

Exploring molecular genetics of bladder cancer: lessons learned from mouse models

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Urothelial cell carcinoma (UCC) of the bladder is one of the most common malignancies worldwide, causing considerable morbidity and mortality. It is unusual among the epithelial carcinomas because tumorigenesis can occur by two distinct pathways: low-grade, recurring papillary tumours usually contain oncogenic mutations in *FGFR3* or *HRAS*, whereas high-grade, muscle-invasive tumours with metastatic potential generally have defects in the pathways controlled by the tumour suppressors p53 and retinoblastoma (RB). Over the past 20 years, a plethora of genetically engineered mouse (GEM) models of UCC have been developed, containing deletions or mutations of key tumour suppressor genes or oncogenes. In this review, we provide an up-to-date summary of these GEM models, analyse their flaws and weaknesses, discuss how they have advanced our understanding of UCC at the molecular level, and comment on their translational potential. We also highlight recent studies supporting a role for dysregulated Wnt signalling in UCC and the development of mouse models that recapitulate this dysregulation.

Introduction

Urothelial cell carcinoma (UCC) of the bladder is a significant health problem, with an incidence that is continuing to rise. In terms of overall cancer frequency, it is ranked ninth, with an estimated 356,600 new cases diagnosed annually worldwide (<http://info.cancerresearchuk.org/cancerstats/types/bladder/incidence/index.htm>).

Most UCCs are transitional cell carcinomas (TCCs), arising from the transitional epithelium that lines the bladder. Of these, ~70% of cases are low-grade, papillary, non-invasive tumours that, despite local excision, tend to recur in over 30% of patients. There is controversy regarding whether these papillary tumours progress to invasive disease; some studies propose that progression occurs in a maximum of 10-20% of cases (Knowles, 2001). However, whether this is true progression or development of de novo tumours remains to be elucidated. Most morbidity and mortality associated with UCC is caused by a high-grade, non-papillary, muscle-invasive form of the disease (20-30% of new TCC cases). These invasive tumours can penetrate deeply through the muscle wall of the bladder and, despite treatment, 50% of patients relapse with tumours that metastasise to distant sites (Williams and Stein, 2004). Treatment fails in 95% of patients with advanced disease, and the 5-year survival rate for metastatic bladder cancer is only 6%. Therefore, therapies guided by preclinical models of aggressive

bladder cancers might provide the scientific basis for novel therapies.

Clinical and pathological studies have found that these two forms of UCC arise through at least two separate mechanisms (Koss, 1992; Wu, 2005). In addition, recent gene expression studies and genomic profiling identified signatures for two molecular subtypes of UCC that stratify into low- and high-grade disease and can independently prognosticate the development of metastasis and disease-specific survival (Lindgren et al., 2008; Lauss et al., 2010; Lindgren et al., 2010). Although this progress has begun to unravel some of the molecular details that underlie UCC, many questions remain. Several mouse models that recapitulate UCC have been developed to better understand the disease, but there remains a paucity of models that represent the high-grade, muscle-invasive and metastatic form. In this review, we describe the current state of play regarding mouse models of UCC, how they have contributed to our understanding of the molecular basis of each form of the disease and their translational impact. We also discuss new technologies in mouse model development and the perspectives for the future of this field.

Diverse molecular pathways of UCC

Clinical and genetic evidence suggests that the two different forms of UCC arise and progress along two distinct pathways (Fig. 1). Accordingly, the two forms are associated with different mutations, affecting proteins involved in the different pathways. Low-grade, superficial UCCs frequently harbour mutations in genes of the RAS pathway (10-15%) or the fibroblast growth factor receptor 3 (*FGFR3*) gene (55-65%), both of which activate the MAPK pathway. Studies have demonstrated that *FGFR3* mutations are mutually exclusive with RAS-pathway mutations in UCC, but that *FGFR3* mutations occur together with *PIK3CA* mutations (Jebar et al., 2005; Kompier et al., 2010; Sjobahl et al., 2011). This indicates that activation of the receptor tyrosine kinase (RTK)-RAS cascade might have an early and crucial role in the tumorigenic pathway leading

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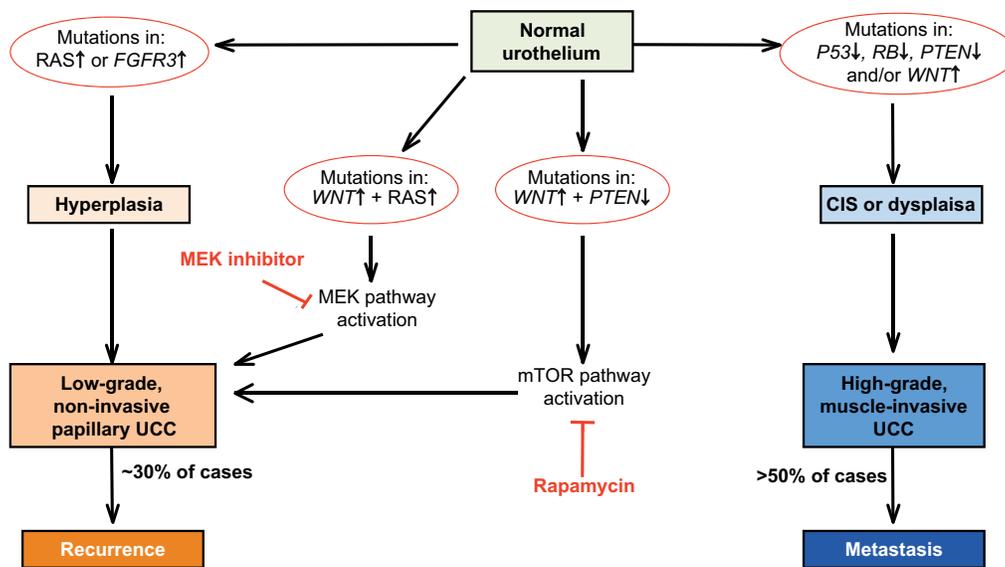


