In Vivo Disruption of an Rb–E2F–Ezh2 Signaling Loop Causes Bladder Cancer

Mirentxu Santos1,2, Mónica Martínez-Fernández1,2, Marta Dueñas1,2, Ramón García-Escudero1,2, Beogna Alfaya1, Felipe Villacampa1,2, Cristina Saiz-Ladera1, Clotilde Costa1, Marta Oteo2,4, José Duarte2,3, Victor Martínez2, Mª José Gómez-Rodríguez2,3, Mª Luisa Martín5, Manoli Fernández3, Patrick Viator6, Miguel A. Morcillo4, Julien Sage5, Daniel Castellano2,3, Jose L. Rodriguez-Peralto6, Federico de la Rosa2,3, and Jesús M Paramio1,2

Abstract

Bladder cancer is a highly prevalent human disease in which retinoblastoma (Rb) pathway inactivation and epigenetic alterations are common events. However, the connection between these two processes is still poorly understood. Here, we show that the in vivo inactivation of all Rb family genes in the mouse urothelium is sufficient to initiate bladder cancer development. The characterization of the mouse tumors revealed multiple molecular features of human bladder cancer, including the activation of E2F transcription factor and subsequent Ezh2 expression and the activation of several signaling pathways previously identified as highly relevant in urothelial tumors. These mice represent a genetically defined model for human high-grade superficial bladder cancer. Whole transcriptional characterizations of mouse and human bladder tumors revealed a significant overlap and confirmed the predominant role for Ezh2 in the downregulation of gene expression programs. Importantly, the increased tumor recurrence and progression in human patients with superficial bladder cancer is associated with increased E2F and Ezh2 expression and Ezh2-mediated gene expression repression. Collectively, our studies provide a genetically defined model for human high-grade superficial bladder cancer and demonstrate the existence of an Rb–E2F–Ezh2 axis in bladder whose disruption can promote tumor development. Cancer Res; 74(22); 6565–77. ©2014 AACR.

Introduction

Bladder cancer is a complex and heterogeneous disease caused by both genetic and environmental factors (1). Most tumors arise from the inner lining epithelial cells of the bladder wall, being more than 90% transitional cell carcinomas. At diagnosis, two major types of transitional cell carcinomas can be identified according to the pathologic characteristics: two thirds of patients present with superficial non–muscle-invasive bladder cancer (NMIBC) tumors, the remaining one third of patients present (or develops) a highly aggressive, muscle-invasive bladder cancer (MIBC) that leads to the death of 50% of patients. In general, NMIBCs have a favorable prognosis and are treated by transurethral resections and intravesical therapy. However, these tumors show a high rate of recurrence, which in some cases can progress into MIBC. This makes necessary a regular surveillance with cystoscopy and urine cytology indefinitely (EAU guidelines; ref. 2). Therefore, NMIBC represents one of the most costly malignancies to health care systems in developed countries (3). In MIBC, surgery, radiotherapy, and chemotherapy are effective treatments for the disease, but there has been little progress in survival in the last 20 years.

The interpretation of the genetic landscape of bladder cancer has largely been influenced by the current clinical (NMIBC vs. MIBC) and pathologic (stage and grade; papillary vs. solid-invasive) classifications (4). Molecular taxonomy provides a distinct view of bladder cancer subphenotypes and their relationship with the disease progression (5). TP53 mutations and RB1 inactivation are more prevalent in MIBC and may favor tumor progression and muscle invasion (6). However,
there is insufficient evidence to use them as independent predictors of poor outcome in the clinical practice of patients with bladder cancer (7). Low-grade NMIBCs are genomically stable, whereas high-grade NMIBCs and MIBCs display genomic instability (8). FGFR3 and PIK3CA mutations are more prevalent in NMIBCs and can also be predictive of local recurrences in NMIBCs (9). Bladder cancer is also characterized by significant alterations in genes involved in chromatin regulation, affecting in particular those genes implicated in histone modification (10).

