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GI GEMs: Genetically Engineered Mouse Models of Gastrointestinal Disease

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Although there are similarities between each segment of the gastrointestinal (GI) tract that distinguish it from other organs, each segment has its own specific functions that are based on unique gene expression patterns that direct responses to the unique environments and stresses of each segment.[1] Consequently, modeling these diseases in the proper GI segment with similar pathologies has led to a variety of genetically engineered mouse (GEM) models. The vast majority of tumors carry multiple genetic alterations that accumulate from initiation through progression. Altered tumor susceptibilities can result also from gene polymorphisms. Animal modeling of the major components of this genetic complexity is possible in GEMs, although the full range of such variation is probably not possible. However, information obtained from xenograft models in which this variation a priori exists, will inform GEM modelers. Oncogenes can be introduced and tumor suppressor genes can be ablated. Hereditary cancer can be modeled through germ-line mutations, and nonhereditary cancer can be introduced in tissue-specific and inducible manners. Multiple gene defects can be combined or added in sequence through a combination of breeding and inducible systems. Polymorphisms can also be introduced in the germ line or in tissue-specific and inducible manners. Similarly, the effects of microenvironment can be functionally tested through genetic combination with gene alterations in those compartments and through alteration of the animal's environment. GEMs with more complex genetic combinations and more highly controlled regulation of tumor suppressor genes and oncogenes are now the predominant GEMs being used for mouse modeling of human cancer (reviewed[2–4]). In the fields of immunology and inflammation, GEMs have been predominant experimental tools, although the genetic complexity of these GEMs is not as great. Because cancer and inflammation are more intimately related in the GI tract than in any other system, the use of animal modeling has been critical for our understanding of inflammatory bowel disease (IBD) and its relationship to tumorigenesis (reviewed[5]).

In this review, a brief history of the development of the mouse genetic engineering field will be followed by a discussion of the genetic complexity being introduced into GEM models of human cancer. Brief discussions of the use of GEMs in GI cancers and IBD are then discussed.

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Conflicts of interest
The author discloses no conflicts.

History of GEMs

Transgenic Mice

Approaches to overexpress exogenous gene sequences in mice were being investigated in the 1970s. Germ-line transmission was first attained with Maloney Murine Leukemia Virus infection[6] and then pronuclear DNA microinjection.[7] Transgene expression, however, was elusive until it was discovered that an intron introduced into the β-globin transgene conferred expression.[8] Copy-dependent β-globin transgene expression was achieved by inclusion of a distant cis-acting locus control region.[9] Excellent reviews on transgenic animals and their usefulness for understanding gene function are available.[10,11]

Embryonic Stem Cells and Gene Targeting

Mouse teratocarcinoma or embryonal carcinoma cell lines[12] were first cultured from spontaneous teratomas that had been shown previously to contain pluripotent cells through their ability to be serially transplantable in the mouse.[13,14] Blastocyst injection of these cells produced chimeric animals, but germ-line transmission was too infrequent to be useful as an approach to transgenesis.[15,16] The breakthrough came with the isolation of primary pluripotent stem cells (later to be known as embryonic stem [ES] cells) from the inner cell mass of mouse blastocysts.[17,18] These cells colonized the germ line with useful efficiency[19] and were capable of transmitting transgenes[20] with generational stability.[21] Homologous gene targeting of an endogenous gene in a mammalian cell was first demonstrated in 1985.[22] Gene targeting in ES cells occurred shortly thereafter,[23,24] followed by germ-line transmission of some of those cells.[25,26] Reviews on the early developments in gene targeting are available.[27,28]

