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REVIEW

Recent Progress in Mouse Models for Tumor Suppressor Genes and its Implications in Human Cancer

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Abstract: Gain-of-function mutations in oncogenes and loss-of-function mutations in tumor suppressor genes (TSG) lead to cancer. In most human cancers, these mutations occur in somatic tissues. However, hereditary forms of cancer exist for which individuals are heterozygous for a germline mutation in a TSG locus at birth. The second allele is frequently inactivated by gene deletion, point mutation, or promoter methylation in classical TSGs that meet Knudson's two-hit hypothesis. Conversely, the second allele remains as wild-type, even in tumors in which the gene is haplo-insufficient for tumor suppression. This article highlights the importance of *PTEN*, *APC*, and other tumor suppressors for counteracting aberrant PI3K, β-catenin, and other oncogenic signaling pathways. We discuss the use of gene-engineered mouse models (GEMM) of human cancer focusing on *Pten* and *Apc* knockout mice that recapitulate key genetic events involved in initiation and progression of human neoplasia. Finally, the therapeutic potential of targeting these tumor suppressor and oncogene signaling networks is discussed.

Keywords: tumor suppressor gene, mouse model, PTEN, AKT, APC, ATM, CHK2, VHL

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Introduction

We recently reviewed recent progress in tumor suppressor gene studies using transgenic and knockout mouse models, focusing on *Rb*, *p53*, *Ink4a*/*Arf*, *Brca1/2*, and their related genes.[1] Numerous other TSGs that are altered in human cancers have also been isolated and characterized. In this review, we discuss recent progress in mouse models for TSGs focusing on *Pten* and *Apc*, which are frequently used as mouse models for prostate/breast and colon cancers, respectively.

The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is a major survival pathway activated in cancer. The phosphatase and tensin homolog deleted in the chromosome 10 (PTEN) tumor suppressor encodes a phosphatidyl inositol 3′ phosphate phosphatase that negatively regulates the PI3K signaling pathway. Deletions or loss of function of *Pten* results in tumor formation in various tissues. Thus, *PTEN*, similarly to the *p53* and *retinoblastoma (Rb)* genes, is now thought to be a central player in tumor suppression. Because global *Pten* deletion is embryonic lethal, *Pten* was demonstrated to be a TSG by analyzing $Pten^{+/-}$ mice in 1990s.[2,3] Tissue-specific, total deletion *Pten* models were created using $Pten^{loxP/loxP}$ mice,[4] which have shown to be very useful for demonstrating the preventive role of *Pten* in prostate and other cancers. $Pten^{+/-}$ mice continue to be useful because many human cancers lose one allele in the *PTEN* locus while retaining the wild-type locus and because reduced expression, but not a total loss, of PTEN is observed in many human cancers.

Mouse models of familial adenomatous polyposis (APC) contain a heterozygous mutation on the *Apc* TSG at the genomic DNA level. APC plays a major regulatory role in the Wnt signaling pathway through its interaction with β-catenin. *Apc* knockout mice are thus valuable tools for studying intestinal carcinogenesis, since most human sporadic cancers have mutations inactivating *APC*. One study involving a mouse model found that $Apc^{+/-}$ mice develop cancers principally in the small intestine, while humans develop mainly colorectal cancers. Intestinal tumors are now discovered in the distal colon and rectum in recently created, more sophisticated mouse models that recapitulate human disease using a Cre-loxP strategy to achieve *Apc* inactivation in which exon 14 is specifically deleted. Tissue-specific, drug-inducible *Apc* knockout mouse models using Cre-ER mice have also been developed. The details of this progress are explained below. Representative in vivo studies using *Atm*, *Chk2*, and *Vhl* knockout mice are also reviewed. The gene names, affected pathways, related human diseases, and mouse models reviewed in this paper are summarized in Table 1 with references.

PTEN

One of the most frequently observed genetic alterations in advanced human cancers as detected by loss of heterozygosity (LOH) assays have been found in chromosome 10q23. Extensive studies on this candidate tumor suppressor gene, designated as *PTEN*, have been validated in a variety of tumors.[5] Frequent genetic inactivation of *PTEN* as detected by LOH occurs in glioblastoma, endometrial cancer, pancreatic cancer, and prostate cancer, and reduced expression has been observed in many other tumor types such as melanoma,[6] lung,[7,8] and breast cancer.[9] High frequency of 10q23 LOH was found in brain metastases of lung and breast tumors.[5]

To clarify the role of the PTEN-PI3K/Akt pathway, Terakawa et al studied expression of these proteins in patients with endometrial cancer. Thirty-seven (36%) of 103 endometrial cancers were PTEN-negative according to immunohistochemical staining, and the level of phosphorylated Akt was significantly higher in PTEN-negative cases than in positive cases.[10] Survival time for PTEN-positive patients was significantly longer than for patients whose tumors were PTEN-negative or showed reduced immunohistochemical staining. When patients underwent chemotherapy, the survival rate for PTEN-positive cases was significantly higher than that for PTEN-negative or -heterogeneous cases.[10] Gonzalez-Angulo et al[11] showed that PTEN-negative breast cancer is found in 30% of primary tumors and 25% in metastatic tumors. Among patients with non-small-cell lung cancer (NSCLC), 38% had *PTEN* deletions/mutations.[12] Interestingly, less than 10% of intragenic *PTEN* mutations and two homozygous deletions were reported in 22 cases primary SCLC.[7] Conversely, LOH of the *PTEN* locus has been reported in all histologic types of primary lung cancer.[13] Notably, more than 33% of *PTEN* allelic deletions occurred before lung metastasis, and allelic loss at the primary site and that at the metastatic site of each patient were identical, suggesting that loss of *PTEN* contributed to metastasis.[13] Deletion of *PTEN* occurs in ~30% of primary and up

to 70% of advanced human prostate cancers and is associated with aggressive metastatic potential, poor prognosis, and androgen independence.[14–16] Suzuki et al[17] reported that 10 of 18 cases of metastatic prostate cancer had *PTEN*$^{+/-}$ locus at presentation, while the same allele was lost in metastatic sites in 9 of 10 cases. In summary, deletions of *PTEN* and simultaneous activation of the PI3K/Akt pathway are associated with poor prognosis and therapy resistance in endometrial, breast, lung, and prostate cancers.

In addition to deletion and loss-of-function mutations, there is accumulating evidence that epigenetic suppression of *PTEN* levels by miRNA contributes to tumorigenesis in various tissues. Considering the importance of *PTEN* gene dosage for cancer susceptibility,[18] the ability to compete for miRNA and thus regulate expression levels of PTEN protein reveals the significance of the *PTEN1* pseudogene (located on chromosome 9p13.3) for oncogenesis.[19,20] PTEN expression and function may also be regulated at the post-translational level.[21]

Germline mutations of *PTEN* have been reported in three genetic syndromes: Cowden disease (multiple hamartoma syndrome), Lhermitte-Duclos disease (dysplastic gangliocytoma of the cerebellum), and Bannayan-Zonana syndromes (also known as Ruvalcaba-Myhre-Smith syndrome).[22] These autosomal dominant disorders have pathological/clinical features in common, such as multiple hamartomas and increased susceptibility to breast/thyroid cancers, as well as brain tumors. The human *PTEN* locus encodes 403 amino acids dual-specificity phosphatases that recognize lipids and protein. A missense mutation in *PTEN*, PTEN-G129E, which was observed in Cowden disease, specifically removes the ability of PTEN to recognize inositol phospholipids as substrates, indicating that loss of lipid phosphatase activity is responsible for the etiology of the disease.[23] *PTEN* loss causes accumulation of phosphatidylinositol (3,4,5)-triphosphate (PIP3) at the plasma membrane, which then recruits 3-phosphoinositide dependent protein kinase-1 (PDK1) and protein kinase B (PKB)/Akt (Fig. 1). Next, PDK1 and target of rapamycin complex 2 phosphorylate and activate PKB/Akt. Activated PKB/Akt phosphorylates a wide spectrum of substrates, including Bad, a BH3-only protein of the Bcl2 family, the Forkhead transcription factors FKHR, FKHRL1, and AFX, and glycogen synthase kinase-3β (GSK-3β) (Fig. 1). Via these substrates, activated PKB/Akt regulates various biological processes, including survival, metabolism, and proliferation. A major PTEN function is to maintain a low threshold of cellular PIP-3, antagonize PI3K signaling, and activate PKB/Akt and mTOR pathways (Fig. 1).

