Ras mutation cooperates with $\beta$-catenin activation to drive bladder tumourigenesis

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Mutations in the Ras family of proteins (predominantly in H-Ras) occur in approximately 40% of urothelial cell carcinoma (UCC). However, relatively little is known about subsequent mutations/pathway alterations that allow tumour progression. Indeed, expressing mutant H-Ras within the mouse bladder does not lead to tumour formation, unless this is expressed at high levels. The Wnt signalling pathway is deregulated in approximately 25% of UCC, so we examined if this correlated with the activation of MAPK signalling in human UCC and found a significant correlation. To test the functional significance of this association we examined the impact of combining Ras mutation (H-RasQ61L or K-RasG12D) with an activating $\beta$-catenin mutation within the mouse bladder using Cre-LoxP technology. Although alone, neither Ras mutation nor $\beta$-catenin activation led to UCC (within 12 months), mice carrying both mutations rapidly developed UCC. Mechanistically this was associated with reduced levels of p21 with dependence on the MAPK signalling pathway. Moreover, tumours from these mice were sensitive to MEK inhibition. Importantly, in human UCC there was a negative correlation between levels of p-ERK and p21 suggesting that p21 accumulation may block tumour progression following Ras mutation. Taken together these data definitively show Ras pathway activation strongly cooperates with Wnt signalling to drive UCC in vivo.

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Urothelial cell carcinoma (UCC) of the bladder is the fifth commonest cancer in the world.1 The majority (75%) of these tumours are non-invasive, well-differentiated tumours (i.e., Ta/T1) that can be controlled by transurethral resection of the bladder wall. However, up to 70% of the patients with a superficial UCC will have recurrences after its removal, and 10–15% will progress to muscle-invasive UCC. Even in those that do not progress, regular surveillance by cystoscopy is required, making bladder cancer one of the most expensive and labour-intensive cancers to treat.

A number of genetic and epigenetic alterations involved in bladder tumourigenesis have been identified, including activating mutations in FGFR3 and Ras family genes, amplification of ERBB2, and loss of the TP53, RB1 and PTEN tumour suppressors.2–5

H-Ras was the first human oncogene isolated in human UCC, being mutated most often at codon 12, 13 and 61 (ref. 6). As well as becoming constitutively active, mutation can also result in overexpression of the protein because of alternative splicing of the last intron. Despite the controversy regarding the reported mutation frequency rate recent studies indicate that H-Ras mutation occurs in approximately in 30–40% of low-grade papillary and up to 10% of muscle-invasive UCC.7,8

Transgenic models have provided invaluable information regarding the molecular mechanisms behind H-Ras activation.9 Previously published studies by the Wu lab demonstrate that mice carrying a transgene with a H-RasQ61L mutation have early-onset urothelial hyperplasia, with this hyperplasia progressing to low-grade non-invasive papillary tumours. Interestingly, tumour latency depended on transgene number: in mice that had one or two copies of the H-Ras transgene (low-copy), the tumour latency was up to 2 years. Histologically, by 3–5 months the urothelial layer has become hyperplastic (from 3 layers to 6–7 layers). At 8 months, the urothelium contained areas of nodular hyperplasia. By 26 months of age, 63% of mice developed superficial non-invasive UCC. These lesions remained non-invasive during the follow-up period. In contrast, mice harbouring ‘high-copy’ numbers of the H-Ras transgene (30–48 copies) succumbed to death by 5 months of age. The mice had evidence of significantly enlarged bladders and associated bladder outflow obstruction (hydronephrosis and hydroureter). Again these tumours were of a papillary non-invasive histology, with no evidence of muscle invasion or metastases.

