Foxm1 Expression in Prostate Epithelial Cells Is Essential for Prostate Carcinogenesis*

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Background: Foxm1 is up-regulated in prostate adenocarcinomas and its expression correlates with the poor prognosis.

Results: Conditional depletion of Foxm1 in prostate epithelial cells inhibits tumor cell proliferation, angiogenesis, and metastasis.

Conclusion: Foxm1 expression in prostate epithelial cells is essential for prostate carcinogenesis in mouse models.

Significance: Foxm1 may play a key role in the pathogenesis of prostate cancer in human patients.

The treatment of advanced prostate cancer (PCa) remains a challenge. Identification of new molecular mechanisms that regulate PCa initiation and progression would provide targets for the development of new cancer treatments. The Foxm1 transcription factor is highly up-regulated in tumor cells, inflammatory cells, and cells of tumor microenvironment. However, its functions in different cell populations of PCa lesions are unknown. To determine the role of Foxm1 in tumor cells during PCa development, we generated two novel transgenic mouse models, one exhibiting Foxm1 gain-of-function and one exhibiting Foxm1 loss-of-function under control of the prostate epithelial-specific Probasin promoter. In the transgenic adenocarcinoma mouse prostate (TRAMP) model of PCa that uses SV40 large T antigen to induce PCa, loss of Foxm1 decreased tumor growth and metastasis. Decreased prostate tumorigenesis was associated with a decrease in tumor cell proliferation and the down-regulation of genes critical for cell proliferation and tumor metastasis, including Cdc25b, Cyclin B1, Plk-1, Lox, and Versican. In addition, tumor-associated angiogenesis was decreased, coinciding with reduced Vegf-A expression. The mRNA and protein levels of 11β-Hsd2, an enzyme playing an important role in tumor cell proliferation, were down-regulated in Foxm1-deficient PCa tumors in vivo and in Foxm1-depleted TRAMP C2 cells in vitro. Foxm1 bound to, and increased transcriptional activity of, the mouse 11β-Hsd2 promoter through the −892/−879 region, indicating that 11β-Hsd2 was a direct transcriptional target of Foxm1. Without TRAMP, overexpression of Foxm1 either alone or in combination with inhibition of a p19ARF tumor suppressor caused a robust epithelial hyperplasia, but was insufficient to induce progression from hyperplasia to PCa. Foxm1 expression in prostate epithelial cells is critical for prostate carcinogenesis, suggesting that inhibition of Foxm1 is a promising therapeutic approach for prostate cancer chemotherapy.

Development of cancer is a multistep process involving gain-of-function mutations in oncogenes leading to increased cell proliferation and survival (1). Cancer progression also requires inactivation of tumor suppressor genes that arrest cell proliferation in response to oncogenic stimuli (2). In normal prostate epithelium, the relatively low rate of cell proliferation is balanced by a low rate of apoptosis (3). In contrast, prostatic intraepithelial neoplasia (PIN) and early invasive carcinomas are characterized by an increase in the proliferation rate, whereas advanced and/or metastatic prostate cancers also display a significant decrease in the rate of apoptosis. Altered cell-cycle control is therefore likely to play a role in progression of disease, whereas deregulation of apoptosis may be more important for advanced carcinoma. Published studies have demonstrated significant activation of the PI3K/Akt and ERK mitogen-activated protein kinase (MAPK) signaling pathways in prostate carcinomas (4, 5).

The Foxm1 transcription factor is a member of the Forkhead box (Fox) family of transcription factors and broadly expressed in actively dividing cells (6–9). Activation of the MAPK signaling pathway drives cell cycle progression by regulating the temporal expression of cyclin regulatory subunits that activate their corresponding cyclin-dependent kinases (Cdk) through complex formation. Cdk-cyclin complexes phosphorylate and activate a variety of cell cycle regulatory proteins, including Foxm1 (1, 2, 10). Activated ERK directly phosphorylates the Foxm1 protein, contributing to its transcriptional activation (11).

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‡1 These abbreviations used are: PIN, prostatic intraepithelial neoplasia; PCa, prostate carcinoma; Cre, Cre recombinase; Fox, Forkhead box transcription factor; 11β-Hsd2, 11β-hydroxysteroid dehydrogenase type II; ARF, alternative reading frame; TRAMP, transgenic adenocarcinoma mouse prostate; Dox, doxycycline; qPCR, quantitative PCR; rTCA, reverse tetracycline activator; GC, glucocorticoids.
proliferating cells, expression of the Foxm1 is induced during the G1/S-phase of the cell cycle and its expression continues throughout mitosis. Foxm1 directly stimulates the transcription of genes essential for progression into DNA replication and mitosis, including Cyclin B1, Cdc25B phosphatase, Aurora B kinase, and Polo-like kinase 1 (12). Foxm1 was shown to be essential for diminishing nuclear accumulation of p21Cip1 and p27kip1, proteins that inhibit Cdk2 activity in G1 (13–15). Expression of the alternative reading frame (ARF) tumor suppressor is induced in response to oncogenic stimuli (2). ARF prevents aberrant cell proliferation by targeting Mdm2 to nucleolus and increasing stability of the p53 tumor suppressor (16, 17). The ARF protein also targets E2F1, c-Myc, and Foxm1 transcription factors to the nucleolus, thus preventing the transcriptional activation of their target genes (18, 19). Expression of the ARF protein is lost in a variety of tumors through DNA methylation and silencing of the ARF promoter region (2, 17).

