K-Ras and β-catenin mutations cooperate with Fgfr3 mutations in mice to promote tumorigenesis in the skin and lung, but not in the bladder

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SUMMARY
The human fibroblast growth factor receptor 3 (FGFR3) gene is frequently mutated in superficial urothelial cell carcinoma (UCC). To test the functional significance of FGFR3 activating mutations as a ‘driver’ of UCC, we targeted the expression of mutated Fgfr3 to the murine urothelium using Cre-loxP recombination driven by the uroplakin II promoter. The introduction of the Fgfr3 mutations resulted in no obvious effect on tumorigenesis up to 18 months of age. Furthermore, even when the Fgfr3 mutations were introduced together with K-Ras or β-catenin (Ctnnb1) activating mutations, no urothelial dysplasia or UCC was observed. Interestingly, however, owing to a sporadic ectopic Cre recombinase expression in the skin and lung of these mice, Fgfr3 mutation caused papilloma and promoted lung tumorigenesis in cooperation with K-Ras and β-catenin activation, respectively. These results indicate that activation of Fgfr3 can cooperate with other mutations to drive tumorigenesis in a context-dependent manner, and support the hypothesis that activation of FGFR3 signaling contributes to human cancer.

INTRODUCTION
Urothelial cell carcinoma (UCC) of the bladder is the fifth commonest cancer, with 357,000 new cases diagnosed yearly on a worldwide basis (Parkin et al., 2005). The majority (75%) of these tumors are noninvasive and well differentiated, and can be controlled by transurethral resection of the tumor lesions. However, up to 70% of the patients with a superficial UCC will have recurrences after its removal, and 10-15% will progress to invasive UCC (http://info.cancerresearchuk.org/cancerstats/). Even in cases of no progression, regular surveillance by cystoscopy is required, making bladder cancer one of the most expensive and labor-intensive cancers to manage.

A number of genetic and epigenetic alterations have been identified in bladder tumorigenesis, including activating mutations in fibroblast growth factor receptor 3 (FGFR3) and RAS family genes, amplification of ERBB2, and loss of the TP53, RB1 and PTEN tumor suppressors (Schulz, 2006; Luis et al., 2007; Cordon-Cardo, 2008; Diaz et al., 2008). Among these, somatic mutations in FGF3 have been identified at a high frequency in cases of superficial UCC (60-80%) (Knowles, 2008b). FGFR3 is a receptor tyrosine kinase that is known to mediate the effects of fibroblast growth factors (FGFs). When these activating mutations occur in the germ line, they are known to cause several autosomal dominant human skeletal dysplasia syndromes (Muenke and Schell, 1995). Several studies have shown that somatic mutations of FGFR3 are strongly associated with bladder cancer of a low tumor grade and stage (Billerey et al., 2001; Jebar et al., 2005; Lamy et al., 2006; Lindgren et al., 2006).

Although some previous studies strongly suggest the importance of FGFR3 mutations in tumor formation (Chesi et al., 2001; Logie et al., 2005), thus far they have been uninformative on the precise mechanistic role of FGFR3 mutations in tumor formation and progression. The role of FGF signaling in bladder cancer is not well understood. Generation of a relevant mouse model is essential not only for the investigation of mechanism but also for testing potential therapeutic approaches. In this study, the role of two potent activating mutations of FGFR3 that are found in UCC was assessed in the initiation and development of UCC as a sole driver in vivo, and in synergy with β-catenin (Ctnnb1) and K-Ras mutations.

