Single and Multiple Gene Manipulations in Mouse Models of Human Cancer

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Supplementary Issue: Animal Models of Cancer Biology

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ABSTRACT: Mouse models of human cancer play a critical role in understanding the molecular and cellular mechanisms of tumorigenesis. Advances continue to be made in modeling human disease in a mouse, though the relevance of a mouse model often relies on how closely it is able to mimic the histologic, molecular, and physiologic characteristics of the respective human cancer. A classic use of a genetically engineered mouse in studying cancer is through the overexpression or deletion of a gene. However, the manipulation of a single gene often falls short of mimicking all the characteristics of the carcinoma in humans; thus a multiple gene approach is needed. Here we review genetic mouse models of cancers and their abilities to recapitulate human carcinoma with single versus combinatorial approaches with genes commonly involved in cancer.

KEYWORDS: genetically engineered mouse model, epithelial cancer, genetic manipulation

SUPPLEMENT: Animal Models of Cancer Biology

CITATION: Lehman and Stairs. Single and Multiple Gene Manipulations in Mouse Models of Human Cancer. *Cancer Growth and Metastasis* 2015: 8(S1) 1–15 doi:10.4137/CGM.S21217.

TYPE: Review

RECEIVED: March 27, 2015. **RESUBMITTED:** June 17, 2015. **ACCEPTED FOR PUBLICATION:** June 20, 2015.

ACADEMIC EDITOR: Marc D. Basson, Editor in Chief

FUNDING: Authors disclose no funding sources.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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PEER REVIEW: 7 peer reviewers contributed to the peer review report. Reviewers' reports totaled 1494 words, excluding any confidential comments to the academic editor.

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Introduction

Genetically engineered mouse models (GEMMs) of cancer are useful platforms for understanding the molecular and cellular mechanisms of tumorigenesis. GEMMs allow us to study tumorigenesis and the interaction of tumor cells with the tumor microenvironment. Genetic manipulation in mice allows tumors to develop with concurrent inflammatory, angiogenic, and stromal responses that commonly occur in human cancers. These mouse models also offer utility for preclinical target validation and experimental therapeutics studies. However, the relevance of a mouse model relies on how closely it is able to mimic the histologic, molecular, physiologic, and metastatic characteristics of the respective human cancer.

GEMMs have been instrumental in our continuously evolving knowledge of tumorigenesis, but have historically fallen short of mimicking the expected characteristics of human cancers. A single genetic alteration may lead to a lower tumor penetrance in mice, lower metastatic rates in mice than is typically seen in humans, or no phenotype at all. This is often seen in transgenic models with the expression of a single activated oncogene or loss of a single tumor suppressor gene. These single genetic manipulations are frequently not sufficient to convert the epithelial cells to a malignant phenotype.

The low frequency of cancer that occurs in GEMMs with single gene manipulations may be related to the notion that cancer is a multistep process. For example, *CHD5* is a tumor suppressor gene at human 1p36, a common deletion in cancers of epithelial, neural, and hematopoietic origin.[1] Of the two *CHD5* single-gene-knockout mouse models reported, *CHD5* deficiency was shown to disrupt spermiogenesis, but no tumorigenesis was reported.[2,3] *CHD5*, like other genes studied alone in mouse models, may be a highly important gene in a given cancer. However, due to the multistep nature of cancer, more genetic events throughout the course of the life of the mouse may be necessary for the development of cancer. Furthermore, given the lifespan of a mouse, it may not be feasible in some cases to mimic a human disease that typically takes decades to develop. *K-ras* is another highly mutated gene that, in single gene manipulation models of pancreatic cancer, results in precursor lesions and some metastatic tumors, though with a long latency period and incomplete penetrance.[4,5] These models display more progressive disease, though *K-ras* activating mutations may still need to be combined with other genetic alterations to mimic pancreatic tumorigenesis.

More recently, the paradigm for GEMMs has shifted to studying the interaction of oncogenes with each other, tumor suppressor genes and growth factors, for example, to allow the creation of models more reflective of the human disease. Crossing transgenic strains that harbor these different genetic lesions permits us to assess the contributions of the genetic

events and the requirements for progression to malignancy. While GEMMs with multiple genetic manipulations may still have stochastic tumor formation, they are generally more poised to mimic human cancer. In this review we focus on the abilities of GEMMs to recapitulate human disease with single versus combinatorial manipulations of genes commonly involved in cancer (Table 1). Epithelial cancers account for 80%–90% of all cancer cases and deaths;[6] thus, there is a strong need for mouse models that are able to mimic the tumorigenic properties of these cancers seen in humans.

Breast Cancer

Over 30 years ago, the human oncogene *c-myc* was expressed in the mammary epithelium of transgenic mice under the control of the mouse mammary tumor virus (*MMTV*) promoter, resulting in the development of mammary tumors in a stochastic manner.[7]

Table 1. Modification of genes and phenotypic effects in GEMMs of human cancer.

HUMAN DISEASE	GENE	PHENOTYPE
Breast cancer	*c-myc*	Stochastic mammary tumors
	H-ras	Stochastic mammary tumors
	c-myc/H-ras	Tumor occurrence in 100% of mice
	Cyclin D1	Hyperproliferation in mammary gland; focal mammary tumors at 18 months
	Cyclin D1/Cdk2	Mammary gland hyperplasia and desmoplasia; Heterogeneous mammary tumor formation
	ErbB2	Multifocal adenocarcinomas with lung metastases at 15 weeks in 100% of mice; Focal mammary tumors in 8–12 months with sporadic lung metastases
	ErbB2/p53	Mammary tumors at 5 months with large cellular and nuclear size, increased mitosis and apoptosis
	PyMT	Multifocal adenocarcinomas in 100% of mice at 4 weeks; lung and lymph nodes metastases in >85% of mice at 4 months
	PyMT/Tgfbr2	Shortened tumor latency; increase in number and size of lung metastases
Prostate cancer	*Bcl-2*	Hyperplasia in the ventral lobe
	c-myc	Low grade PIN lesions; some invasive adenocarcinoma in 6–12 months
	c-myc/Akt	Microinvasive adenocarcinoma by 7 weeks
	PTEN	Neoplasia in off-target tissues; early death at 8 months; PIN lesions in 8–10 months
	PTEN/Ink4a/Arf	PIN lesions at earlier age
	PTEN/p27	Adenocarcinoma within 3 months; complete penetrance; invasion
	p53	PIN lesions in luminal epithelium at 20 months
	Rb	PIN lesions in luminal epithelium at 20 months
	p53/Rb	Adenocarcinoma in 8 months; neuroendocrine differentiation; highly invasive
Lung cancer	*SV40*	Lung adenocarcinoma in few months
	K-ras	Focal proliferative lesions in pneumocytes in 1 week; adenomas and adenocarcinomas within 2 months
	K-ras/p53	Hyperplastic lesions in 1–2 weeks; large adenomas or adenocarcinomas with nuclear atypia in 1 month
	K-ras/Ink4a/Arf	Hyperplastic lesions in 1–2 weeks; large adenomas or adenocarcinomas with nuclear atypia in 1 month
	c-myc	Multifocal bronchiolo-alveolar hyperplasia; adenomas; carcinomas; incomplete tumor penetrance; no metastases
	IgEGF	Hyperplasia of alveolar epithelium; incomplete penetrance
	c-myc/IgEGF	Bronchiolo-alveolar adenocarcinoma in 9 months
Colorectal cancer	*Apc*	Tumors in small intestine; Majority of adenomas benign
	K-ras	No effect on intestinal homeostasis
	Apc/K-ras	Accelerated intestinal tumorigenesis; increased invasion; 100% tumor penetrance; macroscopic adenomatous lesions at 6 weeks in the large intestine
Ovarian cancer	*SV40*	Poorly differentiated carcinomas with serous features by 13 weeks
	p53/BRCA2	SEOC between 7 and 11 months
	p53/PTEN	Oviductal lesions and endometrial tumors between 6 and 10 months
	p53/BRCA1/2/PTEN	Reduced latency to SEOC; decreased survival of mice to 5 months; invasive lesions
	ARID1A	No tumor formation in ovarian epithelium
	PIK3CA	Hyperplasia; no tumor formation
	ARID1A/PIK3CA	Primary ovarian tumors