Prostate cancer (PCa) continues to be the most common malignancy diagnosed in American men and the second leading cause of male cancer mortality (20). Major efforts have been directed toward identifying early detection and prognostic markers for PCa. In contrast, significantly less research has been devoted to understanding the molecular mechanisms underlying PCa pathogenesis. Identification of genes regulating the initiation and/or progression of PCa would provide novel targets for diagnosis and treatment of human PCa. The transgenic adenocarcinoma of the mouse prostate (TRAMP) recapitulates multiple stages of human PCa by using the probasin promoter to drive the expression of the SV40 virus large T antigen (Tag) oncoprotein specifically in prostate epithelial cells (21, 22). T antigen inactivates the tumor suppressor proteins retinoblastoma (Rb), p53, and PPARα serine/threonine-specific phosphatase (23), effectively inducing prostate tumors in adult mice. At early stages, TRAMP mice develop prostate epithelial cell hyperplasia and PIN, that progress to histopathologically invasive PCa (21, 24). Both the reproducibility and progressive nature of PCa development in the TRAMP mouse model has provided a greater understanding of the molecular mechanisms involved in PCa development and progression (25).

Increased expression of Foxm1 is observed in tumor cells, inflammatory cells, and stromal cells of numerous human tumors, implying that Foxm1 plays an important role in tumor progression (reviewed in Refs. 12 and 26–31). Indeed, overexpression of Foxm1 using the ubiquitous Rosa26 promoter accelerated tumor growth induced by SV40 T large/small T antigens (29). However, given that expression of the Rosa26-Foxm1 transgene was ubiquitous, the specific requirements for Foxm1 in different cell types of PCa remain unknown. The present study was designed to determine the cell autonomous role of Foxm1 in prostate epithelial cells during formation of prostate adenocarcinomas in vivo.

**Experimental Procedures**

**Transgenic Mice**

**Loss-of-Function of Foxm1 in Prostate Epithelium—Foxm1^fl/fl^ mice (32) were bred with Pb-Cre transgenic mice (33) to generate Pb-Cre^+/Foxm1^fl/fl^ mice. In Pb-Cre^+/Foxm1^fl/fl^ mice (Fig. 1A), Cre recombinase is specifically expressed in prostate epithelial cells under control of rat probasin (Pb) promoter. Pb-Cre^+/Foxm1^fl/fl^ mice were crossed with TRAMP transgenic mice containing Pb-driven SV40-T large and small antigens (5, 6). Pb-Cre^+/Foxm1^fl/fl^/TRAMP mice were fertile with no obvious abnormalities. Pb-Cre^+/Foxm1^fl/fl^, Foxm1^fl/fl^, and Pb-Cre^+/TRAMP, TRAMP littermates were used as controls. Mouse prostate glands were harvested 8 and 23 weeks after birth and used for isolation of total prostate mRNA or for immunohistochemistry. Animal studies were approved by the Animal Care and Use Committee of Cincinnati Children’s Hospital Research Foundation.

**Gain-of-Function of Foxm1 in Prostate Epithelium—** Transgenic mice with doxycycline (Dox)-inducible Foxm1 expression (TetO-GFP-Foxm1-ΔN) were previously generated (34). The TetO-GFP-Foxm1-ΔN mice were bred with Pb-Cre^+/LoxP-stop-LoxP-rtTA(Rosa26)^+/+ mice, the latter of which contained a reverse tetracycline activator (rtTA) knocked into Rosa26 locus (35). In Pb-Cre^+/LoxP-stop-LoxP-rtTA(Rosa26)^+/* TetO-GFP-Foxm1-ΔN^+/+ mice (abbreviated as Pb-Foxm1-ΔN), Dox treatment results in prostate epithelial-specific expression of the Foxm1-ΔN transgene due to excision of LoxP-stop-LoxP cassette by the Cre recombinase. To induce Foxm1-ΔN expression, mice were given Dox in food chow beginning at 4 weeks of age and kept on Dox until the end of the experiment. Dox-treated TetO-Foxm1-ΔN littermates lacking the Pb-Cre transgene were used as control for Pb-Foxm1-ΔN transgenic mice. Further controls included Pb-Cre^+/TetO-Foxm1-ΔN^+/+ or Pb-Foxm1-ΔN mice without Dox treatment.

Pb-Foxm1-ΔN mice were bred with p19ARF^−/− mice to generate Pb-Foxm1-ΔN/p19ARF^−/− mice. Pb- Foxm1-ΔN/p19ARF^−/− mice. To induce Foxm1 transgene, mice were given Dox at 6 weeks of age. Homozygous Pb-Foxm1-ΔN/p19ARF^−/− mice developed aggressive lymphomas without Dox treatment (data not shown) and were not used to study prostate carcinogenesis. Heterozygous Pb-Foxm1-ΔN/p19ARF^−/+ mice were treated with Dox for 23 weeks and harvested along with control Pb-Foxm1-ΔN and p19ARF^−/+ mice. The orthotopic model of prostate cancer, which uses Myc-CaP tumor cells, was previously described (36).

**Quantitative Real-time RT-PCR**

Total RNA was prepared from prostate, and analyzed by qRT-PCR using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). Samples were amplified with TaqMan Gene Expression Master Mix (Applied Biosystems) combined with inventoried TaqMan mouse gene expression assays: Foxm1, Mm00514924_m1; β-Actin, Mm00607939_g1; Cdc25b, Mm00494449_m1; Cyclin B1, Mm00838401_g1; Pik1, Mm00440924_g1; Vegfa, Mm00437304_m1; Foxf1, Mm00487497_m1; Hif1a, Mm00468869_m1; Spdef, Mm01306245_m1; Igf-1, Mm00439560_m1; Fzd1, Mm00445405_s1; Cxcl4, Mm01996749_s1; Ar, Mm00442688_m1; Cxcl12, Mm00442688_m1; p19ARF, Mm00494449_m1. Human (transgenic) Foxm1-ΔN was amplified using Hs0153543_m1 primers. Reactions were analyzed in triplicates and expression levels were normalized to β-Actin. Cells were transfected using following Foxm1-specific siRNAs: siRNA #1, 5’-GGA CCC UCU CCC UUA CUU U-3’;
siRNA #2, 5′-UAA UUA UGU CAA GAU GAU GAU-3′; siRNA #3, 5′-CCA UUA GGA AGA AGA AAU-3′ (Dharmacon Research, Lafayette, CO). Cells were also transduced with shFoxm1 lentivirus (clone ID: V3LMM_457621, Thermo Fisher Scientific, Pittsburgh PA).

