

# CD24 expression is important in male urothelial tumorigenesis and metastasis in mice and is androgen regulated

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**Overexpression of CD24, a glycosyl phosphatidylinositol-linked sialoglycoprotein, is associated with poor outcome in urothelial carcinoma and contributes to experimental tumor growth and metastasis. However, the requirement for CD24 (*Cd24a* in mice) in tumorigenesis and spontaneous metastasis from the orthotopic site remains uncharacterized. Using *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine induction of invasive and metastatic bladder cancer, we show that *Cd24a*-deficient male mice developed fewer bladder tumors than C57BL/6 control male mice. Evaluating only mice with evidence of primary tumors, we observed that *Cd24a*-deficient male mice also had fewer metastases than wild-type counterparts. In parallel observations, stratification of patients based on CD24 immunohistochemical expression in their tumors revealed that high levels of CD24 are associated with poor prognosis in males. In female patients and mice the above observations were not present. Given the significant role of CD24 in males, we sought to assess the relationship between androgen and CD24 regulation. We discovered that androgen receptor knockdown in UM-UC-3 and TCCSUP human urothelial carcinoma cell lines resulted in suppression of CD24 expression and cell proliferation. Androgen treatment also led to increased CD24 promoter activity, dependent on the presence of androgen receptor. In vivo, androgen deprivation resulted in reduced growth and CD24 expression of UM-UC-3 xenografts, and the latter was rescued by exogenous CD24 overexpression. These findings demonstrate an important role for CD24 in urothelial tumorigenesis and metastasis in male mice and indicate that CD24 is androgen regulated, providing the foundation for urothelial bladder cancer therapy with antiandrogens.**

carcinogenesis | genetically engineered mice

**B**ladder cancer represents a significant public health burden (1) and is the most costly cancer to treat in the Medicare population (2). Bladder cancer presents as both superficial (non-invasive) and invasive tumors (3); the invasive phenotype heralds metastasis in nearly half of diagnosed patients. An emerging molecule of interest in bladder cancer is CD24 (4, 5). CD24 is a glycosylated, mucin-like protein (6) which is rarely expressed in normal tissue (7). However, CD24 is detected in a wide range of carcinomas and has prognostic significance (8). CD24 is integral to tumor cell proliferation and anchorage-independent growth (4, 9), both hallmarks of cancer development (10). Additionally, CD24 is linked to tumor cell invasion (11) and metastatic progression (5, 12). Notably, *Cd24a*-deficient mice have no increased incidence of spontaneous cancer (13–15).

We sought to evaluate the contribution of CD24 in bladder cancer incidence and progression within a defined genetic model by exposing *Cd24a*-deficient mice to a carcinogen. Because carcinogens, such as those found in cigarette smoke (16), are the primary causative agents for bladder cancer, using a carcinogenesis-based animal model approximates the spontaneous tumor formation and progression of this disease. To initiate this carcinogenesis, we fed mice *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine

(hereafter, OH-BBN), a carcinogen known to induce bladder cancer selectively (17, 18). Molecular characterization of OH-BBN-induced tumors reveals that their genetic profile resembles that of invasive human bladder cancer (19, 20). Notably, this model system also accurately recapitulates the increased incidence of bladder cancer found in males (21). This predilection to males persists despite considering differential exposure to environmental and occupational risk factors such as cigarette smoking and aromatic amines (16, 22), the primary risk factors for bladder cancer in humans (23). Interestingly, a recent meta-analysis suggests a role for additional factors in the etiology of human bladder cancer (24), such as hormonal effects, that could explain the male preponderance of bladder cancer (3:1 ratio) (1, 16) observed in human epidemiology studies (21, 25).

Accordingly, the results from our study suggest that CD24 plays a significant role in bladder tumorigenesis and metastasis, especially in males. These data also implicate CD24 as an important androgen-regulated effector in male human bladder cancer and thus provide a rationale for novel therapeutic approaches targeting the androgen receptor (AR) in male bladder cancer.

## Results

**Bladder Tumor Incidence Is Reduced in *Cd24a*-Deficient Mice.** To study the effect of CD24 deficiency on the incidence of bladder tumors, we exposed paired cohorts of *Cd24a*-deficient and WT C57BL/6 control mice to water containing OH-BBN, ad libitum (Fig. 1A). Results from serial micro-computed tomography (mCT) imaging with subsequent necropsy and pathologic evaluation suggested that 16-, 24-, and 28-wk assessment points following OH-BBN initiation were optimal for stratifying tumor progression (Fig. 1B). The incidence of cancer was lower in *Cd24a*-deficient mice (29%) than in WT mice (45%) at 16 wk (Fig. 1C) and remained lower in *Cd24a*-deficient mice at 24 and 28 wk. Bladder tissues from *Cd24a*-deficient and WT mice, when matched for tumor grade, displayed no discernible histopathological differences (Fig. 1D).

Author contributions: D.T. designed research; J.B.O., K.H.K., S.T., M.D.N., M.A.H., and S.C.S. performed research; J.B.O., K.H.K., J.E.D., S.T., M.D.N., M.A.H., S.C.S., H.F.F., M.C., and D.T. analyzed data; and J.B.O., K.H.K., J.E.D., S.T., M.D.N., M.A.H., S.C.S., H.F.F., M.C., and D.T. wrote the paper.

The authors declare no conflict of interest.

