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Pathology of Rodent Models of Intestinal Cancer: Progress Report and Recommendations

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Abstract

In October 2010, a pathology review of rodent models of intestinal neoplasia was held at The Jackson Laboratory. This review complemented 2 other concurrent events: a workshop on methods of modeling colon cancer in rodents and a conference on current issues in murine and human colon cancer. We summarize the results of the pathology review and the committee's recommendations for tumor nomenclature. A virtual high-resolution image slide box of these models has been developed. This report discusses significant recent developments in rodent

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RS: Workshop participant; drafted manuscript

JS: Workshop participant, revised manuscript critically

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modeling of intestinal neoplasia, including the role of stem cells in cancer and the creation of models of metastatic intestinal cancer.

Keywords

Intestinal neoplasms; pathology; genetically engineered mice; disease models; animal

In 2000, a panel of 7 pathologists and 4 basic scientists met at The Jackson Laboratory in Bar Harbor, Maine. The group participated in a Mouse Models of Human Cancers Consortium-sponsored symposium, focused on intestinal neoplasia, and a workshop on techniques for modeling intestinal cancer in mice, sponsored by The Jackson Laboratory. After reviewing 17 different models of murine intestinal cancer and comparing representative lesions with those of prototypical human colorectal cancers, the panel developed guidelines for nomenclature of intestinal tumors rodents and criteria for distinguishing invasive carcinomas from herniations of non-neoplastic epithelium in rodent models. The findings and recommendations were published in a consensus report in *Gastroenterology* in 2003 ¹.

Since 2000, new developments in modeling human gastrointestinal (GI) cancers, including reports of convincing models of metastatic disease and new models derived from epithelial stem cell populations, have resulted in major advances in the field. The pathology of new rodent models of intestinal cancer was revisited in 2010 by a panel of pathologists and basic scientists. It was generally agreed that a “multiple pathways” hypothesis of intestinal cancer had largely replaced the sequential genetic model for human colorectal cancer.

The goals of this workshop in 2010 were: to examine the pathology of new rodent models of intestinal neoplasia and reach a consensus among a group of expert pathologists regarding the findings, to gauge the progress made in the intervening decade toward modeling human intestinal cancer, to assess the utility of the original recommendations regarding nomenclature, and to explore the creation and ongoing curation of a digital slide box of rodent models that would be accessible to investigators worldwide.

The models reviewed at the 2000 meeting were summarized in Supplemental Table 3 of the 2003 report ¹ and the models reviewed at the 2010 meeting are summarized in Table 1. Not all existing mouse models of intestinal tumors were discussed at the 2010 meeting (many have been reviewed recently by Mark Taketo and Winfried Edelmann²), and a number have since been developed. These include additional reports of mismatch-repair–and phosphoinositide 3-kinase–induced tumors ^{3–6}. There was little discussion of the effects of the microbiome on tumorigenesis or the use of orthotopic or xenograft tumors. Reports on these topics have been recently published ^{7–10}.

Update on Mouse Pathology Nomenclature

Most of the nomenclature recommendations from the 2000 Mouse Histopathology Workshop ¹ have been adopted by the research community, with the notable exception of the “gastrointestinal intraepithelial neoplasia” (GIN) terminology for small precursor lesions. This terminology was initially recommended to parallel similar recommendations by the World Health Organization (WHO) in 2000 for use in human diagnostic pathology¹¹. However, although “intraepithelial neoplasia” is used by some countries, the term is not used routinely by medical pathologists in the USA or Europe. The working group convened to update the 4th edition of the WHO classification was unable to reach a consensus on a single term for non-invasive neoplastic lesions of the digestive system ¹².

The WHO Classification of Tumours of the Digestive System, published in 2010, has also broadened the definition of intraepithelial neoplasia to include all precursor lesions, whether or not “traditional morphologic features of neoplasia” are identified¹². Since the intraepithelial neoplasia terminology is not universally applied to human GI neoplasia, and has also not been widely adopted for lesions in animal models (for example, small pre-invasive neoplastic lesions have been termed small or unicyptal adenomas, individual transformed crypts, or GIN), the panel agreed that although the GIN terminology is still acceptable, it is no longer recommended for use in characterizing intestinal neoplastic lesions in animal models. In alignment with new WHO recommendations and paralleling the nomenclature used for human intestinal neoplasms, the terminology, outlined in Table 1 of the 2003 recommendations¹, and updated here (Supplemental Table 1; hyperplasia, aberrant crypt foci (ACF), adenoma, herniation, and adenocarcinoma) is endorsed. The criteria for the categories other than GIN remain unchanged from the 2003 recommendations¹.

Major areas of discussion at the workshop included application of the original criteria to distinguish invasive adenocarcinomas from herniations of non-neoplastic or non-invasive crypts (a common problem in inflammation-associated models of intestinal neoplasia), definition of the term intra-mucosal carcinoma, and assessment of serrated architecture. The original criteria for invasion, which included presence of desmoplastic stroma, sharp, irregular or angulated glands, lateral spread of invasive crypts relative to the mucosal surface component, and cell loss from the invading mucosa¹, were reviewed and endorsed by the panel.

The term intra-mucosal carcinoma is applied to neoplastic lesions in the human large intestine that show invasion of neoplastic cells into the lamina propria, or into, but not through, the muscularis mucosae (Figure 1A), without involvement of the submucosa; it is recommended for similar lesions in animal models. Intra-mucosal carcinomas often share cytologic features with invasive carcinomas, such as cribriform architecture with intra-luminal accumulation of tumor and inflammatory cell debris (dirty necrosis). The term carcinoma in situ is not used for human glandular intestinal tumors and is discouraged because of its imprecision.