**ChIP Assay**

TRAMP C2 mouse prostate adenocarcinoma cells were transfected with either CMV-Foxm1 or CMV-empty plasmids using the Neon transfection system following the manufacturer’s protocol (Invitrogen). 24 h after transfection, cells were cross-linked by addition of formaldehyde, sonicated, and used for immunoprecipitation with a Foxm1 rabbit polyclonal antibody (C-20, Santa Cruz, CA) as described previously (37). DNA fragments were between 500 and 1000 bp in size. Reversed cross-linked ChIP DNA samples were subjected to qPCR using oligonucleotides specific to promoter regions of mouse **H11002**. PCR products was cloned into a pGL2-Basic firefly luciferase (LUC) reporter plasmid (Promega, Madison, WI) and verified by DNA sequencing. A dual luciferase assay (Promega) was performed 24 h after transfection indicating that tumors developed from non-recombined epithelial cells (Fig. 2C, bottom panel). Furthermore, deletion of Foxm1 prevented the development of mouse PIN at early stages.

**Results**

Conditional Deletion of Foxm1 from Prostate Epithelial Cells—To investigate the role of Foxm1 in prostate epithelial cells during PCa formation, we generated transgenic mice containing LoxP-flanked exons 4–7 of the Foxm1 gene (Foxm1\(^{12/12}\)) and the Probasin-Cre transgene (Pb-Cre\(^{12/12}\)/Foxm1\(^{12/12}\) mice). In Pb-Cre\(^{12/12}\)/Foxm1\(^{12/12}\) mice (Fig. 1A), Cre recombinase is specifically expressed in prostate epithelial cells under control of rat probasin (PB) promoter (33). To induce prostate carcinogenesis, Pb-Cre\(^{12/12}\)/Foxm1\(^{12/12}\) mice were crossed with TRAMP mice containing PB-driven SV40-Tag transgene (22, 24). Pb-Cre/Foxm1\(^{12/12}\)/TRAMP mice were fertile with no obvious abnormalities. To determine the efficiency of Foxm1 deletion, total prostate RNA from 8-week-old Pb-Cre/Foxm1\(^{12/12}\)/TRAMP male mice was examined by qRT-PCR. Compared with control Foxm1\(^{12/12}\)/TRAMP tissues, a 90% decrease in Foxm1 mRNA was only observed in prostate tissue, but not in lung tissues from Pb-Cre/Foxm1\(^{12/12}\)/TRAMP mice (Fig. 1B), demonstrating that the deletion of Foxm1 was prostate-specific. The number of Foxm1-positive epithelial cells in Pb-Cre/Foxm1\(^{12/12}\)/TRAMP prostates was greatly reduced, confirming the efficient Foxm1 deletion by the Cre recombinase (Fig. 1C, upper panel). Moreover, the total number of Ki-67 positive cells and the number of Ki-67/Foxm1 double-positive epithelial cells were significantly decreased in Pb-Cre/Foxm1\(^{12/12}\)/TRAMP prostates (Fig. 1D, bottom panel), consistent with a down-regulation in cell proliferation. There were no differences in the number of apoptotic cells in Foxm1\(^{12/12}\)/TRAMP and Pb-Cre/Foxm1\(^{12/12}\)/TRAMP prostates (data not shown).

**Foxm1 Deficiency in Prostate Epithelial Cells Prevents Adenocarcinoma Formation Caused by SV40 T-antigen**—To determine whether Foxm1 expression in prostate epithelial cells was critical for prostate carcinogenesis, Foxm1\(^{12/12}\)/TRAMP and Pb-Cre/Foxm1\(^{12/12}\)/TRAMP mouse prostates were analyzed at 23 weeks of age. The weights of the prostate glands from control Foxm1\(^{12/12}\)/TRAMP and TRAMP groups of mice were 2.3 ± 2.7 ± 2.3 g, respectively (Fig. 2A). In contrast, a significant 20-fold decrease in prostate weight was observed in Foxm1-deficient Pb-Cre/Foxm1\(^{12/12}\)/TRAMP mice (prostate weight 0.12 ± 0.03 g, p < 0.01) (Fig. 2A). The mRNA levels of Foxm1 were decreased in Pb-Cre/Foxm1\(^{12/12}\)/TRAMP prostates, a finding consistent with efficient Foxm1 deletion (Fig. 2B). Histo-pathological analysis of the H&E-stained sections demonstrated that only 8% (1 of 12) of Pb-Cre/Foxm1\(^{12/12}\)/TRAMP mice developed adenocarcinomas compared with 90% (9 of 10) of TRAMP mice and 80% (8 of 10) of Foxm1\(^{12/12}\)/TRAMP mice (Table 1 and Fig. 2C, upper panel). All tumor lesions, including those that developed in Foxm1-deficient Pb-Cre/Foxm1\(^{12/12}\)/TRAMP prostates, stained positively for the Foxm1 protein, indicating that tumors developed from non-recombined epithelial cells (Fig. 2C, bottom panel). Furthermore, deletion of Foxm1 prevented the development of mouse PIN at early stages.