RESULTS

Targeting of the Fgfr3 mutations in the bladder
In order to achieve urothelial-specific conditional expression, mice expressing Cre recombinase driven by the mouse uroplakin II (UroII) promoter were selected. UroII is expressed throughout the urothelial layers in mice (Mo et al., 2005). The UroII Cre promoter has been reported to successfully drive the expression of proteins that lead to bladder tumor formation, including SV40 large T antigen and H-Ras (Zhang et al., 2001). To demonstrate Cre-driven recombination in the urothelium, we crossed the UroII Cre line with Z/EG reporter mice (Novak et al., 2000). Visualization with the Olympus OV100 whole-mouse fluorescent imaging system showed that recombination had occurred in the urothelial lining of the bladder, as well as in the ureters (supplementary material Fig. S1A).
We then bred UroIICre+ mice to lines carrying Fgfr3 K644E and Fgfr3 K644M mutations (murine equivalent of human K652E and K652M, respectively) (Iwata et al., 2000; Iwata et al., 2001). Both of these mutations have been found in human UCC (Knowles, 2008a) and are known to highly activate the kinase activity of the receptor (Iwata et al., 2001). Both lines carry a neomycin resistant gene (neo) flanked by loxP sites in the intron prior to the exon with the K644 mutation. This neo insertion is known to suppress the expression of the Fgfr3 mutant allele in the absence of Cre recombination. In the presence of Cre, the neo gene is excised, allowing expression of the mutant Fgfr3 protein. In the offspring (UroIICre+Fgfr3+/K644E and UroIICre+Fgfr3+/K644M), the pattern and approximate levels of Fgfr3 protein expression were similar to those of the age-matched wild-type mice (supplementary material Fig. S1B,C,F).

**Fgfr3 mutation alone does not drive tumorigenesis of the bladder**

To investigate the role of Fgfr3 mutations as a driver of UCC, we aged the UroIICre+Fgfr3+/K644E and UroIICre+Fgfr3+/K644M mutant mice to 18 months (tumor phenotype is summarized in supplementary material Table S1). Neither of these lines developed urothelial hyperplasia, dysplasia or carcinoma (Fig. 1E,I). After 2 hours of in vivo incorporation of 5-bromo-2-deoxyuridine (BrdU), no apparent positivity was observed, indicating that little or no cell proliferation took place in the urothelium either in the wild-type or in the mutant cohorts (Fig. 1F,J). We next examined some of the well-known core signaling pathways downstream of FGF. A strong upregulation of expression of phosphorylated extracellular-signal-regulated kinases 1 and 2 (pERK1/2) was observed in both UroIICre+Fgfr3+/K644E and UroIICre+Fgfr3+/K644M mutants compared with wild type (Fig. 1G,K and supplementary material Fig. S2). This was accompanied by an upregulation of Sprouty2 levels (Fig. 1H,L and supplementary material Fig. S2). No significant pAKT(Ser473) or p-mTOR staining was present in the Fgfr3 mutant cohorts, similar to wild type (supplementary material Fig. S3).

Next, we addressed whether Fgfr3 mutations require other mutations for the formation of UCC. FGFR3 and H-RAS mutations are reported to be mutually exclusive events in human UCC (Jebar et al., 2005). Previous studies that have examined K-RAS mutations in a mixture of human bladder cancer have suggested a wide variation in frequency (4-29%) (Uchida et al., 1995; Olderoy et al., 1998; Ayan et al., 2001). In order to address how much of the FGFR3 signaling activities are augmented by additional upregulation of the ERK-MAPK pathway, we examined the role of oncogenic K-Ras G12D (Jackson et al., 2001) in UroIICre+Fgfr3+/K644E K-RasG12D/+ mice (n=23) (supplementary material Table S1). Upon aging to 12 months, no UroIICre+Fgfr3+/K644E K-RasG12D/+ mice developed urothelial hyperplasia, dysplasia or carcinoma (Fig. 1M) and no apparent changes in BrdU incorporation were observed (Fig. 1N).

**Fig. 1. Fgfr3 mutation is not the sole driver of tumorigenesis in the bladder.**

The samples are from 12-month-old wild-type (A-D), UroIICre+Fgfr3+/K644E (E-H), UroIICre+Fgfr3+/K644M (I-L), UroIICre+Fgfr3+/K644E K-RasG12D/+ (M-P) and UroIICre+Fgfr3+/K644E β-catenin exon3/+ (Q-T) mice. Hematoxylin and eosin (H&E) staining showed no apparent lesions in the urothelium in all Fgfr3 mutants (A,E,I,M) except for the compound model with β-catenin exon3/+ (Q), in which hyperplasia was observed (red arrowhead; also in S,T). No significant cell proliferation was observed (B,F,J,N) except for the area of hyperplastic lesion in UroIICre+Fgfr3+/K644E β-catenin exon3/+ mice (black arrowhead) (R). Upregulation of pERK1/2 (G,K,O,S) and Sprouty2 (H,L,P,T) was observed in Fgfr3 mutant mice, compared with wild type (C,D, respectively). Scale bar: 200 μm.
Similar to the single mutants, a strong upregulation of pERK1/2 (Fig. 1O) as well as an accompanying upregulation of Sprouty2 (Fig. 1P) was observed. Comparable levels of upregulation of pERK1/2 and Sprouty2 were observed in the UroIICre⁺Fgfr3⁺/K-RasG12D/+ urothelium (data not shown). Minimal levels of upregulation of pAKT(Ser473) and p-mTOR were observed in the UroIICre⁺Fgfr3⁺/K-RasG12D/+ cohorts (supplementary material Fig. S3).