Mouse models of global *Pten* loss

To examine the role of *Pten* in organ development and tumor suppression, Di Christofano et al[2] knocked out *Pten* in mice using a conventional procedure. Global *Pten*-loss resulted in early embryonic lethality between E6.5–E9.5. They reported that *Pten*-deficient ES cells formed aberrant embryoid bodies and exhibited an altered ability to differentiate into endodermal, ectodermal, and mesodermal tissues, as well as an overgrowth in the cephalic and caudal regions.[2,3] PTEN induces p27^{KIP1} expression, which, in turn can negatively regulate cell cycle progression.[24] *Pten*-null mouse embryos display regions of increased cell proliferation. *Pten*-deficient immortalized mouse embryonic fibroblasts exhibit decreased sensitivity to cell death in response to a number of apoptotic stimuli, which is accompanied by constitutively elevated protein levels and phosphorylation of PKB/Akt.[25] Their results indicated that PTEN may act as a tumor suppressor by negatively regulating the PI3K/PKB/Akt signaling pathway.[25]

Since global biallelic loss of *Pten* is embryonic lethal, mice with a heterozygous deletion of *Pten* have been used to study the role of *Pten* loss in systemic disease. Di Cristophano et al[26] reported that nearly all *Pten*$^{+/-}$ mice developed a lethal polyclonal autoimmune disorder between 4 and 5 months of age, affecting the submandibular, axillary, and inguinal lymph nodes. The mice died of renal failure before 12 months of age. Interestingly, these mice had 'clinical' features similar to those found in *Fas*-deficient mice.[27] For instance, Fas-mediated apoptosis was significantly subverted in *Pten* heterozygous mice, and T lymphocytes from these mice showed reduced activation-induced cell death and increased proliferation, indicating that Pten is an essential mediator of the Fas response and also a regulator of autoimmunity.[26,27] Consistently, Heindl et al[28] reported lymphoid/thymus hyperplasia, hyperplastic tonsils, autoimmune lymphocytic thyroiditis, autoimmune hemolytic anemia, and colitis in patients

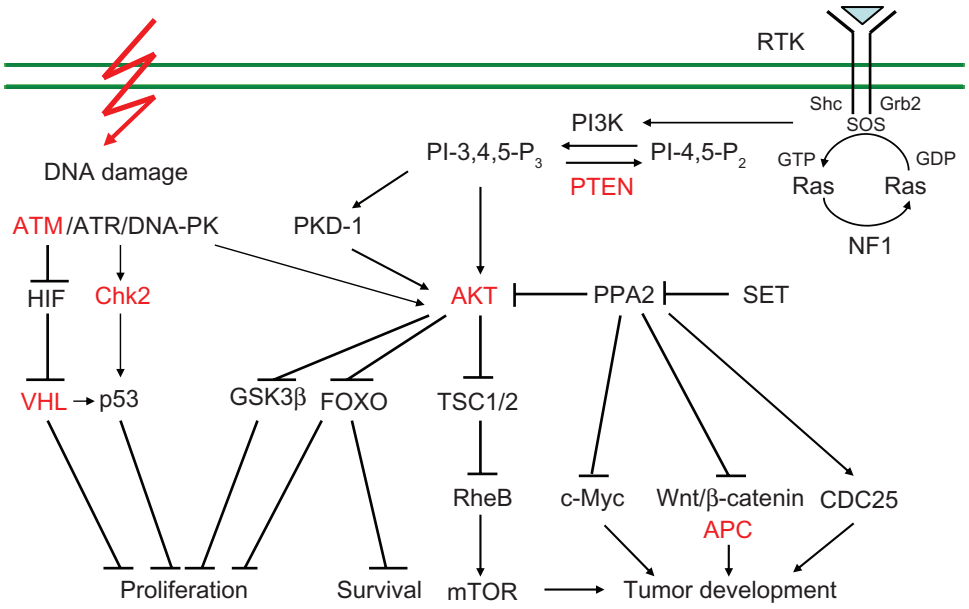

Figure 1. Roles of molecules explained in this review in signal transduction. The PI3K-Akt pathway is a major survival pathway activated in cancer. The PTEN tumor suppressor is the major brake of the pathway and a common target for inactivation in somatic cancers. In various human cancers, *PTEN* is frequently found to be mutated, deleted, or epigenetically silenced. This review highlights the networking of PTEN with other inhibitors of the pathway, relevant to cancer progression. PTEN constitutes the main node of the inhibitory network, and a series of convergences at different levels in the PI3K-Akt pathway, starting from those with growth factor receptors, are described. PTEN exerts enzymatic activity as a phosphatidylinositol-3,4,5-trisphosphate (PIP(3)) phosphatase; thus, opposing the activity of PI3K, the concerted actions to increase the availability of PIP(3) in cancer cells, relying either on other phosphoinositide enzymes or on the intrinsic regulation of PTEN activity by other molecules are discussed. Particularly, the synergy between PTEN and the circle of its direct interacting proteins will be brought forth in an attempt to understand both the activation of the PI3K-Akt pathway and the connections with other parallel oncogenic pathways. PI3K is a major signaling hub downstream of HER2 and other receptor tyrosine kinases. PI3K activates AKT, serum/glucocorticoid regulated kinase, phosphoinositide-dependent kinase 1 (PDK1), mammalian target of rapamycin (mTOR), and several other molecules involved in cell cycle progression and survival. Recent new findings have demonstrated that PTEN also plays a critical role in DNA damage repair and DNA damage response. Phosphorylation of CHK2 is triggered by DNA damage induced by ionizing radiation (IR) or hydroxyurea (HU). Functional ATM protein is required for CHK2 phosphorylation following IR, but not HU treatment.
Notes: It has been reported that PTEN dosage is essential for neurofibroma development and malignant transformation. Loss of *Pten* in combination with overexpression of the *K-ras* oncogene is an important step in malignant peripheral nerve sheath tumor development. pVHL directly associates with and stabilizes p53 by suppressing Mdm2-mediated ubiquitination and nuclear export of p53. Moreover, upon genotoxic stress, pVHL causes acetylation of p53, which ultimately leads to increased p53 transcriptional activity and p53-mediated cell cycle arrest and apoptosis. Understanding the interplay between the modulators of the PI3K-Akt pathway in cancer will lead to the design of novel therapeutic approaches with increased efficacy in the clinic.
Abbreviations: SET, suppressor of variegation, enhancer of zeste, and trithorax; PPA2, protein phosphatase A2; FOXO, forkhead box O; Tsc1, tuberous sclerosis 1; RheB, ras homologue enriched in brain; mTOR, mammalian target of rapamycin.

with *PTEN* mutations. Functional analysis of lymphoid tissue revealed increased signaling through the PI3K-AKT pathway. Thus, PTEN reduced activity affects homeostasis of human germinal center B cells by activating PI3K-AKT signaling.[28]

Pten$^{+/-}$ mice (>6 months old) developed a range of tumors that were similar the spectrum of tumors observed in patients with Cowden syndrome.[23,29] Half of *Pten*$^{+/-}$ females developed mammary tumors, whereas all had endometrial hyperplasia, and there was a high incidence (22%) of endometrial cancer. Hamartomatous tumors of the gastrointestinal tract, as well as prostate and adrenal neoplasia, were also frequently observed. Most tumors in *Pten*$^{+/-}$ mice showed loss of the wild-type allele of the *Pten* locus, which matched the definition of a classical tumor suppressor gene. PKB/Akt was hyperphosphorylated in tumor cells of *Pten*$^{+/-}$ mice, indicating that Pten suppresses tumorigenesis by inhibiting the PKB/Akt survival pathway.[30]

PTEN and prostate cancer (PCa)

The prostate cancer phenotype in *Pten*$^{+/-}$ mice is represented by intraepithelial neoplasia (mouse PIN) that never progresses to invasive prostate cancer.[3,21,31,32] To examine the role of *PTEN* loss in prostate cancer, compound genetically engineered mice (GEM) were generated by crossing *Pten*$^{+/-}$ mice with several knockout and transgenic models. Double transgenic mice were obtained by crossing *Pten*$^{+/-}$ mice with transgenic adenocarcinoma of mouse prostate (TRAMP) mice, with prostate-specific expression of SV40 under the rat *probasin* promoter. These mice developed invasive prostate cancer and died at a mean

Table 1. Gene names, affected pathways, related human diseases, and mouse models, with references.