**Statistical Analysis**

We used Microsoft Excel to calculate S.D. and statistically significant differences between samples using the Student’s t test. p values < 0.05 were considered statistically significant.
of prostate carcinogenesis (8 weeks, Table 1). Altogether, these results indicate that Foxm1 expression in prostate epithelial cells is required for prostate carcinogenesis in vivo and that depletion of Foxm1 in prostate epithelial cells is sufficient to inhibit development of PCa in the TRAMP mouse model.

Deletion of Foxm1 Down-regulated Cell Proliferation and Decreased Expression of Cell-cycle Regulatory Genes—Immunohistochemical analysis determined that Ki-67 and pH 3 expression was decreased in 23-week-old Pb-Cre/Foxm1fl/fl TRAMP prostate tissue sections, confirming that deletion of
Foxm1 expression decreased cell proliferation (Fig. 3A). Quantitative analysis determined that the numbers of Ki-67-positive and pH3-positive cells were significantly decreased in Pb-Cre/Foxm1fl/fl/TRAMP prostate sections as compared with control Foxm1fl/fl/ TRAMP tissue (Fig. 3A). Foxm1 deficiency in Pb-Cre/Foxm1fl/fl/ TRAMP prostates was associated with decreased mRNA levels of Cdc25b, Cyclin B1, and Plk-1 (Fig. 3B), all known transcriptional targets of Foxm1 (40). Because Cdc25b, Cyclin B1, and Plk-1 are critical for proliferation of tumor cells, their decreased expression can contribute to decreased proliferation and reduced carcinogenesis in Pb-Cre/ Foxm1fl/fl/ TRAMP mice.

FIGURE 1. Conditional deletion of Foxm1 from prostate epithelial cells.

A, schematic drawing of Cre-mediated deletion of the Foxm1-floxed allele in vivo. Expression of Cre-recombinase in Pb-Cre/Foxm1fl/fl/ TRAMP mice causes the deletion of exon 4–7 encoding the DNA binding domain and transcriptional activation domain of the Foxm1 protein. B, efficient deletion of Foxm1 in prostate epithelial cells is shown by qRT-PCR. Total prostate RNA and total lung RNA was prepared from 8-week-old Pb-Cre/Foxm1fl/fl, Foxm1fl/fl/ TRAMP, and Pb-Cre/Foxm1fl/fl/ TRAMP mice. β-Actin mRNA was used for normalization. Data represent mean ± S.D. of three independent determinations using prostate tissue from n = 5–10 mice in each group. C, H&E staining shows prostate adenocarcinoma in Foxm1fl/fl/ TRAMP mice (left panel) and PINs in Pb-Cre/Foxm1fl/fl/ TRAMP prostate (right panel, black arrows).

FIGURE 2. Deletion of Foxm1 from prostate epithelial cells prevents prostate carcinogenesis. A, experimental Pb-Cre/Foxm1fl/fl/ TRAMP and control Foxm1fl/fl/ TRAMP mice were sacrificed at 23 weeks of age. Prostate glands were collected and weighed. Deletion of Foxm1 significantly decreased (p < 0.05) the weight of prostate glands at 23 weeks of age (top panel). Mean weights of prostate glands (± S.D.) were calculated from 10 to 12 mouse prostates per group. An asterisk (*) indicates a p value < 0.05. B, efficiency of Foxm1 deletion in prostate epithelial cells is shown by qRT-PCR. Total prostate RNA was prepared from Pb-Cre/Foxm1fl/fl, Foxm1fl/fl/ TRAMP, and Pb-Cre/Foxm1fl/fl/ TRAMP mice. β-Actin mRNA was used for normalization. Data represent mean ± S.D. of three independent determinations using prostate tissue from n = 5–10 mice each group. C, H&E staining shows prostate adenocarcinoma in Foxm1fl/fl/ TRAMP mice (left panel) and PINs in Pb-Cre/Foxm1fl/fl/ TRAMP prostate (right panel, black arrows).
VEGF Expression and Angiogenesis Are Compromised in Pb-Cre/Foxm1fl/fl/TRAMP Prostates—Because angiogenesis plays an essential role in prostate cancer progression and VEGF-A is a main angiogenic factor produced by tumor cells (41), we compared the levels of VEGF-A in Pb-Cre/Foxm1fl/fl/TRAMP and control prostates. At 23 weeks, VEGF-A mRNA was decreased significantly (p < 0.01) in prostate tissues of Pb-Cre/Foxm1fl/fl/TRAMP mice as shown by real time qRT-PCR (Fig. 4A). The tumors in control TRAMP mice were well vascularized as demonstrated by immunostaining for endothelial-specific protein Flk-1 (Fig. 4B, middle). In contrast, aberrant angiogenesis did not occur in Foxm1-deficient prostates (Fig. 4B, right). siRNA-mediated depletion of Foxm1 caused a significant reduction in Vegf-a mRNA in cultured TRAMP C2 mouse prostate adenocarcinoma cells in vitro (Fig. 4C), a finding consistent with regulation of Vegf-a by Foxm1 in pancreatic tumor cells (42). Thus, reduced Vegf-a levels may inhibit prostate carcinogenesis in Pb-Cre/Foxm1fl/fl/TRAMP mice.