Mounting evidence is present for a role of Wnt pathway activation in human bladder cancer development, including an association between the accumulation of nuclear β-catenin and reduced patient survival (Kastritis et al., 2009). We recently reported the first in vivo evidence of β-catenin activation leading to the formation of UCC when combined with other tumor suppressor mutations, such as a loss of Pten (Ahmad et al., 2011a). In addition, we have also shown that the β-catenin mutation can cooperate with H-Ras mutation to drive superficial bladder cancer (Ahmad et al., 2011b). In order to test whether there is cooperation between the FGF and Wnt pathways in bladder tumorigenesis, we examined the role of β-catenin activating mutations (Harada et al., 1999) in UroIICre⁺Fgfr3⁺/K-RasG12D/+ mice (n=27) (supplementary material Table S1).

In order to drive deregulated Wnt signaling, we used mice that carry a dominant allele of the β-catenin gene in which exon 3 is flanked by loxP sequences (Harada et al., 1999). In these mice, Cre recombinase deletes exon 3, which contains the residues that are phosphorylated by GSK3β. Thus, β-catenin will accumulate in the nucleus and drive Wnt signaling (Moon et al., 2004). Although areas of hyperproliferation were observed in the bladders of UroIICre⁺Fgfr3⁺/K-RasG12D/+β-cateninexon3/+ mice from approximately 3 months of age in 100% of mice (n=20), equivalent lesions were also found in the UroIICre⁺β-cateninexon3/+ urothelium (Ahmad et al., 2011a). In both groups these lesions incorporated BrdU (Fig. 1R); however, they never progressed further (examination up to 12 months) (Fig. 1Q). Once again, upregulation of pERK1/2 and Sprouty2 was observed in this model (Fig. 1S,T). The areas of hyperproliferation that were observed in UroIICre⁺β-cateninexon3/+ mice showed comparable levels of upregulation of BrdU, pERK1/2 and Sprouty2 in the lesions (data not shown), indicating that Fgfr3 mutations are not contributing to urothelial hyperplasia in UroIICre⁺Fgfr3⁺/K-RasG12D/+β-cateninexon3/+ mice. No upregulation of pAKT(Ser473) or p-mTOR was observed in this model (supplementary material Fig. S3). Taken together, these data suggest that K-Ras and β-catenin activating mutations do not cooperate with Fgfr3 mutation to drive UCC or to enhance signaling.

Skin papilloma formation in UroIICre⁺Fgfr3⁺/K-RasG12D/+ mice

In contrast to observations in the urothelium, by 1 year of age, 44% (9/23) of the UroIICre⁺Fgfr3⁺/K-RasG12D/+ cohort developed papilloma (Fig. 2A; supplementary material Table S1, Fig. S4). The tumors reached 1 cm in diameter by a median of 220 days (mean 249 days). Upon crossing of the UroIICre⁺Fgfr3⁺/K-RasG12D/+ mice to the Z/Eg reporter line, a strong GFP signal was observed within papillomas, indicating that the tumor formation was due to sporadic Cre recombination in the tumor (Fig. 2G). By contrast, no papilloma was observed in the UroIICre⁺K-RasG12D/+ cohort aged up to 18 months (n=20), indicating that Fgfr3 mutation cooperates with K-Ras mutation to drive papilloma formation.