In an attempt to reproduce human tumors, multiple genetically engineered mouse models have been generated (11). In these, the expression of large TAg from SV40 [leading to the elimination of all retinoblastoma (Rb) family members and p53] can induce bladder aggressive tumors in transgenic mice (11, 12). This is in contrast with the absence of bladder tumors upon Rb1 ablation in the urothelium (13). In this regard, we did not observe bladder tumor development in large cohorts of mice bearing the specific elimination of Rb gene directed by keratin K14Cre expression (14), which is active in the adult mouse urothelium (15), even in the absence of p107 (16), E2F1 (17), or p130 (18). These data indicate the existence of large overlapping roles for the Rb family members in bladder urothelial cells, similar to that reported for other mouse epithelial cells (19). To circumvent this possible problem and to specifically delete Rb family function in the urothelium of adult mice, we took advantage of a mouse strain bearing floxed alleles of Rb1 and Rb2 genes and whole deficiency in Rbm1 gene (Rb1<sup>F/F</sup>·p130<sup>F/F</sup>·p107<sup>−/−</sup>; ref. 20). Conditional gene deletion in the urothelium was induced by delivering an AdenoCre into the bladder lumen of adult male mice (13). Here, we show that the ablation of all three Rb family members in the mouse bladder urothelium leads to tumor development. The molecular characteristics of these triple knockout mouse tumors combined with their genomic characterization provide new possible molecular mechanisms of bladder cancer development. Collectively, the present data support a molecular connection between Rb/E2F and Ezh2 that may explain human NMIBC recurrence and progression.

Retinoblastoma family-deficient bladder tumor mouse model

All the animal experiments were approved by the Animal Ethical Committee and conducted in compliance with Centro de Investigaciones Energéticas, Medioambientales y Tecno-

ology (CIEMAT) Guidelines. Rb1<sup>F/F</sup>·p130<sup>F/F</sup>·p107<sup>−/−</sup> mice were generated by breeding Rb1<sup>F/F</sup>·p107<sup>−/−</sup> (16) and p130<sup>F/F</sup> (20) mice. Adenovirus expressing Cre recombinase was obtained from University of Iowa’s Vector Core Facility (www.uiowa.edu) and surgically delivered to the bladder lumen as previously described (13). At the time of sacrifice, tissues were collected and processed as previously reported (16, 17, 21).

Tissue microarray

The construction and analysis of tissue microarray containing the human samples has been reported elsewhere (9). At least two representative duplicate cores for each case were scored.

Immunohistochemistry

Immunohistochecmical analyses of human and mouse were performed essentially as previously described (9). Antibodies used were: anti-Ezh2 (Abnova; mAb9542 diluted 1:200), anti-K5 (Covance; diluted 1:500), anti-K8 (TROMA, University of Iowa; rat mAb diluted 1:10), anti-laminin (Sigma; 19393 diluted 1:100), anti-p63 (Santa Cruz Biotechnology; mAb 4A4), anti-pRb (Santa Cruz Biotechnology; sc105 diluted 1:50), and anti-p130 (Santa Cruz Biotechnology; sc317 diluted 1:50). Signal was amplified using avidin-peroxidase (ABC Elite Kit; Vector Labs), and peroxidase was visualized using 3,3′-diaminobenzidine as a substrate (DAB kit, Vector Labs). Negative control slides were obtained by replacing primary antibodies with PBS (data not shown). Scoring of the results and selection of the thresholds, internal controls for reactivity of each antibody, and tissue controls for the series were done according to previously published methods (9). Mice were injected intraperitoneally with bromodeoxyuridine (BrdUrd; 0.1 mg/g weight in 0.9% NaCl; Roche) 1 hour before sacrifice. BrdUrd incorporation was monitored in formalin-fixed sections using an anti-BrdU antibody (Roche) as described (16, 17, 21).

Determination of FGFR3 and PIK3CA gene mutation

The presence of mutations in the PIK3CA and FGFR3 genes was assessed in tumor gDNA by PCR test (Qiagen) and/or by snapshot technique has been reported elsewhere (9).

RT-qPCR

Total RNA was isolated from mouse and human samples and analyzed by RT-qPCR as previously described (9, 16, 17, 21, 22). Briefly, total RNA was isolated using miRNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions and DNA was eliminated (RNase-Free DNase Set Qiagen). Reverse transcription was performed using the Omniscript RT Kit (Qiagen) and a primer mix specific for all genes of interest in the case of human samples, and oligo dT primer for the mouse samples, using 10 ng and 1 μg of total RNA, respectively. PCR
Immunoblot was performed essentially as described elsewhere (22). Deposited in GEO (GSE38264). Mouse to human comparison procedures (see Supplementary Information). Datasets have been performed using the Affymetrix HuGene-1_0-st-v1 microarray.