The fact that the bladder tumours in ‘low-copy’ mice developed localised, superficial papillary tumours with a much longer latency, suggests, in the absence of overexpression, H-Ras, activation requires a secondary event, either genetic or epigenetic, to fully induce bladder tumours.10 Conversely few studies have looked at K-Ras mutations in human bladder cancer, although those that have suggest a wide variation in frequency (4–29%).11–13 Interestingly, a study by Vageli et al demonstrated the K-Ras oncogene was
overexpressed in 15 of the 26 (58%) samples although there was no classification of tumour stage. As yet there have been no studies investigating targeting K-Ras mutation to the murine bladder in an equivalent manner to H-Ras.

Although the human data regarding Wnt pathway activation is quite controversial, it is now becoming clear that the pathway is activated in a proportion of UCCs. As yet segregation of Wnt pathway high tumours into the non-invasive, papillary pathway or the muscle-invasive pathway has not been performed, although Wnt pathway activation has been associated with a poor prognosis and resistance to therapy. We have previously shown that activation of the Wnt signalling pathway in the bladder of mice alone fails to drive UCC, however, it strongly cooperates with PTEN loss to drive tumourigenesis.

p21 is downregulated in the majority of urothelial carcinomas that harbour p53 mutation. Similar to p53, loss of p21 expression is also associated with a higher recurrence and lower survival rates than those tumours with maintained p21 expression levels, irrespective of tumour grade and pathological stage. It was also noted that maintenance of p21 expression appeared to negate the effects of p53 alterations on UCC progression. Moreover, Shariat et al. demonstrated that positive p21 expression was independently associated with lower UCC recurrence and a slower progression to carcinoma in situ (CIS) (with no muscle-invasive disease), possibly by p53-independent modulation of p21. Thus far there have been no functional studies to investigate whether p21 modifies UCC tumourigenesis in vivo.

Therefore, given the observation that Ras mutation is inefficient at driving bladder carcinogenesis alone in the mouse, the aim of this study was to test whether Ras overexpression or mutation cooperates with deregulated Wnt signalling to promote UCC in the murine urothelium.

**Results**

**Human UCC demonstrate correlation between Wnt and Ras activation.** In human urothelial cancer, a number of studies have suggested Wnt signalling is important. Of particular note is the demonstration that nuclear β-catenin is associated with a poor prognosis, and methylation of the inhibitors of Wnt signalling, the SRFPs, act as markers of a bad prognosis. Indeed the methylation of these proteins has been suggested as a marker of invasive bladder carcinoma.

We have previously shown that in human UCC upregulation of the Wnt pathway (nuclear β-catenin) is associated with activation of the PI3K-pAKT pathway (loss of PTEN/ upregulation of pAKTSer473). Given this strong correlation, we wanted to investigate which other oncogenic/tumour-suppressor pathways were associated with Wnt signal activation. Using the same tissue microarray of 80 human bladder cases: 60 UCC (transitional cell carcinomas), and 20 benign controls (Folio Biosciences, Columbus, OH, USA) we chose to investigate the MAPK pathway. Using the histoscore technique, we demonstrated a significant correlation between upregulation of β-catenin and activation of pERK1/2 (n= 24/56, Pearson correlation coefficient = 0.6769, P< 0.0001 (two-tailed), Minitab v15, Minitab Inc., PA, USA) (Figure 1). Interestingly, these tumours appear to be a different subset to the ones expressing high levels of p-AKT as no correlation between levels of p-ERK and p-AKT was observed (Pearson correlation coefficient = 0.083, P = 0.45, Minitab v15).