Gene	Pathways	Association with human diseases	Mouse models	References
PTEN	PI3K-Akt p53	Endometrial cancer Prostate cancer Breast cancer T cell lymphoma	Global knockout, lethal *Pten*$^{loxP/loxP}$; *Pb-Cre* *Pten*$^{hy/+}$	2 to 83 43 to 45 71
ATM	Chk2-p53 HIF-VHL	Chronic lymphocytic leukemia	Global knockout	84 to 100
Chk2	p53	Ovarian, breast, and colorectal cancers (LOH)	Global knockout	101 to 108
APC	WNT/β-catenin	Colon cancer	global knockout, lethal *Min (Apc*$^{+/-}$*)* *Apc*$^{flox/flox}$; *Fabpl-Cre, Villin-Cre, Ah-Cre*; *Villin-Cre-ERT2*; *tetO-P(hCMV)-Cre* *Apc*$^{3lox14/+,}$ *Apc*$^{2lox14/+}$	109 to 141 132 to 139 140,141
VHL	p53	Hemangiomas in the cerebellum, retina renal cell carcinoma	Global knockout, lethal *Vhl*$^{loxP/loxP}$; *Albumin-Cre*	142 to149 148,149

age of 185 days, versus TRAMP mice that died at a mean age of 226 days.[33] To address the role of p27^{kip1}, compound transgenic mice heterozygous for *Pten*$^{+/-}$ and with homozygous or heterozygous knockout of *p27*^{kip1} were generated.[24] *Pten*$^{+/-}$; *p27*$^{kip1-/-}$ mice with complete *p27* loss developed prostate cancers and died on average at 15 weeks, whereas double heterozygous *Pten*$^{+/-}$; *p27*$^{kip1-/-}$ mice died on average at 35 weeks.[24] Surprisingly, another group reported inhibition of prostate tumor development in triple knockout mice homozygous null for *p27* in the *Pten*$^{+/-}$ and *Nkx3.1*$^{+/-}$ background, whereas mice heterozygous for the *p27*-null allele showed enhanced prostate carcinogenesis. This dose-dependent effect was attributed to cyclin D1, which was elevated in *p27*$^{kip+/-}$ and down-regulated in *p27*$^{kip1-/-}$ mice.

p27^{kip1} inhibits activation of Cdk4 and Cdk2, each of which induce phosphorylation and degradation of Rb. Thus, inactivation of Rb (either by SV40 expression in TRAMP mice or loss of *p27*^{kip1}) may cooperate with *Pten* loss by accelerating the cell cycle in prostate epithelial cells. Indeed, loss of p27^{kip1} protein was correlated with poor outcomes in prostate cancer,[34,35] and loss of *Rb* has been recently associated with castration-resistant metastatic prostate cancer.[36]

NKX3.1 is a homeobox gene localized on the chromosome 8p21 region, which is deleted in 80% of prostate cancers. Knockout of *Nkx3.1* in mouse prostate resulted in prostate epithelial hyperplasia and dysplasia that did not progress to invasive carcinoma.[37] However, *Nkx3.1*$^{+/-}$;*Pten*$^{+/-}$ mice develop invasive adenocarcinoma accompanied by lymph node metastases.[38] One possible mechanism underlying the synergism between loss of *Pten* and of *Nkx3.1* is that loss of *Nkx3.1* mimics c-Myc overexpression, as it has recently been shown that Nkx3.1 and Myc share target genes.[39] In fact, combined prostate-specific loss of one *Pten* allele and overexpression of c-Myc synergistically accelerate acquisition of the prostate cancer phenotype.[40] However, the precise nature of targets of either Nkx3.1 and Myc that synergize with the loss of *Pten* remains elusive. Furthermore, hyperactivation of Akt signaling has been suggested as a possible mechanism of cooperativity between heterozygous loss of *PTEN* and *NKX3.1*.[41] Increased activation of Akt signaling was also proposed as a major mechanism of synergy between heterozygous loss of *Pten* and knockout of Akt phosphatase PH domain leucine-rich repeat protein phosphatase 1 (PHLPP1).[42] However, elevated Akt activity may be necessary, but not sufficient, for producing the advanced prostate cancer phenotype, as effects of prostate-specific expression of constitutively active Akt were limited to hyperplasia.[43]

Although compound transgenic mice with a *Pten*$^{+/-}$ background were used to recreate the advanced

prostate cancer phenotype, they do not reflect tumors with homozygous loss of *PTEN*. To study the role of homozygous *Pten* loss in prostate cancer, $Pten^{p-/-}$ mice with prostate-specific knockout were generated by crossing $Pten^{loxP/loxP}$ mice with transgenic mice that expressed Cre recombinase under the *probasin* promoter.[4,44] These mice showed increased prostate epithelial cell proliferation at 4 weeks and developed prostate intraepithelial neoplsia (PIN) at 6 weeks and invasive prostate adenocarcinoma at 9 weeks. After 12 weeks, metastases were observed in the lymph nodes and lungs in 5 of 11 mice. In contrast, other groups reported lower overall the rates of invasive cancer and metastases in $PbCre^{+/-}$;$Pten^{loxP/loxP}$ mice with a C57BL/6 genetic background.[45,46] A similar model of prostate-specific *Pten* knockout used Cre recombinase under the prostate-specific antigen (*PSA*) promoter.[47] In this model, development of prostate cancer was substantially delayed; prostate hyperplasia developed at 12 weeks, followed by mouse PIN at 7 months and carcinoma 10–14 months. Unlike in *the probasin-Cre* model, lymph node metastases were detected in only one 16-month-old mouse.

Most mechanistically interesting results are from combined *Pten*;*p53* knockout mice that substantially accelerated the development of prostate tumors. The average tumor weight in double knockout mice increased 32-fold compared to age-matched $Pten^{p-/-}$ mice, yet these mice did not develop metastases.[46] Analysis of the PTEN-p53 interaction revealed that loss of Pten and subsequent activation of the PI3K/Akt pathway leads to increased expression of p53 and to p53-induced senescence. This up-regulation of p53 is in striking contrast to glioblastoma, lung cancer, and breast cancer cells, in which PI3K/AKT signaling negatively regulates p53 by accelerating Mdm2-mediated ubiquitination and degradation.[47–52] The mechanisms of p53 stabilization by *Pten*-loss in prostate cells remain to be elucidated. Increased p53 expression and senescence were not affected by the simultaneous loss of *Pten* and $p19^{Arf}$, a well-documented upstream regulator of p53 expression.[53]

The existence of a negative feedback loop induced by activation of PI3K/Akt pathway in prostate cells was confirmed by another group using compound GEM mice with heterozygous $Pten^{+/-}$ knockout and homozygous knockout of Akt phosphatase PHLPP1.[54] Increased activation of Akt in these mice led to compensatory induction of PHLPP2 orthologs and induction of p53 and senescence. These observations suggest that inactivation of *p53* by deletion of mutation is necessary for prostate tumorigenesis driven by the PTEN/PI3K/Akt pathway.

Another compound mouse model explored various combinations of *Pten* loss, *p53* loss, and expression of c-Myc. Deletion of *Pten* together with either c-Myc expression or *p53* loss resulted in invasive prostate cancer within 6 months.[55,56] Reminiscent of Pten/p53 co-regulation, loss of *Pten* leads to activation of the TGFβ/Smad4 signaling axis in advanced prostate cancer. Combined knockout of *Pten* and *Smad4* in mice led to development of locally metastatic prostate cancer.[57] Furthermore, a combination of *Pten, p53* and *Smad4* knockouts led to bone metastases in 12% of mice, whereas expression of active telomerase in a *Pten*/*p53* knockout produced bone metastases in 100% of cases.[58]

In addition to c-Myc, several compound models have been developed to examine the effects of oncogene expression in Pten-negative prostates. Accelerated prostate cancer development was observed when HER2,[59] active K-Ras,[60] SOX9,[61] ERG,[62] and Bmi1[63] were expressed in prostate-specific *Pten* knockout mice. Loss of *Nkx3.1* and increased expression of Myc, ERG, Bmi1, and Sox7 as well as elevated activation of the Ras/MAPK pathway have been documented in advanced prostate cancers in men.

In summary, existing compound GEM models reflect the genotypes and phenotypes of various stages of prostate cancer and are useful for understanding the molecular mechanisms driving prostate cancer progression to advanced metastatic disease. For example, induction of p53 and senescence by complete *Pten* knockout explains the mostly heterozygous loss of *PTEN* during early stages of prostate cancer and it is thought that in advanced cancers, p53 loss precedes loss of the second *PTEN* allele. Analysis of patients' samples from primary and metastatic prostate cancer confirmed reduced expression or inactivation of *p53* in metastatic tumors with increased PI3K/Akt signaling due to loss of *PTEN* or *PHLPP*.[64]

Metastatic growth has been reported in original studies describing prostate-specific *Pten* knockout,[65] but this was not confirmed in subsequent reports.[58] Recently, metastases formation has been shown in compound mouse models that combine loss of *Pten*,

p53, and *Smad4*[57] or loss of *Pten* and expression of constitutively active Ras^{G12}.[65] It remains unknown if recently emerged genetically engineered mouse models (GEMM) of metastatic prostate cancer will respond to androgen ablation similarly to metastatic prostate cancer in men.