Whole transcriptome analysis

Genome-wide transcriptome experiments were performed using the Affymetrix HuGene-1_0-st-v1 microarray or Mo Gene-1_0-st-v1 at the Genomics Facility of the Cancer Research Center (Salamanca, Spain) using standard procedures (see Supplementary Information). Datasets have been deposited in GEO (GSE38264). Mouse to human comparison was performed essentially as described elsewhere (22).

Immunoblot

Immunoblotting was performed as described previously (16, 21). Briefly, dissected bladder tumors were disrupted by freeze-thawing cycles in lysis buffer [200 mmol/L4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.9, 25% glycerol, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L ethylene glycol tetraacetic acid, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L Na3VO4, 1 mmol/L NaPPi, 1 mmol/L Na2VO3, 2.5 mmol/L dithiothreitol] and centrifuged to obtain supernatant containing total protein. Thirty-five micrograms of protein per sample was resolved in SDSPAGE and transferred to nitrocellulose membranes (Amersham). Membranes were blocked with 5% non-fat milk in TBS and incubated with the appropriate antibodies diluted in TBS-0.5% BSA. Secondary antibodies were purchased from Jackson ImmunoResearch. Super Signal West Pico Chemiluminescence Substrate (Pierce) was used according to the manufacturer’s recommendations to visualize the bands. Antibodies used are against E2F1, E2F2, Erk1/2 (Thr202/Tyr204-P), p19Arf (Abcam); p53 (Novocastra); and Stat3-Tyr817-P and Stat3-Tyr805-P and S6-P (Cell Signaling). Loading was controlled by using an anti-actin antibody (Santa Cruz Biotechnology).

Statistical analysis

Comparisons were performed using the Wilcoxon–Mann–Whitney test (for two groups) and the Student t test for paired samples showing normal distribution. Survival analyses (recurrence-free or tumor progression in recurrence) according to various variables were performed using the Kaplan–Meier method, and differences between the patient groups were tested by the log-rank test. Discrimination between samples showing increased or decreased gene expression was made using the median.

Results

Ablation of all Rb family members causes bladder tumor development

To study the functional consequences of Rb family loss in the urothelium of adult mice, conditional gene deletion mediated by delivering an AdenoCre into the bladder lumen (13) of adult male Rb+/p130+/p107+/- mice (20) was used. We observed the development of bladder lesions in all the AdenoCre-infected mice (n = 18, Fig. 1A and B) but not in control mice (noninfected littermates or infected with AdenoGFP, n = 30). The presence of these lesions was easily monitored by CT (Supplementary Fig. S1) and in some cases was accompanied by hematuria. The histologic analysis of these lesions revealed papillary masses growing inward to the bladder lumen (Fig. 1A and Supplementary Fig. S1), displaying histologic features of high-grade non–muscle-invasive carcinoma. The mouse bladder tumors were keratin K5-positive, keratin K8-negative (Fig. 1C), displayed high proliferation (analyzed by BrdUrd incorporation; Fig. 1C), and invaded the basal lamina (as determined by laminin staining Fig. 1C). When mice were followed up to 1 year after infection (Fig. 1B), no visible metastases were detected.

We observed the ablation of all Rb family members (Supplementary Fig. S2) and the increased expression of E2F1, 2, and 3a (Fig. 1D) in these tumors, by both immunoblotting and RT-qPCR analyses (Supplementary Fig. S3). In addition, tumors were characterized by the upregulation of p19Arf and p53 and the activation of Erk, S6, and Stat3 signaling pathways, as demonstrated by immunoblot analysis using phospho-specific antibodies (Fig. 1D). In contrast, tumors did not show increased Akt activity (Fig. 1D).