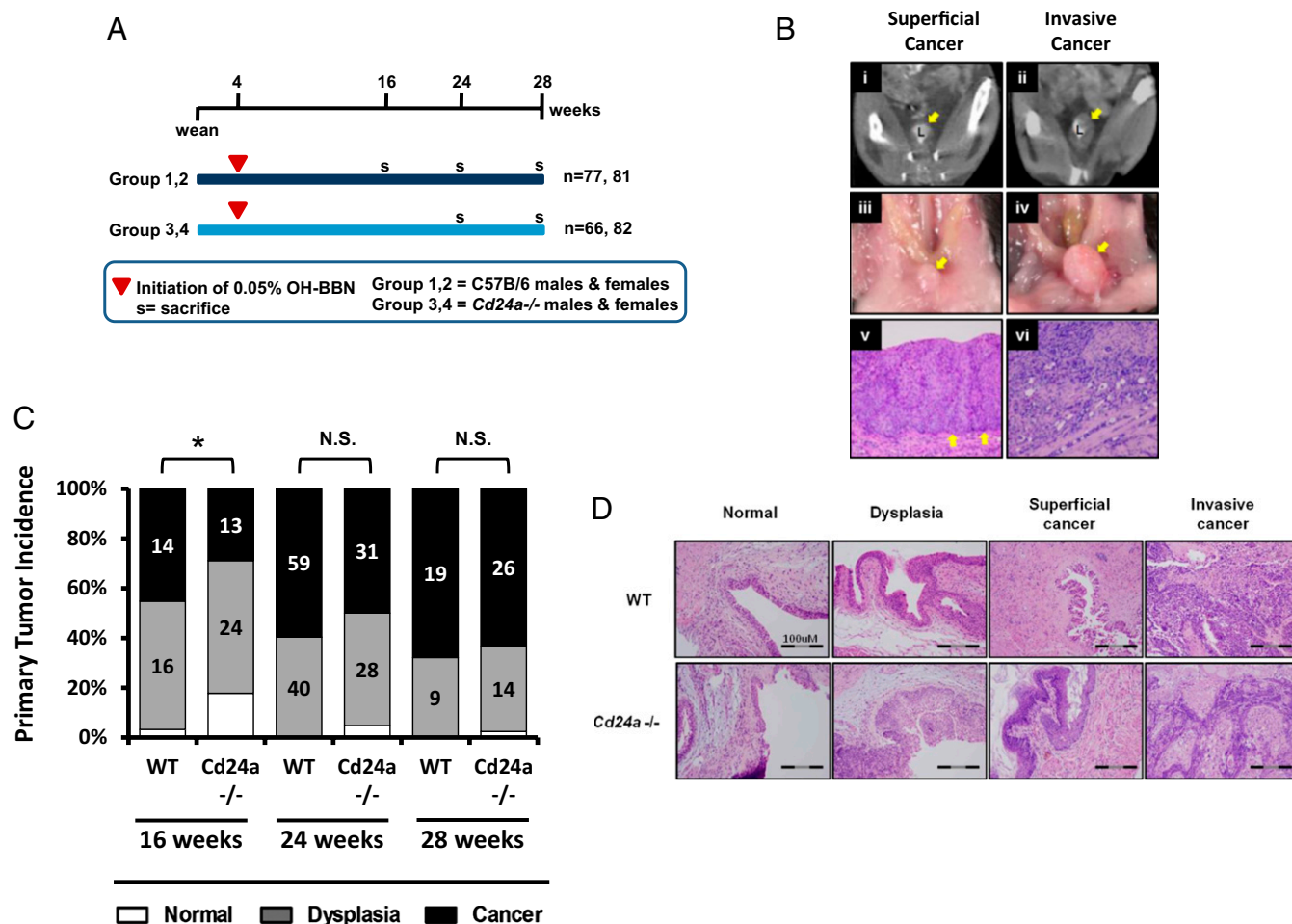
This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper can be found in GenBank database [JN565036 (UM-UC-3 CD24 promoter); JN565037 (J82 CD24 promoter); JN565040 (LNCaP CD24 promoter); JN565041 (LuL-2 CD24 promoter); and JN565042 (EJ CD24 promoter)].

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See Author Summary on page 20788 (volume 109, number 51).

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1113960109/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1113960109/-DCSupplemental).



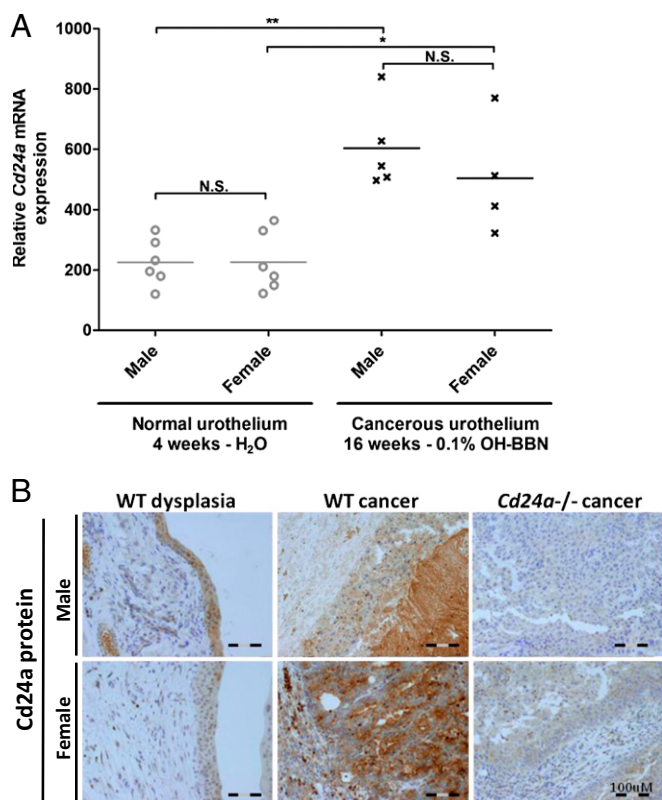
**Fig. 1.** Germline *Cd24a* loss is associated with reduced incidence of OH-BBN-induced bladder cancer. (A) Schematic of the experimental design for assessing bladder tumor incidence. WT and *Cd24a*-deficient (*Cd24a*<sup>-/-</sup>) mice were started on OH-BBN ~4 wk after weaning. Successive mCT imaging of pilot study animals suggested the use of assessment points at 16, 24, and 28 wk for best assessment of tumor development, so cohorts were analyzed for the presence of bladder tumors at those time points. (B) Corresponding mCT scans (Top), necropsy images (Middle), and histological preparations (Bottom) collected at 24 wk. Images *i*, *iii*, and *v* are of a superficial (i.e., noninvasive) bladder tumor; *ii*, *iv*, and *vi* are of invasive tumor. Arrows indicate the bladder wall in *i*–*iv* and the basement membrane in *v*. L, lumen of bladder. (C) Comparison of tumor incidence over time in populations of WT and *Cd24a*-deficient mice using binary and logistic regression models revealed that the incidence of malignancy was significantly higher in WT mice than in *Cd24a*-deficient mice. \* $P < 0.05$ . N.S., not significant. (D) Representative H&E-stained bladder tissues displayed no differences in histology between grade-matched WT and *Cd24a*<sup>-/-</sup> tissues.