From a translational standpoint, the most valuable models would be mouse models that faithfully represent metastatic disease and mimic responses to therapies for advanced prostate cancer in men. Considering the recent expansion of GEM as a model of prostate cancer, it is somewhat surprising that only two groups have systematically studied the therapeutic effects of androgen ablation in the $Pten^{-/-}$ model. Castration of $Pten^{p-/-}$ mice led to massive apoptosis of prostate epithelial cells, yet unlike in prostates of wild-type mice, a population of proliferative prostate cells positive for stem cell markers (p63 and casein kinase 5) in primary tumors were detected.[65] A better response was achieved when anti-androgen therapy (MDV3100 or castration) was combined with an mTOR inhibitor.[66] In the compound $Pten^{+/-}$;$Nkx3.1^{-/-}$ mice, prostates were completely resistant to castration. At 48 h post-castration, $Pten^{+/-}$;$Nkx3.1^{-/-}$ mice did not display significant apoptosis and maintained a high proliferation index in contrast to wild-type mice.[67] Taken together, these studies suggest that loss of Pten function is sufficient for establishing androgen-independence in prostate cancer and providing a favorable background for engagement of other oncogenes in tumorigenesis.

PTEN and breast cancer

Bose et al[68] analyzed the allelic loss of *PTEN* in breast cancer using microsatellite markers spanning the 10q23 region. Although no LOH was found in pure intraductal carcinomas (0/20 cases), LOH was observed in 40% (17/42) of invasive carcinomas ($P = 0.0005$). Interestingly, in situ lesions were also found in invasive tumors displaying LOH. Allelic loss was significantly associated with loss of the estrogen receptor ($P = 0.011$). They concluded that loss of 10q23 is strongly associated with tumor progression.[68] Garcia et al[69] investigated allelic losses in microsatellites of the 10q23 region and their correlations with pathologic parameters in 105 breast carcinomas. LOH in at least one marker of five in the *PTEN* region was found in 30% of tumors. *PTEN* LOH was associated with age, lymph node metastases, higher histological grade, and progesterone receptors. These results suggest the *PTEN* and/or neighbor genes are functionally related to breast cancer, likely influencing the development of histological features associated with poor prognosis.[69] Although PTEN protein is deficient in 30% of breast cancer cases,[9] recent studies have shown that PTEN protein is down-regulated in more than 50% of sporadic breast tumors, indicating the relevance of PTEN dose in the pathogenesis of the disease.[70,71] Alimonti et al analyzed *Pten* hypomorphic mice (ie, mice that express Pten at lower levels than wild-type animals, known as $Pten^{hy/+}$), expressing 80% normal levels of Pten.[71] $Pten^{hy/+}$ mice developed a spectrum of tumors, with mammary tumors occurring at the highest penetrance. Notably, all mammary tumors analyzed retained two intact copies of *Pten* and maintained Pten levels above the levels found in heterozygous mice. Thus, subtle down-regulation of Pten altered the steady-state biology of the mammary tissues and expression profiles of genes involved in cell proliferation, as well as the development of cancer in some tissues.

Constitutive activation of the phosphatidylinositol 3-kinase (PI3K) pathway either through loss of *PTEN* or mutation of PI3K catalytic subunit alpha (PIK3CA) occurs frequently in human cancers. Saal et al[72] identified *PIK3CA* mutations in 26% of human breast cancer specimen and cell lines. They reported an association between *PIK3CA* mutations and retention of *PTEN* protein expression.[72] Interestingly, *PIK3CA* mutations and *PTEN* loss are nearly mutually exclusive in breast cancer, suggesting that deregulated phosphatidylinositol-3,4,5-triphosphate (PIP(3)) is critical for tumorigenesis in breast cancers and that loss of PIP(3) activity by inactivating either *PIK3CA* or *PTEN* relieves the selective pressure for targeting other genes.[72]

Heterozygous inactivation of *Pten* leads to formation of basal-like mammary tumors in mice,[73] and loss of PTEN protein expression is appreciably associated with the basal-like breast cancer subtype in humans. Additionally, Saal et al[73] reported frequent *PTEN* mutations, particularly in *BRCA1*-deficient breast cancers. These data suggest a specific synergism between the dysfunction of BRCA1-nediated DNA repair and PTEN loss in the pathogenesis of basal-like breast cancers.[73]

PTEN overexpression in the mammary epithelium inhibits cell growth, differentiation, and secretion.

In contrast, overexpression of the proto-oncogene *Wnt-1* in mammary epithelium leads to mammary hyperplasia focal mammary tumors. To explore the possibility that PTEN interferes with Wnt-induced tumorigenesis, Zhao et al[74] created mice that ectopically express PTEN and Wnt-1 in mammary glands using the *mouse mammary tumor virus* (*MMTV*) promoter. PTEN overexpression resulted in significant reduction of Wnt-1-induced tumors with delayed onset of mammary tumorigenesis. Thus, PTEN can inhibit the Wnt-1-induced mammary tumorigenesis at premalignant stages by blocking the AKT pathway and by reducing IGF-1 receptor levels in the mammary gland.[74] This study identified PI3K/AKT as a therapeutic target for treating mammary cancer and presumably other types of cancers.

Overexpression and/or amplification of the *HER2* receptor tyrosine kinase gene and inactivation of *PTEN* are two important genetic events in human breast carcinogenesis. To address the biological impact of conditional inactivation of *Pten* on ErbB2-induced mammary tumorigenesis, Schade et al[75] generated a novel transgenic mouse model using the *MMTV* promoter to simultaneously express activated ErbB2 and Cre recombinase in the same mammary epithelial cells using the same bicistronic transcript with an internal ribosome entry sequence placed between the two cDNA sequences (*MMTV-NIC*). Inactivation of *Pten* in the mammary epithelium of the *MMTV-NIC* mice dramatically accelerated the development of multifocal, highly metastatic mammary tumors, which exhibited the pathology representing an ErbB-2-type pattern showing solid nodular growth of intermediate cells with central necrosis. *Pten*-deficient/*NIC*-induced tumorigenesis was also associated with increased angiogenesis. Inactivation of *Pten* in the *MMTV-NIC* mouse model resulted in hyperactivation of the PI3K/Akt signaling pathway. However, tumors obtained from *Pten*-null/*MMTV-NIC* mice displayed histopathological and molecular features of the luminal subtype of primary human breast cancer. Their study provided a valuable tool for testing the efficacy of therapeutic agents targeting *PTEN* deficiency and HER2 activation.[75]

PTEN and trastuzumab therapy

Trastuzumab (trade name, Herceptin) is a monoclonal antibody that interferes with the HER2/neu receptor that is often overexpressed in human breast cancer. Its main use is to treat certain breast cancers to induce cell death. Nagata et al[76] reported that PTEN activation contributes to trastuzumab's anti-tumor activity. Trastuzumab treatment quickly increased PTEN membrane localization and phosphatase activity by reducing PTEN tyrosine phosphorylation via Src protein tyrosine kinase inhibition. Consistently, reducing PTEN in breast cancer cells by anti-sense oligonucleotide DNA was associated with trastuzumab resistance *in vitro* and *in vivo*. Patients with PTEN-deficient breast cancers had significantly poorer responses to trastuzumab-based therapy than those with normal PTEN. Thus, PTEN deficiency is a powerful predictor of trastuzumab resistance. PI3K inhibitors rescued PTEN loss-induced trastuzumab resistance, suggesting that PI3K-targeting therapies can be used to overcome this resistance.

Association of trastuzumab therapy resistance with non-receptor protein tyrosine kinase SRC activation has been reported by two different groups.[77,78] Liang et al reported that the erythropoietin receptor (EpoR) was coexpressed with HER2 in a significant percentage of human breast cancers.[77] Simultaneous treatment of cells with *rh*EPO and trastuzumab reduced the cellular response to trastuzumab both in vitro and *in vivo*. They found that Jak2-mediated activation of Src and inactivation of PTEN were underlying mechanisms through which *rh*EPO antagonizes trastuzumab-induced therapeutic effects.[77] Zhang et al[78] reported that increased SRC activation conferred considerable trastuzumab resistance in breast cancer cells and correlated with trastuzumab resistance in patients. Indeed, targeting SRC in combination with trastuzumab sensitized resistant cells to trastuzumab and eliminated such tumors in vivo, suggesting the potential clinical application of this strategy for overcoming trastuzumab resistance.