The papillomas in the UroIICre⁺Fgfr3⁺/K-RasG12D/+ cohort incorporated BrdU (Fig. 2B). In terms of signaling, tumor formation was associated with a robust activation of pERK1/2 expression in the absence of Sprouty2 upregulation (Fig. 2C,D). This is distinct from the observation in the urothelium of these mice (Fig. 1P), where Sprouty2 upregulation was seen (Fig. 2D), suggesting that the negative feedback counteracting the oncogenic ERK-MAPK pathway has not occurred in this line. Levels of pAKT(Ser473) and p-mTOR were unchanged (Fig. 2E,F). We also compared the signaling profiles observed in these papillomas of Fgfr3 mutants with those of papillomas from UroIICre⁺K-RasG12D/+Ptenfl/+ mice, which regularly develop papillomas (8/19; 42%) (supplementary material Fig. S5A). We found a similar increase of BrdU incorporation (supplementary material Fig. S5B). However, in contrast to UroIICre⁺Fgfr3⁺/K-RasG12D/+ papillomas, only mild upregulation of nuclear and cytoplasmic pERK1/2 was observed in the lesion, verified by the Histoscore quantification (n=3, P<0.001 and 0.05, respectively) (supplementary material Fig. S5C,D). Sprouty2 was also mildly upregulated (supplementary material Fig. S5D). Furthermore, the ERK downstream effectors pElk and Pea3...
were downregulated (supplementary material Fig. S5E,F), indicating that overall ERK was not very high in this model. Conversely, we found much higher levels of pAKT(Ser473) staining in papillomas of the UroIICreK-RasG12D+/Ptenfl/+ mice in comparison to UroIICreFgf3r3K644E K-RasG12D/+ mice (n=3, P<0.001; supplementary material Fig. S5G,H,K,L).

**Formation of lung tumors in UroIICreFgf3r3K644E β-catenin exon3/+ mice**

Although there was no papilloma formation, 36% (10/27) of the UroIICreFgf3r3K644E β-catenin exon3/+ mice developed lung tumors by 1 year of age (supplementary material Table S1 and Fig. S4). Imaging with the OV100 microscope showed a strong GFP signal in the lung, indicating that the tumor formation was due to sporadic Cre recombination in the tumor (supplementary material Fig. S6A). These tumors resembled most closely solitary fibrous tumors (SFTs) of the lung (supplementary material Fig. S6B). SFTs are mesenchymal neoplasms of subendothelial origin that can be found mostly in the pleura but also in extraserosal sites, such as the lung, mediastinum, liver, head and neck, and deep soft tissues of the extremities. Most SFTs behave as slowly growing, painless masses (Kouki et al., 2008). No lung tumors were observed in the extremities. Most SFTs behave as slowly growing, painless masses (Kouki et al., 2008). No lung tumors were observed in the extremities.

**Elevation of ERK downstream signaling is associated with tumor formation in the bladder and in papilloma**

Given the difficulty of assigning ERK pathway activity in vivo, we next examined the expression of two known targets of ERK: Pea3 and pELK (O’Hagan et al., 1996; Cruzalegui et al., 1999). In the bladder of UroIICreFgf3r3K644E β-catenin exon3/+ mice, the levels of Pea3 and pElk1 were mildly upregulated (Fig. 3C,D) when compared with wild-type bladders (Fig. 3A,B). By contrast, in papillomas from UroIICreFgf3r3K644E K-RasG12D/+ mice, Pea3 and pElk1 expression was highly upregulated (Fig. 3E,F). Quantitative reverse-transcriptase PCR (RT-PCR) experiments showed that, in UroIICreFgf3r3K644E K-RasG12D/+ papillomas (n=2), the relative expression level of the Pea3 transcript normalized by actin was increased by 120-fold compared with that in wild-type bladders (n=4), with the average threshold cycle (Ct) values of 9.37 and 16.28±0.88 in papilloma and wild-type bladders, respectively. By contrast, in the difference in the expression levels of Pea3 between the bladders of UroIICreFgf3r3K644E β-catenin exon3/+ mice (n=3) and wild type was not detected at the transcript level (average Ct = 15.76±0.97 in UroIICreFgf3r3K644E β-catenin exon3/+, statistically not significant). This is in accordance with our hypothesis that the upregulation of pERK1/2 in the absence of Sprouty2 leads to an overall increase in ERK signaling in papillomas of UroIICreFgf3r3K644E K-RasG12D/+ mice (Fig. 2C,D). By contrast, we suggest that Sprouty2 upregulation is sufficient to suppress the overall ERK signaling levels in the bladders of UroIICreFgf3r3K644E β-catenin exon3/+ mice (Fig. 1G,H). We have also examined Pea3 and pELK1 levels in two established bladder tumor models, UroIICreH-RasQ61L and UroIICreβ-catenin exon3/+ H-RasQ61L (Ahmad et al., 2011b). In these models, both Pea3 and pElk1 levels are highly upregulated in the bladder tumors (Fig. 3G-I), indicating that strong activation of the ERK-MAPK pathway might be one of the pathways that drives bladder tumor formation.