Foxm1 Deficiency in Prostate Epithelial Cells Alters Expression of Genes Critical for Prostate Carcinogenesis—To identify additional Foxm1-regulated target genes, we examined the expression of several genes critical for prostate cancer progression and metastasis. Total RNA was isolated from excised prostate glands and used for real time RT-PCR analysis. In Pb-Cre/Foxm1fl/fl/TRAMP prostates, mRNAs encoding the 11β-Hsd2, Fzd1, Igf1, AR, Cxcl12, and Cxcr3 genes known to mediate carcinogenesis (43–45) were all decreased, whereas Hif-1α, Spdef, and Foxf1 mRNA levels did not change (Fig. 5A). Consistent with the in vitro data (Fig. 1B), knockdown of Foxm1 in cultured TRAMP C2 tumor cells decreased mRNA levels of the cell-

| Mice                        | 8 Weeks of age | 23 Weeks of age |
|-----------------------------|----------------|-----------------|
|                             | Mice with PIN | Number of mice with PIN out of total number | Mice with prostate carcinoma | Number of mice with prostate carcinoma out of total number |
| Pb-Cre/Foxm1fl/fl/TRAMP     | 0%            | 0/7             | 0%                          | 1/12            |
| TRAMP                       | 100%          | 9/9             | 90%                         | 9/10            |
| Foxm1fl/fl/TRAMP            | 100%          | 8/8             | 80%                         | 8/10            |

**FIGURE 3.** Deletion of Foxm1 decreased cell proliferation and reduced expression of cell-cycle regulatory genes. A, decreased proliferation of tumor cells was observed in Pb-Cre/Foxm1fl/fl/TRAMP prostates as demonstrated by reduced numbers of Ki-67-positive and PH3-positive tumor cells. Mouse prostate glands were harvested 23 weeks after birth and used for immunohistochemistry with Ki-67 and PH3 antibodies. Magnification is ×100. Counting of Ki-67-positive and PH3-positive cells were performed using 5 random fields in each of 6 individual mice per group and scored blindly. Data represent mean ± S.D. A p value < 0.01 is shown with an asterisk (*). B, decreased mRNA levels of Cdc25b, Cyclin B1, and Plk-1 in Pb-Cre/Foxm1fl/fl/TRAMP prostates was found by qRT-PCR. β-Actin mRNA was used for normalization. Data represent mean ± S.D. of three independent determinations using prostate tissue from n = 5–10 mice in each group. A p value < 0.05 is shown with an asterisk.
cycle regulatory genes Cyclin B1, Cyclin D1, Cdc25b, and Plk-1 in addition to 11β-Hsd2, Igf1, AR, and Hif-1α mediators of prostate carcinogenesis (Fig. 5B). Because all these genes are critical for proliferation of prostate tumor cells and formation of prostate cancer, reduced expression of these genes may contribute to reduced prostate carcinogenesis in Pb-Cre/Foxm1^fl/fl^/TRAMP mice. Moreover, depletion of Foxm1 was associated with decreased mRNA expression of Lox and Versican (Fig. 5C), genes critical for tumor metastasis (46, 47). Consistent with these results, the percentage of Pb-Cre/Foxm1^fl/fl^/TRAMP mice with metastasis to the lymph nodes and the lung was dramatically decreased compared with control Foxm1^fl/fl^/TRAMP mice. Taken together, Foxm1 not only regulates expression of genes critical for PCa cell proliferation and TRAMP tumor formation, but also those that promote tumor progression and metastatic disease.

To demonstrate that the role of Foxm1 is not limited to the TRAMP model of prostate cancer, we used Myc-CaP prostate adenocarcinoma cells for the orthotopic injection into mouse prostates. Foxm1 mRNA and protein were increased in Myc-CaP prostate tumors compared with normal prostates (Fig. 6, A and B). Depletion of Foxm1 in Myc-CaP cells by shRNA reduced expression of cell cycle regulatory genes (Fig. 6C) and decreased proliferation of Myc-CaP cells in vitro (Fig. 6D). Thus, Foxm1 induces cell proliferation in both SV40 T-antigen (TRAMP) and c-Myc-transformed tumor cells.

Overexpression of Foxm1 Is Not Sufficient to Induce PCa—Next, we examined whether overexpression of activated Foxm1 mutant protein (Foxm1-ΔN) either alone or in combination with inactivation of tumor suppressor p19^ARF^, a known Foxm1 inhibitor (14, 48), would be sufficient to induce prostate carcinogenesis. To induce Foxm1-ΔN expression specifically in prostate epithelial cells, TetO-GFP-FoxM1-ΔN transgenic mice (49) were cross-bred with Pb-Cre^G6^/loxP-stop-LoxP-rtTA(Rosa26)^G6^/N mice containing a reverse tetracycline activator (rtTA) knocked into Rosa26 locus. Dox treatment of Pb-Cre^G6^/loxP-stop-LoxP-rtTA(Rosa26)^G6^/N mice resulted in prostate epithelial-specific expression of rtTA due to excision of LoxP-stop-LoxP cassette by the Cre recombinase. This strategy resulted in the F1 generation of Pb-Cre^G6^/loxP-stop-LoxP-rtTA(Rosa26)^G6^/N tetO-GFP-FoxM1-ΔN^G6^/N transgenic mice, subsequently referred to as Pb-Foxm1-ΔN mice. Expression of the Pb-Foxm1-ΔN transgene was under Dox control thereby enabling us to study the role of Foxm1 in adult prostate epithelium.

In a second model to study Pb-Foxm1-ΔN overexpression in the presence of p19^ARF^ inactivation, Pb-Foxm1-ΔN mice were cross-bred with p19^ARF^−/− mice. To induce Foxm1 transgene, mice were given Dox at 6 weeks of age. Because homozygous p19^ARF^−/− expression in Pb-Foxm1-ΔN/p19^ARF^−/− mice promoted development of aggressive lymphomas independent from Foxm1 transgene activity (data not shown), these mice could not be used in our study. Therefore, we used heterozygous Pb-Foxm1-ΔN/p19^ARF^−/− mice along with appropriate controls (Pb-Foxm1-ΔN mice and p19^ARF^+/− mice). Prostates were collected following 16, 24, and 40 weeks of Dox treatment. Although p19^ARF^−/−prostates exhibited a normal phenotype (Fig. 7A, left panels), overexpression of the Foxm1-ΔN mutant induced prostate epithelial hyperplasia in regions showing strong immunostaining for the
Foxm1 Requires for Prostate Carcinogenesis