Table 1. (*Continued*)

HUMAN DISEASE	GENE	PHENOTYPE
Pancreatic cancer	*K-ras*	PanIN lesions; some metastatic tumors after 1 year
	Ink4/Arf	No phenotype
	K-ras/Ink4a/Arf	PanIN lesions with rapid progression to highly invasive and metastatic cancer; death by 11 weeks
	K-ras/Ink4a/Arf	Solid pancreatic tumors in 7–12 weeks
	p53	No phenotype
	K-ras/p53	PanIN lesions; well-differentiated PDAC tumors
	SMAD4	No phenotype
	K-ras/SMAD4	PanIN lesions; decreased survival
	K-ras/SMAD4/ Ink4a/Arf	Well-differentiated PDAC tumors
Brain cancer	*EGFRvIII*	No gliomagenesis
	EGFRvIII/Ink4a/Arf	Diffuse brain lesions
	PTEN	Hypertrophy; hyperproliferation; no glioma formation
	PTEN/Ink4a/Arf	Aggressive tumors
	PTC1	Medulloblastoma between 5 and 25 weeks in 14% of mice
	PTC1/p53	Medulloblastoma between 4 and 12 weeks in 95% of mice
	Ink4c	No phenotype
	PTC1/Ink4c	Medulloblastoma between 12 and 36 weeks in 30% of mice
	PTC1/Kip1	Medulloblastoma in 60–70% of mice
Retinoblastoma	*Rb*	No phenotype
	Rb/p107	Unilateral retinoblastoma in 9 months
	Rb/p130	Bilateral retinoblastoma in 4 months with 100% penetrance
Bladder cancer	*Fgfr3*	Urothelial hyperplasia
	Fgfr3/K-ras	Urothelial hyperplasia
	Fgfr3/β-catenin	Urothelial hyperplasia
	H-ras	Hyperproliferation; low-grade, papillary, non-invasive tumors
	p53	Urothelial hyperplasia and dysplasia
	H-ras/p53	Low-grade and high-grade tumors
	PTEN	Urothelial hyperplasia; UCC by 13.5 months
	PTEN	Non-invasive UCC after 10 months in 10% of mice
	PTEN/p53	Bladder tumors with 100% penetrance at 6 months; 60% metastases by 4–6 months
Head and neck squamous cell cancer	*Cyclin D1*	Oral-esophageal dysplasia
	Cyclin D1/p53	Severe dysplasia; invasive oral-esophageal cancer by 5–6 months
	TGFβ1	Hyperproliferation in buccal mucosa, tongue, esophagus
	TGFβRII	No phenotype
	K-ras	Benign papillomas in oral cavity
	TGFβRII/K-ras	Primary tumors within 5 weeks
Gastric cancer	*K-ras*	Dysplasia
	CDH1	No tumor incidence
	CDH1/p53	Invasive cancer in 6–9 months
Liver cancer	*c-myc*	Dysplasia; hepatocellular adenomas; HCC by 12–15 months
	c-myc/TGF-α	HCC at 4 months
	E2F-1	Dysplasia; hepatocellular adenomas; some carcinoma
	c-myc/E2F-1	Acceleration of HCC growth; Neoplastic nodules by 10 months in 100% of mice
	Mdr2	Dysplastic liver nodules in 12–16 months
Esophageal cancer	*Klf5*	Increased proliferation in basal layer of esophagus
	Klf4	Hyperplasia, dysplasia, inflammatory infiltrate by 6 months; invasive tumors at 2 years
	p120ctn	Epithelial dysplasia by 4–6 months; squamous cancer by 9–12 months in 70% of mice

The results suggested that *c-myc* was necessary but not sufficient for tumorigenesis and required a further transforming event, as the authors expected more uniform development of tumor masses in the mammary glands of all mice. The same group also developed a similar mouse expressing the viral *Ras* oncogene (*v-Ha-Ras*). They found that activated *Ras* induced multiple neoplasms in the breast but in a stochastic manner.[8] These transgenic mice, genetically engineered to express dominant oncogenes, were subsequently described as the first "oncomice".[7] Since *c-myc* and *H-ras* are both overexpressed in human breast cancer, the same group then went on to pair *c-myc* with *H-ras*; they demonstrated that, in comparison to mice expressing *H-ras* alone in which *H-ras* is not sufficient for full malignant transformation, the combination of *c-myc* and *H-ras* expression together in the same animals is highly carcinogenic.[9] Coexpression of *c-myc* and *H-ras* causes a greater than threefold increase in the kinetics of tumor occurrence, with tumors occurring in all mice.[9] These experiments laid the foundation for the future use of mouse model systems to examine single- and multi-gene effects in breast carcinoma.

Since that time, many studies have addressed the role of individual genes in breast cancer tumorigenesis. Many of these studies focus on gain-of-function mutations in oncogenes or loss-of-function mutations in tumor suppressor genes. One of these is *cyclin D1*, which is found within the commonly mutated chromosomal band 11q13,[10] and is amplified in approximately 20% of primary human breast cancers and overexpressed at the protein level in 50% of breast cancers.[11] Studied under the control of the *MMTV* promoter, *cyclin D1* overexpression has been shown to result in proliferation abnormalities in the mammary gland with the development of focal mammary tumors at 18 months of age on average.[12] Given the long latency and focal nature of the mammary tumors, these data suggested that, though *cyclin D1* could promote mammary tumorigenesis, there needed to be additional genetic events for the full development of breast carcinoma. To this notion, further studies have demonstrated that mammary tumor formation induced by activation of Src kinases or ErbB-2 requires mammary epithelial expression of cyclin D1.[13–15] In addition, it has been reported that cyclin D1/cyclin-dependent kinase 2 (Cdk2) complexes are present at a high frequency in breast cancer; thus, Corsino et al (2007) utilized a cyclin D1–Cdk2 fusion protein[16] and expressed it under the control of the *MMTV* promoter. This resulted in mammary gland hyperplasia, desmoplasia, and mammary tumor formation.[17] Tumors from the *MMTV-cyclin D1-Cdk2* transgenic mice are heterogeneous and express luminal and myoepithelial markers consistent with human basal-like breast carcinomas.[18] These results suggest that *cyclin D1* and *Cdk2* together may mediate some of the transforming effects seen with *cyclin D1* alone in human breast carcinomas.