PTEN deficiency in tumor stroma

Tumor stroma is believed to contribute to some of the most malignant characteristics of carcinomas. Signaling between stromal and tumor cells is complex and remains poorly understood. To evaluate the role of *Pten* in the tumor microenvironment of breast cancer, Trimboli et al[79] generated mice containing a mesenchymal-specific *Fsp-Cre* transgene and conditional alleles of *Pten* (*Pten*^{loxP}). They showed that

genetic inactivation of *Pten* in stromal fibroblasts of mouse mammary glands, but not that of epithelial cells, accelerated the initiation, progression, and malignant transformation of mammary epithelial tumors.[79] This occurred simultaneously with reconstruction of the extracellular matrix, inflammatory cell infiltration, and increased angiogenesis. Notably, loss of *Pten* in stromal fibroblasts increased expression, phosphorylation, and recruitment of Ets2 to target promoters known to be involved in these oncogenic processes. *Ets*2 inactivation in *Pten* stroma-deleted tumors inhibited the tumor microenvironment and was sufficient for decreasing tumor growth and progression, indicating that Ets2 is a critical target for this process. Global gene expression profiling of mammary stromal cells identified Pten-specific gene expression that was frequently observed in the tumor stroma of breast cancer patients. These findings identified the Pten-Ets2 axis as a critical stroma-specific signaling cascade that inhibits breast cancer development.[79]

Using proteomic and gene expression profiling, the same group showed that *Pten* loss from mammary stromal fibroblasts activated oncogenic secreted cellular proteins (secretome) that direct the transcriptional reprogramming of other cell types in the microenvironment.[80] Down-regulation of miR-320 and up-regulation of ETS2 were critical events in *Pten*-deleted stromal fibroblasts responsible for inducing this oncogenic secretome, which, in turn promoted tumor angiogenesis and cell invasion. Interestingly, expression of the Pten-miR-320-Ets2-regulated secretome distinguished human normal stroma from that of tumor-derived stroma and correlated with the recurrence of breast cancer. Their work revealed miR-320 as a critical component of the Pten tumor suppressor axis that acts in stromal fibroblasts to reprogram the tumor microenvironment and inhibit tumor progression.[80]

PTEN deficiency in other cancers

Using a conditional Cre-loxP system, Suzuki et al[81] created a T cell-specific deletion of *Pten* ($Pten^{flox/-}$). They reported that T cell-specific deletion of *Pten* caused $CD4^+$ T cell lymphomas by 17 weeks of age. $Pten^{flox/-}$ mice showed increased thymic cellularity due to a defect in negative selection in the thymus. These mice showed elevated levels of B cells and $CD4^+$ T cells in the periphery, spontaneous activation of $CD4^+$ T cells, autoantibody production, and hypergammaglobulinemia.[81] *Pten*-deficient T cells hyperproliferated, were auto-reactive, and secreted increased levels of Th1/Th2 cytokines. They resisted apoptosis and showed increased phosphorylation of PKB/Akt and ERK. PTEN is thus an important regulator of T cell homeostasis and self-tolerance.[81] Backman et al[82] generated a brain-specific deletion of *Pten* to address its role in brain function.[82] Mice homozygous for this deletion ($Pten^{loxP/loxP}$;*Gfap-Cre*) developed seizures and ataxia by 9 weeks and died by 29 weeks. Histopathological analysis showed brain enlargement in $Pten^{loxP/loxP}$;*Gfap-Cre* mice as a consequence of primary granule-cell dysplasia in the cerebellum and dentate gyrus.[82] Brain-specific *Pten* mutant cells showed an increase in soma size and elevated phosphorylation of Akt. Thus, Pten and Akt play an important role in cell size regulation in mammals, and their brain-specific knockout for *Pten* provided an animal model for a human Lhermitte-Duclos disease.[82]

$Pten^{+/-}$ mice have been used as a mouse model to study endometrial carcinoma (EMC). The females develop atypical endometrial hyperplasia, of which ~20% progresses to EMC. Daikoku et al[83] reported that that conditional deletion of endometrial *Pten* results in EMC in all female mice as early as age 1 month with myometrial invasion occurring by 3 months. In contrast, conditional deletion of *p53* in endometrium had little effect within this period of time. However, mice with a combined deletion of *Pten* and *p53* in the endometrium had a shorter life span with more aggressive disease than *Pten*-loss alone. *Pten*-deficient EMC showed elevated Cox-2 and phospho-Akt levels.[83] Thus, this mouse model for endometrium-specific loss of *Pten* will be extremely useful for studying the detailed mechanisms for the initiation and progression of EMC *in vivo*.

ATM

Ataxia-Telangiectasia (A-T) is an autosomal recessive disorder characterized by immunodeficiency, progressive cerebellar ataxia, radiosensitivity, defects in cell cycle checkpoints, and predisposition to lymphoid tumors.[84] A-T is caused by mutations in the *ATM* gene. Thus far, more than 400 disease-causing *ATM* mutations have been identified, with most mutations resulting in truncated or destabilized ATM protein.[85,86] The human *ATM* protein is a 3,056-amino acid protein kinase

that belongs to PI3K-related protein kinase (PIKK) family. In response to double-strand breaks, ATM is activated by autophosphorylation and subsequently phosphorylates or mediates the phosphorylation of various proteins responsible in cell cycle checkpoints activation and DNA repair initiation (Fig. 1). p53 is one of these substrates phosphorylated by ATM at Ser15 and other sites, increasing its stability and activity. p53 is also stabilized by ATM-mediated CHK2 phosphorylation. Active CHK2 phosphorylates Ser20 on p53, inhibiting the p53/MDM2 interaction and stabilizing p53. Double-strand breaks also trigger ATM to phosphorylate MDM2, preventing p53 nuclear transport and degradation.[87] Another important ATM substrate is BRCA1. Both BRCA1 and ATM are found in the BRCA1-associated genome surveillance complex, a large protein complex containing several DNA damage repair proteins. BRCA1 is also involved in the regulation of *p21* and *GADD45* gene expression, which are essential in cell-cycle control. Thus, ATM is a key player monitoring the genomic integrity of the cell.[84,88]

Mice models for A-T have been developed by disrupting the *Atm* gene. These mice have similar characteristics as in A-T patients including a predisposition to develop thymic lymphomas.[89] $Atm^{+/-}$ mice generated by gene targeting[90–92] are healthy, but exhibit increased sensitivity to sublethal doses of IR, as manifested by decreased survival and premature graying of the hair.[93] This finding is reminiscent of the radiosensitivity of AT patients. $Atm^{-/-}$ mice are viable but display growth retardation, neurologic dysfunction, infertility, small thymus, and defective T lymphocyte maturation, as well as increased sensitivity to γ-irradiation. They are also highly predisposed to cancer, with most dying of widely metastasized malignant thymic lymphomas by 4–5 months of age. Cytogenetic analysis of $Atm^{-/-}$ thymic lymphomas has consistently identified chromosomal abnormalities involving the *T cell receptor* α/δ locus, suggesting that ATM plays a role in V(D)J recombination, and aberrant DNA repair during this process may contribute to genome instability and subsequent tumorigenesis. Two recombinases, RAG-1 and RAG-2, are required for V(D)J recombination, and deficiency for either results in a complete lack of V(D)J recombination. Tumors do not develop in <9-month-old $Atm^{-/-}$; $Rag1^{-/-}$ mice, and the survival of these animals is increased compared to $Atm^{-/-}$ mice.[94] $Atm^{-/-}$;$Rag2^{-/-}$ mice do develop thymomas, but at a lower frequency and with a longer latency than $Atm^{-/-}$ mice.[95] The possible synergy between inactivation of *Atm* and other TSGs has been explored. $Atm^{-/-}$;$p53^{-/-}$ mice exhibit accelerated tumor formation compared to $p53^{-/-}$ or $Atm^{-/-}$ mice, suggesting collaboration between the two mutations.[96] Apoptosis of thymocytes in response to IR is suppressed in $Atm^{-/-}$;$p53^{-/-}$ mice, suggesting that IR-induced apoptosis of $Atm^{-/-}$ thymocytes is p53-dependent.[97] It has been reported that expression of p21 in $Atm^{-/-}$ cells was increased, which serves as a failsafe mechanism for preventing further genomic instability. It was found that $Atm^{-/-}$;$p21^{-/-}$ mice were prone to develop carcinomas and sarcomas and $Atm^{-/-}$; $p21^{-/-}$ cells showed increased aneuploidy. Thus, ATM cooperates with p21 to suppress aneuploidy and subsequent tumor development.[98]