***Cd24a* mRNA and Protein Expression Are Increased in OH-BBN-Induced Bladder Cancer.** Because loss of *Cd24a* appears to protect bladder urothelium from OH-BBN-induced tumorigenesis, we sought to determine if *Cd24a* expression increased in OH-BBN-induced cancers compared with normal bladder tissue. *Cd24a* mRNA levels in urothelium samples from mice exposed to OH-BBN in drinking water were analyzed using quantitative RT-PCR (qRT-PCR). We found that *Cd24a* mRNA levels were significantly elevated in cancerous urothelium from both male and female mice exposed to OH-BBN as compared with normal urothelium ( $P < 0.01$  and  $P < 0.05$ , respectively by Mann–Whitney test) (Fig. 2A). This finding was consistent with other studies (26) that have associated CD24 overexpression with malignant transformation.

In parallel with our assessment of *Cd24a* mRNA levels, we used immunohistochemical analysis to evaluate whether *Cd24a* protein levels were increased as a function of histopathologic findings. Compared with dysplastic tissue from WT mice, WT cancerous urothelium demonstrated significantly higher levels of *Cd24a* staining (Fig. 2B). Urothelium from male and female mice did not express a quantifiable difference in *Cd24a*. Cancerous urothelium from *Cd24a*-deficient mice showed no appreciable staining. Urothelial tissue obtained from WT mice without his-

tologically apparent tumors also showed no evidence of *Cd24a* staining. Thus, *Cd24a* mRNA and protein levels increased as urothelial cells were transformed into a cancerous pathology, consistent with CD24 being a driver for tumorigenesis.

***Cd24a* Deficiency in Male Mice Delays Bladder Malignancy.** Human bladder cancer rates are higher in males (16, 22). Similarly, OH-BBN-induced bladder tumor rates are higher in male WT mice than in female WT mice (21, 25). Consistent with these observations, our studies found that after 16-wk exposure to OH-BBN males had a greater proportion of malignant tumors (56%) and fewer normal results (0%) than females (33% and 7%, respectively) (Table 1). Based on ordinal logistic regression models, these differences were statistically significant ( $P = 0.050$ ). Similar results were found at 24 wk, although the differences were not significant ( $P = 0.059$ ). Expanding these studies to look at the effect of *Cd24a* deficiency on these rates revealed that the cancer incidence was significantly lower in *Cd24a*-deficient male mice than in WT mice at all time points (Table 1). Thus, loss of CD24 is protective against OH-BBN-induced bladder cancer in male mice but not in female mice.



**Fig. 2.** OH-BBN-induced bladder tumors have high levels of *Cd24a* mRNA and protein expression. (A) C57BL/6 WT mice ( $n = 21$ ) were provided with normal drinking water until age 4 wk; then 12 mice were killed, and the remaining nine mice were exposed to drinking water containing OH-BBN ad libitum for an additional 12 wk. Bladders from each cohort were processed for histological and qRT-PCR analysis. *Cd24a* mRNA analysis revealed that cancerous urothelium from the cohort exposed to OH-BBN had significantly more *Cd24a* than comparative control normal urothelium, regardless of the sex of the mice.  $**P < 0.01$ ,  $*P < 0.05$ ; Mann-Whitney test; N.S., not significant. (B) The expression of *Cd24a* protein increases as a function of tumor progression. After exposure to OH-BBN, bladders from WT mice with cancer were found to express higher levels of *Cd24a* protein than dysplastic counterparts ( $n = 17$ ). Cancerous urothelial samples from *Cd24a*-deficient mice were stained similarly to control for antibody specificity ( $n = 4$ ).

#### ***Cd24a* Deficiency Reduces Metastasis in Male Mice Harboring Primary Tumors.**

Previous studies have shown that CD24 expression is higher in nodal metastases than in matched primary tumors (4, 5). To determine whether *Cd24a* deficiency would limit metastatic incidence and burden, new cohorts of *Cd24a*-deficient and WT mice subjected to OH-BBN carcinogenesis were monitored until they reached previously established experimental surrogates of death (Fig. 3A). To ensure that our evaluation of metastatic incidence was independent of tumor occurrence, only mice with evidence of primary bladder cancer by histopathologic analysis were included in our assessment of metastatic frequency. Histological analysis of lung metastasis samples revealed no apparent differences in tumor architecture between metastases obtained from WT and from *Cd24a*-deficient mice of either sex (Fig. 3B). In analyzing metastatic incidence by sex, we found that the incidence of metastasis was higher in male WT mice than in female WT mice (Fig. 3C), similar to previous reports (21). In accordance with data on tumor incidence, we also observed that loss of *Cd24a* resulted in dramatic protection against metastatic incidence. The incidence of metastasis was significantly lower in *Cd24a*-deficient male mice (7%) than in WT (21.7%) male mice ( $P = 0.039$ , one-tailed Wald test). *Cd24a*-deficient female mice did

**Table 1. Histological analysis of bladders from C57B/6 control (WT) and *Cd24a*-deficient mice exposed to OH-BBN**

	WT male, <i>n</i> (%)	<i>Cd24a</i> <sup>-/-</sup> male, <i>n</i> (%)	WT female, <i>n</i> (%)	<i>Cd24a</i> <sup>-/-</sup> female, <i>n</i> (%)
<b>16 wk</b>				
Normal	0 (0)	5 (21)	1 (7)	3 (14)
Dysplasia	7 (44)	11 (46)	9 (60)	13 (62)
Cancer	9 (56 <sup>*,†</sup> )	8 (33 <sup>†</sup> )	5 (33 <sup>*</sup> )	5 (24)
<b>24 wk</b>				
Normal	0 (0)	1 (3)	0 (0)	2 (6)
Dysplasia	14 (30)	16 (54)	26 (49)	12 (38)
Cancer	32 (70 <sup>†</sup> )	13 (43 <sup>†</sup> )	27 (51 <sup>†</sup> )	18 (56 <sup>†</sup> )
<b>28 wk</b>				
Normal	0 (0)	0 (0)	0 (0)	1 (3)
Dysplasia	3 (20)	4 (33)	6 (46)	10 (35)
Cancer	12 (80 <sup>†</sup> )	8 (67 <sup>†</sup> )	7 (54 <sup>†</sup> )	18 (62 <sup>†</sup> )
Total	77	66	81	82

\*Increased proportion of WT males with advanced disease compared with WT females at 16 wk ( $P = 0.05$ ;  $\chi^2$  for trend).

<sup>†</sup>Increased proportion of WT males with advanced disease compared with *Cd24a*<sup>-/-</sup> males ( $P < 0.05$ ;  $\chi^2$  for trend).