Variety of Genetic Modifications Now Possible

The first gene modifications involved transgenes driven by tissue-specific promoters and gene knockouts in which homologous recombination was used in ES cells to replace or add sequences that eliminated gene function (Figure 1*A, B;* (reviewed[10,11,27,28]). A bacteriophage recombination system has been employed to alter gene function in a conditional manner by generating a mouse in which the sequences encoding that function were flanked with recombinase recognition (*LoxP*) sites (Figure 1*C*).[29] This system has been used for both tissue-specific[30] or inducible[31,32] gene ablation (Figure 1*D–G*; reviewed with a wide range of very useful examples[33]). Variations on this theme have been developed in which transgenes could be conditionally activated by floxing (flanking with *LoxP* sites) transcriptional stop elements generally called Lox-Stop-Lox (LSL) sites (Figure 1*H*).[34,35] This approach has been used in lineage tracing studies in development (reviewed for pancreas[36,37]), and for lineage-specific expression of oncogenes in an inducible fashion (reviewed[4]). Reporter systems that are CRE activated include a LacZ reporter, in which an LSL was targeted into the ROSA26 locus so that the *LacZ* gene is expressed only in the presence of CRE,[38,39] and another in which an LSL EGFP fluorescent reporter was targeted into the ROSA 26 locus.[40] Deleter systems in which CRE activity causes removal of the LSL resulting in reporter gene expression include the conventional tissue-specific and doxycycline-inducible Cre transgenes referred to above, and also a tamoxifen-inducible system in which the CRE protein is fused to an estrogen receptor (CRE-ER) such that CRE becomes active only when the fusion protein binds tamoxifen.[41,42] Combination of the Cre/LoxP recombinase system with another recombination system from yeast (Flip/FRT[43]) increases the complexity of conditional gene alterations that can occur. Finally, the development of asymmetric LoxP sites that can irreversibly invert the floxed sequences can be used to conditionally invert sequences on the complementary DNA strand onto the reading strand.[44] These can be used to move a wild-type exon into the opposite strand and a

mutant exon, for example, with a single nucleotide polymorphism from the opposite strand, into the reading frame.

Engineering Genetic Complexity Into Mouse Models of Human Cancer

Hereditary Cancer

Germ-line mutations such as in p53 (Li-Fraumeni syndrome), BRCA genes (breast cancer), mismatch repair (MMR) genes (hereditary nonpolyposis colorectal cancer; Lynch syndrome) can be introduced into mice with simple targeted gene ablation,[45–47] but they do not always serve as good models for the human disease owing either to embryonic lethality in the homozygous state and low phenotype penetrance in the heterozygous state, as in *Brca* gene knockout mice,[46] or a tumor tissue prevalence that is not representative of humans, as in the MMR-deficient *Msh6* knockout mouse.[47] Inherited mutations are often the initiators that establish conditions for additional mutations that then direct the tissue prevalence and progression pathway for tumorigenesis. Consequently, it is necessary to generate tissue-specific or inducible ablation alleles to better model human hereditary cancer.

Sporadic Cancer

Most human cancers are sporadic in that they involve an initiating mutation in a cell, with subsequent accumulation of other genetic changes that drive pathways of progression to malignancy.[48] In mice, the initial tumor suppressor or oncogenic mutation can be generated through conditional gene targeting or transgenesis. Through tissue-specific and inducible systems these mutations can be combined or added in a sequential manner (reviewed[2]). An excellent demonstration of this approach has been applied to a set of tumor suppressor and oncogenes to delineate the tumorigenic processes that they affect.[49] Cell-specific pRb (retinoblastoma protein) inactivation was introduced by a truncated SV40 T antigen transgene whose product inactivates pRb. When combined with inactivations of *Pten*, *p53*, and *E2f1* and transgenic *Kras* activation in astrocytes, prostate, breast, brain, and ovarian cells, a variety of progression pathways for tumorigenesis in each of these cell types was delineated. Knowledge of these cell-type–specific pathways may inform clinical approaches to treatments for each type of cancer.