Ataxia-Telangiectasia patients have an increase risk of lymphoid tumors, while *ATM* mutation carriers have a lower risk of cancer. Sporadic *ATM* mutations occur in 10%–20% of chronic lymphocytic leukemia (CLL) and are often associated with chromosome 11q deletions, which cause the loss of an *ATM* allele. The role of constitutional *ATM* mutations in the pathogenesis of CLL is unknown. Skowronska et al[99] studied the frequency of constitutional *ATM* mutations in either of two CLL cohorts, those with and without a chromosome 11q deletion. They found that constitutional pathogenic *ATM* mutations were increased in patients with chromosome 11q deletions, but not in those without 11q deletions. These results suggest that *ATM* germline heterozygosity does not play a role in CLL initiation, but rather influences rapid disease progression through *ATM*-loss.[99] Recent large epidemiological studies have also shown that *ATM* mutations that cause A-T in biallelic carries are associated with a modest risk of breast cancer in monoallelic carriers.[100]

CHK2

The checkpoint kinase Chk2 plays a key role in inhibiting cell cycle progression in response to DNA damage (Fig. 1). Upon activation by low-dose ionizing radiation (IR), which occurs in an ATM-dependent manner, Chk2 can phosphorylate the mitosis-inducing phosphatase Cdc25C at an inhibitory site, blocking entry into mitosis causing G_2 arrest.[101,102] Chk2

directly phosphorylates p53 on serine 20, which is known to interfere with Mdm2-binding, thus providing a mechanism for the increased stability of p53 in response to DNA damage. Phosphorylation of CHK2 is also triggered by DNA damage DNA-damaging agent hydroxyurea (HU).[103] Notably, ATM is required for CHK2 phosphorylation following IR, but not HU (Fig. 1). CHK2 is thought to be a tumor suppressor protein since *CHK2* mutations are found in some patients with Li-Fraumeni syndrome without *p53* mutation, suggesting that inactivation of *CHK2* predisposes to human cancers and inactivation of *CHK2* and *p53* is mutually exclusive.[104] Additionally, CHK2 interacts with and phosphorylates BRCA1, allowing BRCA1 to restore survival after DNA damage.[105] Takai et al[106] reported that *Chk2*$^{-/-}$ mice were resistant to IR. The IR-induced G1/S cell cycle checkpoint was subverted in embryonic fibroblasts derived from *Chk2*$^{-/-}$ mice. They also reported that p53-dependent transcriptional activation of target genes, such as *p21*^{Cip1} and *Noxa*, was not observed in *Chk2*$^{-/-}$ cells. Thus, Chk2 plays a critical role in p53 function in response to IR by regulating its transcriptional activity.[106]

Unlike *Atm*$^{-/-}$ or *p53*$^{-/-}$ mice, *Chk2*$^{-/-}$ mice were not tumor-prone, although Chk2 suppressed dimethylbenzanthracene-induced skin tumors.[107] Tissues from *Chk2*$^{-/-}$ mice, showed significant defects in IR-induced apoptosis or G_1/S arrest. Interestingly, IR-induced apoptosis was restored in *Chk2*$^{-/-}$ thymocytes by reintroduction of the wild-type *Chk2* gene, but not by a *Chk2* gene in which the sites phosphorylated by Atm and ataxia telangiectasia and rad3(+) related (Atr) were mutated to alanine. Atr may thus selectively contribute to p53-mediated apoptosis. These data indicate that distinct pathways regulate activation of p53, leading to cell cycle arrest or apoptosis.

Thus far, few studies have investigated sporadic cancers for somatic *CHK2* mutations. There have been only a small number of studies examining *CHK2* for promoter methylation, and even fewer studies using a combined genetic and epigenetic approach. However, published studies indicate that *CHK2* behaves as a 'classic' tumor suppressor gene that requires both copies to be inactivated to accelerate tumor growth.[108] Williams et al[108] conducted LOH studies across the *CHK2* locus with breast, ovarian, and colorectal cancers using 18 microsatellite markers spanning chromosome 22. LOH at the *CHK2* locus was observed in 54%, 44%, and 30% of the breast, ovarian, and colorectal cancers, respectively. Among patients showing LOH, most cases had lost the entire chromosome, particularly in ovarian and colon cancers. Since a point mutation or epigenetic inactivation of the retained allele was uncommon, it remains unclear whether *CHK2* acts as a classical TSG in these tumors. Detailed LOH studies with custom-made primers similar to the *CHK2* locus and methylation/mutational analyses are necessary to determine the role of *CHK2* in human carcinogenesis.

Adenomatous polyposis coli (Apc)

Familial adenomatous polyposis (FAP) is an inherited disorder in which numerous polyps are found primarily in the large intestine. These polyps begin as a benign lesion, which will then undergo malignant transformation into colon cancer. Most sporadic and hereditary colorectal tumors show loss of APC function caused by mutation of the *APC* gene. The human *APC* gene is localized on chromosome 5q21 and encodes a 2,843-amino acid protein with a calculated molecular weight of 311.6 kDa that contains several different domains and motifs.[109] The APC protein is involved in a number of cellular processes, including cell cycle progression, apoptosis, cell adhesion, cell migration, microtubule assembly, and cell fate determination.[110] Notably, APC plays a major regulatory role in the Wnt signaling pathway.[111] Binding of Wnt to the Frizzled receptor activates Dishevelled, which inhibits GSK-3β phosphorylation of β-catenin and prevents its proteosomal degradation. The APC protein makes a complex with GSK-3β and axin by interacting with the 20 amino acids and Ser-Ala-Met-Pro repeats. This complex binds to β-catenin in the cytoplasm, which has dissociated from adherens junctions between cells. After initial phosphorylation of β-catenin by casein kinase 1, GSK-3β phosphorylates β-catenin in a second step. This phosphorylation targets β-catenin for ubiquitination and proteasomal degradation, preventing it from translocating into the nucleus where it could transactivate genes that accelerate cell proliferation, such as *cyclin D1* and *c-Myc*. Therefore, by destabilizing β-catenin, APC controls the Wnt signaling pathway and suppresses cell growth.[111] Thus, tumor cells with *APC* mutations contain elevated levels of β-catenin that can be down-regulated by expression of exogenous wild-type APC.[112]

Knockout mouse models for intestinal tumors with *Apc* mutation have been developed. Multiple intestinal neoplasia (*Min*) mice were derived from a C57BL/6 male treated with ethylnitrosourea to induce random germline mutagenesis.[113,114] The mutation was identified in a pedigree analysis established during a mutagenesis project in which C57BL/6 males were treated with ethylnitrosourea, and then mated to AKR females. A progressive, adult-onset anemia was noted in some of the progeny. Anemia appeared to be transmitted as an autosomal-dominant trait. Anemic mice frequently passed bloody feces and had numerous visible tumors in the large and small intestines (1–8 mm). The primary phenotype of mice carrying this mutation appeared to be development of multiple adenomas, which progress to adenocarcinomas of the intestine in older mice with secondary anemia.[113] Homozygous inactivation for *Apc* leads to embryonic lethality before E8.[115–117] Moser et al[117] studied the role of the *Apc* gene in mouse development and found that functional Apc is required for normal growth of inner cell mass derivatives. $Apc^{+/-}$ mice developed multiple intestinal neoplasias in a manner similar to that observed in patients with familial adenomatous polyposis and in Min mice.[113,115,118]

Most both germ-line and somatic mutations at the *APC* gene are clustered in the 5′ half and predict truncation of the protein product.[119] Mutations beyond codon 1600 are rare and appear to result in undetectable levels of the corresponding truncated protein.[120] Therefore, the mutation spectrum observed in the *APC* gene suggests that the carboxyl-terminal domains and part of the β-catenin regulatory domains are critical for its tumor-suppressing function. Smiths et al[121] created a mouse model carrying a targeted mutation at codon 1638 of the mouse *Apc* gene, *Apc1638T*, resulting in a truncated Apc protein encompassing three of the seven 20 amino acid repeats and one SAMP motif, but missing all of the carboxyl-terminal domains thought to be critical for tumorigenesis. $Apc^{1638T/1638T}$ mice survived during the postnatal period, and, most importantly, animals that survived into adulthood were tumor-free, suggesting that the SAMP motif retained in Apc1638T is important for tumor suppression.[121] Their results also indicated that the association with Drosophila discs large, EB1 (end binding 1), and microtubules is less critical for maintaining homeostasis by APC and that proper β-catenin regulation by APC appears to be required for normal embryonic development and tumor suppression.[121]

Other tumor suppressor genes that can modify tumor development in *Apc*-deficient mice have also been examined. TGFβ plays a central role in inhibiting cell proliferation and also modulates processes involving cell invasion, immune regulation, and microenvironment modification. Mutations in the TGFβ type II receptor occur in approximately 30% of colorectal carcinomas. Smad4 (Dpc4) plays a critical role in TGF-β signaling. Mutations in *SMAD4* are found in patients with familial juvenile polyposis, an autosomal dominant condition associated with an increased risk of colorectal cancer.[122] It is an essential gene for embryogenesis since $Smad4^{-/-}$ mutant mice die before E8.5.[123–125] Because both *Apc* and *Smad4* are located on the same chromosome, Takaku et al[124] created compound heterozygotes carrying both mutations on the same chromosome through meiotic recombination. $Apc^{+/-}$;$Smad4^{+/-}$ mice developed an increased number of intestinal polyps compared to simple *Apc* heterozygotes, with an extensive stromal cell proliferation, submucosal invasion, and in vivo transplantability. Similarly, deletion of *Msh2* (DNA mismatch repair gene),[126] *Mlh1* (DNA mismatch repair gene),[127] *Tcf1* (transcription factor),[128] and *p53*[129] in $Apc^{+/-}$ mice showed a synergistic effect on intestinal tumor development.