ErbB2, a member of the epidermal growth factor receptor gene family, has been studied in *MMTV-Neu* transgenic mice with the activated form of the rat homolog of *ErbB2* (*Neu*). These mice develop multifocal adenocarcinomas with lung metastases at approximately 15 weeks of age[19] with 100% tumor incidence. *MMTV-Neu* mice have also been created with overexpression of the unactivated form of *ErbB2*, with the mice developing focal mammary tumors in 8 to 12 months and sporadic secondary metastases to the lung.[20] *ErbB2* and *p53* are overexpressed together in human breast cancers and have been associated with progression of disease.[21] The combination of *ErbB2* and *p53* mutation causes accelerated development of mammary tumors, occurring in the mice around 5 months of age. The tumors have a larger cellular and nuclear size with increased rates of mitosis and apoptosis, consistent with a higher grade of neoplasm. These data indicate cooperativity between *ErbB2* and *p53*.[22]

Expression of the polyomavirus middle T oncogene (*PyMT*) under the control of the *MMTV* promoter in the mammary epithelium of mice is a widely used GEMM and allows the study of breast cancer through four distinctly identifiable stages of tumor progression.[23] In contrast to many single-gene mouse models of breast cancer, expression of *PyMT* resulted in transformation of the mammary epithelium. Development of multifocal mammary adenocarcinomas occurred in 100% of mice as early as 4 weeks of age. Metastases were seen in the lungs and lymph nodes at an incidence greater than 85% within 4 months.[24] Histological analysis of these tumors and metastatic lesions demonstrated similarity to human breast cancers in morphology and biomarker expression.[25]

Even with the ability of the *MMTV-PyMT* GEMM to accurately recapitulate human breast cancer progression, the addition of other genetic manipulations further enhances the model. For example, loss of the type II transforming growth factor-β receptor (*Tgfbr2*) in the context of *PyMT* expression (*MMTV-PyVmT/Tgfbr2*^{MGKO}) results in a shortened median tumor latency and an increase in the number and size of lung metastases.[26]

Breast cancer is a heterogeneous disease that is continuously being uncovered through the use of invaluable tools like GEMMs, which will allow researchers to translate basic science discoveries into clinical advances. With the ongoing discovery of the molecular profiles of breast cancers, new GEMMs with the manipulation of multiple genes are being developed and compared to human tumors with the hope of using GEMMs for drug development.[27]

Prostate Cancer

The first reported GEMM for prostate cancer was established by expressing the SV40 large T antigen oncogene under the control of the C3(1) promoter; this oncogene is known to inactivate p53 and retinoblastoma (Rb) proteins. These mice develop prostatic hyperplasia around 2 to 3 months of age and adenocarcinoma around 7 months of age.[28] The anti-apoptotic protein bcl-2 was examined in the context of development and progression of prostate cancer under the control of the C3(1) promoter. The authors demonstrated that the mice develop hyperplasia in the ventral lobe of the prostate, though with

no malignant transformation, rendering bcl-2 to have little impact on tumor initiation.[29]

c-myc is overexpressed or amplified in 80%–90% of prostate cancers.[30,31] Overexpression of *c-myc* in a transgenic mouse model was found to induce low-grade prostate intraepithelial neoplasia (PIN) lesions, which are formed by cells that proliferate within the prostatic epithelium and disrupt its well-defined architecture. Depending on the promoter used to drive *c-myc* expression, the PINs may or may not progress to invasive adenocarcinomas.[32,33] Mice with progression to invasive adenocarcinoma did so within 6 to 12 months of age.[33] These mice, under the control of the rat probasin ARR2PB promoter, were crossed with mice expressing a constitutively activated myristoylated Akt and accelerated progression of the PINs to microinvasive adenocarcinoma by 7 weeks of age.[34] The occurrence of *c-myc* amplification and PI3K pathway alterations together have previously been implicated through examination of human prostate tumors.[34] The data described above in the mouse indicate that additional events in the PI3K pathway are needed to cooperate with *c-myc* in driving prostate tumorigenesis.

Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) deletion is one of the most frequent genetic alterations in human prostate cancer. Heterozygous mutants of *PTEN* ($PTEN^{+/-}$) typically develop neoplasia in multiple off-target tissues with early death around 8 months of age.[30] Those that do survive show PIN lesions around 8 to 10 months of age, with no advance to invasive adenocarcinoma. In an effort to study *PTEN*, mutation of the gene has been combined with the manipulation of other tumor suppressors, including *Ink4a/Arf*. You et al (2002) demonstrated that $PTEN^{+/-}/Ink4aArf^{+/-}$ mice develop PINs at an earlier age than $PTEN^{+/-}$ alone, though still with no progression to adenocarcinoma.[35] Further crosses of $PTEN^{+/-}$ mice with *p27* null mice show evidence of prostate cancer within 3 months of age of the mice with complete penetrance. These tumors become invasive with no metastases.[36] Taken together, these data indicate a cooperative role of *PTEN* with other genetic events in prostate tumorigenesis.

p53 and *Rb* are altered in at least one-third of prostate cancers.[37] Zhou et al (2006) created single gene transgenic models of either *p53* or *Rb* inactivation in the prostate epithelium.[38] Prostate-epithelium-specific inactivation of either *p53* or *Rb* using B6.D2-Tg (*Pbsn-Cre*)*4Prb* (*PB-Cre4*) mice under the control of the ARR2PB promoter led to PIN lesions in the luminal epithelium around 20 months of age. However, when these mice are crossed to combine *p53* and *Rb* inactivation ($PB\text{-}Cre4;p53^{loxP/loxP}Rb^{loxP/loxP}$), rapid progression within 8 months of age to carcinoma with luminal epithelial and neuroendocrine differentiation is noted. These carcinomas are highly invasive and have gene expression signatures similar to that of human prostate carcinomas.[38] This study demonstrates the necessity of multiple genetic events to occur in the prostate cancer mouse model in order to accurately reflect the human disease and pathogenesis.

To date, the GEMMs created to study prostate cancer have provided much insight into the molecular pathways involved in prostate cancer initiation and progression. Moving forward, expressing or deleting genes in different cell types in a temporally controlled manner will continue to allow us to understand the multistep tumorigenesis process in prostate cancer.

Lung Cancer

Various GEMMs of lung cancer have been developed that target a specific subset of lung epithelial cells, allowing the role of oncogenes and tumor suppressors to be explored. The first oncogene targeted to the lung was the Simian virus large T antigen, resulting in adenocarcinoma of the lung within a few months.[39–41]

One widely used model involves the use of the reverse *tet* transactivator (*rtTA*) to induce activated *K-ras* ($K\text{-}Ras4b^{G12D}$) expression with the addition of doxycycline. Within 1 week, focal proliferative lesions are seen in pneumocytes of mice. Furthermore, within 2 months, the lungs contain adenomas and adenocarcinomas.[42] *K-ras* and *p53* mutations are less commonly found to be associated in lung cancer,[43] though *Ink4a/Arf* methylation has been associated with *K-ras* mutations in human lung cancer.[44] The authors then went on to combine this *K-ras* mutation with either *p53* or *Ink4a/Arf* deficiency. In both cases, hyperplastic lesions were present within 1–2 weeks and progressed to large solid adenomas or adenocarcinomas with mild nuclear atypia. These tumors developed within 1 month, more quickly than with the *K-ras* mutation alone.[42]

c-myc expression under the control of the lung-specific surfactant protein C promoter results in multifocal bronchiolo-alveolar hyperplasia, adenomas, and carcinomas. These mice exhibit incomplete tumor penetrance with no metastases.[45] Transgenic mice expressing a secretable form of the epidermal growth factor (IgEGF), a homologue of TGFα, develop hyperplasia of the alveolar epithelium with incomplete penetrance. In a mouse model expressing both *c-myc* and *IgEGF* together, the mice developed bronchiolo-alveolar adenocarcinoma at an accelerated rate, at an average age of 9 months.[45] These data suggest that *c-myc* and *EGF* cooperate with each other during lung carcinoma formation.

Progress in the detection of genetic alterations found in human lung cancer has resulted in the identification of a growing number of genes important in the disease. Differences exist in lung anatomy and physiology between mice and humans. Therefore, further development of lung cancer GEMMs manipulating these genes in combination is important in gaining a model that is histologically and molecularly similar to human disease. These GEMMs can also be combined with chemical carcinogen-induced methods of lung tumor formation to study lung cancer in an even more relevant context.