Tumors in Rb-deficient mouse resemble human bladder cancer

To further characterize the Rb family mutant mouse bladder tumors and to compare with human bladder cancer, a whole transcriptome characterization of the mouse samples was performed. This analysis revealed a differential expression of 3,053 transcripts (1,849 downregulated and 1,204 upregulated) in tumors compared with normal bladder (Fig. 2A and Supplementary Table S2). Gene ontology characterization of these deregulated transcripts indicated that downregulated genes were involved in oxidoreduction, small GTPases transduction signaling, cell adhesion, and translation processes (Fig. 2B and Supplementary Table S3), whereas the upregulated genes were primarily involved in cell-cycle and DNA repair processes, as would be expected in tumors with high E2F activity (Fig. 2B and Supplementary Table S4). To characterize the putative transcription factors controlling these differentially expressed genes, we performed chromatin immunoprecipitation (ChIP) enrichment analyses (ChEA; ref. 24). These revealed the possible involvement of polycomb repressive group 2 (PRC2) in downregulated genes (Fig. 2C and Supplementary Table S5), whereas the upregulated genes showed a primary involvement of E2F and Myc, together with histone demethylases (Fig. 2C and Supplementary Table S6). Gene set enrichment analysis (GSEA) showed significant overlap of the upregulated and downregulated genes in mouse bladder tumors with various
human bladder datasets and also with stem cell signatures, including genes silenced in embryonic stem cells by H3K27me3 (Supplementary Table S7), thus reinforcing the possible role of PRC2 in gene expression deregulation. The comparative analyses between the downregulated (Fig. 2D, top, and Supplementary Table S8) or upregulated (Fig. 2D, bottom, and Supplementary Table S9) mouse genes and human bladder tumors available in Oncomine database (25) showed a very significant overlapping with multiple external bladder cancer datasets comparing normal versus tumor samples, tumors with poor clinical outcome, and the presence of specific gene mutations in human tumors, including KDM6A and RB mutations. Remarkably, this comparison (Fig. 2D and Supplementary Table S9) also showed a statistically significant overlap between the upregulated mouse genes and upregulated genes in human recurrent bladder tumors and also between the downregulated genes in mouse tumors and multiple datasets identifying genes silenced by PRC2 in stem cells (Supplementary Table S8).

**Increased Ezh2 in Rb family–deficient mouse bladder tumors**

The observed similarities between downregulated genes in mouse bladder tumors and those being silenced by PRC2 prompted us to analyze possible changes in the expression of PRC2 elements, Ezh2, Suz12, and Eed, in the mouse tumors. RT-qPCR analysis (Fig. 3A) showed significantly increased expression of Ezh2 and Suz12 genes, but not Eed in mouse tumors compared with control samples, the increased expression of Ezh2 gene being more relevant. Immunoblot analysis confirmed the increased expression of Ezh2 protein (Fig. 3B), the catalytically active member of the PRC2 complex and responsible for trimethylation of H3K27. Finally, immunohistochemical analysis of Ezh2 expression (Fig. 3D) compared with that of
Figure 2. Transcriptome analysis of mouse bladder tumors. A, heatmap showing the distribution of genes (rows) identifying tumors and normal control uninfected mouse bladder samples. Each column represents a sample. A red (overexpressed) to blue (downregulated) scheme following the above scale limits (in log2 scale) is shown. Numbers on the right denote the number of transcripts of each group (upregulated or downregulated). B, representative Gene Ontology biologic processes categories affecting the functions of the downregulated (top) or upregulated (bottom) genes in mouse bladder tumors. Numbers on the right of each bar indicate the genes on COBP category. C, relative relevance of transcription factors and histone-modifying enzymes in the genes downregulated (top) or upregulated (bottom) in tumors as obtained by ChEA. The relevance of each factor is provided by the P value (in \(-\log_{10}\) scale). Numbers on the right of each bar represent genes bound by each transcription factor from the database. D, summary of relevant overlap between downregulated (top) or upregulated (bottom) genes in mouse bladder triple knockout (TKO) tumors with human bladder tumors from the Oncomine database. The different comparison concepts (tumor vs. normal, mutation type, clinical outcome, and recurrence) are provided for each group. P values were obtained by the exact Fisher test. The number of overlapping genes is shown for each dataset.
urothelium basal layer marker p63 (Fig. 3C) revealed the increased expression of Ezh2 in mouse tumors, but not in the adjacent normal urothelium.