**Ras activation alone does not lead to UCC in the mouse.** To test whether oncogenic Ras mutation would lead to UCC, we utilised mice carrying the constitutively active H-RasQ61L oncogene (‘low-copy’). These ‘low copy’ mice have two copies of the oncogenic rabbit transgene. Although Zhang et al. demonstrated tumour latency from 10 to 26 months of age; in our hands no tumours were observed in our H-RasQ61L mice cohort at killing (12 months of age). As previously published, we noticed global hyperplasia of the 12-month-old H-RasQ61L urothelium compared with wild type (Figures 2a and b). There was little proliferation as observed by Ki-67 staining (Figures 2c and d). We observed upregulation of members of the MAPK family: pERK1/2 and pMEK1/2 in comparison to wild type (Figures 2e and f, g and h, respectively). Importantly, we noticed an upregulation of p21 in the mutant urothelium (Figures 2i and j), without any accompanying nuclear accumulation of p53 (data not shown). When we investigated members of the PI3K-pAKT-p-mTOR (pAKTSer473, p-mTOR and pS6K) signalling pathways, we failed to observe upregulation in the urothelium (Supplementary Figure 1). In terms of Wnt signalling, we observed scattered basal cells with nuclear β-catenin and corresponding scattered induction of the Wnt target gene Sox9 in the basal layer (Supplementary Figure 2). The hyperplasia was evident in the urothelia of 3-month-old H-RasQ61L mice. In this 9-month period, there was no progression to malignancy, despite the thickness of the urothelium increasing moderately (data not shown) suggesting that tumour-suppressor mechanisms were stopping progression.

We also demonstrated that the UrolCRE+ K-RasG12D mice do not develop cancer when aged to 18 months of age (n = 20). These mice carry a point mutation of the K-Ras (K-RasG12D) allele, therefore Cre-mediated recombination leads to deletion of a transcriptional termination sequence (Lox-Stop-Lox) and expression of the oncogenic protein. In contrast to the H-RasQ61L mice, no phenotypic abnormalities were observed in the urothelium of UrolCRE+ K-RasG12D mice. There was little upregulation of pERK1/2 and pMEK1/2 and no upregulation of members of the PI3K-pAKT-p-mTOR pathway (Supplementary Figure 3). There was no evidence of increased Wnt signalling in these urothelium, that is, no nuclear β-catenin or accumulation of Wnt targets such as Sox9 (Supplementary Figure 3).

**Ras activation cooperates with β-catenin to drive UCC formation.** We have previously demonstrated that UrolCRE+ β-cateninK1439exon3 mice develop areas of hyperproliferation in their urothelium from 3 months of ages (increased levels of Ki-67 and BrdU incorporation), but these lesions do not progress when these mice are aged up to 18 months. These lesions have high levels of the tumour-suppressor p21 and PTEN, suggesting that further mutations
are required to progress these lesions. We demonstrated that adding a second mutation (through genetic deletion of PTEN) to these lesions was sufficient to progress it to frank carcinoma.14

Given that our human data suggested a correlation between upregulated Wnt signalling and MAPK pathway activation, and that neither single mutant murine model develops UCC,14 we decided to test the cooperation of β-catenin and Ras activation in vivo by generating the UroICRE⁺β-catenin exon3/exon3H-RasQ61L (n = 29) cohort. Two experiments were performed, with mice either being killed at 3 months of age or aged until tumour development. In contrast to the singly mutant mice, the UroICRE⁺β-catenin exon3/exon3H-RasQ61L mice rapidly developed symptoms of bladder tumourigenesis, namely abdominal swelling, haematuria (blood in the urine) and hunching (Figure 3j). On necropsy, we observed bladder tumours with histological progression to non-invasive papillary carcinomas were observed. We found no evidence of metastasis in any of our models.

Tumours demonstrated upregulation of the proliferation marker Ki-67 (Figure 3b), with the number of proliferating cells within each tumour being much higher than in the UroICRE⁺β-catenin exon3/exon3 mice (Supplementary Figure 4). This may possibly explain why lesions do not progress in the single mutant. We observed significant upregulation of pERK1/2 and pMEK1/2 (Figures 3d and e, Supplementary Figure 5), as well as upregulation of nuclear β-catenin and the Wnt target genes such as Sox9 (Figures 3f and g, Supplementary Figure 5). These tumours demonstrated negligible levels of pAKT and an intact PTEN signal (Figures 3h and i). This in contrast to our previous study where we deleted PTEN in combination with β-catenin activation and tumours have upregulation of p-AKT/p-mTOR and were dependent on mTOR signalling.14 Given the high levels of proliferation within the tumours, we next examined the expression of p21 within the tumours as this is high in premalignant lesions in β-catenin mice and the H-Ras mice alone (Figure 3c). Quantification of p21 levels showed a significant reduction in positivity in the tumours compared