Fig. 1. Important genetic defects that characterise the diverse pathways underlying UCC. Low-grade, non-invasive papillary tumours (70–80% of human UCC cases) are frequently associated with activating mutations in either RAS-pathway components or *FGFR3*, which are thought to be mutually exclusive. High-grade, muscle-invasive tumours (20–30% of human UCC cases) are associated with loss of p53 or RB function. Loss of PTEN function and activation of the Wnt signalling pathway have also been proposed to play a role in muscle-invasive bladder tumours. Mouse model data suggest that Wnt pathway activation can also contribute to non-invasive UCC: in the context of PTEN inactivation, Wnt pathway activation leads to the development of non-invasive bladder tumours that depend on mTOR signalling (as highlighted by responsiveness to the mTOR inhibitor rapamycin). By contrast, Wnt pathway activation in the context of RAS pathway activation leads to the development of non-invasive tumours that depend on MAPK signalling (as demonstrated by their responsiveness to MEK inhibition, but not rapamycin). Further elucidation of the molecular pathways underlying UCC should reveal new therapeutic targets.

to low-grade, non-invasive UCC (Jebar et al., 2005; Kompier et al., 2010). High-grade, muscle-invasive tumours can either originate from carcinoma in situ (CIS) or arise de novo from previously normal bladder urothelium. Over 50% of these muscle-invasive tumours contain mutations in genes encoding the tumour suppressors p53 and/or the retinoblastoma protein (RB). There seems to be redundancy in each of the pathways controlled by these factors, again suggesting that the mutations occur in a mutually exclusive manner (Jebar et al., 2005).

The COSMIC (Catalogue of Somatic Mutations in Cancer) database from the Wellcome Trust Sanger Institute in Cambridge, UK, has confirmed that a number of the mutations mentioned above are present in human UCCs (Fig. 2) (Forbes et al., 2008). Interestingly, a number of mutations (*PIK3CA*, *CDKN2A* and *APC*) do not fit within the pathways that are widely accepted to underlie UCC (illustrated in Fig. 1). Similarly, a recent genome screening study highlighted the role of chromatin remodelling genes in low-grade UCCs (Gui et al., 2011). This suggests that the paradigm shown in Fig. 1 needs revising, and might be more complex and/or heterogeneous. Recent work by the Hoglund group (Lund University, Lund, Sweden) has done much to delineate pathways underlying UCC. By carrying out gene expression, whole-genome array comparative genomic hybridisation (CGH) and mutational analyses of eight genes commonly implicated in UCC on 144 UCC samples, they identified two distinct molecular subtypes that had independent prognostic effects on metastasis and disease-specific survival. Their data show that genomic instability is the most distinguishing genomic feature of the high-grade, muscle-invasive tumours, and

that this feature is dependent on *P53* and/or *MDM2* alterations (Lindgren et al., 2010; Sjodahl et al., 2011).

As our understanding of UCC has grown, mouse models have been developed to investigate many disease-associated mutations and other genes linked with the disease (including *PTEN*, *EGFR* and β -catenin). These models have further advanced our understanding of the disease and should enable exploration of treatment regimes and, ultimately, the translation of preclinical knowledge to improved patient care.

Experimental models of UCC

Success in elucidating the molecular mechanisms underlying UCC using cancer cells in in vitro tissue culture environments has been limited because the stroma and the microenvironment cannot be easily modelled in vitro, and because of problems with long-term propagation of these tumour cells. Moreover, primary culture of bladder epithelial cells is difficult, and there is a continued lack of robust 3D culture models, making it difficult to assess the process of carcinogenesis initiation to malignancy ex vivo. Other confounding issues include how much primary cells or cell lines differ from their origins in terms of their morphology, growth characteristics and other phenotypic features (Gabriel et al., 2007), and it is well known that high passage number is associated with a higher rate of spontaneous mutations, selection processes and senescence. Thus, mouse models will continue to be an important tool to increase our understanding of the molecular basis of UCC.

Traditionally, mouse models of bladder cancer have used either an orthotopic approach (i.e. transfer of primary cells or genetically altered cell lines derived from localised and metastatic disease) or

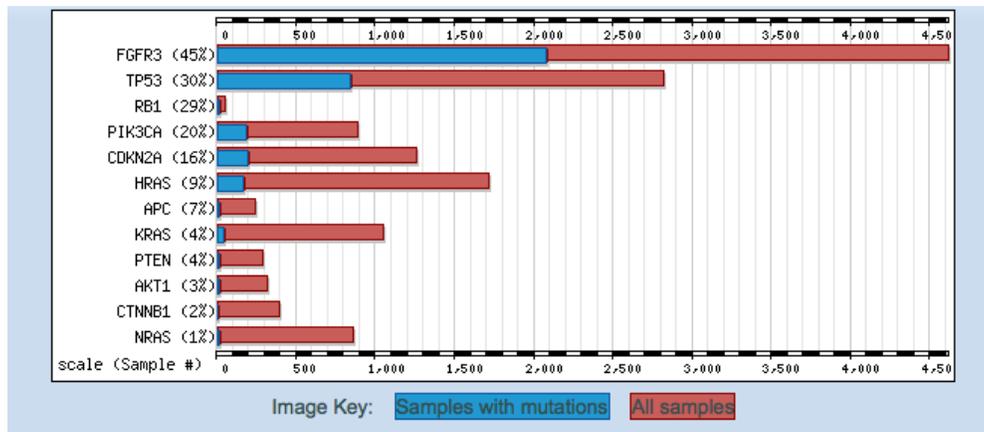


Fig. 2. Mutational profile of UCC (COSMIC Database, Genome Research Limited). The top 12 genes with mutations found in human UCC samples are shown. Red bars represent total number of samples (i.e. differing samples sizes and sets) and blue bars represent the proportion of samples positive for a mutation in each gene. The percentage value represents the mutation frequency. This analysis was not stratified for invasive and non-invasive UCC. Figure reproduced with permission from Genome Research Ltd (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=byhist&ss=bladder&sn=urinary_tract&s=3).

chemical carcinogens. More recently, genetically engineered mouse (GEM) models have been developed that might more closely mimic the human disease at the molecular level. In the sections below, we review each of these types of mouse models and how they have contributed to our understanding of UCC.