Finally, we have examined the ERK-MAPK signaling pathway in human UCC by using a tissue microarray (TMA) that contains 60 UCC and 20 benign controls (Folio Biosciences, OH). Using the Histoscore technique, we found statistically significant increases in both nuclear and cytoplasmic pERK1/2 staining in the UroIICreβ-catenin exon3/+ K-RasG12D/+ cohort compared with the UroIICreFgf3r3K644E β-catenin exon3/+ mice (n=3, P<0.001; supplementary material Fig. S6M,N). Conversely, we found much higher levels of pAKT(Ser473) staining in lung tumors of the UroIICreFgf3r3K644E β-catenin exon3/+ mice in comparison to UroIICreβ-catenin exon3/+ K-RasG12D/+ mice (n=3, P<0.001; supplementary material Fig. S6O,P). Consistent with this,
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and the downregulation of Sprouty2 (Pearson correlation coefficient=0.6596, P<0.0001, two-tailed) (data not shown), indicating that the absence of Sprouty2 upregulation might underlie the mechanism leading to UCC formation in humans. In addition, using Oncomine (https://www.oncomine.org), Lindgren and colleagues demonstrated that, in FGFR3 mutant bladder cancer, there is a downregulation of Sprouty2 at the mRNA level (P=0.015) (Lindgren et al., 2006). In the same study, Sprouty2 mRNA levels were found to be downregulated as the disease progresses from Grade 1 to 3 (P=0.015).

DISCUSSION

We demonstrate here that activating mutations of FGFR3 are unlikely to be the sole initiating factor for UCC and that neither β-catenin exon3/ nor K-RasG12D/+ mutation cooperates with FGFR3 to drive UCC. FGFR3 mutations are thought to be mutually exclusive with H-RAS mutation (Jebar et al., 2005), and FGFR3 is only rarely mutated with p53 (<5%) (Bakkar et al., 2003). Therefore, generation of mouse models that recapitulate superficial UCCs that have FGFR3 mutations might be difficult and will require greater knowledge of the genetic changes that accompany FGFR3 mutations (Jebar et al., 2005; Wu, 2005; Puzio-Kuter et al., 2009).

The study, however, showed that somatic Fgfr3 mutations caused a modest upregulation of the ERK-MAPK pathway in the urothelium, which was accompanied by upregulation of Sprouty2, one of the feedback inhibitors of the ERK-MAPK pathway (Fig. 1). We speculate that upregulation of negative-feedback genes, such as Sprouty2, could be one of the mechanisms by which UCC is normally prevented. In papillomas in UroICre+Fgfr3+/K644EK-RasG12D/+ mice, no concomitant upregulation of Sprouty2 had occurred, potentially leading to uncontrolled activation of the ERK-MAPK pathway in the absence of a negative-feedback mechanism (Fig. 2). In the case of lung tumors in the UroICre+Fgfr3+/R644E β-catenin exon3/ cohort, a strong upregulation of the PI3K-pAKT and Wnt–β-catenin pathways might underlie tumorigenesis (supplementary material Fig. S6). It is tempting to propose that these differential downstream signaling profiles are the underlying drivers of tumorigenesis, with the different oncogenic mutations cooperating with FGFR3 mutation in a context-dependent fashion (supplementary material Fig. S7). It would be interesting to assess in the future whether downregulation of Sprouty2 in our mouse system results in UCC formation. Tissue-specific effects of Fgfr3
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**Translational Impact**

**Clinical Issue**
Urothelial cell carcinoma (UCC) of the bladder is the fifth most common cancer worldwide. Because bladder cancer has a tendency to recur even in the non-invasive cases, it is one of the most expensive and labor-intensive cancers to manage. Fibroblast growth factor receptor 3 (FGFR3) is a receptor tyrosine kinase that is known to mediate the effects of fibroblast growth factors (FGFs). Several studies have shown that mutations in FGFR3 are closely associated with bladder cancer of a low tumor grade and stage. However, whether and how FGFR3 mutations contribute to bladder tumorigenesis is unknown. Thus, generation of a relevant mouse model is essential not only for investigating this issue but also for testing potential therapeutic approaches.