![Graph A: Prostate Tissue](image)

![Graph B: Tramp C2 Cells](image)

![Graph C: % Mice with metastasis](image)

**FIGURE 5.** Deletion of Foxm1 alters expression of genes critical for prostate carcinogenesis. A, qRT-PCR was performed using total prostate RNA isolated from prostate glands of 23-week-old mice. β-Actin mRNA was used for normalization. B, TRAMP C2 cells were transfected with either control siRNA (Mock) or siRNAs specific for Foxm1 mRNA (siFoxm1). We used three distinct siFoxm1s (siFoxm1 #1, #2, and #3, see “Experimental Procedures”). Forty-eight hours after siRNA transfection, total RNA was extracted and analyzed by qRT-PCR. β-Actin mRNA was used for normalization. C, deletion of Foxm1 from prostate epithelium in TRAMP mice decreased prostate cancer metastasis. D, decreased mRNAs of Lox and Versican in Pb-Cre/Foxm1^{fl/fl}/TRAMP prostates were found by qRT-PCR. A p value < 0.05 is shown with asterisk (*).

Foxm1-ΔN transgenic protein at 24 weeks of Dox treatment (Fig. 7A, middle panels). Foxm1 overexpression on a p19_{ras}^{+/+} genetic background did not promote PCA development, but caused atrophy of prostate epithelial tubules as evidenced by smaller size of prostate tubules embedded into fibrotic tissue (Fig. 7A, right panels). Altogether, our data demonstrated that overexpression of activated Foxm1-ΔN in adult prostates induced epithelial hyperplasia, but was insufficient to stimulate progression of epithelial hyperplasia to prostate adenocarcinoma. In contrast to hepatocellular car-
cinomas (50), simultaneous overexpression of Foxm1 and inhibition of the p19ARF tumor suppressor did not induce prostate carcinogenesis in vivo.

Foxm1 Directly Regulates Hsd11b2 Gene Transcription—Foxm1 induced 11β-Hsd2 mRNA and protein in mouse prostates in vivo and in cultured TRAMP C2 cells as shown by qRT-PCR (Fig. 5, A and B). Consistent with these observations, 11β-Hsd2 protein levels were reduced in Foxm1-deficient TRAMP C2 cells (Fig. 8C) and Pb-Cre/Foxm1fl/fl/Tramp prostates (Fig. 8D). We next investigated whether 11β-Hsd2 was a direct transcriptional target of Foxm1 in prostate tumor cells. Two potential Foxm1 binding sites were identified within the −2.69-kb promoter region of the mouse 11β-Hsd2 gene (Fig. 8A). Chromatin immunoprecipitation (ChIP) assay was performed to demonstrate that the Foxm1 protein physically bound to the −706/−873 bp 11β-Hsd2 promoter region, whereas no specific binding of Foxm1 to the −2366/−2480 bp region was observed (Fig. 8A). To identify functional Foxm1-binding sites in the 11β-Hsd2 promoter region, we performed luciferase reporter assays using the constructs as outlined in Fig. 7B. CMV-Foxm1b significantly increased transcriptional activity of the −2.69 kb 11β-Hsd2 promoter region (Fig. 8B). Analysis of deletion mutants revealed that the −1263/−649 bp region of the 11β-Hsd2 promoter was required for full transcriptional activation of 11β-Hsd2 by Foxm1 (Fig. 8B). Site-directed mutagenesis of Foxm1-binding sequences in the −892/−879 region decreased transcriptional activity of the 11β-Hsd2 promoter, indicating that the −892/−879 region is important for transcriptional regulation of the 11β-Hsd2 gene by Foxm1 (Fig. 8B). Thus, Foxm1 directly bound to and induced transcriptional activity of the 11β-Hsd2 promoter, indicating that 11β-Hsd2 is a direct Foxm1 target.

Finally, to determine whether 11β-Hsd2 is important for cellular proliferation in Foxm1-expressing cells, TRAMP C2 tumor cells were transduced with sh11β-Hsd2 lentivirus. Depletion of 11β-Hsd2 was sufficient to decrease cellular proliferation as demonstrated by the reduced number of TRAMP C2 cells in two separate proliferation assays (Fig. 9, A–D). Depletion of 11β-Hsd2 decreased mRNA levels of cell cycle regulatory genes, such as Cyclin D1 and Cdc25b (Fig. 9B), sup-

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**FIGURE 6.** Foxm1 is up-regulated in c-Myc-driven prostate cancer and is required for proliferation of Myc-CaP prostate adenocarcinoma cells. Mouse prostates were harvested 5 weeks after inoculation of Myc-CaP cells and embedded into paraffin blocks or used to isolate RNA. A, Myc-CaP tumors express high levels of Foxm1 protein. Only single Foxm1-positive cell are found in the normal prostates. Prostate tissue sections were stained with antibodies specific for Foxm1. Nuclear fast red was used for counterstaining. Magnification is ×400. B, Foxm1 mRNA is up-regulated in the Myc-CaP tumors. Total RNAs were isolated from prostate glands 5 weeks after inoculation of Myc-CaP cells and examined shown by qRT-PCR. β-Actin mRNA was used for normalization. C, depletion of Foxm1 in Myc-CaP cells decreased mRNA levels of proliferation-specific genes. Myc-CaP cells were transduced with either control or shFoxm1 lentiviruses. β-Actin mRNA was used for normalization. D, depletion of Foxm1 decreased proliferation of Myc-CaP cells in vitro. Control and Foxm1-depleted Myc-CaP cells were seeded in triplicates and counted at different time points using either hemocytometer (left panel) or WST1 Cell Proliferation Reagent (right panel). A p value < 0.05 is shown with asterisk (*).
porting our conclusion that 11β-Hsd2 is important for cellular proliferation in prostate cancer cells.