GI Cancer GEMs

Esophageal Cancer

In Barrett's esophagus, normal squamous epithelium is replaced by metaplastic columnar epithelium with goblet cells and is associated with a 30- to 125-fold increased risk of esophageal adenocarcinoma.[50–52] Zinc deficiency has been linked to esophageal cancer.[53] Squamous epithelial dysplasia of the oral– esophageal tissue was achieved by driving cyclin D1 expression with the Epstein–Barr virus ED-L2 promoter in transgenic mice.[54] Combination with a p53 deficiency led to invasive oral–esophageal cancer.[55] Further treatment of cyclin D1 transgenic mice or $p53^{-/-}$ mice with a zinc-deficient diet or with the esophageal carcinogen *N*-nitrosomethylbenzylamine resulted in esophageal cancer.[56,57] $Apc^{min/+}$, $p53^{-/-}$ and $p27^{-/-}$ mice have undergone esophagojejunostomy with gastric preservation to model jejunoesophageal reflux to determine which genes are important in the development of columnar metaplasia. It was found that loss of either of these genes leads to columnar metaplasia, but only loss of p53 and p27, but not APC, supports tumorigenesis in some of these mice.[58,5]

Expanded use of GEMs to identify the genes involved in formation of columnar metaplasia and progression to esophageal cancer under various nutritional and physiologic conditions should inform diagnostics and treatment.

Gastric Cancer

In mice, *Helicobacter* infection can lead to gastric inflammation and hyperplasia, but not to duodenal ulcer or gastric cancer without additional coupling to carcinogen treatment or other genetic alterations.[60] Because hypergastrinemia is associated with gastric cancer in humans, a transgenic mouse line with an insulin-promoter–driven gastrin gene was developed and infected with *Helicobacter*.[61] By 20 months of age three quarters of the mice had developed gastric cancer. Another set of models were based on the observations that in human gastric cancer there is decreased BMP, increased WNT signaling, and increased levels of prostaglandin (PG)E_2. Several transgenic GEMs were generated all using the cytokeratin K19 promoter to drive transgene expression in the gastric epithelium, and the resulting mice were infected with *Helicobacter* (reviewed[62]). *Noggin* (inhibitor of BMP) transgenics and doubly transgenic *Cox2* and *Pmes* mice (both genes in PGE_2 synthetic pathway) did not develop gastric cancer until triply combined. Similarly, although *Wnt1* transgenics developed preneoplastic lesions, no adenocarcinomas developed until genetically combined with the double transgenic *Cox2* and *Pmes* mice. These GEM models provide insight into the combinatorial approaches that may be useful in gastric cancer therapy.

The role of inflammation in gastric apoptosis and preneoplasia has been investigated using ionizing radiation and *Helicobacter* infection in GEMs with epithelial-specific disruption of I-κB-kinase β/nuclear factor κB signaling.[63] Increased apoptosis was found in response to cellular stress, and accelerated development of dysplasia occurred in *Helicobacter*-infected animals. A role for the myeloid-derived suppressor system in enhancing gastritis and initiating gastric carcinogenesis has been shown in a GEM transgenic line with increased interleukin-1β production.[64] Nuclear factor κB was found, in part, to mediate activation of the myeloid-derived suppressor cells.