Genome-wide transcriptome analysis of human recurrent bladder tumor

These observations indicate that the mouse Rb family mutant tumors represent a bona fide model of human bladder cancer. The pathologic characteristics resemble those of human high-grade NMIBC, a possibility also reinforced by the genomic comparisons. To confirm this, a whole transcriptome study was performed using a series of human NMIBC samples focusing on differential gene expression between recurrent and nonrecurrent tumors. The analysis identified 351 transcripts (162 overexpressed and 299 underexpressed) differentially expressed in recurrent tumors (Fig. 4A and Supplementary Table S10), which also discriminated normal tissue. Remarkably, this classification did not discriminate between Ta and T1 stages, high- and low-grade tumors, or tumors bearing FGFR3 mutations (Fig. 4A). On the contrary, tumors bearing PIK3CA gene mutation were predominantly associated with nonrecurrent samples (Fig. 4A). These findings may explain our previously reported association between PIK3CA gene alterations and reduced recurrence in superficial bladder tumors (9). Gene Ontology (GOBP; Supplementary Table S13) revealed that the upregulated genes in recurrent tumors played a major role in cell-cycle control, proliferation, and proteosomal protein degradation, whereas the downregulated genes displayed an association with ribosome, 3′ untranslated region (UTR)-mediated translational regulation and protein translation. The GSEA also indicated similarities with stem cell transcription and PRC2 silencing (Supplementary Table S13). The comparison of these differentially expressed genes with other human bladder cancer datasets in Oncomine database revealed a significant overlap and trend with previously reported bladder cancer datasets (Supplementary Table S14). Of note, we found that the downregulated genes in recurrent samples of our study were able to identify early recurrence of NMIBC in an external dataset (Fig. 4B; ref. 26). ChEA of these deregulated genes revealed, besides the common involvement of multiple transcription factors in all datasets (Supplementary Tables S15 and S16), a statistically significant involvement of histone-modifying enzymes, affecting histone methyl transferases and demethylases, and including the PRC2 members: Ezh2, Suz12, and Eed (Fig. 4C). In agreement, we also observed a significant overlap with H3K27me3 in downregulated genes in recurrent tumors by GSEA (Supplementary Table S13).

Finally, we integrated our transcriptome data from both mouse and human genes in a common dataset. The unsupervised classification of this common dataset according to (i) the expression of genes discriminating mouse normal and tumor bladder samples (Fig. 4D, top) or (ii) human genes discriminating human recurrent and nonrecurrent NMIBC samples (Fig. 4D, bottom) invariably showed that mouse tumors were clustered with human recurrent tumors. Thus, the mouse bladder tumors initiated by the ablation of all retinoblastoma family members represent a putative model of human recurrent NMIBC.

Increased EZH2 expression in human recurrent bladder tumors

Genomic data in mouse and human bladder tumors analyzed pointed to a major involvement of PRC2, and in particular Ezh2, in the development and recurrence of NMIBC. To further support this, those genes, identified by ChEA to be bound by any PRC2 element or specifically by Ezh2 (n = 38 and 12, respectively), were loaded into Oncomine database. A statistically significant overlap was found with the Lee bladder dataset (26) in the case of PRC2-bound genes (overlapping genes: n = 4 of 38, P = 0.00044, OR, 12.7). Moreover, we also observed a significant overlap with both PRC2- and Ezh2-bound genes in another external dataset (overlapping genes: n = 5 of 38, P = 0.002, OR, 9.1; and n = 3 of 12, P = 0.008, OR, 13.6, respectively; ref. 27). Importantly, even these limited numbers of overlapping genes were able to discriminate patients of NMIBC with high likelihood of recurrence (Supplementary Fig. S4A–S4C). These observations point to a primordial role of Ezh2-mediated gene silencing in NMIBC early recurrence.

We next analyzed expression changes of PRC2 members in a series of human NMIBC samples previously described (9). RT-qPCR analysis revealed a significant increase of EZH2 expression (Fig. 5A) but not of SUZ12 and EED (Supplementary Fig. S5A and S5B) in tumors compared with paired normal bladder samples. The EZH2 gene expression was also higher in recurrent tumors than in nonrecurrent (Fig. 5A). When the patients were stratified according to EZH2 expression in a tissue microarray (Fig. 5C and data not shown), we found that increased expression of EZH2 correlated with tumor recurrence (Fig. 5D, P = 0.000021).