DISCUSSION

Our previous studies demonstrated that aberrant overexpression of Foxm1 in all cell types using ubiquitous Rosa26 promoter accelerated prostate carcinogenesis in TRAMP and LADY transgenic mice (31). Prostate cancer lesions contain a heterogeneous population of cells, which includes epithelial, inflammatory (macrophages, granulocytes), and stromal cells, all of each up-regulate Foxm1 expression during cancer formation (51). Recent studies highlighted the important role of Foxm1 in regulation of cancer-associated inflammation and production of inflammatory cytokines and chemokines (28), suggesting that Foxm1 may indirectly influence prostate carcinogenesis in Rosa26-Foxm1 mice by inducing tumor-associ-
ated inflammation. A specific role of Foxm1 in prostate epithelial cells, the precursors of prostate adenocarcinoma cells, remain unknown. The important contribution of the current study is the establishment that the expression of Foxm1 in prostate epithelial cells is required for prostate carcinogenesis.

Using a novel conditional knock-out mouse line in which Foxm1 was selectively deleted from prostate epithelial cells (Pb-Cre/Foxm1fl/fl/TRAMP mice), we demonstrated that deletion of Foxm1 prevented prostate carcinogenesis in TRAMP mice. The Foxm1 deletion in Pb-Cre Foxm1fl/fl/TRAMP mice occurs

**FIGURE 8.** 11β-Hsd2 is a direct transcriptional target of Foxm1. A, a schematic drawings of the mouse 11β-Hsd2 promoter region (m11β-Hsd2). Location of two potential Foxm1 DNA binding sites are indicated (boxes). The ChIP assay demonstrated that Foxm1 protein directly binds to the −706/−873-bp 11β-Hsd2 promoter region, but not to the −2366/−2480-bp region. Cross-linked chromatin from mock-transfected TRAMP C2 mouse prostate adenocarcinoma cells or TRAMP C2 cells transfected with Foxm1-specific siRNA was immunoprecipitated (IP) with either Foxm1 antibodies or IgG control. Genomic DNA in the IP fraction was analyzed by qPCR. Foxm1 binding to genomic DNA was normalized to IgG control. Diminished binding of Foxm1 to the −892/−879-bp 11β-Hsd2 promoter region was observed after siFoxm1 transfection. B, Foxm1 induces transcriptional activity of the mouse 11β-Hsd2 promoter. Schematically shown the luciferase (LUC) reporter constructs that include either the −2.69-kb mouse 11β-Hsd2 promoter region (I, includes both Foxm1 binding sites) or one of its deletion mutants (II–IV). TRAMP C2 cells were transfected with CMV-Foxm1b expression vector and one of the 11β-Hsd2 LUC reporter plasmids. CMV-empty plasmid was used as a control. Cells were harvested 24 h after transfection. Dual LUC assays were used to determine LUC activity. Deletion of both Foxm1 binding sites (construct IV) prevented activation of the 11β-Hsd2 promoter region by the CMV-Foxm1b plasmid. Site-directed mutagenesis of Foxm1-binding sequences in the −892/−879 region (construct V) decreased transcriptional activity of the 11β-Hsd2 promoter. Transcriptional induction is shown as a fold-change relative to CMV-empty vector (±S.D.). A p value < 0.05 is shown with asterisk (*).

C, Foxm1 knockdown by siRNA decreased 11β-Hsd2 protein levels. TRAMP C2 cells were transfected either with control siRNA (Mock) or with siRNA specific for Foxm1 (siFoxm1). Forty-eight hours after siRNA transfection, protein extracts were analyzed by Western blot. D, decreased 11β-Hsd2 protein expression was observed in Pb-Cre/Foxm1fl/fl/TRAMP prostates by immunohistochemistry. Prostate tissue sections of 24-week-old mice were stained with antibodies specific to 11β-Hsd2. Magnification is ×200.
at the same time as the initiation of prostate cancer through the Pb-driven SV40 T-antigen (TRAMP model). Therefore, it is possible that Foxm1 is important for both cancer initiation and cancer progression. Because the aberrant expression of Foxm1 is found in human prostate adenocarcinomas and its expression correlates with the severity of the disease (29), our findings provide foundation for the development of new therapeutic approaches based on inhibition of Foxm1.

Glucocorticoids (GC) are widely used in combination with other chemotherapeutic drugs to treat prostate cancer (52, 53). GC inhibit multiple steps in prostaglandin cascade, including enzymatic activity of cytosolic phospholipase A2, which releases the Cox substrate arachidonic acid. GC also inhibit the expression of both Cox-2 and microsomal PGE synthase, the terminal enzyme of Cox-2-mediated prostaglandin E2 biosynthesis (54, 55). However, the adverse side effects of immune suppression limit the use of GC in clinic. 11β-Hsd2 is an enzyme involved in metabolism of GC, particularly in the conversion of the active ligand cortisol to the inactive agonist cortisone (44). Cortisol binds to the intracellular glucocorticoid receptor, which then translocate into the nucleus and activates anti-proliferative and anti-inflammatory genes. In the presence of agonist cortisone, the glucocorticoid receptor is not activated and is not providing the anti-proliferative/anti-inflammatory response. Increased expression of 11β-Hsd2 was associated with increased tumor cell proliferation due to increased conversion of cortisol to agonist cortisone (56, 57). In the current study, we demonstrated that 11β-Hsd2 mRNA and protein were increased in the prostate tumors of TRAMP mice, implicating 11β-Hsd2 in proliferation of prostate tumor cells in vivo. Conditional deletion of Foxm1 from tumor cells prevented the up-regulation of 11β-Hsd2, coinciding with decreased carcinoma and reduced proliferation of prostate tumor cells. 11β-Hsd2 is a direct transcriptional target of Foxm1, because Foxm1 directly bound to and increased promoter activity of the mouse 11β-Hsd2 gene through the −706/−873 region. Thus, decreased expression of 11β-Hsd2 in Foxm1-deficient prostate may contribute to decreased tumor cell proliferation and reduced prostate carcinogenesis in Pb-Cre/Foxm1fl/fl TRAMP mice.