Chemically induced carcinogenesis

Spontaneous bladder cancer in mice (and rats) is a rare phenomenon, so intravesical installation of carcinogens is often used to generate models of UCC. The most commonly used carcinogens are *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN), *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) and *N*-methyl-*N*-nitrosurea (MNU). The total dose of these carcinogens has a greater effect when administered as several fractions over multiple days (effects are synergistic rather than additive when the chemicals are given in combination). The grade of cellular atypia and the extent of invasion by these transformed urothelial cells increase as the dose of carcinogen increases and when the experimental period is extended (Oliveira et al., 2006). These models are used particularly for studies of chemoprevention agents, as well as the elucidation of molecular mechanisms (Black and Dinney, 2007).

Because the chemicals are applied directly to the bladder in these models, one advantage is that tumours that are induced are specific to the urothelium. In addition, there is typically a 100% tumour formation rate. The type of UCC observed depends on the carcinogen applied, the background strain of the mouse and the treatment regime. FANFT-induced tumours are predominately UCCs, with a degree of squamous differentiation; in addition, hyperplasia, dysplasia and carcinoma in situ (CIS) of the urothelium has also been reported (Becci et al., 1981). BBN is the most commonly used agent because the pathology induced most closely resembles that of high-grade human UCC, progressing from cellular atypia to CIS and finally to muscle-invasive carcinoma (Kunze et al., 1976). Bladders exposed to intravesical MNU develop progressive neoplastic changes, with the lesions becoming progressively less differentiated with time, developing from CIS through to papillary carcinoma and finally resulting in large bulky muscle-invasive tumours, which have a relatively low potential for metastasis (Kunze et al., 1976). Disadvantages of this system are that tumour induction and progression takes 8-14 months (depending on carcinogen and dosage), and there are safety issues

surrounding the exposure of laboratory and animal unit staff to carcinogens.

Orthotopic models

There are two types of orthotopic models of UCC: xenograft models and syngeneic models. Xenograft models involve the implantation of human bladder cancer cells into a nude (immunodeficient) mouse. Several different TCC cell lines have been used, including KU7, KU-19, T24 and UM-UC3 cells. A major disadvantage of this technique is that the immune response, which is an important factor regulating tumour growth, cannot be assessed because of the immunodeficient nature of the host. Syngeneic models involve the implantation of immunocompetent mice with bladder tumours derived from the same mouse strain. The two cell lines most frequently used for this approach are MB49 cells, which contain a mutation in codon 12 of the *K-ras* gene (from 7,12-dimethylbenzanthracene-induced bladder tumours in the C57BL/6 mouse), and MBT-2 cells, which are deficient for p53 (from FANFT-induced bladder tumours in the C3H/He mouse) (Soloway, 1977; Summerhayes and Franks, 1979; Luo et al., 1999; Wada et al., 2001).

One concern with these models is that the 'take' rate of tumour implantation can vary significantly from as low as 30% up to 100% (Chan et al., 2009). Factors influencing tumour 'take' include tumorigenicity, the number of cells implanted, the duration of implants and pre-treatment conditioning (such as traumatization of urothelial mucosa before inoculation with cells) (Chan et al., 2009).

GEM models

GEM models of UCC (Table 1) are important research tools and have been instrumental in elucidating pathways of bladder cancer (Wu, 2005). GEM models are engineered to recapitulate genetic abnormalities that have been associated with the disease in humans and thus allow studies of these abnormalities in vivo. They allow studies of single as well as compound mutational events involving oncogenes and/or tumour suppressors, in an organ-specific and temporal manner. Mouse models that develop metastasis provide an excellent platform for investigating the specific genetic events involved in progression to a metastatic phenotype, as well as the role of the tumour microenvironment. Despite their many advantages, however, it is important to note that GEM models have