A possible involvement of E2F in Ezh2-deregulated expression

The expression of EZH2 gene is regulated by E2F activity (28). Accordingly, we monitored whether EZH2 expression correlated with the expression of different E2Fs in our series of human bladder tumor samples. This analysis revealed a striking correlation between EZH2 and E2F3a gene expression (Fig. 6A). We also observed that E2F3a expression was significantly higher in tumor than in normal samples and in recurrent than in nonrecurrent tumors (Fig. 6B). Importantly, increased E2F3a expression levels stratified patients with recurrent NMIBC (Fig. 6C). On the other hand, E2F1, 2, and 3b gene expression did not discriminate between normal and tumor samples (Supplementary Fig. S6A) or between recurrent and nonrecurrent human NMIBC (Supplementary Fig. S6B). Furthermore, the expression of E2F1, 2, and 3b genes did not show significant correlation with EZH2 gene expression (Supplementary Fig. S6C, S6E, and S6G) and did not allow statistical stratification of recurrence in our series of patients with NMIBC (Supplementary Fig. S6D, S6F, and S6H). These results indicate that the early recurrence mediated by increased EZH2 expression could be facilitated primarily by increased E2F3a expression. However, a
Figure 3. Mouse bladder tumors display increased Ezh2 expression. A, expression of Ezh2, Suz12, and Eed genes in control (uninfected bladder samples) and mouse bladder tumors as assessed by RT-qPCR (with respect to Tbp). P value was obtained by the Mann–Whitney t test; mean and SEM are denoted in red. B, immunoblot analyses of control (uninfected bladder samples) and mouse bladder triple knockout (TKO) tumors showing the expression of Ezh2 protein. Actin was used to normalize protein loading. C, representative examples of the immunohistochemical analysis of mouse bladder tumor showing the expression of p63. Bottom, higher magnifications of the corresponding areas denoted in top. D, representative examples of the immunohistochemical analysis of mouse bladder tumor showing the expression of Ezh2. Bottom, higher magnifications of the corresponding areas denoted in top. Bar in top, 500 μm; bottom, 150 μm.
Figure 4. Transcriptome studies of human NMIBC recurrence. A, heatmap showing the distribution of genes (rows) identifying recurrent tumor samples. Each column represents a sample. The mutation of PIK3CA or FGFR3 genes is denoted by red and green arrows, respectively. The stage (Ta and T1) and grade (high, H; low, L) for each tumor sample is also provided. A red (upregulated) to blue (downregulated) scheme following the scale limits (in log2 scale) is shown. Numbers on right side denote the number of transcripts characterizing each class. B, Kaplan–Meier distribution of patients in Lee dataset (26) according to the expression levels of the downregulated genes identified in our study. P values were obtained by the log-rank test. n, the number of samples scored of each group. C, relative relevance of the different histone-modifying enzymes in the genes of each quoted group obtained by ChEA. The relevance of each enzyme is provided by the P value. The number on the right of each bar represents the number of specific genes bearing this type of modification and the total number of transcripts on each group: upregulated (left) or downregulated (right) in recurrent tumors. D, dendrograms of mouse human transcriptome comparison upon unsupervised clustering (Pearson correlation and average linkage method) according to the expression of genes differentiating mouse control and bladder tumor samples (top) or genes discriminating human recurrent tumors (bottom).
concerted action of other E2F family members in this process could not be discarded at this point.

**EZH2 affects tumor progression in recurrence**

Besides the high recurrence rate, an important problem in NMIBC is the relatively high rate of recurrent tumors appearing with increased stage and/or grade compared with the primary tumors, indicating that tumor progression upon recurrence occurred. During this study, 11 of 33 patients suffering recurrence in our series also displayed tumor progression. To study whether similar events to those described for recurrence were also affecting tumor progression, the differential gene expression between tumors showing or not tumor progression upon recurrence was studied. This analysis revealed a very limited

Figure 5. EZH2 expression in human NMIBC. A, expression of EZH2 gene as assessed by RT-qPCR (with respect to TBP) in normal versus tumor samples and in recurrent versus nonrecurrent tumors. P values were obtained by the Mann–Whitney t test; mean and SEM are shown. B, representative examples of human tumors showing positive (left) and negative (right) staining for EZH2 protein. Bar, 100 μm. C, Kaplan–Meier distribution of recurrence according to the expression of EZH2 protein form tissue microarrays. P values were obtained by the log-rank test (n, the number of samples in each group).

Figure 6. E2F3a expression in human NMIBC. A, expression of E2F3a gene as a function of E2F3a gene expression as assessed by RT-qPCR in NMIBC samples. The r and P values are provided according the Spearman correlation method. B, expression of E2F3a gene in normal and tumor bladder samples (left) and in recurrent and nonrecurrent bladder tumors (right). P values were obtained by the Mann–Whitney t test; mean and SEM are denoted in red. C, Kaplan–Meier distribution of NMIBC recurrence according to the increased expression of E2F3a gene. P value was obtained by the log-rank test. n, the number of samples scored for each group.
The number of transcripts (Supplementary Table S17). ChEA analysis of these transcripts showed that a significant number of underexpressed genes in tumors showing progression were bound by PRC2 complex elements (Fig. 7A). In addition, these transcripts displayed a significant overlap with external datasets of gene lists regulated by PRC2, including genes with H3K27me3 marks (Fig. 7B). Accordingly, samples of tumors showing progression in recurrence showed also a significant increase in EZH2 and E2F3a expression (Fig. 7C). Moreover, the increased expression of EZH2 protein was an independent predictor of tumor progression in the recurrences of our series of NMIBC samples (Fig. 7D).

