

# Inhibition of Tumor Growth and Progression of LNCaP Prostate Cancer Cells in Athymic Mice by Androgen and Liver X Receptor Agonist

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## Abstract

**Androgen-dependent human LNCaP 104-S tumor xenografts progressed to androgen-independent relapsed tumors (104-Rrel) in athymic mice after castration. The growth of 104-Rrel tumors was suppressed by testosterone. However, 104-Rrel tumors adapted to androgen and regrew as androgen-stimulated 104-Radp tumors. Androgen receptor expression in tumors and serum prostate-specific antigen increased during progression from 104-S to 104-Rrel but decreased during transition from 104-Rrel to 104-Radp. Expression of genes related to liver X receptor (LXR) signaling changed during progression. LXR $\alpha$ , LXR $\beta$ , ATP-binding cassette transporter A1 (ABCA1), and sterol 27-hydroxylase decreased during progression from 104-S to 104-Rrel. These coordinated changes in LXR signaling in mice during progression are consistent with our previous findings that reduction of ABCA1 gene expression stimulates proliferation of LNCaP cells. To test if attenuation of LXR signaling may enhance prostate cancer progression from an androgen-dependent state to an androgen-independent state, castrated mice carrying 104-S tumors were given the synthetic LXR agonist T0901317 by gavage. T0901317 delayed progression from 104-S to 104-Rrel tumors. Based on our *in vivo* model, androgen is beneficial for the treatment of androgen-independent androgen receptor-rich prostate cancer and modulation of LXR signaling may be a potentially useful therapy for prostate cancer.** (Cancer Res 2006; 66(13): 6482-6)

## Introduction

Androgen ablation therapy, proposed by Charles Huggins in 1941, is still the standard therapy for certain prostate cancer patients. However, most patients who receive androgen ablation therapy develop androgen-independent tumors. Previously, we derived androgen-independent human LNCaP 104-R1, 104-R2, and CDXR prostate cancer cells from clonally isolated androgen-dependent LNCaP 104-S cells after androgen deprivation in culture (1–3). These androgen-independent LNCaP cells have elevated androgen receptor expression and express prostate-specific antigen (PSA) upon androgen treatment (1–5). Androgens paradoxically inhibit the proliferation of these cells (1–5) partially by down-

regulating *c-myc* and inducing the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup>, which causes G<sub>1</sub> cell cycle arrest (1–3). Implanted 20 mg testosterone pellets, which provide a serum testosterone level 10-fold higher than that in healthy men and male mice, suppressed growth of 104-R1, 104-R2, and CDXR tumors in athymic mice (3–5). However, it is unclear if a physiologic concentration of serum testosterone can suppress growth of androgen receptor-rich androgen-independent prostate tumors. It has also not been shown whether a pharmacological concentration of testosterone (10-fold higher than the physiologic level) derived from an implanted pellet will suppress growth of androgen-dependent 104-S tumors or androgen-independent relapsed tumors generated in mice. Androgen may regulate proliferation of prostate cancer cells in part by modulating the expression of ATP-binding cassette transporter A1 (ABCA1; ref. 6), which is a target gene of liver X receptor (LXR). Expression of ABCA1 mRNA and protein in androgen-dependent 104-S cells is inhibited by androgen, and knockdown of ABCA1 expression in 104-S cells increases the rate of cell proliferation (6). In LNCaP cells, expression of *LXR $\alpha$* , *LXR $\beta$* , and *ABCA1* genes decreases during prostate cancer progression in cell culture (6), and LXR agonists suppress proliferation of both androgen-dependent and androgen-independent LNCaP cells (7). These results suggest that LXR agonists may affect prostate cancer progression *in vivo*. Therefore, we developed an *in vivo* model of prostate cancer progression using LNCaP cancer cells and determined whether androgen and LXR agonist may inhibit prostate tumor growth and prostate cancer progression in castrated mice.