<sup>\*</sup>No statistically significant difference between WT females with advanced disease and *Cd24a*<sup>-/-</sup> females.

not show a reduction in metastatic incidence relative to WT female mice. There was no statistical difference in metastatic incidence between male and female *Cd24a*-deficient mice (Fig. 3C).

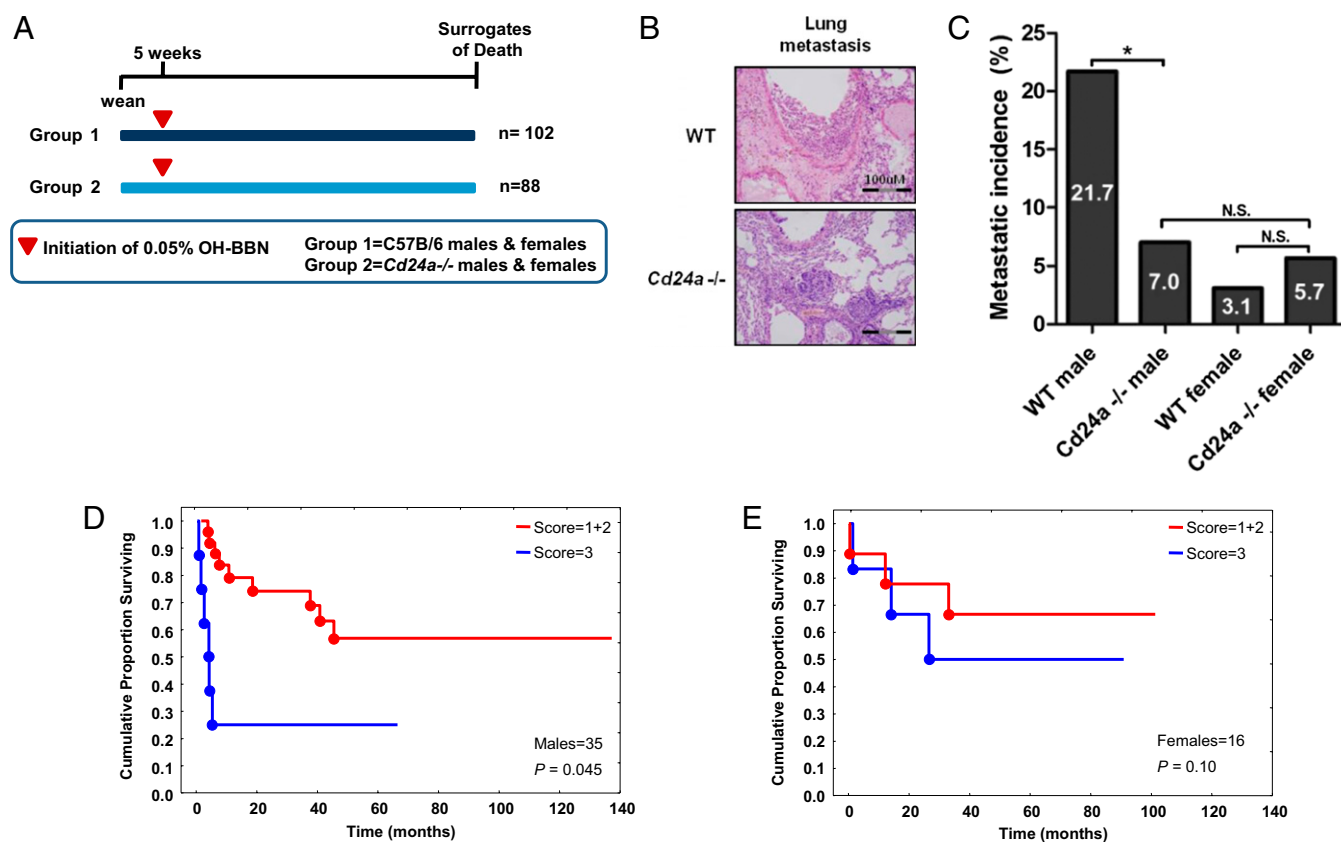
#### **Tumor CD24 Expression Is Prognostic of Outcomes in Bladder Cancer Patients.**

Based on the reduction of OH-BBN-induced bladder metastases in *Cd24a*-deficient mice, we reevaluated a previously reported tissue immunohistochemical analysis of CD24 in human bladder cancer samples (4) to determine if CD24 expression has sex-specific prognostic significance in bladder cancer patients. Male patients with tumors expressing higher levels of CD24 had shorter disease-free survival times than males whose tumors expressed lower levels of CD24 (Fig. 3D), even in a small ( $n = 35$ ) patient cohort ( $P = 0.045$ ). This correlation with high CD24 expression was not as apparent in female patients ( $P = 0.10$ ) (Fig. 3E). Our limited human cohort study corroborates results from the murine metastasis model and supports the hypothesis that CD24 is an important contributor to the development of bladder metastasis in males and, to a lesser extent, in females. Thus, loss of CD24/*Cd24a* appears to protect against both tumorigenesis and metastasis in male mice and in humans.

#### **Androgen-Induced Growth Is Dependent on CD24 Expression in UM-UC-3 Male Bladder Tumor Cells.**

Interestingly, males appear to be more protected than females by loss of CD24/*Cd24a* even though the sexes exhibit similar CD24/*Cd24a* mRNA and protein levels (Fig. 2B). One explanation for this sex difference is that CD24/*Cd24a* may function in a signaling network that is less important in females than in males. Androgen regulation is the most broadly studied of such pathways. Therefore we investigated androgen-mediated cell proliferation in the context of CD24 depletion to assess the importance of CD24/*Cd24a* in androgen-induced cellular growth. UM-UC-3 cells were treated with CD24 siRNA and analyzed for differential growth with alamarBlue following treatment with R1881. Consistent with previous studies (25, 27, 28), R1881 induced the growth of control cells (Fig. 4A). In contrast, neither CD24-silenced cells nor AR-silenced cells exhibited significant changes in proliferation after R1881 treatment. These findings suggest that CD24 has a significant role in androgen-mediated growth in bladder tumors.