![Image of β-catenin, pERK1/2, and p21 expression in Wildtype and UCC samples](image_url)

**Figure 1** Correlation between β-catenin and pERK1/2 in human bladder UCC TMA. IHC of benign and UCC bladder samples revealing no β-catenin, pERK1/2 or p21 expression (a–c). In the UCC samples there was expression of nuclear β-catenin (d) corresponding upregulation of pERK1/2 (b), with scattered expression of p21 (c). Scatterplot demonstrating correlation between β-catenin and pERK1/2 (d). Each core size is 1.5 mm.
upregulation of the MAPK pathway members: pERK1/2 (e) by Ki67 staining in wild-type and mutant mice (c). MAPK signalling pathway. The H-RasQ61L with the wild-type urothelium (a). We noticed significant upregulation of the MAPK pathway members: pERK1/2 (f) and pMEK1/2 (g and h) as well as p21 (i and j) in the mutant mice compared with wild type.

with the hyperplastic urothelium of the single HRAS mutant (Supplementary Figure 6, P-value >0.04, Mann–Whitney) and premalignant lesions from mice where β-catenin is activated alone within the bladder. Again, there was no nuclear accumulation of p53 in these tumours (data not shown).

To assess whether there was a correlation in human UCC between the loss of p21 and high levels of MAPK pathway, we stained our UCC tissue microarrays (TMAs) for p21 levels and correlated these levels with p-ERK1/2. The presence of nuclear p21 was observed in a third of the tumour samples and correlated with high levels of MAPK pathway, accumulation of p53 in these tumours (data not shown).

Finally, to assess if this cooperation was restricted to H-Ras mutation, we generated a cohort UrolIcre⁺ β-catenin⁺/+ /K-RasG12D (n = 25). In contrast to the single mutant mice, the UrolIcre⁺ β-catenin⁺/+ /K-RasG12D (mean survival 185 days, median 200 days) mice phenocopied the

β-Catenin cooperates with Ras to cause UCC. Given this downregulation of p21 in tumours compared with high levels of p21 in the single mutant H-Ras and β-catenin activated bladders, we next wished to test the functional importance. To do this, we intercrossed p21 knockout mice to the β-catenin exon3 mice and asked if this now facilitated tumour progression. The reason for choosing to cross to p21 knockout mice to the β-catenin exon3 mice was that p21 was upregulated in nearly every cell of the early lesions so could be acting as a tumour suppressor in this instance. Thus, we generated the following cohorts: UrolIcre⁺ p21⁻/⁻ and UrolIcre⁺ β-catenin⁺/+ /p21⁻/⁻ (n = 6 and 16, respectively). We then aged mice until tumour development. The UrolIcre⁺ β-catenin⁺/+ /p21⁻/⁻ mice rapidly developed urothelial tumours (mean 237, median 238 days). In comparison, UrolIcre⁺ p21⁻/⁻ mice did not develop tumours and had no urothelial phenotype (Figure 5g). Tumours from the UrolIcre⁺ β-catenin⁺/+ /p21⁻/⁻ mice were sensitive to MEK inhibition because of their dependence on the MAPK signalling pathway.