**Results**
In this paper, the authors test the capacity of FGFR3 activating mutations to drive UCC by creating a mouse model in which mutated Fgfr3 is targeted to the urothelium, using Cre-loxP recombination driven by a urothelium-specific promoter. Their findings demonstrate that activating mutations in FGFR3 are unlikely to be the sole initiating factor for UCC, even in the presence of activating mutations in K-Ras or β-catenin. However, sporadic ectopic Cre recombinase expression in the skin and lung of these mice shows that an activating mutation can cause papillomas (by cooperating with K-Ras) and promote lung tumorigenesis (by cooperating with β-catenin). Furthermore, somatic Fgfr3 mutations cause upregulation of the ERK-MAPK pathway, as well as upregulation of Sprouty2 (a feedback inhibitor of the pathway) in the urothelium, but do not lead to UCC formation. By contrast, the formation of papillomas in mice carrying activating mutations in both Fgfr3 and K-Ras were not associated with upregulation of Sprouty2, potentially leading to uncontrolled activation of the ERK-MAPK pathway in these mice. Therefore, the authors speculate that feedback inhibitors of the FGF signaling pathway might be one of the mechanisms by which UCC is normally prevented.

**Implications and future directions**
These data indicate that activating mutations in FGFR3 can cooperate with other mutations to drive tumorigenesis in a context-dependent manner. However, further studies of how the FGFR3 signaling pathway is dysregulated in UCC are required to enable patient stratification according to risk of progression and recurrence, and to aid in patient selection for single-agent or combination therapies. Moreover, identifying molecular events that cooperate with FGFR3 activating mutations to drive UCC formation will aid in the development of genetic models of UCC to test combinatorial therapies.

**Methods**

**Mice**
Uroplakin II Cre mice (UroIICre+) (Zhang et al., 1999) were intercrossed with mice harboring Fgf3 K644E and Fgf3 K644M (Fgf3+/K644Eneo and Fgf3+/K644Mneo) (Iwata et al., 2000; Iwata et al., 2001), β-catenin exon3/+ (Harada et al., 1999), and K-RasG12D/+ (Jackson et al., 2001) mice. Mice were genotyped by PCR as previously described (Harada et al., 1999; Zhang et al., 1999; Iwata et al., 2000; Iwata et al., 2001; Jackson et al., 2001). Mice were of a mixed background and littersmates were used as control mice. All experiments were carried out in accordance with the Project Licence under Home Office Animal (Scientific Procedures) Act 1986 in the UK.

**Immunohistochemistry**
Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded samples. Upon harvesting, the bladders were emptied of urine before being placed in formalin for overnight fixation and were paraffin embedded. All bladders were processed and cut in the same manner by a single histology technician to aid standardization. For each genotype we stained at least three samples from different mice and took representative images. We used antibodies against: FGFR3 (C-15, Santa Cruz Biotechnology, 1:20, no antigen retrieval), BrdU (347580, BD Biosciences, 1:500, citrate buffer and microwave antigen retrieval), pAKT(Ser473) (#4060, Cell Signaling, 1:100, citrate buffer and microwave antigen retrieval), pERK1/2 (#9101, Cell Signaling, 1:100, citrate buffer and microwave antigen retrieval), Sprouty2 (ab60719, AbCam, 1:300, citrate buffer and microwave antigen retrieval), pAKT(Ser473) (#3787, Cell Signaling, 1:50, citrate buffer and microwave antigen retrieval), β-catenin
(C19220, Transduction Labs, 1:50, Tris/EDTA water bath antigen retrieval with 50 minutes at 99°C), p-mTOR(Ser2448) (#2976, Cell Signaling, 1:100, citrate buffer and microwave antigen retrieval), pElk1(Ser389) (ab28818, Abcam, 1:50, citrate buffer and microwave antigen retrieval) and PEA3 (ab70425, Abcam, 1:200, citrate buffer and microwave antigen retrieval).