Colorectal Cancer

Studies comparing human tumor tissue and normal tissue have highlighted the various key mutations that are commonly

involved in colorectal tumorigenesis; this has been the key in the development of genetically engineered mouse models of colorectal cancer. Colorectal cancers begin with specific molecular alterations in the Wnt-β–catenin pathway, specifically loss of function of the adenomatous polyposis coli (*Apc*) tumor suppressor gene. Apc and Wnt signaling is aberrantly activated by mutation in 90% of human colorectal cancer.[46] The first Apc mouse developed and the most widely used of Apc mutant mice was the multiple intestinal neoplasia (Min) mouse ($Apc^{Min/+}$).[47] Though this model results in high tumor incidence, the tumors develop in the small intestine, which differs from the human disease where the incidence of colorectal tumors is significantly higher than those in the small intestine.[48,49] Another disadvantage of the *Apc* mouse model is that the majority of the adenomas are benign, as deregulation of Wnt signaling is thought to be an important initiator of tumorigenesis but is not sufficient to drive tumor progression.[46] It is thought that the sequential accumulation of mutations in specific genes, including *Apc*, *K-ras,* and *p53*, is necessary to drive the transition from normal epithelium to colorectal cancer,[50] supporting the notion that malignant transformation may require the involvement of other genetic events or signaling pathways in cooperation with *Apc*.

More recently, various groups have undertaken the task of using the *Apc* mutation in mice with the Cre-Lox system to shift tumorigenesis away from the small intestine and into the large intestine using *SRα*, *CDX2*, and *hCA1* promoters.[51–53] Sansom et al (2006) conditionally expressed an oncogenic *K-ras* allele (V12) in the small intestine of adult mice.[54] The authors found that normal intestinal epithelium appeared to be resistant to *K-ras* mutation, as expression of *K-ras* (V12) did not affect intestinal homeostasis in the mouse. However, when they combined this *K-ras* mutation with *Apc* deficiency in the mice, there resulted accelerated intestinal tumorigenesis and increased invasion.[54] Byun et al (2014) also combined inactivation of *Apc* and activation of mutant *K-ras*, but used colon-specific expression of Cre recombinase (AKC mice).[49] The authors used mice with the Apc^{580S} mutant allele,[55] latent activated $LSL\text{-}K\text{-}ras^{G12D}$ mutation,[56] and achieved inactivation of *Apc* and activation of *K-ras* through a cross with carbonic anhydrase 1 (CAC+) mice whereby Cre expression was tied to carbonic anhydrase 1, a gene expressed only in the large intestine.[53] In contrast to many commonly used Apc mouse models, these AKC mice have a tumor penetrance of 100% and develop macroscopic adenomatous lesions as early as 6 weeks of age, only in the large intestine.[49]

A variety of mouse models of human colorectal cancer exist that mimic various aspects of colon carcinogenesis. While chemically induced mouse models are able to mimic sporadic colon cancer and are often used to study dietary influences of carcinogenesis, GEMMs of colorectal cancer have been useful for studying the importance of specific genomic alterations in the development and progression of colorectal cancer.[57] With the development of models with multiple genetic manipulations, these GEMMs will be even more effective for drug sensitivity studies.

Ovarian Cancer

Most ovarian cancers (90%) are epithelial in origin, with the majority of these (70%) being serous epithelial ovarian cancers (SEOCs).[58] The majority of genetically engineered mouse models reported for ovarian cancer have been disappointing in their ability to mimic the features of the human disease, particularly for the predominant SEOCs. Aberrations in *p53*, *BRCA1/2*, and *Rb1* are most common in SEOCs. The first model of SEOC was Amhr2-SV40Tag, in which the small t and large T antigens of SV40 could act together through the inactivation of *p53* and *Rb1*. These mice were able to develop poorly differentiated ovarian carcinomas with serous features by 13 weeks of age.[59]

Since then, a more robust model of SEOC has been developed in which the mutation of *p53* is combined with *BRCA1/2* mutation and *PTEN* loss.[60] Previously, loss of one of these genes alone had not generated a tumorigenic phenotype.[61] However, Perets et al (2013) demonstrated that the combination of mutant *p53* and loss of *BRCA2* results in SEOC in mice between 7 and 11 months of age.[60] The combination of mutant *p53* with *PTEN* loss results in oviductal lesions and endometrial tumors between 6 and 10 months of age. Furthermore, the combination of loss or mutation of *p53*, heterozygous or homozygous loss of *BRCA1/2*, and homozygous loss of *PTEN* results in reduced latency to SEOC and decreased survival of mice to 5 months. The resultant lesions are invasive with an increased Ki-67 proliferative index and an immunohistochemical profile similar to that of human tumors.[60]

More recently, a mouse model of another subtype of epithelial ovarian cancer, namely, ovarian clear cell carcinoma (OCCC), was developed.[62] The authors found that *ARID1A* inactivation was not sufficient for tumor formation in the targeted ovarian epithelium. They also examined the effects of the mutation of the phosphoinositide 3-kinase catalytic subunit (*PIK3CA*). Utilizing the H1047R *PIK3CA* mutation, they used a Cre-inducible $(Gt)Rosa26Pik3ca^{H1047R}$ allele and observed hyperplasia but no tumor formation in the mice. These data suggest the need for additional genetic or mutational events to occur with *ARID1A* or *PIK3CA* to cause ovarian tumorigenesis. Chandler et al (2015) went on to study the potential cooperation between *ARID1A* and *PIK3CA* in $Arid1a^{fl/fl};(Gt)Rosa26Pik3ca^{H1047R}$ mice, finding primary ovarian tumors with histopathology similar to that of human OCCC.[62] These data support the notion that cooperation between *ARID1A* and *PIK3CA* mutations is necessary to induce ovarian cancer. These data also support the need for multiple versus single genetic manipulations in a mouse model in order to accurately mimic the human disease being studied.

Development of a reliable mouse model for epithelial ovarian cancer has met its challenges due to the lack of

a specific promoter for the ovaries, as many promoters are leaky.[63] Data from current GEMMs of ovarian cancer demonstrate that multiple genetic changes are required for ovarian tumorigenesis. Much progress has been made in identifying genetic event combinations that are able to accurately mimic the human disease in a GEMM with hopes for using them for preclinical testing.[58]

Pancreatic Cancer

The earliest attempts to develop genetically engineered mouse models of pancreatic cancer began in the 1980s, with the expression of SV40 T-antigen,[64,65] H-ras,[66] and TGF-β.[67,68] Most of these models did not produce pancreatic intraepithelial neoplasia (PanIN), the most common and well-characterized precursor lesion of pancreatic cancer, or pancreatic ductal adenocarcinoma (PDAC), the most common histological variant of pancreatic cancer.[69]

K-ras mutations are found in more than 90% of human pancreatic cancer, with the most common *K-ras* mutation on codon 12 ($K\text{-}ras^{G12D}$). Grippo et al (2003) overexpressed this common activating mutation in pancreatic acinar cells with resultant PanIN lesions in mice 18 to 24 months of age.[4] Though precursor lesions were present in these mice, no tumors developed, suggesting the requirement of additional genetic alterations for pancreatic tumorigenesis. Since then, many K-ras mouse models have been developed. Jackson et al (2001) established the $LSL\text{-}K\text{-}ras^{G12D}$ mouse that, when crossed with mice expressing a Cre recombinase under the control of either the *Pdx1* or *Ptf1a* promoters, resulted in constitutive activation of *K-ras*, progressive PanIN lesions, and some metastatic tumors after a latency period of more than a year.[5] These models show promise in demonstrating the ability of *K-ras* activation to induce PDAC in mice; however, the long latency period and incomplete penetrance again suggest that additional genetic events may be necessary to increase the incidence and timeline of malignancy progression. Thus, the use of mouse models combining *K-ras* mutations with other genetic manipulations may be required to effectively study human pancreatic cancer.

