cancers in both carcinogenesis models, and the incidence of lesion development is relatively low. Therefore, we have established transgenic rats bearing a probasin promoter/simian virus 40 (SV40) T antigen construct to resolve these problems<sup>6</sup>. This model, the transgenic rat for adenocarcinoma of the prostate (TRAP), features development of high-grade prostatic intraepithelial neoplasia (HGPIN) from 4 weeks of age and androgen-dependent well-moderately differentiated adenocarcinomas with 100% incidences by the age of 15 weeks. These

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**Fig. 1.** Experimental design for experiments 1 (A) and 2 (B) for induction of invasive prostate adenocarcinomas in TRAP rats. The animals used were male TRAP rats that were 6 weeks of age at commencement of the study. Shaded, testosterone propionate, silicone rubber tube (40 mg); arrow, bilateral orchietomy; filled arrowhead, DMAB 50 mg/kg b.w.; open arrowhead, vehicle (corn oil).

characteristics of the TRAP model have been shown to be very suitable for evaluation of strategies for chemoprevention and treatment<sup>7–10</sup>. Microinvasive carcinomas characterized by a budding morphology from acini are observed in an age-dependent manner in TRAP rats, but these lesions are generally only 0.2–0.3 mm diameter in size and take over 35 weeks to develop<sup>11</sup>. We speculated that testosterone administration might be of paramount importance in the induction of invasive carcinoma in our transgenic rats based on our experience with the DMAB combined with TP-induced prostate carcinogenesis model. In the present study, we therefore assessed whether testosterone exposure might result in a high-grade invasive phenotype or metastatic lesions in TRAP rats.

## Materials and Methods

### Chemicals

TP was purchased from Sigma-Aldrich (St. Louis, MO, USA) and DMAB was obtained from Matsugaki Pharmaceutical Co. (Osaka, Japan). The purity of DMAB was >98%. Antibody for androgen receptor (AR) was obtained from Santa Cruz Biotechnology Inc (N-20, Santa Cruz, CA, USA). The antibody for Ki-67 was from Acris Antibodies GmbH (SP-6, Hiddenhausen, Germany).

### Animals

Male heterozygous TRAP rats with a Sprague–Dawley genetic background were obtained from Oriental BioService Inc. (Minamiyamashiro, Kyoto, Japan) and were housed in plastic cages with hardwood chips in an air-conditioned room with a 12 h light/dark cycle at  $23 \pm 2^\circ\text{C}$  and  $50 \pm 10\%$  humidity. Food (Oriental MF; Oriental Yeast Co., Ltd.,

Tokyo, Japan) and tap water were available *ad libitum*. They were acclimatized for 1 week before use. Surgical treatments, such as orchietomy and tube implantation, were carried out under deep isoflurane anesthesia. All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Nagoya City University Graduate School of Medical Sciences.

### Experimental protocols

**Experiment 1:** A total of 24 male TRAP rats aged 6 weeks were randomly divided into four groups. Rats in groups 1 and 2 were treated with bilateral orchietomy at day 0 of the experiment. Those in groups 1–3 underwent subcutaneous implantation of 2-cm-long silicone rubber tubes (Silascon<sup>®</sup>, inner diameter, 0.2 cm; outer diameter, 0.3 cm, Kaneka Medix Corporation, Osaka, Japan) containing 40 mg TP sealed at both ends with silicone rubber sealing compound (KE-42, Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) into the interscapular region from weeks 1 to 4 and from weeks 6 to 16. The TP implants were replaced at 6-week intervals. Rats in groups 1 and 3 were subcutaneously given DMAB at a dose of 50 mg/kg body weight on the second day after TP tube implantation. No treatment was performed in rats of group 4, which served as controls. Animals were euthanized at weeks 16 and 22 after the beginning of the experiment (Fig.1A).

**Experiment 2:** A total of 24 heterozygous male TRAP rats aged 6 weeks were randomly divided into three groups. All were given TP implants in the same manner as in experiment 1 at day 0. The duration of TP administration was different among the groups; that is, TP was administered in experimental weeks 0–3, 5–8 and 10–15 in group 1 and in experimental weeks 0–3 and 5–15 in group 2. Group 3

was continuously administered TP by implants throughout the experiment. The experiment was terminated at week 15 (Fig. 1B).