Table 1. At a glance reference of published GEM models of UCC

Mutation	Promoter	Phenotype	References
<i>Hras</i> ⁺ (low-copy)	<i>UPII</i>	Low grade, non-invasive (long latency)	Zhang et al., 2001
<i>Hras</i> ⁺ (high-copy)	<i>UPII</i>	Low grade, non-invasive (short latency)	Zhang et al., 2001
<i>Egfr</i> ⁺	<i>UPII</i>	Hyperplasia	Cheng et al., 2002
<i>Egfr</i> ⁺ <i>Hras</i> ⁺	<i>UPII</i>	Hyperplasia	Cheng et al., 2002
<i>Fgf3</i> ⁺	<i>UPII</i>	Hyperplasia	Ahmad et al., 2011b
<i>Fgf3</i> ⁺ <i>Kras</i> ^{G12D}	<i>UPII</i>	Hyperplasia	Ahmad et al., 2011b
<i>Fgf3</i> ⁺ <i>β-catenin</i> ^{exon3/+}	<i>UPII</i>	Hyperplasia	Ahmad et al., 2011b
<i>p53</i> ^{fl/fl}	<i>UPII</i>	Hyperplasia and dysplasia	Gao et al., 2004
<i>p53</i> ^{fl/fl}	<i>UPKII</i>	No phenotype	Ayala de la Pena et al., 2011
<i>p53</i> ^{fl/fl} <i>Hras</i> ⁺	<i>UPII</i>	High grade, invasive UCC	Gao et al., 2004
<i>p53</i> ^{fl/fl} <i>Pten</i> ^{fl/fl}	<i>AdenoCre</i>	Invasive, metastatic UCC	Puzio-Kuter et al., 2009
<i>Pten</i> ^{fl/fl}	<i>FabpCre</i>	Low-grade UCC	Yoo et al., 2006; Tsuruta et al., 2006
<i>Pten</i> ^{fl/fl}	<i>Ksp-Cre</i>	UCC of renal pelvis	Qian et al., 2009
<i>Pten</i> ^{fl/fl}	<i>AdenoCre</i>	No phenotype	Puzio-Kuter et al., 2009
<i>Pten</i> ^{fl/fl}	<i>UPII</i>	No phenotype	Ahmad et al., 2011c
<i>Rb</i> ^{-/-}	<i>UPII</i>	No phenotype	He et al., 2009
<i>Rb</i> ^{-/-} <i>Pten</i> ^{fl/fl}	<i>AdenoCre</i>	No phenotype	Puzio-Kuter et al., 2009
<i>Rb</i> ^{-/-} <i>p53</i> ^{fl/fl}	<i>UPII</i>	Invasive UCC after chemical carcinogenesis	He et al., 2009
<i>SV40 T antigen</i> (low-copy)	<i>UPII</i>	Carcinoma in situ	Zhang et al., 1999
<i>SV40 T antigen</i> (high-copy)	<i>UPII</i>	Invasive, metastatic UCC	Zhang et al., 1999
<i>SV40 T antigen</i>	<i>UPKII</i>	Carcinoma in situ	Ayala de la Pena et al., 2011
<i>β-catenin</i> ^{exon3/+}	<i>UPII</i>	Hyperplasia	Ahmad et al., 2011c
<i>β-catenin</i> ^{exon3/+} <i>Pten</i> ^{fl/fl}	<i>UPII</i>	Low-grade, non-invasive UCC	Ahmad et al., 2011c
<i>β-catenin</i> ^{exon3/+} <i>Hras</i> ^{Q61L}	<i>UPII</i>	Low-grade, non-invasive UCC	Ahmad et al., 2011a

several drawbacks. First, tumours that develop in these models tend to be less heterogeneous than human bladder tumours, which might influence tumour progression and metastasis. Second, the cell of origin in a model might not reflect the cell of origin in the human condition. Third, it has been demonstrated that UCC requires multiple mutations, calling into question the relevance of singly mutant UCC models. Finally, certain models have limitations such as long latencies, incomplete penetrance or the requirement of an artificial genetic element (e.g. some models use knock-in endogenous alleles that mutate the allele in of interest, whereas others induce overexpression of an allele at non-physiological levels).

Several approaches are used to create GEM models. The traditional method involves knockout of a gene of interest (e.g. a tumour suppressor) in the whole animal (Capecchi, 1994). Problems with this approach are that it does not allow evaluation of gene function if knockout results in embryonic lethality or premature death and, with non-lethal knockouts, it can be difficult to determine whether an abnormal phenotype is confounded by a developmental defect (Copp, 1995). An approach that circumvents these limitations is a conditional (transgenic) gene knock-in or knockout (e.g. involving the *Cre-loxP* system), which allows studies of gene function in specific cell and tissue types (Nagy, 2000). Tissue-specific *Cre* transgenes have been generated for nearly every tissue in the mouse, including the bladder urothelium. Notably, many GEM models of UCC have used the mouse uroplakin II (*UPII*) promoter, which is expressed in the basal layer of the urothelium,

in which the stem cell niche is thought to reside (Lin et al., 1995). However, recent work by Ayala de la Pena and colleagues demonstrated that, during the original cloning of this promoter, ~1500 bp of the *UPII* promoter region was oppositely inserted between two *SacI* restriction enzyme sites (from -1262 to -2805 from exon 1) (Ayala de la Pena et al., 2011). This might result in changes in the natural patterns of *UPII* expression. Indeed, this *UPII* promoter (GenBank accession number U14421), which has been used to create the majority of GEM models of UCC (including our own) to study the effects of urothelial-specific gene deletions and activations, might not generate an accurate phenotype relevant for understanding UCC. Ideally, the findings generated using all of these published models should be reconfirmed in systems that use the correct promoter. Notably, a recent study suggests that using the correct promoter (referred to as *UPKII*) to create mice expressing the Simian virus 40 (SV40) large T-antigen (which inactivates both *p53* and *Rb*) results in the development of CIS only, with activation of pro-angiogenic factors but not progression to invasive cancer (Ayala de la Pena et al., 2011). By contrast, SV40 mice created using the *UPII* promoter develop muscle-invasive disease (Zhang et al., 1999; Grippo and Sandgren, 2000). This is by no means a unique example of the caveats that researchers must consider when working with GEM models and relating their results to human disease.

In the following sections, we describe GEM models that have been created to study several UCC-associated mutations, and the insight they have provided into the human disease.

HRAS

HRAS was the first oncogene isolated in human UCC and is mutated most often at codons 12, 13 and 61 (Reddy et al., 1982). These mutations cause *HRAS* to become constitutively expressed. Despite the controversy regarding the reported mutation frequency rate, most studies suggest that the *RAS* mutation rate is lower than initially thought and occurs in ~11-13% of UCC cases (Czerniak et al., 1992; Jebar et al., 2005; Kompier et al., 2010). In line with hypotheses regarding pathways underlying the two forms of UCC, two of these studies found no enrichment of *HRAS* mutations in papillary, non-muscle-invasive tumours (Czerniak et al., 1992; Jebar et al., 2005; Kompier et al., 2010).