Pancreatic Cancer

The first pancreatic cancer model was an SV40 T-antigen transgene driven by an insulin promoter (RIP-Tag mouse), which developed β-islet tumors.[65] Preneoplastic ductal lesions developed in an acinar-cell–specific elastase-promoter– driven *Kras*^{G12D} transgenic mice,[66] consistent with the commonly found oncogenic RAS mutations found in human pancreatic cancer. Elastase-driven *Tgf*α transgenic mice (acinar cell compartment), when genetically combined with *p53*$^{-/-}$, developed pancreatic tumors that often carried additional losses of the bi-allelic *Ink4a/Arf* locus or *Smad4* gene,[67] loci commonly mutated in human pancreatic cancer. *Pdx1-Cre LSL-Kras*^{G12D} double transgenic mice, in which the LSL stop signal is removed with a *Cre* transgene driven by the pancreas progenitor-cell–specific promoter from the *Pdx1* gene, developed intraepithelial neoplasias (PanINs).[68] Further genetic dissection of the *Kras*^{G12D}*/Smad4* combination demonstrated that whereas the *Kras*^{G12D} mutation in pancreatic epithelium (*Pdx1/Cre*-driven) resulted in PanINs that slowly developed neoplasias, and conditional ablation of *Smad4* alone in the same tissue had no effect, combination of the 2 resulted in rapid progression to neoplasias.[69] GEMs modeling the increased levels of NOTCH signaling often found in *Kras*^{G12D}-mutant pancreatic tumors suggested that the combination in mature acinar cells induces initiation and progression of acinar-derived PanINs.[70] This study, along with the early *Tgf*α transgenic study,[67] and another study finding that KRAS activation in acinar cells (*Elastase-Cre LSL-Kras*^{G12D} mice) leads to PanINs without a requirement for chronic exocrine injury,[71] reinforce the notion that different pancreatic epithelial cell compartments can transform into Pan-INs. Indeed, insulin-expressing endocrine cells normally refractory to KRAS-activated PanIN formation (*RipCre-ER*™ *LSL-Kras*^{G12D} or *RipCre-ER*™ *LSL-Kras*^{G12D} *Ink4A/Arf* $^{flox/flox}$ mice) can, however, transform in the presence of chronic inflammatory stress (cerulein) into the source of exocrine neoplasias.[72] In summary, these GEMs are providing important clues

as to how *Kras*^{G12D} pancreatic cells, which very rarely develop neoplasias, can transform into 1 of the most deadly cancers in humans.

Colon Cancer

Although there are many GEM colon cancer models, this discussion is restricted to 2 of the most commonly mutated pathways in human colon cancer, APC/*β*-catenin and transforming growth factor (TGF)-*β* pathways. Mutations in the human *APC* gene are found in nearly 90% of human colon tumors. APC deficiency leads to constitutive WNT signaling through inability of APC to retain *β*-catenin in the cytoplasm for degradation, resulting in nuclear translocation where it becomes a transcriptional co-activator with lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors.[73] Mice heterozygous for an *N*-ethyl-*N*-nitrosourea–mediated mutation in the mouse *Apc* gene have multiple intestinal neoplasia (*Apc*$^{min/+}$ mice),[74] and tumorigenesis in these mice is independent of colitis.[75] Unlike in humans, the tumors are primarily in the small intestine. However, if treated with the carcinogen azoxymethane (AOM),[76] or if fed an arginine-rich diet,[77] or if genetically combined with *Smad3*$^{-/-}$ mice,[78] the incidence of colon tumors is increased. An in-depth Gastroenterology review of the *Apc*-based GEM models, including those in which associated mutations found in humans are combined, was recently presented by Taketo and Edelmann.[79]

TGF-*β* pathway disruptions are found in up to 30% of human colon tumors and have been modeled in several GEM strains. If maintained on a predominantly 129 genetic background, *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$ mice develop proximal (cecum and proximal colon) mucinous colon cancer without APC or p53 pathway disruptions.[80,81] However, if immunodeficient *Tgfb1*$^{-/-}$ mice are maintained on a C3H background, they do not develop colon cancer.[81] Similarly, *Smad3*$^{-/-}$ mice, if on a primarily 129 genetic background,[82–84] also develop colon cancer with a preference for proximal colon and a requirement for *Helicobacter*.[82,85] It is not yet clear what the required modifier genes are in the 129 strain or why tumorigenesis is dependent upon *Helicobacter*-induced inflammation. Nor is it clear how TGF-*β* signaling provides tumor suppressor function. It was originally thought that epithelial growth is uninhibited in the absence of TGF-*β*. However, studies in the *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$ and *Villin-Cre Tgfbr2*$^{flox/flox}$ tumor models[80,86] indicate that TGF-*β* tumor suppressor function does not involve growth inhibition.