In both experiments, blood samples were collected from the abdominal aorta under deep anesthesia, and prostates were removed and fixed in formalin. For tissue preparation of prostate glands, four sagittal slices of the ventral prostate, two sagittal samples of the dorsolateral prostate including the urethra, and two transverse slices from each side of the anterior prostate including seminal vesicles were embedded in paraffin. Tissues were processed routinely and stained with hematoxylin and eosin for histopathological examination. Testosterone and estradiol levels in serum were analyzed using radioimmunoassays by a commercial laboratory (SRL, Inc., Tokyo, Japan).

### *Immunohistochemistry*

Deparaffinized sections were incubated with diluted antibodies for AR and Ki-67. The immunohistochemical analysis was performed with a Discovery XT System (Ventana Medical Systems, Tucson, AZ, USA). Incubation with primary antibodies was carried out for 3 hours followed by a one hour incubation with biotinylated anti-rabbit secondary antibody (Vectastain ABC Kit Rabbit IgG, Vector Laboratories, Burlingame, CA, USA) and a DAB detection kit (Ventana Medical Systems) according to the manufacturer's instructions. Sections were counterstained with hematoxylin to facilitate orientation.

### *Immunofluorescence*

Deparaffinized sections were autoclaved at 120°C for 20 min in antigen retrieval solution (Nichirei Biosciences Inc.) and then allowed to cool. Sections were incubated with 1% skim milk for 1 hour at room temperature. For double staining, anti-smooth muscle actin antibodies (1A4, dilution 1:1,000, mouse monoclonal, Dako) and anti-vimentin antibodies (EPR3776, dilution 1:400, rabbit monoclonal, Abcam) were simultaneously added to the slides and incubated for 1 hour at room temperature. After washing the slides with PBS, fluorescein-labeled goat anti mouse IgG (Life Technologies Corporation) and tetramethylrhodamine-labeled goat anti-rabbit IgG (Life Technologies Corporation) were added followed by incubation at room temperature for 1 hour. After washing the slides with PBS, the sections were mounted with Vectashield containing DAPI (Vector Laboratories) and subjected to fluorescence microscopy.

## **Results**

### *Experiment 1*

At week 16, foci of invasive adenocarcinoma with abundant collagenous stroma were found in lateral, dorsal and anterior prostates of groups 1–3 (Fig. 2A, B, D, E), along with minute ventral invasive carcinomas with minimal fibrous stroma. Cancer invasion into perineural spaces was also observed (Fig. 2E). Almost all of infiltrating carcinoma cells expressed AR (Fig. 2C, F). The incidences of

invasive adenocarcinoma varied among the groups, tending to be higher in group 3 in all prostatic lobes (Table 1). Similarly, the number of invasive carcinoma foci was highest in group 3 (Table 1). There were no differences in histopathological characteristics of invasive adenocarcinomas among the groups. Development of small cell carcinomas of the prostate was sporadically noted, but there were no differences in incidence among the groups. No metastasis of cancer lesions to distant organs was found in any of the groups. Noninvasive adenocarcinomas in the ventral, lateral prostates were observed in all rats of groups 1–4.

At week 22, neoplastic lesions of the prostates were completely resolved with massive involution in all rats of groups 1 and 2. This indicated that all of the invasive adenocarcinomas developed in prostate glands were androgen-dependent (data not shown).