## Materials and Methods

**Animals.** Experiments involving mice were approved by the University of Chicago Institutional Animal Care and Use Committee (Chicago, IL). Intact male BALB/c *nu/nu* mice (National Cancer Institute, Frederick, MD) were injected s.c. in both flanks with  $1 \times 10^6$  LNCaP 104-S cells suspended in 0.5 mL Matrigel (BD Biosciences, Franklin Lakes, NJ). Tumors were measured weekly using calipers, and volume was calculated using the following formula: volume = length  $\times$  width  $\times$  height  $\times$  0.52 (4, 5). Testosterone propionate and cholesterol were purchased from Sigma (St. Louis, MO).

**Western blotting analysis.** Samples were prepared from tissue homogenized in 2 $\times$  Laemmli buffer as described (5). Androgen receptor and fatty acid synthase (FAS) levels were determined using antibodies against androgen receptor (AN-21; refs. 1–3, 5) and FAS (Novus, Littleton, CO). Actin (Chemicon, Temecula, CA) was used as a loading control.

**Real-time quantitative PCR.** Tissue RNA was prepared and reverse transcribed into cDNA as described (5). Primers for androgen receptor cDNA quantification were 5'-CGCCCCTGATCTGGTTTTC and 5'-TTCGGA-CACACTGGCTGTACA, and the androgen receptor probe was 5'-TGAG-TACCGCATGCACAAGCTCCG-3'. Primers and probes used for ABCA1,

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LXR $\alpha$ , LXR $\beta$ , sterol response element-binding protein (SREBP)-1a, and SREBP-1c cDNA quantification were described previously (6). Primers for CYP27 cDNA quantification were 5'-CGCAAGGCTGATCCAGAAGT-3' and 5'-CACTCTTCAACTCCCCGTC-3', and the CYP27 probe was 5'-CAAGGTGGTCCTGGCCCCGG-3'. All transcript levels were normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA levels in each sample. GAPDH cDNA levels were determined using the primers 5'-TCGACAGTCAGCCGCATCT and 5'-ACCTTCCCCATGGTGTCTGAG-3' and the probe 5'-CGTCGCCAGCCGAGCCACA-3'. The 5'-end of all probes was labeled with the reporter fluorescent dye carboxyfluorescein. The 3'-end of all probes was labeled with the quencher dye 6-carboxytetramethylrhodamine.

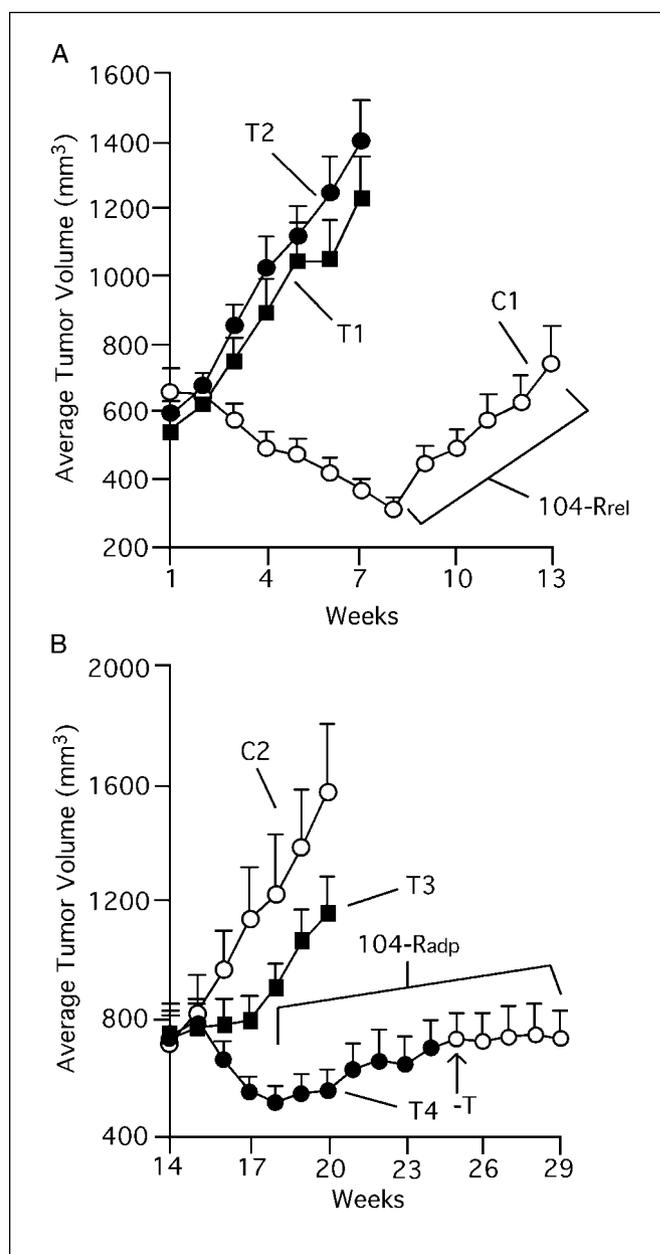
**PSA and testosterone assay.** Mice were bled from the retro-orbital plexus, and PSA and testosterone in sera from mice were assayed as described (5).