Expression profiling of tumors from the 4 main mouse colon cancer models (*Apc*$^{min/+}$, AOM-treated, *Smad3*$^{-/-}$, and *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$ mice) revealed 2 general profiles (*Apc*$^{min/+}$ and AOM; and *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$, and *Smad3*$^{-/-}$). This classification of colon tumors is consistent with the major pathways disrupted in human colorectal cancer, APC, and TGF-*β*, and indicated that the APC-deficient tumors represented an earlier embryonic colon expression pattern and less of an inflammatory pattern than that of the TGF-*β*– deficient tumors.[87] These results combined with the fact that both the *Smad3*$^{-/-}$ and *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$ strains require *Helicobacter* infection and its associated inflammatory response for tumorigenesis, strongly suggest that the tumor suppressor function of TGF-*β* signaling involves regulation of inflammation and immunoregulation of the interaction between the gut mucosa and gut microbiome.

Mice with a TGF-*β* type 2 receptor (*Tgfbr2*) inactivation in which a *Tgfbr2*^{flox} allele is combined with a *Villin* promoter-driven *Cre* transgene (*Villin-Cre*; expressed only in intestinal epithelium) develop duodenal adenomas and few intestinal tumors. However, when genetically combined with mice harboring an *Apc* truncation mutation (*Apc*$^{1638N/+}$), the intestinal epithelial-specific loss of TGF-*β*R2 increased progression of intestinal *Apc*$^{1638N/+}$ adenomas to invasive adenocarcinomas.[86] Similarly, genetic combination of another engineered truncation mutation (*Apc*$^{\Delta 716}$)[88] with a *Smad4* knockout allele led to

increased malignant progression.[89] Finally, conditional ablation of *Smad4* in the T-cell compartment through genetic combination of *Smad4*$^{flox/flox}$ and *Cd4-Cre* transgenic mice led to epithelial tumors throughout the GI tract from the oral cavity to the rectum, whereas conditional ablation in the intestinal epithelial compartment using a *Transthyretin-Cre* transgenic mouse did not.[90] Both this and a previous study in which the G protein subunit alpha i2 was knocked out, causing a thymocyte deficiency, which led to mucinous adenocarcinoma of the colon,[91] indicate the importance of the immunomodulatory microenvironment in tumorigenesis.

Because human *APC* or *TGFβ* mutations usually occur in combination with other mutations, GEMs are also modeling this complexity. Genetic combination of *Apc*$^{Min/+}$ and *Smad3*$^{-/-}$ mice increases distal tumor progression and burden over that found in *Apc*$^{Min/+}$ mice.[78] *Villin-Cre LSL-Kras*^{G12D} mice exhibit colonic epithelial hyperplasia,[92] but when combined with *Ink4a/Arf*$^{-/-}$ mice, the resulting tumors model aspects of human serrated colon cancer.[92] However, when KRAS activation mice are combined with *Apc* mutant mice (*Ah-Cre* [*β*-naphthoflavone-inducible *Cyp1a1* cytochrome p450 promoter] *LSL-Kras*^{G12D} *Apc*$^{flox/flox}$ mice),[93] increased progression and tumor burden occur. With the increasing complexity of these models, colon cancer researchers should be able to better correlate specific gene mutation with phenotypic aspects of colon tumors.