### *Experiment 2*

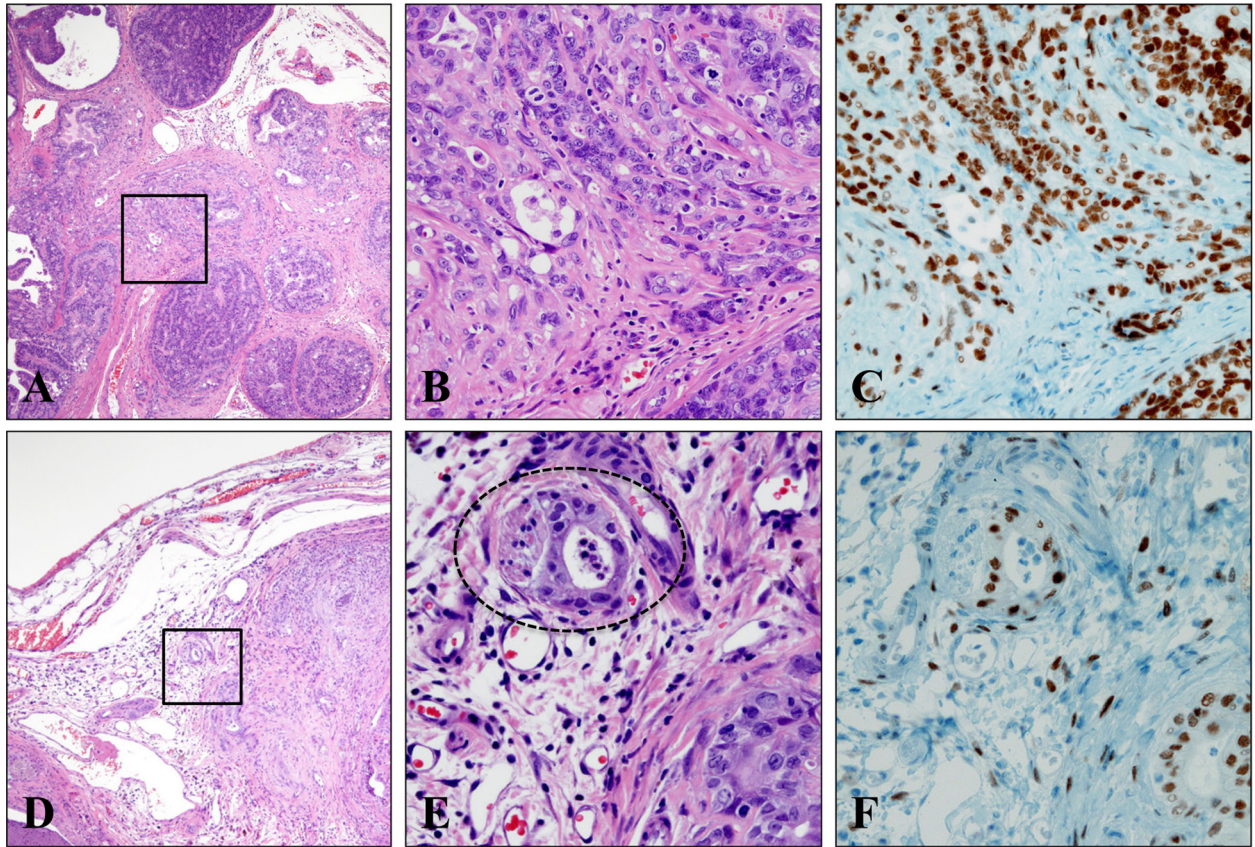
The results of experiment 1 indicated that bilateral orchiectomy or DMAB administration did not deeply contribute to efficient induction of invasive adenocarcinomas. Thus, we focused on whether TP administration/withdrawal was important.

A significant increase of invasive adenocarcinoma development was observed in group 1, and this correlated well with the number of TP administration/withdrawal cycles in the lateral prostates (Table 2). Multicentric development of invasive adenocarcinoma foci with abundant collagenous stroma was found (Fig. 3A–D), and invasive cancer cells were observed in the stroma (Fig. 3E) and were positive for AR protein (Fig. 3F). Some invasive lesions consisted of cells with atrophic features, but more than 50% of these cells were labeled for Ki-67, suggesting that they were high-grade adenocarcinomas (Fig. 3G). Reactive stromal cells surrounding invasive cancers expressed both smooth muscle actin and vimentin and were therefore revealed to be cancer-associated myofibroblasts (Fig. 4). Noninvasive adenocarcinomas in the ventral and lateral prostates were found in all rats of groups 1–3.

## **Discussion**

The TRAP rat features sequential progression from prostatic intraepithelial neoplasias (PINs) to noninvasive adenocarcinomas through prostate epithelial cell-specific expression of the SV40 T antigen regulated by the androgen-dependent probasin promoter. We have applied the TRAP rat model to validate the chemopreventive effects of a variety of chemicals, and cancer development in TRAP rats is very sensitive to chemicals that modulate the AR axis, such as flutamide, finasteride, resveratrol or angiotensin II receptor blockers<sup>7, 9, 12</sup>. These characteristics underly its acceptability to mimic early-stage hormone naïve human prostate cancer without an invasive phenotype.

In the present study, we established a novel rat model for invasive adenocarcinoma of the prostate in TRAP rats by intermittent TP administration (group 1 in experiment 2,



**Fig. 2.** Representative histopathological findings of invasive adenocarcinomas of the lateral prostate in group 3 (A–C) and anterior prostate in group 2 (D–F). Low (A, D) and high (B, E) magnifications of lateral and anterior prostates at week 16 after the beginning of experiment 1. The rectangles in (A) and (D) represent the areas from (B) and (E), respectively. The dotted circle in (E) indicates perineural cancer invasion. (C, F) AR immunohistochemistry.