**Data analysis.** Data are the mean  $\pm$  SD or SE of three experiments or are representative of experiments repeated at least thrice. Unpaired Student's *t* test was used to evaluate the statistical significance of differences between groups with regard to gene expression based on results of real-time quantitative PCR experiments.

## Results and Discussion

To develop an *in vivo* prostate cancer progression model, androgen-dependent LNCaP 104-S cells were inoculated into mice to form androgen-dependent tumors (Fig. 1A). Because the response of 104-S cells to androgens is biphasic (1-3), we determined whether a s.c. implanted 20 mg testosterone propionate pellet could affect the growth rate of 104-S tumors. Serum testosterone level in mice of the T1 (intact mice implanted with 20 mg testosterone propionate pellet), T2 (intact mice without treatment), and C1 (castrated mice) groups was  $33.4 \pm 3.2$ ,  $2.5 \pm 2.0$ , and  $0.1 \pm 0.1$  ng/mL, respectively (week 3 in Fig. 1A). All 104-S tumors in T1 and T2 groups continued growing at a similar rate, indicating that the testosterone propionate pellet, which can suppress the growth of androgen receptor-rich androgen-independent LNCaP 104-R1, 104-R2, and CDXR tumors (1-5), did not affect the growth of 104-S tumors. LNCaP 104-S tumors in mice of group C1 began to regress within a week but started to regrow as androgen-independent relapsed 104-Rrel tumors 8 weeks after castration.

To determine the effect of different serum testosterone levels on 104-Rrel tumor growth, we used pellets with different ratios of testosterone propionate and cholesterol to provide a range of serum testosterone levels. The serum testosterone levels in castrated mice (four-five mice per group) implanted with 20 mg pellets of different testosterone propionate/cholesterol ratios were  $29.7 \pm 4.9$  (pure testosterone propionate),  $7.4 \pm 2.0$  (testosterone propionate/cholesterol = 1:1), and  $1.3 \pm 0.6$  ng/mL (testosterone propionate/cholesterol = 1:9). The serum testosterone level in the last group is close to the average physiologic concentration of serum testosterone in men and male mice. Castrated mice bearing 104-Rrel tumors (Fig. 1A) were separated into three groups (Fig. 1B): C2 (no treatment), T3 [mice implanted with 20 mg testosterone propionate/cholesterol (1:9) pellets], and T4 (mice implanted with 20 mg pure testosterone propionate pellets). All 104-Rrel tumors in the T4 group shrank after 2 weeks, whereas all 104-Rrel tumors in the C2 group continued to grow (Fig. 1B). Tumor growth of 104-Rrel tumors in T3 group stopped but resumed after 4 weeks. Therefore, a serum testosterone level 10-fold higher than the physiologic concentration is necessary to cause regression of androgen-independent prostate tumors in athymic mice.