These data indicated that increased EZH2 expression mediates not only early recurrence but also increased probability of tumor progression in human NMIBC.

Discussion

Bladder cancer is one of the most common cancers in men worldwide making it a current problem in terms of social and medical relevance. The genomic landscape of these tumors may contribute to identify novel targets of possible therapeutic interest. These studies have revealed multiple alterations and mutations in bladder cancer, including chromatin remodeling, cell-cycle, and specific signal transduction pathways (5, 10, 29–33). Regarding cell cycle, RB1 mutations are relevant. However, these studies are biased by the analysis of mainly aggressive MIBC tumors and few examples of NMIBC are included. Nonetheless, even in these few examples, RB1 alterations are present (29, 32). This, together with other possible alterations leading to functional pRb inactivation, such as Cycl or E2F amplification (26, 30, 32), may suggest that Rb-dependent pathway is also of relevance in NMIBC. Our present data showing that the complete ablation of Rb family in vivo leads to urothelial tumors support this possibility.

It has been previously shown that the inactivation of Rb1 gene in mouse urothelium is insufficient to allow spontaneous tumor development (13). Regarding our observations of complete penetrance of bladder cancer development upon Rb family ablation, this apparent discrepancy could be attributed to functional compensation by the other family members, as previously reported in mouse epithelia (19). Our findings are in agreement with the reported results of urothelial expression of T antigen in transgenic mice (12, 34). However, T antigen transgenic mice usually develop muscle-invasive tumors; this

![Figure 7. EZH2-dependent signaling in tumor recurrence and progression. A, relative relevance of the binding of the different PRC2 elements to the downregulated genes in tumors showing progression upon recurrence as assessed by ChEA. The relevance of each enzyme is provided by the P value. B, summary of relevant overlap of the downregulated genes in tumors showing progression upon recurrence and Oncomine database in the concept “literature defined” showing the relevance of PRC2 components. C, expression of EZH2 and E2F3a genes as assessed by RT-qPCR (with respect to TBP) in tumors displaying or not progression upon recurrence. P values were obtained by the Mann–Whitney t test; mean and SEM are denoted in red. D, Kaplan–Meier distribution of tumor progression in recurrences according to increased expression of EZH2. P values were obtained by the log-rank test.](image-url)
could be attributed to impaired p53 tumor-suppressive functions. Such possibility, which is also reinforced by our previous findings indicating that p53 loss in Rb-deficient stratified epithelia facilitates the development of invasive metastatic disease (21, 35), remains to be determined. The pathologic characteristics of the mouse bladder tumors suggest that these mice could represent a bona fide NMIBC model. Also, the mouse tumors display activation of MAPK/Erk, Stat3, and S6 pathways, which have been previously involved in human bladder cancer (36–38).

In view of the findings in the mouse model, we aimed to use it as a tool to gain a better understanding of the molecular mechanisms responsible for the initiation, recurrence, and progression of bladder cancer. This would also provide potential biomarkers. In a first approach, we performed a whole transcriptome analysis. This reinforced the similarities between mouse and human tumors and provided a possible mechanism for the upregulation and downregulation of genes in the mouse tumors involving E2F and PRC2. We found that the removal of Rb family led to overexpression of E2F family members, and these mouse tumors invariably showed increased Ez2 expression. Furthermore, our GSEA and ChEA data indicated a predominant role for E2Fs, in particular E2F3a, in gene upregulation in mouse tumors. Given that (i) E2F3 is frequently amplified and overexpressed in human bladder cancer (39–42), (ii) the inactivation of the Rb pathway is required in addition to E2F3 overexpression in human bladder carcinogenesis (40), (iii) E2F3 is a primary regulator of EZH2 expression (28), and (iv) there is emerging evidence supporting the RB–E2F3–EZH2 pathway as a key oncogenic axis in cancer development and aggressiveness (43), our findings provide a new molecular mechanism for bladder tumor development. Importantly, various EZH2 inhibitors have been developed and are being tested in various preclinical models (44–46). The possibility that these inhibitors may impair bladder tumor growth in our Rb family–deficient model will be the subject of future investigations. In addition, the identification of possible overlapping mutations between human NMIBC and our mouse model will provide further evidence of the relevance of this model for preclinical studies.