Transgenic models have provided invaluable information regarding the molecular mechanisms behind *HRAS* activation, with much of this work being carried out in the Wu lab (Wu, 2005). In the first instance, this group targeted expression of a constitutively active rabbit *Hras* to the urothelium using the *UPII* promoter (Zhang et al., 2001). This mutation induced early onset urothelial hyperproliferation, which progressed to low-grade, papillary, non-invasive tumours. Interestingly, tumour latency was dependent on transgene copy number. In mice that had one or two copies of the *Hras* transgene (low-copy), tumour latency was almost 12 months, and lesions remained non-invasive during a 26-month follow-up period. By contrast, mice with 30-48 copies of the *Hras* transgene (high-copy) succumbed to death by 5 months of age. Again, these tumours were papillary and non-invasive, with no evidence of muscle invasion or metastases. The fact that the bladder tumours in low-copy mice developed with a much longer latency suggests that, in the absence of overexpression, secondary events, either genetic or epigenetic in nature, are required to fully induce bladder tumorigenesis. These mice have been instrumental in demonstrating that *RAS*-pathway activation is sufficient to lead to UCC of the low-grade non-invasive papillary type. This is a crucial point that must be considered when comparing this model to human UCC, because non-muscle invasive tumours in humans are no more enriched in *RAS* mutations than muscle-invasive tumours (i.e. *RAS*-pathway mutations occur in both forms). By contrast, *Hras* mutations do not seem to produce a muscle-invasive form of bladder cancer in mice.

Epidermal growth factor receptor

Epidermal growth factor receptor (EGFR) is overexpressed in 40-60% of human UCC cases at both the mRNA and protein level (Neal and Mellon, 1992). In chemical carcinogen models of UCC, administration of exogenous EGF significantly increased the frequency of orthotopically transplanted bladder tumours in rats (Fujimoto et al., 1996).

The Wu lab also targeted expression of functionally active *Egfr*, again using the *UPII* promoter (Cheng et al., 2002). The bladders in these mice became hyperplastic, but did not progress to develop tumours. When they combined this *UPII*-driven expression of *Egfr* with the activated *Hras* transgene in a compound transgenic mouse, there was no synergism between the two mutations, strengthening the redundancy argument because both are in the same signal transduction cascade.

FGFR3

Several studies have shown that somatic mutations in *FGFR3* are strongly associated with bladder cancer of a low tumour grade and

stage (Billerey et al., 2001; Jebar et al., 2005; Lamy et al., 2006; Lindgren et al., 2006). To test whether *FGFR3*-activating mutations can act as a 'driver' of UCC, we targeted the expression of mutated *Fgfr3* to the mouse urothelium using the *UPII* promoter (Ahmad et al., 2011b). These mice did not show urothelial dysplasia nor urothelial tumorigenesis (up to 18 months of age) alone or when *Fgfr3* mutations were introduced together with *Kras*- or β -catenin-activating mutations (see below). Interestingly, owing to sporadic ectopic Cre recombinase expression in the skin and lungs of these mice, the *Fgfr3* mutation caused papillomas or promoted lung tumorigenesis in cooperation with *Kras* or β -catenin activation, respectively. These results indicate that activated *Fgfr3* cooperates with other mutations to drive tumorigenesis in a tissue-dependent manner. It seems that, when activated *Fgfr3* is expressed in the urothelium, a compensatory response involving a MAPK inhibitor such as *Sprouty2* might be evoked, which might prevent tumorigenesis. Notably, there is evidence that *Sprouty2* prevents *Kras*-induced lung cancer (Sutterluty et al., 2007), although such an effect has not yet been reported in human bladder cancer. In summary, despite evidence indicating that activating mutations in *FGFR3* can act as a driver mutation in UCC, our *Fgfr3* mutant mice did not initiate tumorigenesis. This finding might reflect the fact that initial hypotheses regarding the molecular genetics of UCC initiation and progression (Fig. 1) are premature.

p53

p53 is a nuclear phosphoprotein that acts as a gatekeeper at the G1-S checkpoint of cell cycle progression and is crucial for controlling urothelial cell growth and maintaining genomic stability (Levine, 1997). Mutations and/or deletions in the *P53* gene are among the most common genetic changes found in human UCC (Wu, 2005). Usually, one allele is mutated and thus non-functional while the other is deleted, suggesting that p53 is non-functional in human UCC. Consistent with these findings is that p21 (also known as WAF), an important downstream target of p53, is downregulated in the majority of urothelial carcinomas that harbour a *P53* mutation (Stein et al., 1998; Lu et al., 2002). In line with *P53* data, loss of p21 expression is also associated with disease progression (Stein et al., 1998).

p53 abnormalities are much more prevalent in invasive UCC (>50%), as well as their precursor CIS lesions, compared with the non-invasive form (Cordon-Cardo et al., 1994; Spruck et al., 1994; Wagner et al., 1995; Orntoft and Wolf, 1998; Hartmann et al., 2002), suggesting that loss of p53 plays a role in invasive UCC. Nuclear accumulation of p53 (often signifying mutation of *P53*) is significantly associated with a decreased overall survival in patients with organ-confined disease (Esrig et al., 1994). In the same study, multivariate analysis that was stratified according to grade, pathological stage and lymph node status demonstrated that increased nuclear p53 accumulation was an independent predictor of recurrence-free and overall survival ($P < 0.001$) (Esrig et al., 1994).

A mouse with mutation of *p53* targeted to the urothelium using the *UPII* promoter developed urothelial hyperplasia and dysplasia without progression to frank carcinoma (Gao et al., 2004). More recent work on p53 by the Abate-Shen group has reinforced the idea that the development of UCC has no single driver mutation, but requires multiple mutations (Puzio-Kuter et al., 2009). Using an adenovirus expressing Cre recombinase delivered directly into

the bladder, simultaneous *p53* and *Pten* deletion (*p53^{fl/fl} Pten^{fl/fl}*) resulted in bladder tumours with 100% penetrance at 6 months, with metastasis to local lymph nodes and distant sites, including spleen, liver and diaphragm (60% by 4-6 months) (Puzio-Kuter et al., 2009). The histology of these tumours closely resembled that of CIS and invasive tumours found in human UCC. The tumours had markedly increased levels of phosphorylated mTOR (p-mTOR) and, when rapamycin (an mTOR inhibitor) was given to these mice, tumour regression occurred (Puzio-Kuter et al., 2009; Seager et al., 2009). Importantly, when given to mice at the CIS stage, rapamycin treatment abrogated the progression to invasive tumorigenesis.