In addition to *K-ras* activating mutations, the most common genetic aberrations in human PDAC include inactivation of the tumor suppressor genes *Ink4/Arf*, *p53*, and *SMAD4*. Studies have shown that inactivation of each of these genes alone in the mouse pancreas results in no phenotype and must be combined with mutant *K-ras* to induce PDAC.[69] However, conditional deletion of *p16Ink4a* and *p19Arf* in the pancreas using Pdx1-Cre, in combination with the $K\text{-}ras^{G12D}$ mutation, causes an increase in PDAC progression in mice. PanIN lesions develop and rapidly progress to highly invasive and metastatic cancers that resemble human disease, with a proliferative stromal component and propensity to advance to a poorly differentiated state. In this study, death occurred in all mice by 11 weeks of age.[70] In a similar study using the $LSL\text{-}K\text{-}ras^{G12D}$*;Pdx1-Cre;Ink4a/Arf* mouse model, solid pancreatic tumors were observed in mice 7 to 12 weeks of age and had histological features similar to human disease, including a high Ki-67 proliferative index and large, highly atypical cells.[71]

p53 mutations occur in 50%–75% of human PDAC,[69] with a common mutation being $Trp53^{R172H}$. Hingorani et al (2005) combined the previously created $LSL\text{-}K\text{-}ras^{G12D}$*;Pdx1-Cre* mice with $LSL\text{-}Trp53^{R172H}$ mice and were able to demonstrate many similarities of this mouse model to human PDAC. $Trp53^{R172H}$ and $K\text{-}ras^{G12D}$ cooperate in these mice to promote the development of PanIN lesions as well as well-differentiated PDAC tumors with molecular heterogeneity and genomic instability.[72]

The tumor suppressor gene *SMAD4* is inactivated in more than 50% of human PDAC.[69] Loss of *SMAD4* was shown to promote the progression of $K\text{-}ras^{G12D}$-initiated PDAC when crossed with $LSL\text{-}K\text{-}ras^{G12D}$*;Pdx1-Cre* mice. *SMAD4* depletion resulted in decreased survival of mice and formation of PanIN lesions.[73] Upon further examination of the implication of Smad4, Bardeesy et al (2006) looked at the effect that loss of *SMAD4* had in mice harboring *K-ras* mutations as well as loss of *p16Ink4a/p19Arf*. These experiments showed that the loss of *SMAD4* promotes the rapid formation of well-differentiated PDAC tumors with an increased expression of epithelial markers.[73]

Effective therapies for pancreatic cancer are lacking; therefore, there is a great need for GEMMs that can faithfully mimic human disease and be used for discovering therapeutic strategies for pancreatic cancer. Many models of pancreatic cancer have been developed that focus in some way on *K-ras* mutations and, when combined with other genetic mutations, accurately represent the histology of human disease. Future studies will be focused on identifying the utility and value of these models in therapeutic discovery.[74]

Brain Cancer

Gliomas are the most common forms of primary brain tumors of which glioblastoma multiforme (GBM) is the most aggressive.[75] Constitutive EGFR activation is found in approximately 40% of primary GBM. This constitutive activation can result from mutant variant EGFRvIII able to signal constitutively in the absence of ligand.[75] Holland (2000) developed an *in vivo* glia-specific gene transfer system expressing the avian retrovirus ALV subgroup A receptor TVA under the *Nestin* promoter, allowing *EGFRvIII* to be transferred via a replication-competent ALV splice acceptor (RCAS) vector.[76] Overexpression of constitutively activated EGFR in the resultant transgenic mice (RCAS-*EGFR*) did not cause any brain abnormalities and did not lead to gliomagenesis.[76] These data led the authors to conclude that EGFR mutation alone is not sufficient to cause glioma and additional genetic events are necessary. Holland (2000) then went on to infect RCAS-*EGFR* into mice with *INK4a-ARF* deletion (RCAS-*EGFR*;*tv-a-*$INK4a\text{-}AR^{-/-}$). These mice developed diffuse brain lesions with histologic similarities to gliomas,[77] suggesting that multiple

genetic mutations are necessary for the development of glioma-like lesions.

PTEN mutation is found in ~30% of GBMs; however *PTEN* loss alone does not appear to induce glioblastoma formation.[78,79] Fraser et al (2004) generated mice with expression of Cre recombinase and conditional deletion of *PTEN* under the control of the glial fibrillary acidic protein (GFAP) promoter ($PTEN^{loxP/loxP}$*;GFAP-Cre*). *PTEN* loss in these mice resulted in hypertrophy and hyperproliferation of astrocytes but no glioma formation.[80] Wei et al (2006) generated similar *PTEN* knockout mice (*PTENf/f;hGFAP-Cre*), demonstrating increased brain mass that correlated with increased astrocyte cell proliferation and early death by 6 weeks of age. These data suggested to the authors that *PTEN* inactivation alone may contribute to gliomagenesis progression but needs additional events to initiate the process.[81] To address this issue, Zhu et al (2008) examined the effects of *PTEN* deletion with *INK4a/ARF* loss and constitutively active *EGFRvIII*. These mice displayed the formation of aggressive tumors with histologic and molecular similarities to human gliomas,[82] confirming the need for cooperation between these genes in gliomagenesis.

Medulloblastoma (MB) is the most common malignant childhood brain tumor and is a development-associated embryonal tumor of the cerebellum.[83] The developmental signaling pathway Sonic hedgehog (Shh) has been a primary area of focus in MB studies, particularly a negative regulator of this pathway, patched 1 (PTC1).[83] Goodrich et al (1997) demonstrated that homozygous deletion of PTC1 caused embryonic lethality in mice and that $Ptc^{+/-}$ mice developed MB typically between 5 and 25 weeks of age, though tumors occurred in only 14% of the animals.[84]

It was later found that crossing $Ptc^{+/-}$ mice with *Tp53*-deficient mice ($Ptc^{+/-};Tp53^{+/-}$) increased MB incidence to over 95% with a latency of 4 to 12 weeks of age,[85] demonstrating that multiple genetic events may be required to model MB tumorigenesis. Further supporting this notion, Uziel et al (2005) showed that either homozygous or hemizygous deletion of *Ink4c* on a $Ptc^{+/-}$ background increased MB incidence to 30% with a tumor latency of 12 to 36 weeks. *Ink4c* deletion was not enough on its own to cause MB formation, suggesting cooperation between genes.[86] Similar to the results seen with *Ink4c*, homozygous or hemizygous deletion of another cyclin-dependent kinase inhibitor, *Kip1*, in $Ptc^{+/-}$ mice increased MB tumor incidence to 60%–70%.[87]

Most brain tumors exhibit remarkable molecular heterogeneity.[88] GEMMs of brain cancers that have multiple genetic manipulations have come close to being able to represent the molecular variability in these diseases. A future direction of GEMMs in translational research of brain tumors involves continuing to develop relevant models that can be implemented in therapeutic studies that will be more advantageous than many of the current xenograft systems used in preclinical drug testing.