One of the limitations of the $Apc^{Min/+}$ mouse model is that it only develops benign polyps. Fas is a member of the tumor necrosis family, and defects in the Fas-mediated apoptotic pathway have been reported in colorectal cancer. Guillen-Ahlers et al[130] crossed $Apc^{Min/+}$ mice with *Fas*-deficient (Fas^{lpr}) mice to study the effect of *Fas*-loss in intestinal carcinogenesis. $Apc^{Min/+}$;Fas^{lpr} mice showed a dramatic increase in tumor burden relative to $Apc^{Min/+}$ mice and invasive lesions at advanced ages. $Apc^{Min/+}$;Fas^{lpr} intestinal tumors showed an increase in cellular proliferation, but negligible changes in apoptosis, while p53 increased at early ages. $Apc^{Min/+}$;Fas^{lpr} mice are thus be a better model of advanced intestinal carcinoma than the original $Apc^{Min/+}$ model.[130] Compound knockout mice created by Oshima et al[131] contributed to novel drug screening for colon cancer. Two cyclooxygenase isozymes (COX-1 and COX-2) catalyze the conversion of arachidonic acid to prostaglandin H2. *COX-2*

deficiency dramatically reduced the number and size of the intestinal polyps.[131] Furthermore, treating Apc *ApcΔ716* mice with a novel COX-2 inhibitor (MF tricyclic) reduced the polyp number more significantly than with sulindac, which inhibits both COX-1 and 2. These results provided direct genetic evidence that COX-2 plays a key role in intestinal carcinogenesis and indicated that novel COX-2-selective inhibitors can be used as therapeutic agents for colorectal cancer rather than sulindac.[131]

Intestine-specific inactivation of *Apc* in mice

To overcome the problem of embryonic lethality of global *Apc* loss, $Apc^{flox/flox}$ mice have been developed by targeting the *Apc* gene in which LoxP sites were introduced to flank exon 14.[132] Upon expression of Cre recombinase, exon 14 of the *Apc* gene was deleted, resulting in a frameshift mutation at codon 580 (Apc^{580S}). Homozygous $Apc^{580S/580S}$ mice were phenotypically normal in the absence of Cre recombinase. However, when homozygous $Apc^{580S/580S}$ mice were infected with Cre-expressing adenovirus, multiple rectal adenomas developed at 3 months of age.[132] Genomic analyses of adenomas revealed the homozygous deletion of the Apc^{580S} locus (Apc^{580D}), demonstrating that *Apc* is a classical TSG that follows Knudson's two-hit hypothesis.

Recently, several different systems were developed to obtain intestine-specific expression of Cre recombinase. Among the more commonly used mouse models are the *Fabpl-Cre*, *Villin-Cre*, and *Ah-Cre* transgenic models.[133–137] A 35-nucleotide sequence in the liver *fatty acid-binding protein* gene (*Fabpl*) has been identified. Fabpl interacts with nuclear proteins present in the adult mouse liver, kidney, stomach, small intestine, and colon. The binding site for Fabpl consists of a direct heptad repeat (TTCTGNNTT) separated by five nucleotides.[138] Both heptads are required for formation of stable complexes with nuclear proteins in gel mobility shift assays. The *Fabpl-Cre* model has a promoter element comprising nucleotides −596 to +21 of the rat liver fatty acid binding protein gene.[133] This mouse showed Cre recombinase expression in small intestines and colonic epithelial cells, beginning from E13.5. Fabpl-Cre expression and recombination were maintained in both epithelia throughout adulthood. The same research group also generated an inducible version of the *Fabpl-Cre* mouse.[133] They used *Fabpl* regulatory elements to direct expression of a reverse tetracycline-regulated transactivator (*rt*TA). Another transgene encodes Cre under the control of *tet* operator sequences and a minimal promoter from human cytomegalovirus [*tetO-P(hCMV)-Cre*]. In the absence of a doxycycline (dox), no basal recombination was detectable in the gut of adult tri-transgenic mice: *Fabpl-rtTA*, *tetO-P(hCMV)-Cre* plus a floxed reporter gene. After 4 days of oral administration of dox, recombination of the reporter was observed in the small intestinal and colonic epithelium. After dox withdrawal, the recombined locus persisted for at least 60 days, indicating that recombination had occurred in epithelial cell progenitors of the intestine.[133]

Villin is an actin-bundling protein found in the apical brush border of absorptive intestinal epithelium. The *Villin-Cre* model, established by two different groups as an intestine-specific Cre expression system, is well-characterized. A 9-kb mouse *Villin* regulatory region was used to drive Cre recombinase expression in intestinal epithelial cells.[134] Expression of the transgene was also found in epithelial cells of the proximal tubules of kidney cells. Cre expression from the *Villin* promoter was switched on in the intestinal epithelial cells from E12.5. Similarly, another group developed a 12.4-kb *Villin* promoter-driven *Cre* mouse in which Cre was specifically expressed in intestinal epithelial cells.[135]

Recently, a tissue-specific, inducible *Villin-Cre* expression system was developed that utilized tamoxifen-driven Cre recombinase expression under the control of the mouse *villin* promoter (*Villin-Cre-ERT2*).[136] Cre was expressed under the control of a 9-kb regulatory region of the murine villin gene (*Villin-Cre*). Genetic recombination was initiated at E9 in the visceral endoderm and by E12.5 in the entire intestinal epithelium. Cre expression was maintained throughout adulthood. After tamoxifen treatment, Cre-estrogen receptor (Cre-ER) was detectable throughout the digestive epithelium which persisted for 60 days after tamoxifen administration, indicating that epithelial progenitor cells had been targeted. The *Villin-Cre* and *Villin-Cre-ERT2* mice are valuable tools for studies of cell lineage allocation and gene function in developing and adult intestines.[136] One limitation of this model using Cre-ER is that the rapid and continuous renewal of the intestinal

epithelium creates an obstacle for efficient and persistent conditional gene modifications. If Cre-ER is expressed under the control of a *Villin* promoter that is only active in differentiating intestinal epithelial cells, the kinetics of recombination may be too slow to achieve expression of the protein since these cells are rapidly lost. Moreover, if high levels of protein are expressed in the crypt cells, and if the proteins have long half-lives, ablation of the gene in villi will not deplete these cells for the target protein. Therefore, it is crucial to target the stem cell compartment using a different promoter.[136] Cre expression was detectable in tissue lysates from the small intestine, but to a lesser extent in the large intestine, which must be addressed before this system can be used as an inducible mouse model for human colon cancer by crossing with $Apc^{flox/flox}$ mice.

The *AhCre* transgenic model was created as an inducible system in which Cre expression was controlled by the *cytochrome P-450* promoter controlled by β-naphthoflavone.[137] The *AhCre* mouse model was used to conditionally delete the *Apc* gene by crossing it with $Apc^{flox/flox}$ mice.[138] $AhCre^{+};Apc^{flox/flox}$ mice showed loss of crypt-villus architecture, replaced by a distinct crypt-like phenotype and altered differentiation patterns. There also was induction of the Wnt signaling pathway with nuclear localization of β-catenin. However, the mice became visibly morbid within 5 days of β-naphthoflavone administration and were sacrificed.[139] These mice showed a severe reaction in the intestinal epithelial cells to homozygous deletion of the *Apc* gene. However, it was not possible to use this system as a disease model of intestinal polyposis due to early death of compound mice.