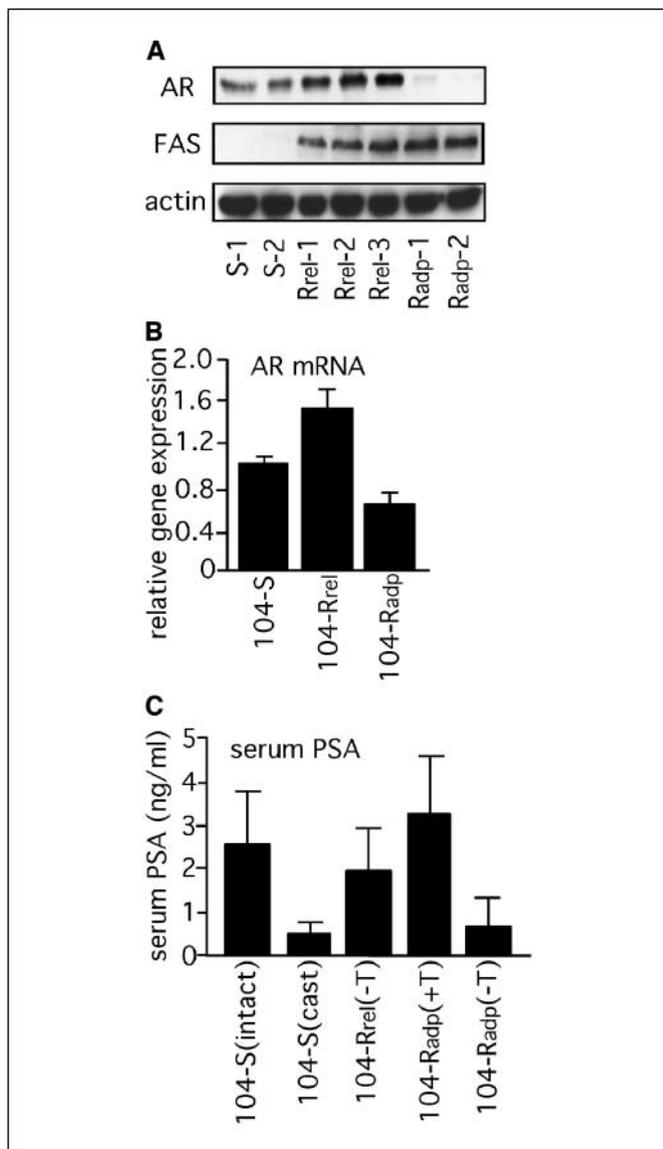


**Figure 1.** Progression of androgen-dependent LNCaP 104-S tumors to androgen-independent 104-Rrel tumors and androgenic growth suppression of 104-Rrel tumors. **A**, mice were injected s.c. with androgen-dependent 104-S cells. After allowing tumors to grow for 7 weeks, mice were separated into three groups and the time was designated as week 1 in the figure. The three groups, including (T1) intact mice implanted with 20 mg testosterone propionate pellets (■, 7 mice with 12 tumors), (T2) intact mice with no treatment (●, 14 mice with 19 tumors), and (C1) the castrated mice group (○, 24 mice with 36 tumors). **B**, mice in the castrated C1 group in (A) at the 14th week were separated into three groups, including a (C2) control group (○, 6 mice with 9 tumors), a (T3) low-dosage androgen treatment group that received a s.c. implant of a 20 mg testosterone propionate/cholesterol (1:9) pellet (■, 9 mice with 12 tumors), and a (T4) high-dosage androgen treatment group that received a s.c. implant of a 20 mg pure testosterone propionate pellet (●, 10 mice with 12 tumors). In the T4 group, new testosterone propionate pellets were implanted at week 22, and testosterone propionate pellets were removed at week 25. Points, mean of tumor volumes; bars, SE.