Retinoblastoma

The development of retinoblastoma typically begins with an inherited germline mutation in the *Rb1* gene of children. Various groups developed mouse models with an inactivated *Rb* gene ($Rb^{+/-}$).[89,90] Although children with heterozygous germline mutation of *Rb* develop retinoblastoma, these mice did not. It was later found that, in order to recapitulate the histopathological and molecular features of human neuroblastoma, more than one Rb family member needed to be inactivated since *Rb1* mutation alone failed to cause retinoblastoma.

Donovan et al (2006) used *Chx10-Cre* to inactivate *Rb* and *p107*, a retinoblastoma-like gene that can function as a tumor suppressor. These mice developed visible retinoblastoma, though with a long latency of 9 months and only unilateral presentation.[91] Inactivation of *Rb* was also combined with inactivation of another retinoblastoma-like gene, *p130*, using *Chx10-Cre*. These mice developed bilateral retinoblastomas with 100% penetrance in ~4 months.[92]

One of the challenges in the development of GEMMs for this germline disease is that the molecular pathways that are deregulated in human retinoblastoma are not so in mice.[93] Despite this, GEMMs are still a valuable tool in the study of retinoblastoma to understand the genes and interactions that can contribute to the tumorigenesis of the disease.

Bladder Cancer

Urothelial cell carcinoma (UCC) is unique among epithelial carcinomas, as tumorigenesis occurs by two distinct pathways: low-grade, papillary tumors contain oncogenic mutations in *Fgfr3* and *H-ras*, while high-grade, muscle-invasive tumors typically have defects in *p53*, *Rb*, and *PTEN*.[94]

It has been hypothesized that *Fgfr3*-activating mutations can act as a driver of UCC. Ahmad et al (2011) targeted the expression of mutated *Fgfr3* to the mouse urothelium under the control of the *UPII* promoter, with resultant urothelial hyperplasia but no evidence of dysplasia or tumorigenesis in the mice. *Fgfr3* and *K-ras* mutations have been found to be mutually exclusive in human bladder cancer patients;[95] however, when the authors paired the *Fgfr3* mutation with *K-ras* ($K\text{-}ras^{G12D}$) or *β-catenin* ($\beta\text{-}catenin^{exon3/+}$) activating mutations, they found similar results to those described above, indicating *Fgfr3* is not involved in initiating UCC tumorigenesis.[94]

Mutations in the *H-ras* oncogene cause it to become constitutively expressed, and Zhang et al (2001) targeted expression of constitutively active *H-ras* to the urothelium, causing early onset hyperproliferation that progressed to low-grade, papillary, noninvasive tumors.[96] Tumor latency depended on copy number of the *H-ras* transgene, though they demonstrated that low copy number mice develop tumors with a much longer latency of approximately 12 months. These data suggest that in the absence of *H-ras* overexpression, secondary genetic events are required to fully cause bladder tumorigenesis.[96]

Mutation and/or deletion of *p53* are common in human UCC and can occur with *H-ras* mutations.[97] Gao et al (2004) used the *UPII* promoter to target mutated *p53* to the urothelium of mice, with resultant urothelial hyperplasia and dysplasia but no UCC. However, when they crossed *p53* knockout mice with activated *H-ras* transgenics, they found bladder tumors of both low-grade and high-grade nature.[98] This suggests that loss of *p53* is not enough to promote bladder tumorigenesis, but needs an event like *H-ras* activation with which to cooperate.

Deletion of *PTEN* occurs frequently in invasive UCC, with reports showing *PTEN* loss in up to 94% of advanced UCC.[99] Results obtained in *PTEN*-null genetically engineered mouse models of bladder cancer have been inconsistent, particularly with the use of different promoters. Using a Fabp-Cre system, Yoo et al (2006) deleted *PTEN* and demonstrated urothelial hyperplasia and UCC by 13.5 months of age.[100] Tsuruta et al (2006) used the same mouse model and showed noninvasive UCC in 10% of mice after more than 10 months.[99] These groups hypothesized that the long latency periods seen in these studies could be due to the requirement of additional genetic events to drive urothelial tumorigenesis. More recently, Puzio-Kuter et al (2009) used an adenovirus expressing Cre recombinase delivered directly to the bladder to simultaneously delete *PTEN* and *p53* ($p53^{fl/fl}$; $Pten^{fl/fl}$). The combinatorial deletion of *PTEN* and *p53* in mice resulted in bladder tumors with 100% penetrance at 6 months of age that histologically resembled human invasive UCC tumors. Furthermore, 60% of the mice also developed metastases to local lymph nodes and distant sites by 4 to 6 months.[101] These data suggest that the development of UCC requires multiple mutations and that there is a need to combine these mutations in a mouse model to generate a relevant model to human UCC.

The 5-year survival rate of a metastatic bladder cancer patient is only 6%.[102] While various mouse models, as outlined above, have been developed that are contributing to a better understanding of the initiation and progression of UCC, there remains a need for models that represent the muscle-invasive metastatic form of the disease. Concerns with UCC GEMMs, including long latency or incomplete penetrance, may be resolved through the continued development of GEMMs with multiple genetic manipulations and may be useful for preclinical therapeutic studies.

Head and Neck Squamous Cell Carcinoma

The first report of a GEMM for head and neck squamous cell carcinoma (HNSCC) was of a model developed by Opitz et al (2002) for oral-esophageal cancer.[103] In this study, the Epstein–Barr *ED-L2* promoter (*L2*) was used to specifically target genes to the oral-esophageal squamous epithelium. *L2-cyclin D1* mice were developed and showed only oral-esophageal dysplasia with *cyclin D1* overexpression. Clinical studies have found a correlation between cyclin D1 and p53 expression and lymph node metastases.[104] Therefore, these *L2-cyclin D1* mice were crossed with *p53*-deficient mice ($L2D1^{+}/p53^{+/-}$), and severe dysplasia and invasive oral-esophageal cancer resulted by 5–6 months of age.[103] Since then, an inducible transgenic model of HNSCC has been developed using the progesterone receptor system in mice to induce expression of TGFβ1, causing hyperproliferation in the buccal mucosa, tongue, and esophagus.[105] The same group went on to use a similar technique to knock out *TGFβRII* in the buccal tissue, tongue, esophagus, and forestomach of mice; no phenotype or pathological changes were observed in comparison to controls. However, when *TGFβRII* deletion was combined with a *K-ras* mutation ($K\text{-}ras^{G12D/+}/TGF\beta RII^{-/-}$), mice developed primary tumors within 5 weeks.[106] A tetracycline-inducible system has also been used for conditional expression of the $K\text{-}ras^{G12D}$ mutant. This study reported the presence of benign papillomas in the oral cavity of these mice.[107] Though *K-ras* may play a causal role in HNSCC, oncogenic *K-ras* is not sufficient for malignant progression to HNSCC and requires other genetic events to occur.

Genetically engineered mouse models of HNSCC thus far have demonstrated that multiple genetic events are required to histologically and molecularly mimic human disease. Continued development of mouse models with multiple genetic manipulations will allow researchers to validate and test the role of newly discovered drugs for HNSCC. Future studies will also focus on the utility of mouse models to study site-specific interactions of oncogenes and tumor suppressors in the head and neck region.[108]

Gastric Cancer

Many mouse models of gastric cancer involve the use of *Helicobacter pylori* infection and carcinogen treatments, though a variety of GEMMs have been established. The first transgenic mouse models to be created were the insulin-gastrin mice.[109] These mice overexpress amidated gastrin and show progression to gastric dysplasia and invasive gastric cancer around 20 months of age.[110] *K-ras* transgenic mice are commonly used, and it has been shown that use of the K19 promoter to drive expression of the *K-ras-V12* mutant results in recruitment of inflammatory cells and the development of dysplasia. These data suggest that *K-ras* plays a role in gastric carcinogenesis initiation.[111] In a study by Shimada et al (2012), loss of expression of the *CDH1* gene that encodes for E-cadherin resulted in no tumor incidence. However, when combined with *p53* knockout, invasive cancer composed of poorly differentiated cells and a histologic similarity to human tumors was detected in mice from 6 to 9 months of age.[112] Though *CDH1* and *p53* loss have not been found to occur together in hereditary gastric cancer,[113] these data suggest that *CDH1* plays a role in gastric cancer, but may require additional mutations.