**Quantification of IHC staining**

For each tissue section (n=5), the percentage of positive immunoreactivity in the nucleus and cytoplasm was evaluated with 40× magnification objectives. Staining intensity was categorized into three categories: 0, 1, 2 or 3, denoting negative, weak, moderate or strong staining, respectively. The final Histoscore was calculated from the sum of (1 × % weakly positive tumor cells) + (2 × % moderately positive tumor cells) + (3 × % strongly positive tumor cells), with a maximum Histoscore of 300 (Kirkegaard et al., 2006). Statistics were performed using the Student’s t-test (Minitab).

**Human TMA**

The TMA (Folio Biosciences, OH) consisted of 60 UCC and 20 benign bladder cores with data that consisted of patient sex, age and tumor grade. Slides were scanned using the Aperio slide scanner.

**RNA isolation and quantitative RT-PCR**

Bladders and papilloma tissues were collected in RNAlater RNA stabilization reagent and RNA was isolated using the RNeasy Mini Kit (Qiagen). DNase digest was performed using the DNAfree Kit (Qiagen). Reverse transcription was carried out using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR primers for actin and Fgfr3 (BioRad) were used for amplification. PCR products were separated on a 1% agarose gel. PCR primers for actin and PEA3 were incubated with chromosomal DNA amplified from cell lines using the Green qPCR Kit (Finnzymes) on a Chromo4 Real-Time PCR cycler (BioRad). PCR primers for actin and PEA3 were obtained from Qiagen (Quantitect qPCR Primer Assay).

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**COMPETING INTERESTS**

The authors declare that they do not have any competing or financial interests.

**AUTHOR CONTRIBUTIONS**

IA., L.B.S., M.F. and C.-A.M. performed the experiments, M.M.T. and X.-R.W. supplied the materials. We thank BICR Services, Biological Services Unit, and Colin Nixon and his histology department. We thank the ‘Think Pink’ charity for the purchase of the Aperio slide scanner and the Slidepath software.

**REFERENCES**

Ahmad, I., Morton, J. P., Singh, L. B., Radulescu, S. M., Ridgway, R. A., Patel, S., Woodgett, J., Winton, D. J., Taketo, M. M., Wu, X. R. et al. (2011a). beta-Catenin activation synergizes with PTEN loss to cause bladder cancer formation. Oncogene 30, 178-189.

Ahmad, I., Patel, R., Liu, Y., Singh, L. B., Taketo, M. M., Wu, X. R., Leung, H. Y. and Sansom, O. J. (2011b). Ras mutation cooperates with beta-catenin activation to drive bladder tumourigenesis. Cell Death Dis. 2, e124; doi:10.1038/cddis.2011.7.

Andino, L., Cagle, P., T. Murer, B., Lu, L., Popper, H., Galateau-Salle, F., Sienko, A. E., Barrios, R. and Zander, D. S. (2006). Pleuropulmonary desmoid tumors: immunohistochemical comparison with solitary fibrous tumors and assessment of beta-catenin and cyclin D1 expression. Arch. Pathol. Lab. Med. 130, 1503-1509.
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Lindgren, D., Liedberg, F., Andersson, A., Chebil, G., Gudjonsson, S., Borg, A., Mansson, W., Fioretos, T. and Hoglund, M. (2006). Molecular characterization of early-stage bladder carcinomas by expression profiles, FGFR3 mutation status, and loss of IgG. Oncogene 25, 2685-2696.

Logie, A., Dunois-Larde, C., Rosty, C., Levrel, O., Blanche, M., Ribeiro, A., Gasc, J. M., Jorcano, J., Werner, S., Sastre-Garau, X. et al. (2005). Activating mutations of the tyrosine kinase receptor FGFR3 are associated with benign skin tumors in mice and humans. Hum. Mol. Genet. 14, 1153-1160.

Luis, N. M., Lopez-Knowles, E. and Real, F. X. (2007). Molecular biology of bladder cancer. Clin. Transl. Oncol. 9, 5-12.

Mo, L., Cheng, J., Lee, E. Y., Sun, T. T. and Wu, X. R. (2005). Gene deletion in urothelium by specific expression of Cre recombinase. Am. J. Physiol. Renal Physiol. 289, F562-F568.

Moon, R. T., Kohn, A. D., De Ferrari, G. V. and Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. Nat. Rev. Genet. 5, 691-701.