Apc exon 14-specific deletion models

Colnot et al[140] used a Cre-loxP strategy to develop a new model of germline *Apc* invalidation in colon carcinogenesis in which exon 14 was deleted. They first created $Apc^{3lox14/+}$ male mice with triple loxP sites, which were then crossed with transgenic *Meu-Cre* females to delete both *exon* 14 and the *hypoxanthine phosphoribosyltransferase* cassette, which were then mated with C57BL/6 mice and analyzed at generation 6 backcross.[140] Deletion of exon 14 generated a frameshift mutation at AA580 that disrupted the *Apc* gene. They compared the phenotype of $Apc^{\Delta 14/+}$ mice to that of the classical $Apc^{Min/+}$ mice. The main phenotypic difference was a shift of the tumors from the small intestines to the distal colon and rectum. Importantly, mice raised in conventional conditions developed more colon cancers than those raised in pathogen-free conditions. Thus, the severity of the colorectal phenotype in *Apc*-deficient mice is partly due to the particular mutation of *Apc* exon 14, but also to environmental parameters. All lesions, including early lesions, revealed *Apc* LOH and loss of *Apc* gene expression with accumulation of β-catenin and its target genes cyclin D1 and c-Myc. The $Apc^{\Delta 14/+}$ model is thus a useful new tool for studying the molecular mechanisms of colorectal tumorigenesis.[140]

In a more recent study, Haigis et al[141] crossed an $Apc^{2lox14/+}$ mouse with a *Fabpl-Cre* mouse to generate *Fabpl-Cre*; $Apc^{2lox14/+}$ compound mice. These mice had pedunculated, focal adenocarcinomas in the colon that had both low- and high-grade tumorigenic regions. Expression of $K\text{-}Ras^{G12D}$ in the colonic epithelium stimulated cell proliferation in a mitogen-activated protein kinase (MEK)-dependent manner. In contrast, $N\text{-}Ras^{G12D}$ did not alter the growth properties of the colonic epithelium, but was able to confer resistance to apoptosis. In *Apc*-mutant colon tumors, activation of K-Ras led to defects in terminal differentiation and expansion of putative stem cells within the tumor epithelium, which was associated with reduced signaling through the MAPK pathway. They also showed that human colon cancer cells expressing mutant K-Ras were hypersensitive to inhibition of Raf, but not MEK. These studies demonstrated clear phenotypic differences between mutant K-Ras and N-Ras, suggesting that the oncogenic phenotype of mutant K-Ras may be independent of MEK.[141]

Carcinogen treatment of mice does generate colonic neoplasia, but these mice show specific gene expression patterns that may not represent the entire range of human colorectal cancers. Most mouse models for human colon cancer targeting *Apc* faithfully replicate the characteristics of their human counterparts. However, most genetic mouse models manipulating *Apc* generate tumors predominantly in the small intestine, in contrast to human colon cancers, where tumors are found in the colonic epithelium. The recently developed $Apc^{\Delta 14/+}$ model is thus a useful tool for studying the molecular mechanisms of colorectal tumorigenesis.

von Hippel-Lindau Gene (*VHL*)

von Hippel-Lindau disease is a dominantly inherited cancer syndrome associated with the formation of hypervascularized neoplasms of the kidney, retina, pancreas, adrenal gland, and central nervous system.[142] *VHL* inactivation, either through sequence alterations or promoter methylation in tumor DNA, was reported in 86.6% of clear cell renal cell carcinoma cases.[143] *VHL* mutations are also observed in sporadic renal cancers. Germ-line mutations in the von Hippel-Lindau tumor suppressor disease are associated with a high risk of retinal and cerebellar hemangioblastomas, renal cell carcinoma (RCC), and, in some cases, pheochromocytoma.[144] Additionally, somatic mutation or epigenetic inactivation of the *VHL* gene occurs in most clear cell RCCs. The VHL protein plays a critical role in regulating proteasomal degradation of the HIF transcription factor, and *VHL* inactivation results in overexpression of many hypoxia-inducible mRNAs including vascular endothelial growth factor (VEGF). The *VHL* gene product is part of a ubiquitin ligase complex that targets the alpha-subunits of the heterodimeric transcription factor hypoxia-inducible factor (HIF) for polyubiquitylation, and hence, proteasomal degradation, when oxygen is available. VHL-defective clear cell renal carcinomas overproduce a variety of mRNAs that are under the control of HIF, including the mRNAs that encode VEGF, platelet-derived growth factor B, and transforming growth factor α.[145]

Roe et al[146] reported that pVHL directly associates with and stabilizes p53 by suppressing Mdm2-VHL induced an interaction between p53 and p300 and acetylation of p53, ultimately leading to increased p53-mediated transactivation and cell cycle arrest/apoptosis (Fig. 1). These results suggest that the tumor suppressor pVHL has the unexpected function of up-regulating the tumor suppressor p53. pVHL was found to associate with ATM and increase Ser-15 phosphorylation of p53, which was further confirmed by the finding that pVHL blocks Mdm2-mediated degradation and nuclear export of p53. From this point of view, the activity of pVHL is very close to that of Dmtf1 tumor suppressor.[147] pVHL is likely to indirectly block Mdm2-mediated degradation of p53 by recruiting ATM and mediating ATM-dependent Ser-15 phosphorylation of p53. Although p53 is known to directly associate with ATM, the finding that pVHL itself can associate with both ATM and p53 indicates that pVHL likely functions to enhance the ATM-p53 connection. Based on the observation that p53 stabilization is drastically reduced in *VHL*-deficient RCC cells in response to genotoxic stress, the authors suggested that pVHL serves as an important component in the formation of the ATM-p53 complex.

Gnara et al[148] targeted *Vhl* in mice and reported that $Vhl^{+/-}$ mice appeared phenotypically normal, while $Vhl^{-/-}$ embryos died between E10.5–E12.5 due to a lack of placental vasculogenesis. Thus, it was not possible to study the role of *VHL* in tumor suppression in the global knockout model. Haase et al[149] later generated a conditional *Vhl*-null allele (2-lox allele) and constitutional *Vhl*-null allele (1-lox allele) by using Cre-lox technology. In contrast to the previous report, mice with the heterozygous 1-lox *Vhl*-null allele developed cavernous hemangiomas of the liver, which was associated with liver cell steatosis and focal proliferations of small vessels. Conversely, liver-specific deletion of *Vhl* using mice with 2-lox allele and Albumin-*Cre* resulted in severe steatosis, blood-filled vascular cavities, and foci of increased vascularization within the liver. HIF-2α and VEGF expression was increased in $Vhl^{-/-}$ hepatocytes. Thus, targeted inactivation of *Vhl* in mice can mimic clinical features of the human disease and underscore the importance of the Vhl protein in the regulation of hypoxia-responsive genes *in vivo*.[149] It remains still unknown why VHL patients develop a spectrum of tumors, whereas only liver tumors are observed in some $Vhl^{+/-}$ mice.

In preclinical models, down-regulation of HIF-α, particularly HIF-2α, is both necessary and sufficient for renal tumor suppression by VHL. These observations are relevant to the demonstrated clinical activity of VEGF antagonists in clear cell renal carcinoma and form a foundation for the testing of additional agents that inhibit HIF, or HIF-responsive gene products, in this disease.

Conclusions

The use of GEMM that mimic human diseases has been crucial for validating the importance of signaling pathways disrupted in lung, breast, prostate, and other cancers. These models will form a basis for developing novel therapies to treat human patients with cancer. The advantages of the GEMM include:

(1) the mice used in these studies are immunocompetent compared to xenograft models using nude mice, and thus the tumor microenvironment in humans can be reproduced in a mouse model; (2) specific genetic alterations that are present in human cancers can be reproduced in an inducible, tissue-specific manner; (3) it is possible to obtain a large amount of tumor materials with specific genetic alterations from a living animal for pathological and molecular genetic analyses; and (4) novel therapeutic approaches can be tested at various stages of tumor development. Conversely, the disadvantages of GEMM include: (1) the complexity of the human cancers with multiple genetic alterations are not always reproduced in mice; it is necessary to cross several different strains of transgenic/knockout mice to reproduce multiple genetic alterations found in humans in mice, but crossing mice for more than three strains is very time- and labor-consuming; (2) mouse tumors are often different from human cancers (eg, the stromal tissue for mouse mammary tumors is adipose tissue while that for human breast cancer is mostly fibrous) and thus may not predict the therapeutic responses of human cancers. Generally, several mouse tumors can be cured with novel chemotherapeutic agents, but the same is not true for human cancers since there is not a direct correlation in therapeutic responses between mice and humans; (3) current GEMMs have been created on mixed genetic backgrounds, and thus must be backcrossed for 6–8 generations to make the mice congenic before being applied to preclinical trials of candidate therapeutic drugs; and (4) GEMM should be maintained in a quarantine facility where continuous monitoring is required to keep the conditions pathogen-free. Thus, the maintenance cost for these mice is very high. Addressing these challenges will be required for promoting more rapid and broad use of GEMMs for novel drug discovery against cancer.

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Author Contributions

Wrote the first draft of the manuscript: KI, PT. Contributed to the writing of the manuscript: KI, EF, PT. Agree with manuscript results and conclusions: KI, EF, PT. Jointly developed the structure and arguments for the paper: KI, EF, PT. Made critical revisions and approved final version: KI, EF, PT. All authors reviewed and approved of the final manuscript.

Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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