H. pylori infection and carcinogen treatment have classically been used to establish gastric cancer mouse models that exhibit similar phenotypes to human disease. Mouse models displaying an aggressive metastatic phenotype that is optimal

for preclinical studies are lacking. Therefore, multiple genetic manipulations in mice or multiple manipulations in combination with carcinogen treatment will be the focus of studies in an effort to develop a reliable model of gastric cancer.

Liver Cancer

Sandgren et al (1989) showed that *c-myc* transgenic mice (*Alb-c-myc*) display hyperproliferation, dysplasia, and hepatocellular adenomas in the liver, but no development of carcinoma prior to 18 months of age.[114] Using the same model, Santoni-Rugiu et al (1996) demonstrated that development of hepatocellular carcinomas (HCCs) occurs by the age of 12–15 months. When *c-myc* was combined with TGF-α, carcinomas developed at an accelerated rate in the double transgenic mice as early as 4 months of age. The tumors had histopathologic features similar to human disease, and these data demonstrate the cooperative effects of the combination of *c-myc* and *TGF-α*.[115]

Overexpression of *c-myc* has also been combined with that of the transcription factor *E2F-1* and targeted to the liver in a transgenic mouse model.[116] Either *c-myc* or *E2F-1* alone in a transgenic mouse causes dysplasia, hepatocellular adenoma, and some evidence of carcinomas. However, the combined expression of *c-myc* and *E2F-1* causes acceleration in the hepatocellular carcinoma growth and 100% of mice have neoplastic nodules by 10 months of age with evidence of malignant transformation.[116]

Mdr2 is a phospholipid flippase that promotes biliary secretion of phospholipids and protects the biliary epithelium from bile acids. Defects in Mdr2 are associated with cholestasis, biliary fibrosis, or cirrhosis.[117] Mdr2 knock-out mouse models have been used to study these diseases as well as hepatocarcinogenesis. Katzenellenbogen et al (2006) found that Mdr2 knock-out (Mdr2-KO) mice develop dysplastic liver nodules with a long latency period of 12–16 months. Further analysis of the Mdr2-KO tumors demonstrated alterations in genes and pathways important in human HCC, raising the question about the role of these genes as well as the utility of studying these genes in combination with Mdr2 deficiency in a mouse model to more closely replicate human HCC.[118]

Chronic hepatitis B (HBV) or hepatitis C (HCV) viral infections account for more than 80% of HCC;[119] however, mouse models to study HCC in the context of these viral infections are lacking. Heckel et al (1990) developed a transgenic mouse in which the urokinase-type plasminogen activator (*uPA*) gene was expressed under the control of the human albumin promoter (*Alb-uPA* mice). These mice exhibited chronic stimulation of hepatocyte growth and were shown to be susceptible to HBV and HCV infections after human hepatocyte engraftment.[120–122] Tesfaye et al (2013) went on to extend this model by crossing transgenic mice carrying the *uPA* gene under the control of the major urinary protein promoter (*MUP*) onto a *SCID/Beige* background (*MUP-uPA*). These mice allowed an 8-month window for engraftment with human hepatocytes and infection with HBV or HCV.[123] The advances made in developing a transgenic mouse model in which to infect HBV or HCV will be useful for future study of HBV- or HCV-derived HCC.

GEMMs of HCC have been useful in studying the roles and interactions of genes in hepatocarcinogenesis and the multistep nature of HCC. Moving forward, the focus of many studies is the development of a mouse model that is able to recapitulate human disease with utility for preclinical therapeutic studies.[124] These models may involve the manipulation of multiple genes in a tissue-specific and time-controlled manner, and with potential for being combined with carcinogen treatment.

Esophageal Cancer

Esophageal cancers, both esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC), are common worldwide with poor prognoses. Current models used to study the tumorigenesis of esophageal cancer are primarily orthotopic or surgical mouse models,[125,126] as few genetically based mouse models exist. Goldstein et al (2007) developed a model with the expression of Kruppel-like factor 5 (*Klf5*), a transcription factor expressed in proliferating cells of the gastrointestinal tract epithelia.[127] Expression of *Klf5* throughout the esophageal epithelia of mice driven by the *ED-L2* promoter caused no esophageal dysplasia or cancer. The mice did exhibit increased proliferation in the basal layer of the esophagus, though expression of another KLF family member, *Klf4*, inhibited this proliferation. This led the authors to conclude that Klf5 regulates proliferation in esophageal epithelial cells, but is not sufficient to maintain proliferation in the esophagus.[127] Since *p53* is the most common genetic alteration in ESCC, a xenograft model of ESCC was developed to look at the potential cooperation between *Klf5* and *p53*. Esophageal keratinocytes with *Klf5* knock down and $p53^{R175H}$ mutation, but not either alone, formed tumors in *SCID/NCr* mice, and these tumors were characteristic of invasive squamous cell carcinoma.[128] Tetreault et al (2010) targeted *Klf4* to esophageal epithelia under the control of the *ED-L2* promoter in mice. These mice developed hyperplasia, dysplasia, and inflammatory infiltrate in the esophageal epithelia and lamina propria by 6 months of age, and invasive ESCC by 2 years of age.[129] The authors concluded that inflammation plays a considerable role in the development of ESCC, though additional genetic events are also most likely required.

P120-catenin (*p120ctn*) is a tumor suppressor that is downregulated or lost in 35%–60% of ESCC patients.[130,131] Stairs et al (2011) generated a conditional knock-out mouse model of *p120ctn* using the *L2* promoter to delete *p120ctn* specifically in the squamous oral cavity, esophagus, and forestomach of mice ($L2Cre;p120^{loxP/loxP}$).[131] The mice developed epithelial dysplasia at 4–6 months of age and severe dysplasia with squamous cancer by 9–12 months of age, mimicking the progression to neoplasia seen in human ESCC patients. Approximately 70% of mice developed tumors during this

time frame, though no metastases were present.[131] These data suggest that deletion of *p120ctn* creates a useful model in which to study ESCC, though it exhibits a long tumor latency period and is not highly invasive, indicating that further genetic events may be necessary to fully recapitulate human ESCC.

GEMMs of esophageal cancer are severely lacking. Environmental factors and genetic alterations have been identified as playing important roles in this disease. However, the combination of genetic events needed for the development and progression of esophageal cancer has yet to be fully unraveled. Therefore, genetic mouse models that are able to mimic esophageal cancer are crucial for the study of this very lethal disease and development of effective therapeutics.

Renal Cancer

To date, no transgenic mouse model has been established to study renal cancer, which is aggressive and difficult to treat. Pollard et al (2007) inactivated mouse fumarate hydratase (Fh1) in the kidney to mimic hereditary leiomyomatosis and renal cell cancer (HLRCC) and found that Fh1 mutants developed renal cysts that overexpressed Hif1α and Hif2α at similar levels to renal carcinomas from HLRCC.[132]

Despite advances in the understanding of renal cancer biology, renal tumors remain difficult to treat partly due to the fact that animal models of the disease are lacking. The development of GEMMs that are able to accurately mimic human renal carcinoma will allow further understanding of important genes and interactions occurring in the development and progression of the disease, leading to the use of mouse models to evaluate therapeutic strategies.