Although several of the studies mentioned above have studied the role of *p53* deletion in bladder cancer, mouse studies looking at the effect of mutant *p53* in the formation of UCC have not been reported. Recent work by our group has demonstrated the essential role of mutant *p53* in overcoming senescence and driving metastasis in a mouse model of pancreatic cancer (Morton et al., 2010). It would be interesting to see whether mutant *p53* has a similar effect in the context of UCC.

PTEN

The phosphatase and tensin homology (*PTEN*) gene, located on human chromosome 10, is a lipid phosphatase that dephosphorylates phosphoinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P₃; also known as PI3P]. This 55-kDa protein antagonises the activity of phosphoinositide 3-kinase (PI3K) and prevents it from activating downstream proliferation and survival signals, especially the phosphorylation of AKT, collectively leading to growth inhibition (Dahia, 2000). The PTEN-PI3K-AKT pathway has also been implicated in UCC: evidence suggests that deletion of *PTEN* is rare in non-invasive UCC but occurs frequently in invasive UCC (Puzio-Kuter et al., 2009). Another study reported that a reduction or loss of PTEN protein expression was observed in 42% of non-invasive and 94% of advanced UCC cases. The degree of reduction of PTEN expression correlated with stage and grade (Tsuruta et al., 2006). As discussed earlier, activating *PIK3CA* mutations seem to occur more frequently in papillary compared with muscle-invasive urothelial cancers, whereas *PTEN* mutations display the opposite pattern (Kompier et al., 2010; Sjodahl et al., 2011). This would suggest that *PIK3CA* and *PTEN* do not have overlapping functions in this context. In addition, although *PTEN* seems to be downregulated in a large proportion of muscle-invasive tumours, the frequency of genomic loss seems to be much lower, so there is probably a functional difference in outcome between downregulation and deletion (Tsuruta et al., 2006; Sjodahl et al., 2011). Indeed, *PTEN* has been implicated in DNA repair, a function that might be important for preventing the development of muscle-invasive cancer (Keniry and Parsons, 2008).

Unfortunately, the results obtained with different lines of *Pten*-null mice have been inconsistent. This is probably due to (1) differences in background strains of the mice, (2) different bladder-specific promoters and (3) the use of different *Pten* floxed alleles; hence, the various results must be interpreted with caution. Yoo and colleagues deleted exons 4-5 of the *Pten* gene using the *FabpCre* system, whereby the expression of Cre recombinase is placed directly under the control of transcriptional regulatory elements from a fatty acid-binding protein gene (*Fabp*); resulting in recombination in all of the cell layers of the urothelium and

intestinal epithelium by embryonic day 16.5). These mice exhibit urothelial hyperplasia and eventual UCC by 13.5 months of age (Yoo et al., 2006). In a study that apparently used the same *Pten*-null model (*FabpCre Pten^{fl/fl}*), the mice developed non-invasive UCC in 10% of cases after a long latency (>40 weeks) (Tsuruta et al., 2006). The long latency and low tumour rate could be because of the slow proliferation rate within the urothelium and the requirement for additional genetic events to drive urothelial carcinogenesis. More recently, using the same *Pten*-floxed mouse and the *Ksp-Cre* promoter (which results in *Pten* deletion specifically in renal epithelial cells), Qian and colleagues observed UCC of the renal pelvis in 57% of mice at 12 months (Qian et al., 2009). Interestingly, they also found upregulation of p-mTOR in these tumours, again suggesting that inhibitors of this pathway, such as rapamycin, might be promising therapeutic agents.

RB

It has been reported that individuals with germline mutations of *RB1* have a higher risk of developing epithelial carcinoma, particularly UCC (Fletcher et al., 2004). Loss of heterozygosity (LOH) of *RB1* is also prevalent in invasive UCC (Cairns et al., 1991). In these scenarios, RB is dysfunctional and cannot inhibit the E2F family of transcription factors, leading to an increase in cell proliferation. Mutation of both *p53* and *RB* occur in over 50% of invasive UCC cases, with patients having an increased rate of recurrence and/or progression, and a worse overall survival than patients with single gene mutations (Grossman et al., 1998). However, inactivating mutations in *RB* occur in only 29% of human UCC cases (Fig. 2), and the true figure is potentially less than this, given the small number of samples analysed (and even if the analysis were restricted to muscle-invasive tumours only). In keeping with this idea, mice with targeted urothelial expression of SV40 develop CIS and subsequent invasive tumours (Zhang et al., 1999; Grippo and Sandgren, 2000). Notably, the CIS in this model evolved to high-grade papillary UCC before progressing to muscle-invasive disease. However, as noted above, it was more recently shown that urothelial targeting of SV40 using the correct *UPKII* promoter results in CIS without progression to invasive disease (Ayala de la Pena et al., 2011). Finally, whether a similar course of disease also occurs in humans with inactivation of both *p53* and *RB* is not known.

A recent study concluded that inactivation of *RB*, in combination with either *PTEN* or *p53* inactivation, in mouse bladder did not lead to a discernible phenotype (Puzio-Kuter et al., 2009). Similarly, work by the Wu lab demonstrated that urothelial-specific deletion of both copies of *Rb* failed to accelerate urothelial proliferation (He et al., 2009). Instead, it activated a 'failsafe' signature by profoundly activating the *p53* pathway and led to apoptosis. Deletion of *p53* in these *Rb*-null urothelial cells also did not remove the barrier to tumorigenesis, with only 2% of mice progressing to non-invasive tumours. These double-mutant mice (null for both *p53* and *Rb*) are, however, extremely sensitive to chemically induced carcinogenesis. After 10 weeks of treatment with 0.01% BBN, the mice developed early-onset muscle-invasive UCC in 50% of cases, compared with 0% in mice carrying either mutation alone. This mouse study suggests that loss of both *p53* and *RB* is necessary for progression of UCC, but insufficient to initiate invasive UCC. It is presumed that other genetic alterations are required to initiate invasive UCC.