Muenke, M. and Schell, U. (1995). Fibroblast-growth-factor receptor mutations in human skeletal disorders. Trends Genet. 11, 308-313.

Naski, M. C., Wang, Q., Xu, J. and Ornitz, D. M. (1996). Graded activation of fibroblast growth factor receptor 3 by mutations causing achondroplasia and thanatophoric dysplasia. Nat. Genet. 13, 233-237.

Ng, T. L., Gown, A. M., Barry, T. S., Cheang, M. C., Chan, A. K., Turbin, D. A., Hsu, F. D., West, R. B. and Nielsen, T. O. (2005). Nuclear beta-catenin in mesenchymal tumors. Mod. Pathol. 18, 68-74.

Novak, A., Guo, C., Yang, W., Nagy, A. and Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis 28, 147-155.

O’Hagan, R. C., Tozer, R. G., Symons, M., McCormick, F. and Hassell, J. A. (1996). The activity of the Ets transcription factor PEA3 is regulated by two distinct MAPK cascades. Oncogene 13, 1323-1333.

Olderoy, G., Daehlin, L. and Ogrend, D. (1998). Low-frequency mutation of Ha-ras and Ki-ras oncogenes in transitional cell carcinoma of the bladder. Anticancer Res. 18, 2673-2678.

Parkin, D. M., Bray, F., Ferlay, J. and Pisani, P. (2005). Global cancer statistics, 2002. CA Cancer J. Clin. 55, 74-108.

Puzio-Kuter, A. M., Castillo-Martin, M., Kinkade, C. W., Wang, X., Shen, T. H., Matos, T., Shen, M. M., Cordon-Cardo, C. and Abate-Shen, C. (2009). Inactivation of p53 and Pten promotes invasive bladder cancer. Genes Dev. 23, 675-680.

Qing, J., Xu, D., Chen, Y., Chan, P., Li, H., Wu, P., Marsters, S., Stawicki, S., Tien, J., Tota, K. et al. (2009). Antibody-based targeting of FGFR3 in bladder carcinoma and t(4;14)-positive multiple myeloma in mice. J. Clin. Invest. 119, 1216-1229.

Rakheja, D., Molberg, K. H., Roberts, C. A. and Jaiswal, V. R. (2005). Immunohistochemical expression of beta-catenin in solitary fibrous tumors. Arch. Pathol. Lab. Med. 129, 776-779.

Schulz, W. A. (2006). Understanding urothelial carcinoma through cancer pathways. Int. J. Cancer 119, 1513-1518.

Uchida, T., Wada, C., Ishida, H., Egawa, S., Ao, T., Yokoyama, E. and Koshiba, K. (1995). Infrequent involvement of mutations on neurofibromatosis type 1, H-ras, K-ras and N-ras in urothelial tumors. Urol. Int. 55, 63-67.

Woenckhaus, M., Klein-Hitpass, L., Gregmeier, U., Merk, J., Pfeifer, M., Wild, P., Bettstetter, M., Wuesch, P., Blaszyk, H., Hartmann, A. et al. (2006). Smoking and cancer-related gene expression in bronchial epithelium and non-small-cell lung cancers. J. Pathol. 210, 192-204.

Wu, X. R. (2005). Urothelial tumorigenesis: a tale of divergent pathways. Nat. Rev. Cancer 5, 713-725.

Yamaguchi, U., Hasegawa, T., Masuda, T., Sekine, S., Kawai, A., Chuman, H. and Shimoda, T. (2004). Differential diagnosis of gastrointestinal stromal tumor and other spindle cell tumors in the gastrointestinal tract based on immunohistochemical analysis. Virchows Arch. 445, 142-150.

Zhang, Z. T., Pak, J., Shapiro, E., Sun, T. T. and Wu, X. R. (1999). Urothelium-specific expression of an oncogene in transgenic mice induced the formation of carcinoma in situ and invasive transitional cell carcinoma. Cancer Res. 59, 3512-3517.

Zhang, Z. T., Pak, J., Huang, H. Y., Shapiro, E., Sun, T. T., Pellicer, A. and Wu, X. R. (2001). Role of Ha-ras activation in superficial papillary pathway of urothelial tumor formation. Oncogene 20, 1973-1980.