p21 upregulation blocks β-catenin driven UCC. Given the UrolIcre⁺ β-catenin⁺/+ /H-RasQ61L tumours were driven by MAPK, given the elevated levels of pMEK1/2 and pERK1/2 and not PI3K (low levels of pAKT) (Figure 4a). This is converse to our UrolIcre⁺ β-catenin⁺/+ /Pten⁻/⁻ mouse tumours that we have previously demonstrated are dependent on PI3K and mTOR signalling (and not MAPK) (Figure 4a). Thus, to test whether MAPK signalling was driving our mutant Ras bladder tumours we treated 12-month-old UrolIcre⁺ β-catenin⁺/+ /H-RasQ61L mice that had palpable tumours and haematuria with PD184352, MEK1/2 inhibitor, (n = 3) or vehicle (n = 3). We also treated our UrolIcre⁺ β-catenin⁺/+ /H-RasQ61L mice, which we have shown previously, are dependent on mTOR signalling with this same regime. A significant regression of tumour bulk between the vehicle and MEK inhibitor treated mice (P = 0.04, Mann–Whitney) and no significant change in tumour size or proliferation. Taken together, these data suggest UrolIcre⁺ β-catenin⁺/+ /H-RasQ61L tumours are sensitive to MEK inhibition because of their dependence on the MAPK signalling pathway.
Discussion

Although UCC can be classified in two major subtypes (superficial and invasive), with H-Ras and FGRF3 mutations being associated with superficial cancer and RB and p53 mutation being associated with invasive cancers, very little is known how these pathways drive bladder carcinogenesis in vivo. It was thus of some surprise that H-Ras mutation was not sufficient to drive bladder carcinogenesis in the mouse unless overexpressed. In a similar scenario, we have data to suggest that FGFR3 mutation is not sufficient to drive bladder carcinogenesis in the mouse (Ahmad, unpublished data). Moreover, it has recently been shown that PTEN and p53 only drive bladder tumourigenesis when combined.22

The high propensity for bladder carcinogenesis in humans may thus reflect the relatively low turnover of the bladder epithelium (in comparison with the intestinal epithelium) and the exposure of the bladder epithelium to carcinogens, which allow multiple mutations to occur which provokes tumourigenesis. It is thus critical to identify these other signalling pathways that cooperate with these presumably initiating mutations to drive cancer.

In this study, we show that deregulation of Wnt signalling strongly cooperates with the activation of Ras to drive tumourigenesis. First, we are able to demonstrate a correlation between upregulation of β-catenin and pERK1/2 in our TMA. Unfortunately there was no segregation into UCC subtype, limiting our ability to predict whether this activation occurs in superficial papillary and or muscle-invasive UCC. We are currently addressing this concern by expanding our human cohort. As our murine tumours are not invasive, it would suggest that human tumour would develop via the non-invasive/papillary UCC pathway; however, other mutations could then occur to drive invasive UCC.
Figure 4  MAPK, but not PI3K signalling, is upregulated in UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 H-Ras\textsuperscript{Q61L} bladder tumours. Immunoblotting demonstrates upregulation of pMEK1/2 and pERK1/2 in the UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 H-Ras\textsuperscript{Q61L} tumours, but not pAKT, which is upregulated in the UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 Pten\textsuperscript{fl/fl} (but not pMEK1/2 or pERK1/2). We demonstrate that MEK inhibition can regress the UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 H-Ras\textsuperscript{Q61L} tumours in terms of size and proliferation (b–c) (P < 0.05, Mann–Whitney). We see no difference in the UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 Pten\textsuperscript{fl/fl} tumour size nor proliferation after MEK inhibition (b–c). We also notice a reduction in pERK1/2 and pMEK1/2 protein levels on IHC in the UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 H-Ras\textsuperscript{Q61L} tumours (d–e) (P < 0.05, Mann–Whitney). U\textsubscript{B} H-Ras\textsuperscript{Q61L} (UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 H-Ras\textsuperscript{Q61L}) and U\textsubscript{B} Pten\textsuperscript{fl/fl} (UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 Pten\textsuperscript{fl/fl})

UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 p21\textsuperscript{−/−}

Figure 5  Histology of UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 p21\textsuperscript{−/−} mouse. H&E reveals bladder tumour (a) with upregulation of Ki67, \textbeta\textcatenin and pERK1 (b–d). There is minimal upregulation of p53 and p21 (e and f). Black bar measures 200 \textmu m (20 \times magnification). Kaplan–Meier curves of tumour-free survival of UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 p21\textsuperscript{−/−} and UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 p21\textsuperscript{−/−} cohorts (g). p21 (UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 p21\textsuperscript{−/−}) and Bcatenin p21 (UroICRE\textsuperscript{\textbeta}\textcatenin\textexon3/exon3 p21\textsuperscript{−/−})
It should be noted that the H-Ras and the K-Ras mice used in
this study are different in respect to knock-in/overexpression
status. We utilised Lox-Stop-Lox K-Ras conditional mouse strain,
in which expression of oncogenic K-Ras \( G^{12D} \) is controlled by a removable transcriptional termination
Stop element.\(^2\) As the endogenous K-Ras locus is targeted in
the K-Ras\( G^{12D} \) strain, only endogenous levels of oncogenic
K-Ras\( G^{12D} \) are expressed (not overexpression). In contrast,
the H-Ras model is an overexpression of the oncogenic
rabbit transgene (point mutation at codon 61 of the second
exon converting CAG (encoding glutamine) to CTG (leucine)).\(^9\) Transgene copy number varied from two copies
(low copy, UCC from 10 to 26 months of age) to 48 copies
(high copy, UCC from 4 months of age). This may account
for the difference in phenotypes of the Ras mutations alone,
namely no phenotype in the \( UroICRE^{-}-K-Ras^{G12D} \) and pronounced hyperplasia in the overexpression \( H-Ras^{Q61L} \)
model. However, it is difficult to make any definitive conclu-
sions because we are dealing with different Ras alleles.
In anticipation we are currently repeating these experiments
with a Lox-Stop-Lox H-Ras\( G^{12V} \) conditional mouse strain,
which will allow us to make direct comparisons. However,
despite these differences in the Ras protein (\( H-Ras^{Q61L} \)
or K-Ras\( G^{12C} \)) and the levels of expression of the mutant,
the cooperation of the either K-Ras or H-Ras mutations
were comparable. This suggests that increasing levels of
MAPK signalling in the context of activating Wnt signalling
will drive bladder carcinogenesis. This was further
confirmed by tumour regression after inhibition of the MAPK
signalling cascade.

Interestingly, despite modelling all these mutations in the
murine urothelium, these tumours remain non-invasive and
do not metastasize despite extended follow-up. This is
consistent with tumours that have H-Ras mutations in
humans. These tend to remain superficial and despite
recurrence rarely progress to metastasis. Therefore, the
model presented here still reflects a useful model of human
disease for preclinical testing. It is interesting to note the
tumours that develop following H-Ras mutation and \( \beta \)-catenin
activation have increased MAPK signalling (although not
p-AKT/p-mTOR activation), while tumours that have PTEN
loss and \( \beta \)-catenin activation show little MAPK pathway and
instead have high levels of p-AKT/p-MTOR.\(^1\) We show that
as a result these tumours have differential sensitivity to MEK
and mTOR inhibition. As a result these models may be
relevant for human tumourigenesis as we show that there is a
subset of patients that have a correlation between high
\( \beta \)-catenin and high p-AKT and this group is distinct from those
patients that show high levels of p-ERK and \( \beta \)-catenin.\(^1\) Moreover,
given neither PTEN nor H-Ras mutation alone
leads to P13 kinase or MAPK pathway activation respectively
\( \text{in vivo} \), it argues in the bladder strong feedback loop pathways
exist so deregulated signalling is not instigated. Thus,
activation of Wnt signalling in this case is cooperating to drive
either MAPK or P13 kinase pathway activation depending on
the cooperating mutation. To this end, it is interesting to note
previous studies have linked Wnt pathway activation to
increase MAPK signalling.\(^23,24\) Moreover, this may also help
to explain why multiple mutations are required to drive cancer
within the murine bladder.