These data reinforce the idea that transformation of the urothelium requires multiple mutations. Initially, Rb loss provokes a p53-mediated apoptotic response. This response is muted when RB-deficient urothelial cells are also p53 null. Urothelial cells that are null for both p53 and Rb are therefore much less capable than those defective for Rb only to mount an apoptotic response following treatment with genotoxic agents such as BBN and are more vulnerable to transformation.

The role of Wnt signalling in UCC Human UCC

The Wnt pathway is known to control many events during embryonic development, and it regulates homeostatic self-renewal in a number of adult tissues. Mutations in components of this pathway are associated with various cancers, owing to the influence of the Wnt pathway on processes such as cell fate, proliferation and motility (Polakis, 2000; Oving and Clevers, 2002). For example, it is well established that germline and somatic mutations of APC – encoding a key component of Wnt signalling that acts as a scaffold protein for the pathway's key intracellular mediator, β -catenin – are found in most colorectal cancers (Kinzler et al., 1991; Cottrell et al., 1992; Rubinfeld et al., 1993). However, in the case of UCC, controversy surrounds whether the disease is associated with somatic mutations in key components of the Wnt signalling pathway, including APC (Miyamoto et al., 1996; Bohm et al., 1997; Stoehr et al., 2002; Urakami et al., 2006a) and β -catenin (Shiina et al., 2001; Shiina et al., 2002; Burger et al., 2008). Miyamoto and colleagues found LOH of the APC locus in only 6% of 16 UCC tumours analysed (Miyamoto et al., 1996), whereas another study reported LOH for the APC locus in 50% of 30 tumours analysed (Bohm et al., 1997). In a larger study, Stoehr and colleagues found LOH in 10% of 72 tumours, but no APC mutations were found in 22 tumours or four cell lines, apart from a common single nucleotide polymorphism (SNP) at codon 1493. The COSMIC database detected APC mutations in 12% of 93 samples analysed. In all of these studies, both non-invasive and invasive tumours were included and analysed as a single entity.

It appears that mutations of different proteins in the Wnt pathway could contribute to UCC. Many earlier studies used immunohistochemistry to demonstrate upregulation of β -catenin (Shimazui et al., 1996; Garcia del Muro et al., 2000; Nakopoulou et al., 2000; Zhu et al., 2000; Kashibuchi et al., 2006). In addition, Urakami and colleagues have shown that CpG hypermethylation silences the region encoding Wnt inhibitory factor-1 (Wif-1), an inhibitor of the Wnt signalling pathway, and that this silencing is a frequent event in bladder tumorigenesis (Urakami et al., 2006b). Recently, Kastiris and colleagues demonstrated missense (13%) and frameshift (3%) deletions in the APC protein adjacent to the β -catenin binding sites in bladder tumours (Kastiris et al., 2009). They also found that either APC mutations or β -catenin accumulation was associated with a shorter disease-free interval and shorter disease-specific survival in a multivariate analysis. In another study, epigenetic silencing of the four secreted frizzled receptor proteins (SFRPs), which are antagonists of the Wnt signalling pathway, was demonstrated as an independent predictor of invasive bladder cancer (Marsit et al., 2005). In a cohort of 355 patients, a linear relationship between the magnitude of risk of invasive disease and the methylation of more SFRP genes was observed; invasive disease risk and SFRP gene methylation correlated

with a reduction in overall survival ($P < 0.0003$). Overall, these studies suggest a key role for dysregulated Wnt signalling in invasive bladder cancer (Fig. 1). Indeed, in our own studies, we found that Wnt signalling was activated in approximately a third of clinical UCC samples (Ahmad et al., 2011c; Ahmad et al., 2011a). A significant correlation between activation of the Wnt pathway and mutually exclusive activation of either the PI3K or MAPK signalling pathways was observed ($P < 0.0001$). Therefore, future mechanistic studies into the potential involvement of Wnt pathway activation in the initiation of bladder tumorigenesis are warranted.

Modelling mutations of the Wnt pathway in mice

The accumulation of human data suggesting a role for Wnt in UCC prompted us to examine whether dysregulation of this pathway could induce the disease in mice. To drive dysregulated Wnt signalling, 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). On addition of *UPEII-Cre*, exon 3 was deleted, causing β -catenin to accumulate in the urothelial cells and activate Wnt signalling in a bladder-specific manner (Moon et al., 2004). Expression of this activated form of β -catenin led to the formation of localised hyperproliferative lesions in the bladder epithelium by 3 months, which did not progress to malignancy (Ahmad et al., 2011c). Furthermore, *UroIICRE+ β -catenin^{exon3/+}* mice showed a marked upregulation of the PTEN tumour suppressor protein that seemed to be a direct consequence of activating Wnt signalling in the bladder, suggesting a potential compensatory mechanism that could prevent progression to invasive disease.

To investigate potential cooperative effects, we combined *UroIICRE+ β -catenin^{exon3/+}* mice with transgenic mice engineered to activate either the PI3K (*Pten^{fl/fl}*) or MAPK (*Hras^{Q61L}*) pathway specifically in the urothelium. Both gene combinations induced low-grade, non-invasive UCC. The *UroIICRE+ β -catenin^{exon3/+} Pten^{fl/fl}* tumours contained increased activation of the AKT pathway and their growth was dependent on mTOR signalling (i.e. regression occurred with rapamycin treatment). By contrast, the tumours in *UroIICRE+ β -catenin^{exon3/+} Hras^{Q61L}* mice, although phenotypically similar, required MAPK signalling (i.e. regression occurred with MEK inhibition, but not with rapamycin) (Ahmad et al., 2011c; Ahmad et al., 2011a).