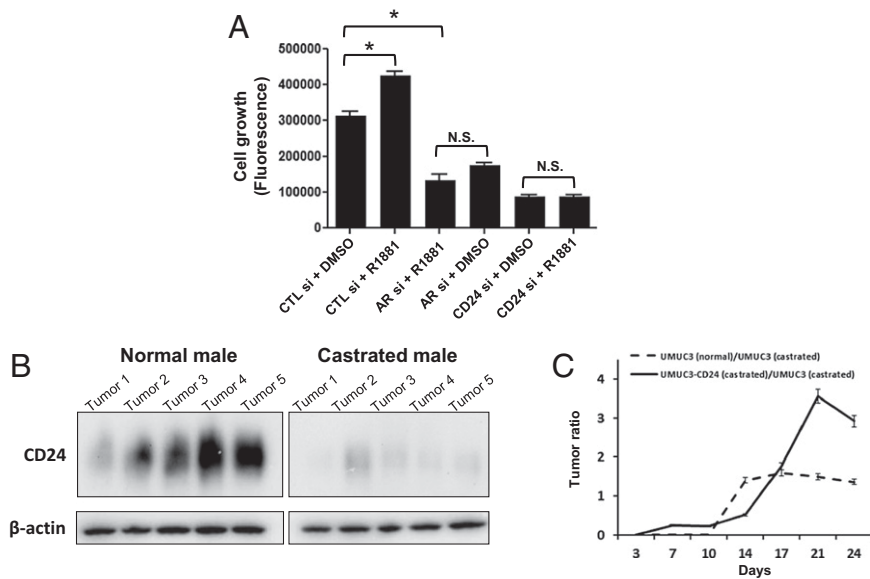
**Fig. 3.** Loss of *Cd24a* decreases metastasis in male mice. (A) Schematic of the experimental design for assessing metastatic incidence. WT ( $n = 102$ ) and *Cd24a*-deficient ( $n = 88$ ) mice were provided OH-BBN until they reached previously established surrogates of death (cachexia  $>20\%$  of control animal weight, lethargy/behavioral changes, or overt respiratory/general distress). (B) Lung metastatic tissues from *Cd24a*-deficient and WT mice were stained by H&E and showed similar histological architecture. (C) Of animals with documented primary tumors, WT males had higher rates of metastasis (21.7%) than WT females (3.1%). Additionally, *Cd24a*-deficient males had decreased metastatic incidence (7.0%) relative to their WT counterparts.  $*P = 0.039$ ; one-tailed Wald test. Comparisons of WT and *Cd24a*-deficient females showed no statistically significant difference (N.S.) in metastasis. (D) Evaluation of a previously reported tissue microarray with immunohistochemical staining for CD24 (4). Stratification of disease-free survival data from human bladder cancer patients as a function of patient sex and CD24 expression level. The red line indicates a score of 1 or 2; the blue line indicates a score of 3. [Scoring was described previously (4)]. Kaplan–Meier analysis suggests that CD24 is an independent prognostic factor for disease-free survival in males ( $P < 0.05$ ).

To assess whether androgen signaling mediates CD24 expression *in vivo*, we used a previously developed xenograft model of bladder cancer (5). Normal and castrated male mice were injected s.c. with  $5 \times 10^5$  UM-UC-3 cells, which express moderate levels of CD24 (5). Tumors resected from control male mice after 4 wk showed distinctly higher expression of CD24 than tumors resected from castrated male mice (Fig. 4B). Thus, *in vivo*, in the absence of androgen (i.e., with castration), CD24 expression is reduced dramatically. Additional analysis of these mice revealed that tumors generated in normal male mice were larger than tumors grown in castrated male mice (Fig. 4C, dotted line). To investigate further the *in vivo* functional impact of CD24 as an effector of androgen-mediated tumor growth, we compared xenografts from parental UM-UC-3 cells and from UM-UC-3 cells that stably overexpress CD24 (5). In this setting, overexpression of CD24 could restore tumor growth characteristics that previously had been reduced by castration (Fig. 4C, solid line). Thus, CD24 appears to have a pronounced functional role as a downstream effector in androgen-mediated growth signaling in bladder cancer.

**CD24 mRNA and Protein Expression Are Androgen Dependent in Male Bladder Cancer Cells.** Previous studies using a similar OH-BBN carcinogenesis model suggest that both AR and androgen signaling function in the development of bladder cancer, both *in vitro* and *in vivo* (25). Given that *Cd24a* deficiency resulted in

fewer malignant bladder tumors in male mice and that AR depletion or blockade reduced tumor growth in a manner dependent on CD24 expression, we questioned whether CD24 may function as part of an androgen-signaling cascade. To address this question, three bladder cancer cell lines were analyzed based on their AR expression status: AR-expressing UM-UC-3 and TCCSUP cells and HTB9 (5637) cells that do not express AR (25). Activation of AR signaling using R1881 (synthetic androgen) treatment in UM-UC-3 and TCCSUP cells led to a 2.5- and twofold induction of AR protein, respectively (Fig. 5A). Strikingly, R1881 treatment also led to a 2.2- and 3.1-fold induction in CD24 protein expression. Treatment of the cell lines with Casodex or siRNA to silence AR expression reduced both AR and CD24 protein expression in UM-UC-3 and TCCSUP cells. Furthermore, R1881 treatment failed to induce CD24 expression in AR-silenced UM-UC-3 and TCCSUP cells. These observations strongly suggest that androgen signaling controls CD24 expression. The expression of CD24 in AR-null HTB9 cells was not affected by R1881-, Casodex-, or siRNA-mediated AR silencing (Fig. 5A).

To assess whether these androgen-induced changes in CD24 protein levels also occurred at the mRNA level, qRT-PCR was performed on total RNA from the UM-UC-3 cells used above for immunoblotting. Here, we observed that R1881 significantly stimulates CD24 mRNA expression in UM-UC-3 (Fig. 5B) and



**Fig. 4.** Tumor growth promoted by androgen signaling is dependent on CD24 in vitro and in vivo. (A) Relative growth of UM-UC-3 cells as assessed by alamarBlue 72 h after indicated treatments and siRNA transfections. Treatment with R1881 increases growth compared with DMSO. Transfection of cells with either AR or CD24 siRNA inhibits R1881-induced growth. \* $P < 0.05$ . (B) Xenograft in vivo tumorigenesis model. Normal and castrated male nude mice ( $n = 10$ ) were injected s.c. with  $5 \times 10^5$  UM-UC-3 cells. After 4 wk, tumors were resected and evaluated for CD24 protein expression with human CD24-specific antibodies. Tumors isolated from normal male mice expressed higher levels of CD24 than tumors resected from castrated male mice. (C) Normal and castrated male nude mice ( $n = 10$ ) were injected s.c. with  $5 \times 10^5$  UM-UC-3 cells that had been vector transfected or CD24 transfected as described previously (5). Graph represents tumor size over time. Data show that castration can reduce growth of UM-UC-3 tumors and that stable exogenous expression of CD24 in UM-UC-3 cells can rescue this growth reduction. Error bars indicate SEM.