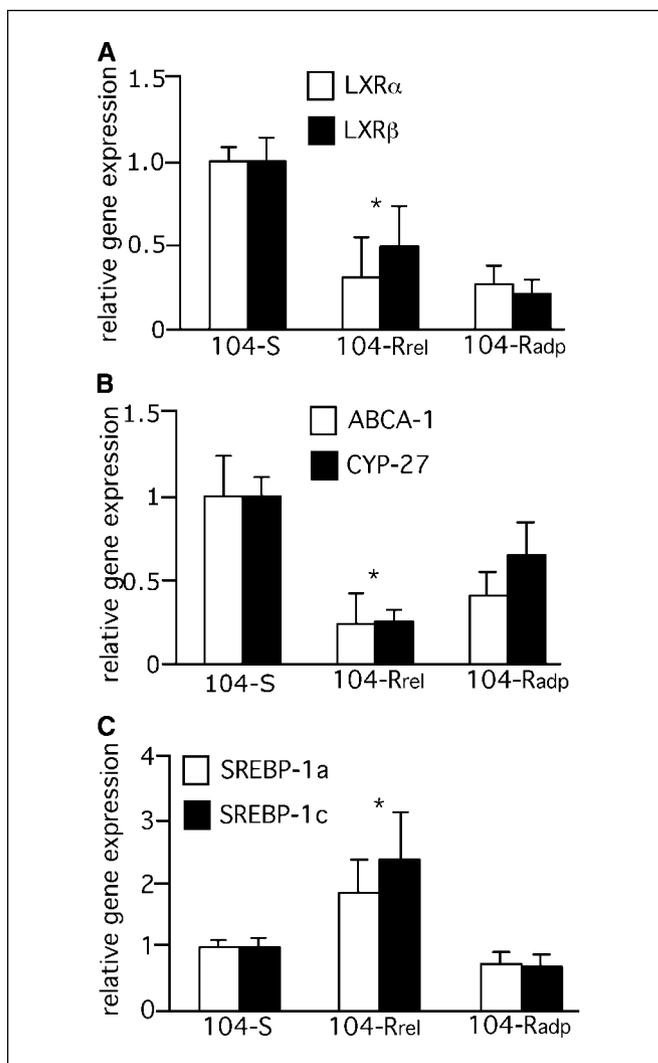
All 104-Rrel tumors in the T4 group eventually adapted to androgenic suppression and resumed growth as adapted 104-Radp tumors. 104-Radp tumors grew at a much slower rate compared with the growth rate of 104-S and 104-Rrel tumors, and removal of

androgen seemed to retard the growth of 104-Radp tumors, suggesting that 104-Radp tumors are androgen stimulated.

Increased androgen receptor expression is observed in prostate cancer cells of many patients developing androgen-independent relapsed tumors (8, 9). *In vitro*, elevated androgen receptor expression in androgen-independent LNCaP cells is necessary for androgenic suppression of the proliferation of these cells (3). Androgen receptor protein (Fig. 2A) and mRNA (Fig. 2B) in tumors



**Figure 2.** Androgen receptor, FAS, and PSA expression in tumor xenografts. Tumor and serum samples were obtained from mice in the experiment described in Fig. 1. A, androgen receptor (AR), FAS, and actin protein levels in androgen-dependent 104-S tumors (S-1 and S-2; T2 group in Fig. 1A; week 7), androgen-independent relapsed 104-Rrel tumors (Rrel-1-Rrel-3; C1 group in Fig. 1A; week 13), and adapted 104-Radp tumors (Radp-1 and Radp-2; T4 group in Fig. 1B; week 29) were assayed by Western blot. B, expression level of androgen receptor mRNA in tumors analyzed in (A) was determined by quantitative real-time PCR. C, serum PSA level of intact mice with 104-S tumors (T2 group in Fig. 1A; week 6), castrated mice with 104-S tumors (C1 group in Fig. 1A; week 3), 104-Rrel tumors without testosterone propionate (C1 group in Fig. 1A; week 13), 104-Radp tumors with pure testosterone propionate (T4 group in Fig. 1B; week 24), and 104-Radp tumors without testosterone propionate (testosterone propionate removed from T4 group mice in Fig. 1B; week 29). Columns, mean for three to eight serum samples; bars, SD.