Given the milieu of the bladder, other mutations may occur
in the recurrent tumours that may then drive metastasis. It is
likely that these may be in the p53 tumour suppressor as these
mutations are common in metastatic bladder cancers and
co-deletion of PTEN and p53 led to metastatic bladder cancer.
The precise mechanism for this is unclear, in many cancers
 genomic instability is associated with metastasis although it is
still unclear how functionally important this is. A number of
groups (including our own) have shown that may also be
dominant gain of function properties to p53 mutations that
may also drive metastasis \( \text{in vivo} \).\(^25,26\)

Mechanistically, it appears that p21 may block tumour
progression within the bladder. In our study, we show
p21 activation in both \( UroICRE^{+}\beta\text{-catenin}^{\text{exon3/exon3}} \) and
\( H-Ras^{Q61L} \) bladders and this is associated with failed
progression to cancer. We have recently shown in a model
of pancreatic cancer that p21 loss accelerates tumourigenesis
following K-Ras\( G^{12D} \) mutation and identifies a subset of a
patient with poor prognosis.\(^27\) Thus, in both scenarios we
show that p21 can functionally block tumourigenesis and is
downregulated in human patients. Given the upregulation of
p21 in \( H-Ras^{Q61L} \) bladders, further studies intercrossing p21
knockout mice to these \( H-Ras^{Q61L} \) transgenic mice should be
performed and are currently underway in the laboratory. As
yet it is unclear the precise mechanism of p21 downregulation
\( \text{in vivo} \) as it is rarely mutated though mutation or down-
regulation of upstream factors such as p53 or Lkb1 could
account for a downregulation of this pathway. Our working
hypothesis is that in bladder cancer, p21 induction would
be observed early in precursor lesions (CIS) and low-grade
tumours, while the more aggressive and muscle-invasive
tumours will show loss of p21 and thus there may be
multiple ways to obtain p21 downregulation during bladder
carcinogenesis.

In summary, we have functionally the tested the ability of
Ras and Wnt pathway to cooperate to drive bladder
carcinogenesis and find that they robustly cooperate to
promote non-metastatic bladder tumours. Given there is a
subset of human patients that show high levels of p-ERK and
\( \beta \)-catenin, it suggest that combinatorial targeting of the MAPK
and Wnt pathways may be efficacious in these patients.

Subjects and Methods

Mice. Uroplakin II Cre mouse (\( UroICRE^{+}\))\(^28\) were intercrossed with mice
harbouring \( \beta\text{-catenin}^{\text{exon3/exon3}} \), \( K-Ras^{G12D} \), \( H-Ras^{Q61L} \) and p21\(^C0\) in combinations
as described below.\(^2,21,28-30\) Mice were genotyped by PCR as previously
described.\(^2,21,28-30\) Mice were of a mixed background and littermates were used
as controls. All experiments were carried out in accordance with UK animal
regulations.

When bladders were excised they were all emptied of urine, before being placed
in formalin for overnight fixation before paraffin embedding. All bladders were
processed and cut in the same manner by a single histology technician to all
standardisation.

Immunohistochemistry. IHC was performed on formalin fixed, paraffin-
embedded samples. For each genotype, we stained at least three samples from
different mice and took representative images for this paper. We used antibodies
against: Ki-67 (VP-RM04, Vector Labs (Burlingame, CA, USA), 1: 100, citrate buffer
and water bath antigen retrieval – 50 min at 99°C), Pten (H9559, Cell Signalling
(Danvers, MA, USA), 1: 100, citrate buffer and water bath antigen retrieval – 50 min
at 99°C), pAKT(Ser473) (#3787, Cell Signalling, 1: 50, citrate buffer and microwave
antigen retrieval), \( \beta\text{-catenin} \) (C19220, Transduction Labs (Franklin Lakes, NJ, USA),
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