TCCSUP cells (Fig. 5C), whereas siRNA silencing of AR prevents this increase ( $P < 0.05$ ). Together these results suggest that androgen and AR function in controlling expression of CD24 at the point of transcription.

**AR Activation Stimulates CD24 Promoter Activity in Human Bladder Cancer Cells.** To test the hypothesis that androgen controls CD24 transcription we generated luciferase reporter constructs containing roughly 1 kbp and 2 kbp of the CD24 promoter upstream from the start codon. UM-UC-3 cells were transiently transfected with each of these reporter constructs and incubated with or without R1881 for 24 h. Although none of the reporter constructs exhibited an increase in basal activity relative to control (pGL4), R1881 treatment induced a 2.8-fold increase in the activity of a 1,896-bp CD24 promoter (Fig. 6A). R1881 also induced an increase in the activity of a prostate-specific antigen (PSA) promoter construct, as expected (29). The ability of R1881 treatment to induce CD24 promoter activity also was observed in TSSCUP cells (Fig. 6A).

Interestingly, the shorter CD24 promoter construct (1,139 bp) did not exhibit any change in activity following R1881 treatment, suggesting that an androgen-response element (ARE) lies between 1,139 and 1,896 bp upstream of the CD24 start codon. Analysis of this region of the CD24 promoter by Genomatix MatInspector software suggested the presence of an ARE in this region. We subsequently mutated six base pairs within this element and tested the ability of R1881 to promote activity. We found that R1881 induction was abolished in the mutated reporter in both UM-UC-3 and TCCSUP cell lines (Fig. 6B), suggesting that androgen-activated AR directly activates CD24 transcription. As a further test of this hypothesis, we investigated the ability of R1881 to increase CD24 promoter activity in the absence of AR. Treatment of both UM-UC-3 and TCCSUP with AR siRNA abrogates the R1881-dependent increase in CD24 promoter activity (Fig. 6C). Together these in vitro studies suggest a model in which androgen activation of AR results in an AR- and ARE-dependent increase in CD24 promoter activity.

To test this model using the endogenous AR and CD24 promoter, we used ChIP on cells treated with or without R1881. Nonspecific mouse IgG and histone H3 antibodies served as negative and positive controls, respectively. We found that AR was associated with the aforementioned ARE within the CD24 promoter but only in an R1881-dependent manner (Fig. 6D). Similarly, AR was associated with the known ARE in the promoter of

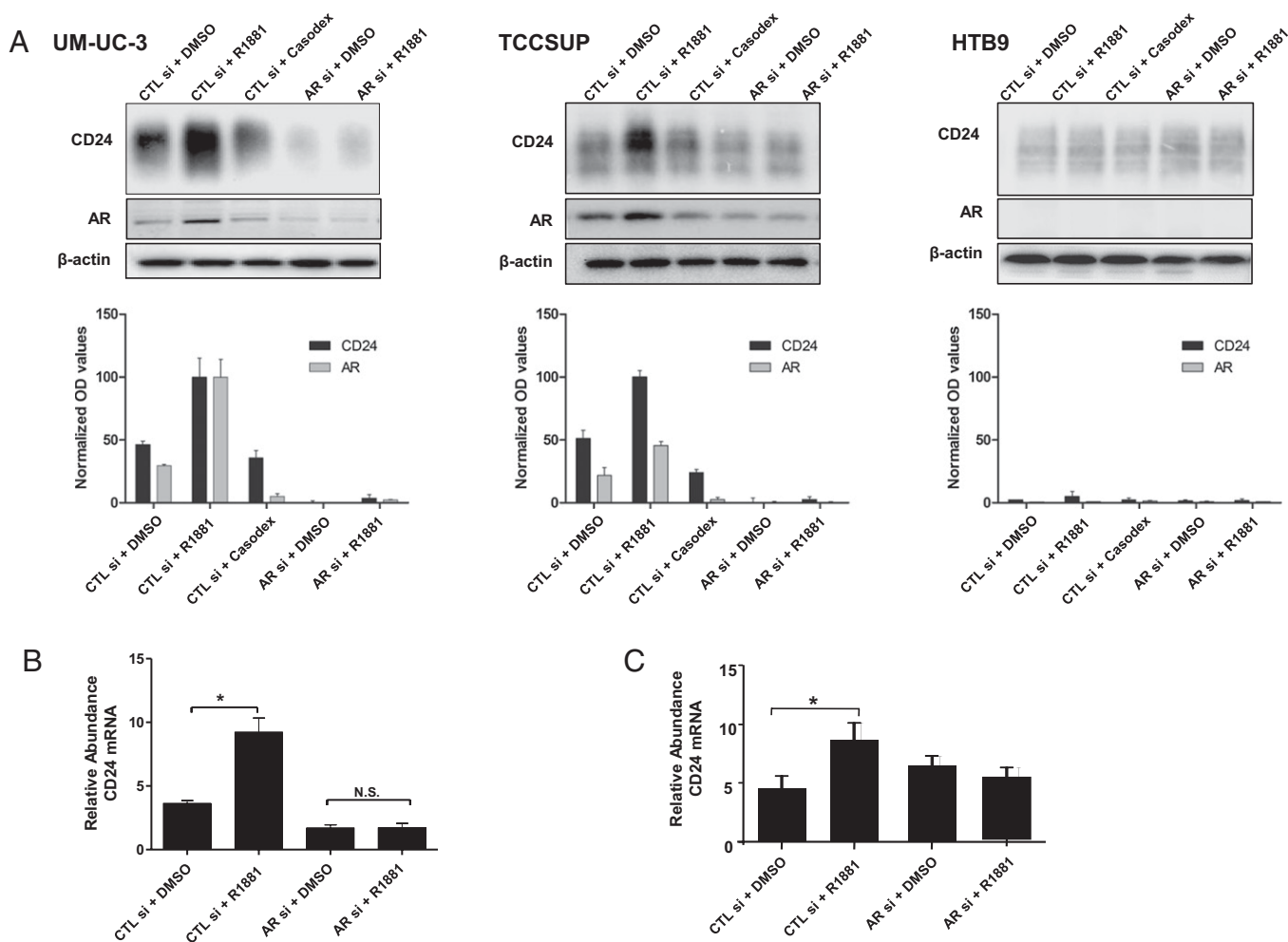
PSA. However, AR was not associated with a region of the CD24 promoter that did not contain a computationally predicted ARE. Taken together, these findings demonstrate that there is an ARE about 1.5 kbp upstream from the CD24 transcription start site and that androgen-activated AR forms a complex with this DNA element to promote an increase in CD24 transcription.