**Figure 3.** Expression of LXR signaling-related genes in tumor xenografts. RNA was prepared from tumors described in Fig. 2. 104-S, S1 and S2; 104-Rrel, Rrel-1 to Rrel-3; 104-Radp, Radp-1 and Radp-2. Gene expression was determined by quantitative real-time PCR for mRNA for LXRα and LXRβ (A), ABCA1 and CYP27 (B), and SREBP-1a and SREBP-1c (C). Statistically significant differences in gene expression were found between 104-S and 104-Rrel. \*,  $P < 0.05$ , assayed by *t* test.

increased during the progression from 104-S to 104-Rrel tumors but decreased during the transition from 104-Rrel to 104-Radp tumors, consistent with results obtained previously *in vitro* (2). The increase in androgen receptor expression level during progression from 104-S to 104-Rrel tumors, therefore, correlates with the suppression of the growth of 104-Rrel tumors by androgen (Fig. 1B). Androgen receptor down-regulation in 104-Radp tumors may release relapsed tumors from growth suppression caused by androgen.

PSA is the most common marker used for detecting prostate cancer growth in patients and decreases after androgen ablation therapy but increases again when androgen-independent prostate tumors develop (10). Castration of mice carrying 104-S tumors in the C1 group caused a decrease in serum PSA (Fig. 2C), but serum PSA levels increased when 104-Rrel tumors developed. When 104-Rrel tumors progressed to 104-Radp (Fig. 1B), removal of the testosterone propionate decreased serum PSA level (Fig. 2C),

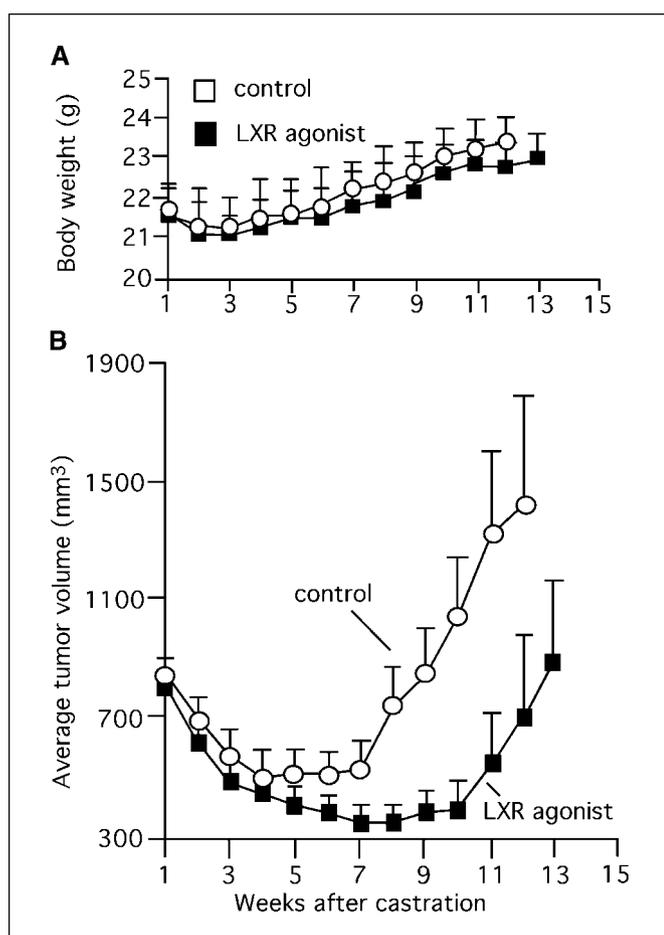
indicating that androgen receptor was still active in 104-Radp tumors.

The LXR signaling pathway plays an important role in homeostasis of fatty acids and cholesterol (11). Expression of FAS increases during progression of CWR22 prostate xenografts (12). FAS expression increased during progression from 104-S to 104-Rrel tumors (Fig. 2A) and remained high in 104-Radp tumors, suggesting that FAS might be important for advanced prostate tumor growth. LXR $\alpha$ , LXR $\beta$ , and ABCA1 mRNA in tumor xenografts all decreased during the progression from 104-S to 104-Rrel tumors and remained low in 104-Radp tumors (Fig. 3A and B). Sterol 27-hydroxylase (CYP27) is a member of the cytochrome P450 superfamily (13). 27-Hydroxycholesterol and cholestenic acid, products of CYP27, are endogenous ligands for LXR (14, 15). The expression of the CYP27 gene decreased during progression from 104-S to 104-Rrel tumors (Fig. 3C) as well. SREBP-1c (16), one isoform of SREBP-1 gene, belongs to a family of transcriptional regulators controlling the metabolic pathways for lipogenesis and cholesterol synthesis. SREBP expression increases in prostate cancer cells during progression from an