In conclusion, over the past 2 decades GI GEMs have become important tools in modeling human GI diseases. With respect to GI cancers, the variety of genetic manipulations that are now available allow us to introduce single mutations that model simple hereditary cancers, combinations of tumor suppressor knockouts and oncogene transgenics to model tumors with multiple mutations, sequential addition and subtraction of oncogenes and tumor suppressor genes to investigate mechanisms of tumor progression, polymorphisms thought to alter tumor susceptibility, and genetic alterations in microenvironments. With these GEMs, we can also probe the mechanisms underlying the effects of environmental stresses on tumorigenesis. With respect to GI inflammatory diseases GEMs with immunoregulatory defects can be used to model IBDs, and combinatorial genetics allow us to determine which of these pathway disruptions are sufficient and which are contributory. Finally, the IBD models should enable us to dissect the disrupted regulatory pathways underlying dysbiosis and the conversion of commensal to pathogenic bacteria. Finally, in all of these cases, GI GEMs can be used for preclinical testing of diagnostic and treatment therapies for GI diseases, including antibiotic and probiotic approaches.

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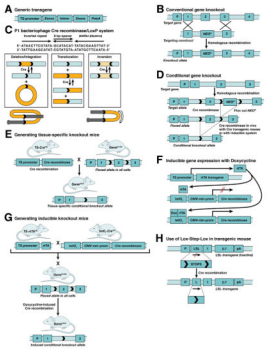

Figure 1.

Mouse genetic engineering: common procedures. (*A*) Conventional transgene with promoter, exons, ≥1 intron, and poly A stop signal. (*B*) Conventional scheme for knocking out a gene in ES cells using homologous recombination. In this scheme, the neomycin resistance selectable marker gene *NeoR* replaces Exon 2 when homologous recombination occurs. (*C*) In the *CreILoxP* recombination system from PI bacteriophage, the CRE recombinase protein recognizes the *LoxP* recognition sites, and, depending on the orientation of the 8-bp spacer, can delete sequences between or translocate sequences at the *LoxP* sites if the spacers are in the same orientation, or it can invert the sequences between the sites if the spacers are in opposite orientation. A diagram of the CRE/LoxP reaction is given. (*D*) Conditional knockout scheme in which both exon 2 and the *NeoR* marker genes are flanked by *LoxP* recognition sites (floxed). The *NeoR* marker gene can be removed in the ES cells or in vivo. In the animal, CRE recombinase is supplied either through breeding with a *Cre* transgenic (Cre^{TG}) mouse in a tissue-specific (TS) manner, as in (*E*), or an inducible marmer, as in (*G*). Often, the marker gene is flanked with FLP recognition sites and removed with FLP recombinase (not shown; see text) either in vitro or in vivo. (*E*) Animal breeding scheme to generate a conditional knockout mouse in a tissue-specific manner where the $Gene^{FLOX}$ mouse is made from the type of construct shown in (*D*). Upon tissue-specific expression of the *Cre* transgene, exon 2 is removed only in that tissue. (*F*) Inducible Cre transgenic scheme.[31] In this particular scheme, rtTA is a chimeric gene expression transactivator composed of (i) the DNA binding domain of a mutant tetracycline repressor (tetR) that binds DNA only in presence of the tetracycline analog doxycycline (Dox), and (ii) the gene expression trans-activation domain of the herpes simplex virus *VP*16 gene. The *rtTA* transgene is expressed in a tissue-specific manner, but the *Cre* transgene, being driven by a tandem set of7 tet operators (*tet07*) coupled to a cytomegalovirus (CMV) minimal promoter, is not expressed until the animal is treated with Doxycycline so that it can complex with the rtTA protein allowing it to bind to the *tetR* element and transactivate *Cre* transgene expression. (*G*) Breeding scheme associated with the inducible scheme (shown in *F*). It requires genetic combination of the $TS\text{-}rtTA^{TG}$ and $tet07\text{-}Cre^{TG}$ doubly transgenic animals with the floxed gene-targeted gene ($Gene^{FLOX}$). When the genetically combined offspring are treated with doxycycline, the target gene will become ablated. (*H*) LSL cassette contains transcriptional and translational stop signals that block expression of the transgene. CRE recombinase deletes the LSL sequences so that transgene expression is initiated. The LSL sequences can also be inserted in opposite orientation so that CRE recombination turns off an active transgene.