**Table 1.** Incidence and Multiplicity of Invasive Adenocarcinoma (at Week 16, Experiment 1)

Group	No. of rats	Ventral		Lateral		Dorsal		Anterior	
		Incidence (%)	No. of foci	Incidence (%)	No. of foci	Incidence (%)	No. of foci	Incidence (%)	No. of foci
1	5	1 (20)	0.20 ± 0.45	4 (80)	1.80 ± 1.64	1 (20)	0.20 ± 0.45	3 (60)	0.80 ± 0.84
2	4	2 (50)	1.00 ± 1.15	3 (75)	3.75 ± 3.78	1 (25)	0.25 ± 0.50	3 (75)	2.75 ± 3.10
3	4	4 (100)	4.00 ± 1.41 <sup>a,b</sup>	4 (100)	6.00 ± 2.45 <sup>c</sup>	3 (75)	1.00 ± 0.82	4 (100)	2.00 ± 0.82
4	5	2 (40)	0.40 ± 0.55	1 (20)	0.20 ± 0.45	0	-	2 (40)	0.40 ± 0.55

<sup>a</sup> P<0.001 vs groups 1 and 4; <sup>b</sup> P<0.01 vs group 2; <sup>c</sup> P<0.05 vs group.

**Table 2.** Incidence and Multiplicity of Invasive Adenocarcinoma (Experiment 2)

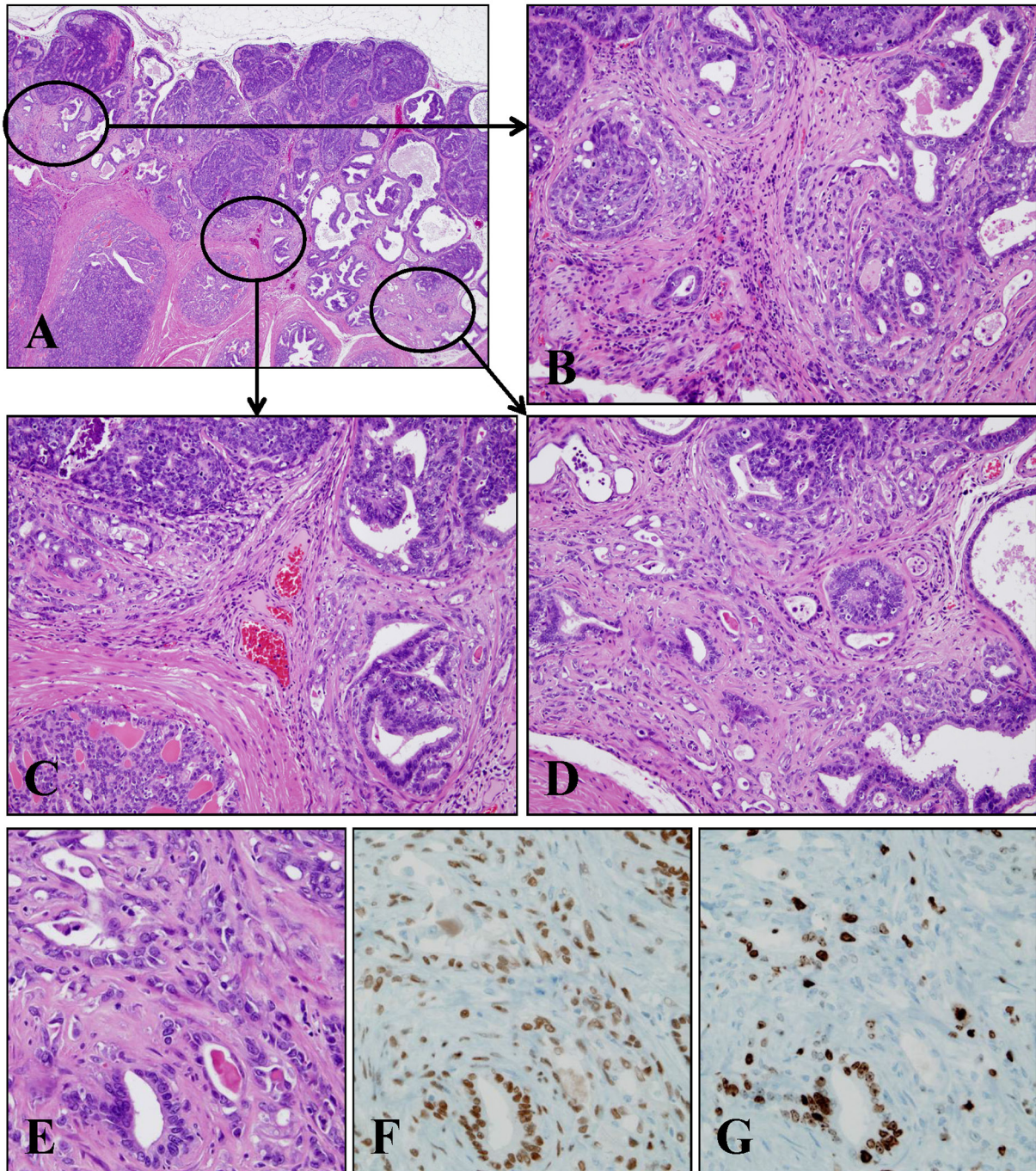
Group	No. of rats	Ventral		Lateral		Dorsal		Anterior	
		Incidence (%)	No. of foci	Incidence (%)	No. of foci	Incidence (%)	No. of foci	Incidence (%)	No. of foci
1	9	8 (89)	1.78 ± 1.10	8 (89)	4.22 ± 2.59**	2 (22)	0.44 ± 1.01	6 (67)	1.11 ± 1.05
2	9	7 (78)	1.67 ± 1.41	5 (56)	0.89 ± 0.93	0	-	7 (78)	0.78 ± 0.44
3	6	5 (83)	3.17 ± 1.72	2 (33)	0.33 ± 0.52	0	-	1 (17)	0.17 ± 0.41