androgen-dependent to an androgen-independent state (17). Unlike other LXR signaling-related genes, the mRNA level of SREBP-1c increased during the progression from 104-S tumors to 104-Rrel tumors (Fig. 3C), similar to the change of SREBP-1a during progression. SREBP-1a is another isoform of SREBP-1, but it is not a target gene of LXR. The different pattern of mRNA expression between SREBP-1c and other LXR target genes may reflect the fact that other pathways, such as androgenic signaling (18), dominate the regulation of SREBP-1 expression during prostate cancer progression.

These coordinated changes in LXR signaling during progression *in vivo* are consistent with our previous findings that suppression of ABCA1 expression by androgen enhanced proliferation of androgen-dependent tumor cells in culture (6). Thus, under androgen-depleted conditions, ABCA1 is active and growth of androgen-dependent 104-S tumors is inhibited. During progression, the surviving androgen-independent 104-Rrel tumor cells may escape from ABCA1 suppression by down-regulating expression of LXR genes. Because the expression of many LXR signaling-related genes was lower in 104-Rrel tumors than in 104-S tumors, we hypothesized that attenuation in LXR signaling may assist prostate cancer progression from an androgen-dependent to an androgen-independent status. Based on this hypothesis, treatment with LXR agonist may stimulate ABCA1 as well as LXR signaling and retard the progression of prostate cancer in mice. To test this hypothesis, we investigated the effect of the potent synthetic LXR agonist T0901317 on our *in vivo* progression model. Previously, we showed that treatment of intact mice carrying 104-S tumors with T0901317 induces expression of the LXR target gene ABCA1 in tumors as well as retards growth of 104-S tumors (7). Mice with 104-S tumors were castrated and then gavaged with 10 mg/kg T0901317. Compared with the control group, there was no significant body weight loss or other observable side effects in the LXR agonist-treated mice (Fig. 4A). However, the development of 104-Rrel tumors after castration was delayed for 4 weeks (Fig. 4B) in mice treated with T0901317. Because treatment of LNCaP cells with a LXR agonist decreases the percentage of S-phase cells and increases the expression of p27<sup>Kip1</sup> (7), it is possible that the retardation of progression from 104-S to 104-Rrel tumors in mice by LXR agonist is in part due to inhibition of proliferation of LNCaP cancer cells in tumors. The growth rate of relapsed 104-Rrel tumors in control group mice and LXR agonist-treated mice was similar (Fig. 4B), indicating that LXR agonist has no suppressive effect on growth of 104-Rrel tumors, possibly due to the reduced expression levels of LXR-related genes on these tumors.

Based on the results from our *in vivo* progression model, administration of androgen at a concentration 10-fold higher than the physiologic concentration may be an effective treatment of patients with advanced prostate cancer cells expressing elevated levels of androgen receptor. The expression of LXR-related genes changed during progression, and the LXR agonist T0901317 retarded prostate cancer progression, suggesting that the LXR signaling may be another useful target for prostate cancer treatment.



**Figure 4.** Effect of the LXR agonist T0901317 on development of androgen-independent 104-Rrel tumors. Mice were injected s.c. with androgen-dependent 104-S cells. After allowing tumors to grow for 9 weeks, mice were castrated and separated into an LXR agonist treatment group (■, nine mice with 15 tumors) and a control group (○, nine mice with 13 tumors). T0901317 (10 mg/kg in sesame oil) and vehicle (sesame oil only) were given by gavage 5 days weekly starting at week 1. Body weight (A) and tumor volumes (B) of mice in control (○) and LXR agonist treatment groups (■) were expressed as the mean  $\pm$  SD (A) and mean  $\pm$  SE (B).

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