## Discussion

CD24 is a cell-surface protein that functions as a mediator of proliferation, invasion, and motility in multiple types of cancer cell lines from multiple tissues (4, 11, 12, 30, 31). Previous efforts to investigate the role of CD24 in the primary tumor setting have been limited to using exogenous overexpression and suppression in xenograft models. In this study, the role of CD24 in bladder tumor formation and metastasis was analyzed using *Cd24a*-deficient mice to model better the natural history of bladder cancer in a more physiologic setting. Mice were exposed to OH-BBN, a validated and commonly used model of bladder cancer induction (17, 21). Treatment of mice with OH-BBN resulted in progressive neoplastic development similar to that seen in prior reports (19, 21, 32). We observed fewer malignant lesions in *Cd24a*-deficient mice than in WT mice, demonstrating that *Cd24a* deficiency inhibits tumor development in an OH-BBN model of bladder cancer. This finding is consistent with a role of CD24 as a driver of bladder cancer and is supported by previous in vivo data showing that CD24-specific antibodies offer therapeutic benefit against bladder tumor xenograft formation (5). Interestingly, further analysis of the OH-BBN data by sex revealed that only males showed significant decreases in tumor incidence with loss of *Cd24a*.

Investigating the effect of *Cd24a* deficiency on metastatic incidence revealed that loss of *Cd24a* in male mice resulted in a nearly threefold decrease in metastatic incidence relative to WT male mice. This finding was independent of the observed reduction of tumor incidence, because only mice with evidence of bladder tumors were included in this metastasis analysis. This important finding demonstrates that CD24 plays a role in both cancer formation and metastasis. A similar trend was seen in female mice when metastatic incidence and loss of *Cd24a* were analyzed, but the results were not statistically significant.

Assessment of human tumor samples revealed a similar relationship between CD24 expression and metastatic recurrence after radical primary therapy: High CD24 expression in tumors from males was associated with a reduction in disease-free survival. Because metastasis is rarely curable and metastasis of bladder



**Fig. 5.** CD24 mRNA and protein expression is regulated by androgen. (A) UM-UC-3, TCCSUP, and HTB9 cells were transfected with control (CTL si) or AR siRNA (AR si) for 48 h before being treated with DMSO, R1881 (1 nM), or Casodex (30  $\mu$ M). After 24 h, lysates were collected and analyzed for CD24 and AR protein expression. OD values obtained with Alpha Innotech software (normalized for protein loading using  $\beta$ -actin staining) are plotted below the corresponding immunoblots. Representative blots from three separate experiments are shown. (B and C) Total RNA extracted from UM-UC-3 (B) and TCCSUP (C) cells in A was analyzed for CD24 expression by qRT-PCR. Treatment with R1881 increases CD24 expression, an effect that is abrogated when cells are transfected first with AR siRNA. \* $P < 0.05$ .

tumors is strongly correlated with death in patients with bladder cancer, this observation is consistent with the mouse metastasis data. A similar trend was observed in female patients. Taken together, these findings show that CD24/*Cd24a* expression levels in males correlate to a high degree with metastatic recurrence and that this protein is important in tumor formation. CD24/*Cd24a* expression levels do not correlate with tumor formation rates in female mice but may play a role in metastasis in female mice and humans. The ability of CD24 to mediate metastasis is consistent with prior data on this molecule (5, 11, 12, 33).

The finding that CD24/*Cd24a* affects tumor formation and metastasis differentially in males and females is surprising, particularly given the similar expression levels of CD24a mRNA and protein in male and female normal and malignant murine urothelium. In males, loss of *CD24a* in mice or a lower level of CD24 in human tumor samples correlates with protection against both tumor formation and metastatic recurrence. Although the data suggest that female mice appear to gain protection from metastasis development with lower *CD24a* levels, this protective effect was not observed during primary tumor formation. This observation suggests that the role of *CD24a* in tumor formation is less important in females and may be muted by a more dominant, female-

specific pathway or that the downstream effectors of CD24 found in males are not expressed or functional in females.

That hormones may play a role in regulating sex-specific tumorigenesis in bladder cancer was shown by Miyamoto et al. (25), who used the OH-BBN model to demonstrate that both androgen signaling-mediated pathways and AR are important in promoting tumorigenesis. Moreover, they reported that androgen signaling may regulate the expression of molecules involved in angiogenesis and metastasis. Given our observations that *Cd24a*-deficient male mice are preferentially protected from OH-BBN-induced tumorigenesis and metastasis, we decided to investigate whether CD24 is responsive to androgen regulation. Because multiple groups have demonstrated AR expression in normal urothelium and AR signals in bladder cancer cells from both males and females (34–36), our model system provides an excellent platform for investigating the ability of androgen and AR to regulate CD24 expression. We discovered that androgen signaling increases CD24 promoter activity, yielding higher CD24 mRNA and protein expression. Furthermore, diminished tumor xenograft growth as a result of depleted androgen was offset by exogenous overexpression of CD24 in vivo. Because CD24 depletion reduces cellular proliferation to the same extent as AR knockdown, our results suggest that the ability of androgen and AR to promote tumorigenesis in males is dependent on CD24 ex-





treatment with R1881 or Casodex. UM-UC-3 cells obtained from ATCC were cultured and injected s.c. into the flank regions of castrated or normal NCr<sup>nu/nu</sup> mice according to previously described protocols (5).

**Carcinogenesis Treatment Protocol.** Male and female 7-wk-old *Cd24a*-deficient mice and WT C57BL/6 control mice were supplied ad libitum with tap water containing 0.1% OH-BBN (TCI America); bottles were refreshed twice a week. Mice were inspected weekly and observed for signs of distress associated with bladder lesions, including hematuria and firm bladders. Cohorts of treated *Cd24a*-deficient and WT mice were killed at 16-, 24-, and 28-wk time points. To evaluate disease-specific survival, animals were killed when they exhibited IACUC-accepted humane surrogates of death (cachexia >20% of control animal weight, lethargy/behavioral changes, or overt respiratory/general distress). We harvested bladder, periaortic lymph nodes, liver, lung, and kidney tissues. A portion of each tissue was preserved in phosphate-buffered 10% formalin and paraffin embedding for eventual sectioning and staining with H&E. The remaining tissues were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for RNA and protein analyses.