\*\* P<0.01 vs groups 2 and 3.

shown in Fig. 1). The invasive carcinomas induced simulate human prostate cancer in several respects, such as perineural invasion and multicentric lesion development. To investigate mechanisms of prostate cancer progression, we pre-

viously combined administration of both DMAB and TP<sup>5</sup>. While several experiments were conducted with the aim of increasing the incidence of invasive cancer and shortening the experimental period, none exceeded the DMAB + TP





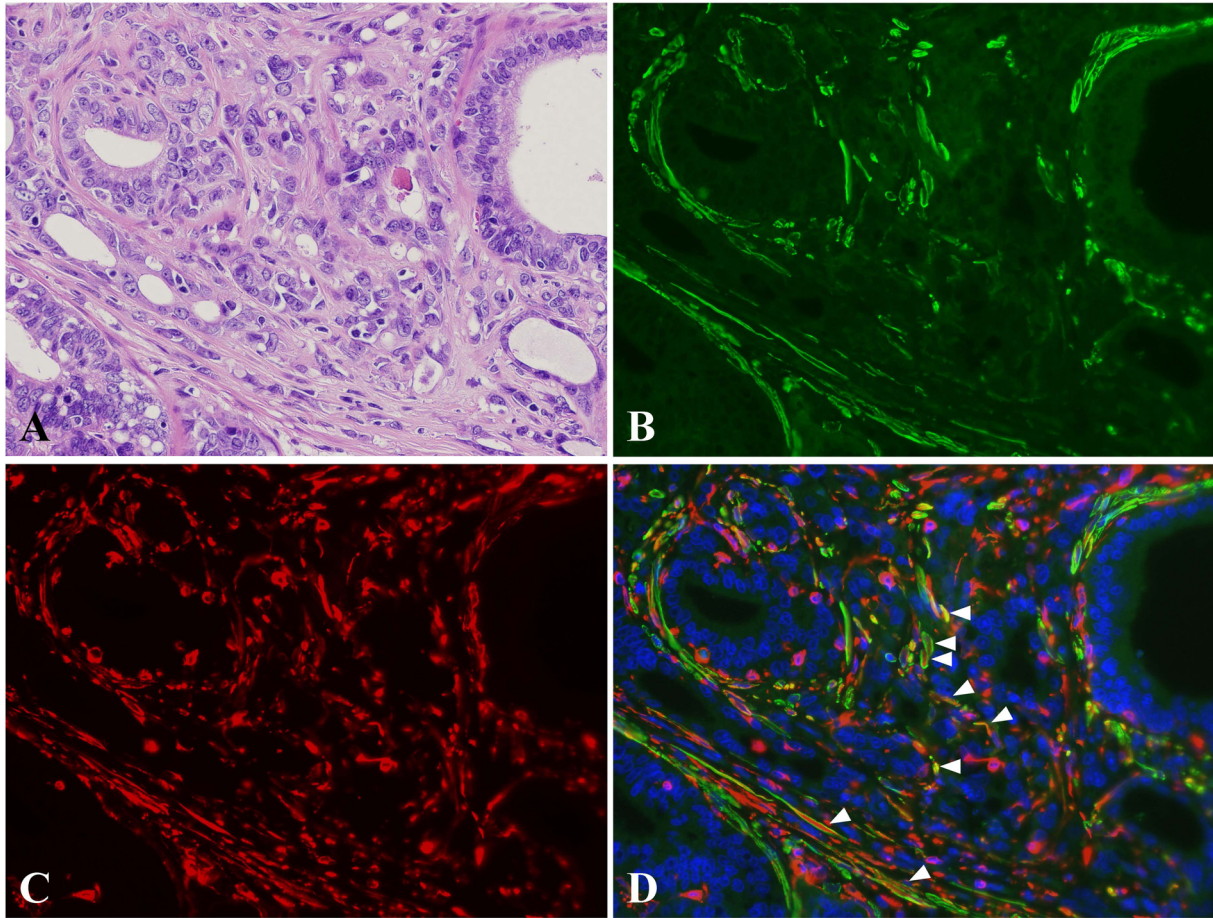
**Fig. 3.** Multicentric development of invasive adenocarcinomas in the lateral prostate in group 1 at week 15 after the beginning of experiment 2 (A–D). High magnifications of invasive adenocarcinoma with H&E (E), AR (F) and Ki-67 (G) staining.