Thus, using transgenic models, either PI3K or MAPK pathway activation can synergise with Wnt signalling to drive low-grade, non-invasive UCC in vivo, leading to tumours with differing molecular and treatment profiles (Fig. 1). These tumours mirror human UCCs with respect to pathway activation and mutational profile; however, some human data also suggest that Wnt pathway activation can contribute to invasive disease. Although targeting the Wnt pathway could be effective for treating UCC cases in which dysregulation of this pathway drives disease, some cases would not respond to cell-surface Wnt inhibitors; for example, cell-surface Wnt inhibitors would not affect cells carrying mutations in β -catenin or with increased methylation of SFRPs.

Outlook

Mouse models developed thus far have provided researchers with new insights into the molecular mechanisms and pathways involved in bladder cancer. However, many limitations remain. There is a paucity of models that recapitulate muscle-invasive metastatic UCC

that would be invaluable for preclinical therapeutic studies. In human UCC, metastases are usually observed in bone, lung and liver, whereas mouse models of the disease show metastasis to lung and liver. Why spontaneous osseous lesions are not observed in mice remains to be elucidated. This could be due to various reasons, including fundamental physiological differences between humans and mice (e.g. pelvic venous drainage), the inability to accurately detect metastasis in mice or the possibility that the genetic defects created in currently available GEM models are insufficient to initiate bone metastasis, suggesting that a linear progression of mutations contributes to this outcome in humans. Furthermore, given the short lifespan of mice, it is possible that the bladder tumours induced in GEM models are too aggressive and thus do not allow enough time for metastatic lesions to develop before the animals must be sacrificed owing to primary tumour burden. The removal of the primary tumour bulk in these mice might allow more time for metastasis to develop.

There are few published preclinical trials on potential UCC therapies using mouse models. Our work and that of the Puzio-Kuter group has assessed the action of pharmacological agents such as rapamycin and MEK inhibitors in GEM models (Kinkade et al., 2008; Seager et al., 2009; Ahmad et al., 2011c; Ahmad et al., 2011a). As additional genes and biomarkers are identified through genetic studies of human UCC, it will be essential to develop new mouse models based on these findings. It is hoped that models developed in the future will mimic human bladder tumorigenesis more closely and will offer attractive platforms for preclinical trials with new therapies.

The next generation of mouse models of UCC will apply inducible systems, allowing specific genes to be turned on and off in a bladder-specific manner for defined time periods, and to be expressed at different levels, enabling a better understanding of what occurs in the human disease. In addition, other approaches involving unbiased somatic mutagenesis will aid the search for genes that contribute to the initiation and/or progression of UCC. For example, in vivo transposon-based methods can help to identify biologically relevant genetic lesions involved in tumorigenesis (Collier et al., 2005; Dupuy et al., 2005). This type of system is ideal for identifying events that collaborate and/or synergise with mutations in *HRAS*, *FGFR3*, *PTEN* and components of the Wnt signalling pathway to drive UCC in vivo.

The application of new technologies will also help to advance UCC research. For example, in vivo imaging of tumours (Box 1) has revolutionised the way that researchers can use mouse models of cancer, allowing more sophisticated studies of disease progression and of the effects of therapeutic interventions (Weissleder, 2002). In addition, recent developments in approaches for large-scale sequencing (e.g. next-generation sequencing), means that these technologies are now so widely available that we will soon be able to sequence a complete human genome for US\$1000 or less (Pfeifer and Hainaut, 2011). Applying this technology to cancer genome sequencing will have major implications for understanding how the normal genome evolves to a cancerous state, help to identify possible biomarkers and therapeutic targets, and move us closer to the dream of personalised medicine. However, the information revealed by cancer genome sequencing has also highlighted the simplicity of currently available mouse models: it is no longer enough to compare a mouse model and a primary

Box 1. Applying in vivo imaging to mouse models of cancer

Ultrasound imaging is an approach that allows regular and convenient assessment of disease progression and treatment response through monitoring tumour size non-invasively (Ahmad et al., 2011c). Both computer tomography (CT) and magnetic resonance imaging (MRI) have been successfully employed in the analysis of tumour growth and drug effects in mouse models of other cancers, but not yet in models of UCC (Huse and Holland, 2009; Martiniova et al., 2010). These modalities do not provide functional information, however. Thus, positron emission tomography (PET), which provides three-dimensional imaging data on the dynamics of labelled biological molecules and metabolites, is becoming more widely used to assess tumours at the functional level (Yang et al., 2006; Garrison et al., 2007; Riemann et al., 2008; Pantaleo et al., 2009). This technique is effective for functionally assessing tumours because their metabolism is often dysregulated compared with normal tissue. PET can also be used to visualise tumours at early stages, to quantify therapy-induced metabolic changes and to identify micro-metastases. Finally, new anti-cancer compounds and probes can be labelled to allow investigation of their pharmacokinetics and pharmacodynamics non-invasively in mouse models. As these techniques become more widely available, we envisage their usage in mouse models of UCC, complementing the advancing molecular knowledge of the disease.

human tumour at the level of tissue and cellular phenotypes. Thus, the introduction of affordable genetic and genomic profiling has raised the benchmark for validating mouse models at the genetic level.

Nevertheless, mouse models of UCC will continue to provide invaluable information on the biology, initiation and progression of the disease, aid in the identification of relevant biomarkers, and facilitate preclinical studies of new anti-cancer agents. It is hoped that the findings of mouse studies can ultimately be clinically translated to improve the diagnosis, management and treatment of patients with UCC.

COMPETING INTERESTS

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

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