**CD24 Promoter, Reporter Assays, and Computational Binding-Site Predictions.** Two previously published CD24 promoter sequences (42, 43) showed considerable mismatch between each other and a partial promoter from GenBank. Therefore we cloned and sequenced the CD24 promoter from several distinct human cell lines to identify a common sequence for our studies as described (44). The sequences can be found in GenBank with the following accession numbers: UM-UC-3 CD24 promoter, JN565036; J82 CD24 promoter, JN565037; LNCaP CD24 promoter, JN565040; LuL-2 CD24 promoter, JN565041; and EJ CD24 promoter, JN565042.

In this study we used the promoter from UM-UC-3 cells called "CD24-1896," representing  $-1936$  to  $-30$  of CD24 5' UTR from UM-UC-3 cells. Use of Genomatix MatInspector software (Genomatix Software GmbH) on CD24-1896 suggested that an ARE is located at  $-1501$  to  $-1475$ . Mutation of this site was executed according to instructions from the site-directed mutagenesis protocol (Stratagene). Primers were AREmS: 5'-CTA-ACG-AGA-GTG-TTC-TGC-AAA-ACT-GAA-ATT-TAA-ACA-AAT-TTG-AGT-CCA-TAC-TAG-GTT-TGG-TCT-CTG-3' and AREmAS: 5'-CAG-AGA-CCA-AAC-CTA-GTA-TGG-ACT-CAA-ATT-TGT-TTA-AAT-TTC-AGT-TTT-GCA-GAA-CAC-TCT-CGT-TAG-3'. The PSA promoter construct has been described previously (45). DNA transfections of UM-UC-3 or TCCSUP cells were carried out using Lipofectamine (Invitrogen) and 1  $\mu\text{g}$  of plasmid. For specific experiments, siRNA (20 pmol) against AR or luciferase was cotransfected with DNA. After 24 h cell medium was replaced with medium containing charcoal-stripped serum. Cells then were treated with R1881 (10 nM) or vehicle (ethanol). After 24 h cells were lysed and assayed for luciferase activity according to the manufacturer's instructions (Promega). Luciferase values were normalized to cell number generated from Cyquant analysis (Life Technologies).

**ChIP.** UM-UC-3 and TCCSUP cells were grown to 70% confluence in two 15-cm dishes per treatment. Medium was removed, and fresh medium containing charcoal-stripped serum and R1881 or EtOH was added for 2 h. Then, according to instructions in the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling), paraformaldehyde was added directly to cell medium (1% final concentration) and quenched with 1 M glycine at room temperature, and cells ( $4 \times 10^7$ ) were collected. The chromatin was sheared using Micrococcal

Nuclease and sonicated for three 10-s pulses at 60% output and 50% duty cycle. Overnight ChIP was set up using 10  $\mu\text{g}$  of chromatin and antibody (histone H3, AR, or control IgG). After purification of the immunoprecipitated DNA, three-step qRT-PCR was used to quantify the DNA levels using an iQ5 thermocycler and iQ SYBR Green Supermix (Bio-Rad). Primers used were CD24-ARE S: 5'-TGA-ACC-AAA-AAT-ACT-AAC-GAG-AGT-G-3'; CD24-ARE AS: 5'-AGC-AAA-CAG-CAG-AGA-CCA-AA-3'; CD24-upstream S: 5'-TCA-AGT-GTG-AAA-ATG-TTC-TC-3'; CD24-upstream AS: 5'-CGA-AGT-GTT-CCT-GTT-TAA-AT-3'; PSA-ARE S: 5'-CTG-CCT-TTG-TCC-CCT-AGA-T-3'; and PSA-ARE AS: 5'-AAC-CTT-CAT-TCC-CCA-GGA-CT-3' (46). The input cycle threshold (Ct) values were adjusted to 100% efficiency by subtracting 5.64 from each Ct value because the starting input fraction was 2% of the total immunoprecipitation. All Ct values subsequently were adjusted to percent of input using the equation  $100 \times 2^{(\text{adjusted input Ct} - \text{sample Ct})}$ .

**Imaging and Histological Analysis of Murine and Human Tissues.** Serial mCT scans were acquired 10 min after the administration of 0.3 mmol/kg Gd-diethylene triamine pentaacetic acid (DTPA) (Magnevist). The images were acquired using 512 slices of a  $512 \times 512$  pixel 2D matrix and a reconstructed pixel element with a spatial resolution of 0.17  $\text{mm}^3$ . Murine tissues were examined as a series of 20- $\mu\text{m}$  sections and were reviewed by a pathologist (H.F.F.) blinded to genotype origins of the sample mouse tissues. Histological tissue architectures were classified as normal, dysplasia, and cancer (encompassing carcinoma in situ). Samples of murine tissues were analyzed with immunohistochemical analysis using rat anti-mouse *Cd24a* antibody (M169 clone; BD Pharmingen). CD24 immunohistochemistry on human tissues, the specific tissue microarray used, and stratification techniques were reported previously (4).

**Statistical Analysis.** Binary and proportional odds ordinal logistic regression models (47) were used to estimate the effects of *CD24a* deficiency vs. WT, sex, and time (e.g., 16 wk vs. 24 wk) on the incidence of normal, dysplasia, and cancer cells in bladder tissue samples. The binary and ordinal models included main effects for *CD24a* deficiency vs. WT, sex, and time and all two-way interactions. The score test was used to check the proportional odds assumption, resulting in a *P* value of 0.28, indicating that the data displayed no significant deviation from the proportional odds assumption. Logistic regression models also were used to assess metastatic incidence by sex and *Cd24a* deficiency. Specific comparisons were made with one-sided Wald tests because of our hypothesis that *Cd24a* deficiency would limit metastases. The log-rank test was used to correlate patient disease-free survival with CD24 expression levels. For the luciferase assay and ChIP data, a mixed-model ANOVA with a random effect for date of replicate experiment was used, and the Levene's test was used to check for homogeneity of variances among groups. Preplanned contrasts were used to test for significant differences between EtOH- and R1881-treated groups. Statistical analysis was carried out with Prism (GraphPad Software); for the binary and ordinal models, SAS 9.2 (SAS Institute, Cary, NC) was used.

**ACKNOWLEDGMENTS.** We thank Drs. Christopher Moskaluk and Pat Praemojago for their technical support. This study was supported by National Institutes of Health (NIH) Grant CA075115 (to D.T.) and NIH Cancer Research Training in Molecular Biology Award 5T32CA009109 (to J.B.O.).

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