To confirm these findings and to validate the mouse model as a suitable tool for understanding human NMIBC, we carried out a whole transcriptome analysis in human bladder cancer samples. Furthermore, as recurrence and progression are common in this type of human tumors and constitute a severe clinical problem, this study was designed to determine possible molecular factors affecting recurrence in NMIBC. The metagenomic analyses also reinforced the extreme similarities between mouse and human recurrent tumors. We also observed that recurrence is coupled to gene upregulation mediated by E2F activity and downregulation associated with increased PRC2 activity. Importantly, we could also validate the role of PRC2 and EZH2-mediated gene silencing in NMIBC recurrence in external datasets, indicating that increased Ez2 expression and activity could act as a predictor of early recurrence. Furthermore, we could also associate the increased EZH2 expression and activity with progression upon recurrence in human NMIBC. In this regard, the increased expression of EZH2 had been previously reported associated with increased malignancy in bladder cancer (47, 48). In agreement, KDM6A (which catalyzes the H3K27 demethylation and thus acting in opposite manner to EZH2) had also been described as frequently mutated in bladder cancer, in some cases, in association with RB1 gene mutation in high-stage or–grade human bladder tumors (29, 30, 33). Future research, aimed to detect possible overlapping mutations in these genes in our mouse model, will provide further data of the relevance of this model for preclinical studies. Nonetheless, to the best of our knowledge, this is the first report associating EZH2 activity with NMIBC recurrence and progression.

Collectively, these findings could be highly relevant in the clinical management and therapy of human bladder cancers. The possible therapeutic use of EZH2 inhibitors in the management of bladder cancer is a crucial aspect that merits future research. In this regard, the mouse model here described arises as an essential and invaluable tool for such analyses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Authors’ Contributions
Conception and design: D. Castellano, J.L. Rodriguez-Peralto, F. de la Rosa, J.M. Paramio
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Santos, M. Martínez-Fernández, M. Dueñas, B. Alaffya, F. Villacampa, C. Costa, M. Otero, J. Duarte, V. Martínez, M.J. Gómez-Rodríguez, M.L. Martín, M. Fernández, M.A. Morcillo, J. Sage, J.L. Rodríguez-Peralto
Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): M. Santos, M. Martínez-Fernández, M. Dueñas, R. García-Escudero, M. Otero, M.A. Morcillo, D. Castellano, J.M. Paramio
Writing, review, and/or revision of the manuscript: M. Martínez-Fernández, M. Dueñas, R. García-Escudero, F. Villacampa, C. Costa, M. Otero, M.J. Gómez-Rodríguez, P. Viator, M.A. Morcillo, J. Sage, D. Castellano, J.L. Rodríguez-Peralto, J.M. Paramio
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.J. Gómez-Rodríguez, P. Viator
Study supervision: F. Villacampa, D. Castellano, J.L. Rodríguez-Peralto, F. de la Rosa, J.M. Paramio
Other (discussed the results and comments on the manuscript): M. Santos

Acknowledgments
The authors want to particularly acknowledge the patients enrolled in this study for their participation and the Biobancos.es. They appreciate F.X. Real from the CNIO for his suggestions and critical reading of the article.

Grant Support
The study was funded by the following: MICINN grants SAF2012-34378 and SAF2011-26212-C02-01; Comunidad Autónoma de Madrid grants S2006/BIO-0232 and S2010/BMD-2470 (Oncoypece Programs); MSc grants ISCIII-RETIC RD06006200029 and RD12/0063/0009; and from Fundación Sandra Herra to J.M. Paramio. Grants AP99782012 and 40100017 from MMA Foundation to M. Dueñas and M.L. Martín, respectively. MSc grants ISCIII-FIS PI12/01959 to M. Santos. M. Martínez-Fernández is funded by a ‘Juan de la Cierva’ research fellowship (IJC-2010-06167) from MICINN. Work done on Rb family mutant mice in the Sage lab (J. Sage and P. Viator) is funded by the NIH (R01 CA114102). Biobank is supported by Instituto de Salud Carlos III.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


In Vivo Disruption of an Rb–E2F–Ezh2 Signaling Loop Causes Bladder Cancer

Mirentxu Santos, Mónica Martínez-Fernández, Marta Dueñas, et al.