model in terms of the cancer incidence<sup>13–16</sup>. The new prostate carcinogenesis model documented here is characterized by invasive adenocarcinoma development at a high incidence in a short period without carcinogen administration. This rat model should enable us to investigate candidate chemopreventive agents for therapeutic effects as well as chemopreventive properties against prostate cancer.

We found that invasive adenocarcinoma incidences became greater as we increased the TP administration/with-

drawal cycles for the TRAP rats. In our previous studies, testosterone induced invasive prostate adenocarcinomas in a dose- or duration-dependent manner after prostatic carcinogen treatment<sup>14, 15</sup>. However, continuous administration of testosterone alone earlier proved unable to cause development of invasive cancer with abundant reactive stromal tissue in the TRAP model<sup>6, 11</sup>. The present results thus lead us to speculate that physiological destruction of the normal acinar structure with stromal cell proliferation by androgen





**Fig. 4.** Immunofluorescence analysis of an invasive adenocarcinoma in a lateral prostate. H&E (A). Immunofluorescence for smooth muscle actin (green, B) and vimentin (red, C), and a merged image (D). Blue, DAPI. Myofibroblasts coexpressing smooth muscle actin and vimentin are indicated by the arrowhead in (D).

depletion plays an important role in the induction of invasive adenocarcinomas.

The process of primary cancer invasion, which initiates metastasis, is multifactorial and multistep and requires alteration of cell adherence, proteolytic degradation of extracellular matrix elements and tumor cell migration through tissue. Accumulating evidence has shown that stromal-epithelial interactions play critical roles in cancer progression<sup>17–19</sup>. The reactive tumor stroma mainly composed of cancer-associated fibroblasts (CAFs) including myofibroblasts, which are the predominant subpopulation of CAFs, is known to contribute to cancer development and progression<sup>18–20</sup>. Growth of myofibroblasts is reported to be stimulated by androgen<sup>21</sup>. TGF $\beta$  is one of the growth factors overexpressed in the prostate of rats after androgen ablation by orchiectomy<sup>22</sup>. TGF $\beta$ 1 induces reactive oxygen species production via enhancement of NOX4 expression and may underly fibroblast-to-myofibroblast differentiation in the prostatic stroma<sup>23</sup>, while myofibroblasts per se contribute to the production and activation of TGF $\beta$ 1 and stromal cell-derived factor-1 (SDF-1)/CXCL12 by autocrine signaling loops<sup>24</sup>. Phosphoglycerate kinase-1 (PGK1), a downstream

molecule of CXCL12-CXCR4 signaling, is upregulated in myofibroblasts, and this is involved in the enhanced proliferation and invasion of prostate cancer cells through activation of MMP, AKT and ERK pathways<sup>25</sup>.

In conclusion, TP administration/withdrawal cycles appear to be of paramount importance to induction of invasive adenocarcinomas in the TRAP rat prostate. Our new rat prostate carcinogenesis model for invasive adenocarcinoma should provide opportunities to investigate molecular mechanisms of prostate cancer progression and may serve as a useful preclinical model for evaluating *in vivo* efficacy of preventive and therapeutic agents in terms of the tumor microenvironment.

**Disclosure statement:** The authors have no conflicts of interest.

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