Conclusions

Genetically engineered mouse models are valuable and essential tools for studying the in vivo aspects of human cancer development. These models have increased our understanding of human malignancies, and have aided in the identification of new biomarkers and testing of therapeutics for diseases. To fully understand the impact of the many genetic changes that occur in the tumorigenic process, it is critical for the mouse models to accurately mimic the human disease.

One of the points highlighted in this review, with regard to some of the GEMMs used in the study of human cancers, is that expression of a single activated oncogene or loss of a single tumor suppressor gene is sometimes not sufficient to convert an epithelial cell to a malignant phenotype. Indeed, there is a need across the spectrum of epithelial cancers for mouse models that utilize a combination of genetic manipulations in order to more closely recapitulate human cancer. Furthermore, many of the cancers highlighted here, including esophageal cancer and renal cancer, are among the most aggressive and deadly cancers and yet lack any highly invasive mouse model with which to study the diseases. Here, there is an even greater need for the development of GEMMs that precisely recapitulate the human condition with the use of combinatorial genetic manipulations.

In addition to the consideration of single or multiple genetic manipulations to develop a GEMM that closely mimics human disease, the technology used to develop the mouse models must be taken into account, with the time and cost of generation of a GEMM in mind. Conventional technology to create GEMMs has relied on homologous recombination in embryonic stem cells. More recently, the gene-editing tool CRISPR/Cas9 (clustered regularly interspaced palindromic repeats/CRISPR associated protein 9) has been used. CRISPR/Cas9 enables modification of a genome at any specific location directly in embryos, eliminating embryonic stem cell work and providing more flexibility. CRISPR/Cas9 tools allow faster model generation; whereas typical embryonic stem cell technology usually takes a year or more to generate a mouse model, CRISPR/Cas9 can take as little as 5 months and thus can be more cost effective in the end.[133] This is of particular interest for drug discovery, where decreasing model development time would be invaluable. Additionally, an advantage of CRISPR/Cas9 is that, after the injection of embryos, the resulting offspring consist solely of modified cells. This is in contrast to conventional methods using embryonic stem cells, which produce chimeric mice composed of both modified and unmodified cells that can be subsequently bred to homozygosity. There are some concerns with the repair mechanisms of double-stranded breaks introduced by the CRISPR/Cas9 system in experiments involving homologous recombination, as well as mosaicism in founder animals.[134]

Another alternative to traditional methods of creating a GEMM is the use of RNA interference through expression of short hairpin RNAs (shRNAs) to manipulate gene expression. One way shRNAs can be expressed in mice is through the use of lentiviral vectors. Lentiviral particles are delivered to the embryo, but the major difference between lentiviral vector shRNAs and traditional embryonic stem cell methods of generating GEMMs is that the use of lentiviral vectors will cause the numbers of integration copies to vary between progeny, as each provirus integrates independently. Traditional pronuclear DNA injection results in transgene insertion into a single locus, which will pass to the next generation containing many copies of the transgene.[135] Through the use of shRNAs in a mouse model, it is possible to modulate the expression levels of multiple cancer-related genes or silence mutated genes. Concerns with this technology exist in that RNA interference results in a knockdown of gene expression instead of complete inhibition, and shRNAs can have nonspecific effects and repression of nontarget genes. Additionally, the *Pol III* promoter frequently used to express shRNA is robust and ubiquitously expressed, which may result in embryonic lethality depending on the gene being silenced.[135]

One further consideration in the development of GEMMs is that most human cancers are genetic mosaics since cancer cells harbor mutations that are absent in normal

cells within the same body. Genetic mosaics techniques in mice allow one to make individual cells or groups of cells homozygous for a mutation(s) of interest at specific points in the development of a mouse. Mosaics in mice are typically created using *Cre-loxP*-mediated intrachromosomal recombination to attain a conditional knockout. Two *loxP* sites are inserted flanking an important part of a candidate gene by homologous recombination in embryonic stem cells. The Cre recombinase then dictates the spatial and temporal specificity of the loss of the candidate gene.[136,137] The mosaic analysis with double markers (MADM) system is a technology that uses the *Cre/loxP* recombination system to allow simultaneous green fluorescent protein labeling and gene knockout in clones of somatic cells or isolated single cells in a mouse model.[137] This system will aid in the analysis of mosaic mouse models and complex diseases resulting from genetic mutations.

The cost, time required to generate GEMMs, and differences between species in disease development can sometimes limit the use of GEMMs for investigating novel genetic interactions in tumorigenesis. Thus, continuous development of novel strategies to successfully modulate the mouse genome in an efficient manner is important in advancing our understanding of human disease. While the use of mouse models that incorporate multiple genetic modifications have gotten us closer to being able to mimic the characteristics of human cancers, none of the models addresses the issue of the timing of the genetic events that occur during human carcinogenesis. The timing and order in which each genetic event may occur in the tumorigenic process could have significant impact on the biologic activity and the secondary mutation spectrum of each cancer and its malignant phenotype. It can be unclear when studying multiple genes as to which genetic event occurs first in the development of a human cancer. Whether a gene is an early initiating event or late event in the tumorigenic process is important, as the genes may differentially influence cancer development and progression. Therefore, the timing of the genetic events, in addition to combinatorial genetic mouse model approaches, is important as we further develop our *in vivo* systems to model human disease. This provides an increased utility for the models to be used not only to study many aspects of cancer biology, but also gene cooperation, metastasis, and mechanisms of sensitivity and resistance to drug therapies.

The development of preclinical GEMMs has been invaluable in furthering our understanding of human diseases and testing new therapeutics. GEMMs that incorporate multiple genetic manipulations would be of particular utility when interrogating the involvement of potential therapeutics that specifically target the genes and downstream pathways of interest. The clinical efficacy of some cancer therapeutics is limited by the development of acquired resistance, which typically occurs within 3–12 months after beginning therapy.[138] This drug resistance can be due to secondary genetic mutations that arise, sometimes leaving limited therapeutic options for patients. GEMMs using combinatorial gene manipulation would allow these mutations to be incorporated into one model. It has been shown, for example, that in metastatic colorectal cancer patients who are treated with and respond to the EGFR monoclonal antibodies cetuximab or panitumamab, secondary *K-ras* mutations cause drug resistance in approximately 50% of patients.[139] For patients who did not have a secondary *K-ras* mutation arise but became resistant to the anti-EGFR antibodies, it was found that the proto-oncogene *Met* became amplified in their tumors and circulation, conferring resistance to the anti-EGFR therapy and causing drugs to fail.[139] GEMMs with multiple genetic aberrations would have great utility here, where a model manipulating *EGFR* and *Met*, could potentially be used to develop a third generation of EGFR drugs to overcome secondary resistance.

Genetically engineered mouse models of human cancer have played a crucial role in understanding various aspects of tumorigenesis in a way that other experimental systems cannot. Using a combinatorial approach to genetic manipulation in mouse models has only further enhanced our ability to answer experimental questions about genetic mutations and interactions that lead to human disease, particularly human cancer. Advances such as these will continue to be made that allow a better understanding of the mechanisms of tumorigenesis, and therefore the identification of better diagnostic and therapeutic strategies.

Author Contributions

Wrote the first draft of the manuscript: HLL. Contributed to the writing of the manuscript: DBS. Jointly developed the structure and arguments for the paper: HLL, DBS. Made critical revisions and approved final version: HLL, DBS. All authors reviewed and approved of the final manuscript.

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