Based on our studies with Pb-Foxm1−ΔN mice, it appears that Foxm1 alone is not sufficient to initiate prostate tumorigenesis. The Pb-Foxm1−ΔN mice developed PINs, but the PINs did not progress into prostate adenocarcinomas. These data are consistent with previous studies demonstrating that overexpression of Foxm1 in pulmonary type II cells did not induce the formation of lung tumors (49). One of the explanations of resistance to form tumors in Pb-Foxm1−ΔN prostate is that

**FIGURE 9.** Depletion of 11β-Hsd2 decreased proliferation of prostate adenocarcinoma cells. TRAMP C2 cells were transduced with sh11β-Hsd2 lentivirus. A, decreased 11β-Hsd2 mRNA was found in sh11β-Hsd2 transduced cells by qRT-PCR. β-Actin mRNA was used for normalization. B, depletion of 11β-Hsd2 decreased Cyclin D1 and Cdc25b mRNAs. C, depletion of 11β-Hsd2 decreased cellular proliferation. Control and 11β-Hsd2-depleted TRAMP C2 cells were seeded in triplicates and counted at different time points using hemocytometer. D, decreased proliferation of 11β-Hsd2-depleted cells is shown by WST1 cell proliferation assay. Control and 11β-Hsd2-depleted TRAMP C2 cells were seeded in triplicates and processed at different time points. A p value of 0.05 is shown with an asterisk (*).
p19\textsuperscript{ARF} tumor suppressor, a known inhibitor of Foxm1 (14, 48), prevents tumor-promoting properties of Foxm1. In fact, we found that expression of p19\textsuperscript{ARF} was increased in \textit{Pb-Foxm1-N}\textsuperscript{N} prostates. Loss of p19\textsuperscript{ARF} function is a critical event for tumor promotion as evidenced by extinguished expression of the p19\textsuperscript{ARF} protein in a variety of tumors through DNA methylation and silencing of the p19\textsuperscript{ARF} promoter region (2). Expression of the ARF tumor suppressor is induced in response to oncogenic stimuli and prevents abnormal cell proliferation through stabilization of p53 tumor suppressor (59). p19\textsuperscript{ARF} also mediates p53-independent cell cycle arrest through the targeting of FoxM1, E2F1, and c-Myc transcription factors to the nucleolus, thus preventing their transcriptional activation of cell cycle regulatory genes (12, 14, 18, 40, 58). The p19\textsuperscript{ARF} protein inhibited Foxm1 transcriptional activity during development of hepatocellular carcinoma (14).

To overcome inhibition of Foxm1 by the p19\textsuperscript{ARF} tumor suppressor, we created transgenic mice overexpressing Foxm1-\textDelta\textsuperscript{N} in prostate epithelial cells in \textit{Pb-Foxm1}\textsuperscript{N}\textsuperscript{N}/p19\textsuperscript{ARF\textsuperscript{+/−}} background. However, those mice succumb to lymphomas earlier than they developed prostate cancer. Genetic inactivation of one p19\textsuperscript{ARF} copy in \textit{Pb-Foxm1}\textsuperscript{N}\textsuperscript{N} prostates was insufficient to induce prostate carcinogenesis. We also found that overexpression of Foxm1-\textDelta\textsuperscript{N} alone or in the absence of one copy of tumor suppressor p19\textsuperscript{ARF} induced epithelial hyperplasia, but was insufficient to stimulate progression of epithelial hyperplasia to prostate adenocarcinomas. These data suggest that either a complete inactivation of p19\textsuperscript{ARF}, as was achieved in liver tumors (50), or deregulation of other tumorigenic pathways is necessary to promote oncogenic properties of Foxm1 in prostate epithelium. Consistent with this hypothesis, Foxm1 synergized with K-Ras (49) and \beta-catenin (39) to induce progression of lung and brain cancer, respectively. Interestingly, Foxm1-overexpressing prostate tubules in \textit{Pb-Foxm1}\textsuperscript{N}\textsuperscript{N}/p19\textsuperscript{ARF\textsuperscript{+/−}} mice underwent a complete atrophy with obliteration of lumen. These results indicate that Foxm1/p19\textsuperscript{ARF} signaling is critical for normal homeostasis in prostate epithelium.

In summary, Foxm1 expression in prostate epithelial cells is required for prostate carcinogenesis induced by SV40 T antigens. Decreased prostate carcinogenesis in Foxm1-deficient mice was associated with decreased proliferation of tumor cells, diminished angiogenesis, and reduced expression of Cdc25b, Cyclin B1, Plk-1, VEGF-a, Lox, and Versican, all of which are critical for cellular proliferation and tumor metastasis. mRNA and protein levels of 11β-Hsd2, an enzyme playing an important role in tumor cell proliferation, were reduced in Foxm1-deficient PCa tumors \textit{in vivo} and in Foxm1-deficient prostate cancer cells \textit{in vitro}. Foxm1 bound to and increased promoter activity of the mouse 11β-Hsd2 gene, indicating that the 11β-Hsd2 is a direct transcriptional target of Foxm1. Our results suggest that inhibition of Foxm1 is a promising therapeutic approach for prostate cancer chemotherapy in human patients.

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