The morphologic characteristics of serrated architecture have not been clearly defined in animal models, and the panel agreed that none of the models reviewed developed neoplasms that were morphologically similar to human serrated intestinal neoplasms. The term serrated has been used to describe villiform hyperplastic lesions in mice¹³, but these lesions do not closely resemble the serrated lesions found in human colon (hyperplastic polyps, traditional serrated adenomas, and sessile serrated adenomas). The panel agreed that the term serrated should be reserved for lesions showing crypts or other glandular structures with saw-tooth or stellate architecture, as observed in human traditional and sessile serrated adenomas (Figure 1B).

Morphology of Intestinal Neoplasms, Rodent Models, and Comparison with Human Lesions

The panel reviewed the morphologic findings in a number of animal models of intestinal neoplasia developed in the 10 years since the first Histopathology Workshop; those that have been published are listed in Table 1. Glass slides were digitally scanned and high-resolution images are available for review at <http://mmgint.org>. The models reviewed were divided into the following broad groups: rodents exposed to carcinogens, models related to alterations in genes associated with putative stem markers, other rodent models such as the *Pirc* rat, complex models with alterations in genes involved in more than 1 carcinogenic pathway, and models showing putative metastatic lesions. Although azoxymethane (AOM)

and other chemical carcinogens have been widely used for a number of years, there is new information about the relationship between background strain and morphology of AOM-induced lesions¹⁴. Many of the other models reviewed at the workshop have greatly advanced studies of human colorectal carcinoma, by altering multiple genes to create more aggressive tumors that can invade and metastasize.

Rodent Exposure to Carcinogens

AOM-Induced Flat Lesions

Histological assessments of AOM-induced tumors have revealed strain-dependent effects on formation of specific tumor morphologies. Of the strains that have greater than 10% tumor penetrance in response to a standardized dose of AOM¹⁵, KK/HIJ, FVB/NJ, 129S6/SvEvTAC, and C57L/J strains predominantly develop pedunculated tumors, whereas NOD/ShiLtJ, MOLF/EiJ, BTBR T+tf/J, SM/J, CBA/J, ILN/J, SWR/J, and ICR/HaROS strains predominantly develop flat or sessile tumors that lack a stalk^{15, 16}. A/J, DBA/2J, and P/J develop similar numbers of tumors of both morphologies. Although few tumors in the AOM model show evidence of invasion, invasive tumors are observed in AOM-susceptible KK/HIJ, MOLF/EiJ, and A/J strains. Deeply invasive pedunculated tumors, as assessed by observation of displaced crypts in the stalk or into the muscularis propria, are rarely observed in this model. Slides examined by the panel showed a range of neoplastic lesions, from small flat adenomas with low-grade dysplasia (Figure 1C), to non-pedunculated adenocarcinomas that invade all layers of the intestinal wall (Figure 1D).

AOM-Induced Mucin-Depleted Foci: Early Lesions in Colon Carcinogenesis

Much effort has been spent on identifying morphologically distinctive pre-invasive mucosal lesions to help elucidate the early steps of colon carcinogenesis. Colonic preneoplastic lesions have been mostly studied in rodents exposed to carcinogens such as 1,2-dimethylhydrazine (DMH) or its metabolite AOM, which induce colonic carcinogenesis and produce tumors with features similar to sporadic colon cancers in humans¹. ACF identified in these models have been widely used as biomarkers of colon carcinogenesis. Although ACF correlate with late events of carcinogenesis in many rodent studies^{17–19} not all ACF represent dysplastic lesions²⁰, prompting concerns about their utility as surrogate markers for intestinal neoplasia. Efforts to identify preneoplastic lesions better correlated with the process of carcinogenesis have led to characterization of so-called mucin-depleted foci (MDF), first described in 2003 in colons of rats exposed to AOM²¹, and in a dose-related manner with DMH administration²² to different strains of mice. The lesions have also been identified in humans at high risk for adenomas and colorectal carcinoma²³. MDF may be preneoplastic markers, in that they decrease in response to inhibitors of carcinogenesis (such as caloric restriction, synbiotics, or polyethylene glycol), and increase in response to tumor promoters such as cholic acid, high-fat diets, or red meat^{24, 25}.

MDF can be visualized in unsectioned colonic mucosal samples stained with reagents that identify mucin, such as the combination of Alcian blue and high iron diamine or neutral red²⁶. They can also be identified in colons already processed by methylene blue staining to detect ACF²¹, allowing direct comparison of the frequency of each type of lesion in individual samples. When visualized with mucin stains, MDF appear as distinct patches of crypts that can be shorter and smaller than normal and have lower levels of MUC2, the most abundant mucin in the colon. In addition, they lack intestinal trefoil factor (a marker of the goblet cell lineage)²⁷, and have many dysplastic features on histological examination²¹ (Figure 2A). Slides examined by the panel revealed multiple small dysplastic lesions, often comprising only 4 to 5 crypts and minimally elevated above the adjacent mucosa. Alcian

blue stain showed abundant goblet cells in adjacent morphologically normal crypts, but not in MDF.

The number of MDF (about 8 per animal) observed in animals following carcinogen exposure is much lower than the number of ACF (about 200 per colon)²². Similar to human colorectal adenomas, MDF have constitutive activation of WNT signaling, with decreased levels of β -catenin at the plasma membrane and increased cytoplasmic and nuclear β -catenin.²² *Adenomatous polyposis coli* (*Apc*) and *β -catenin* gene mutations were detected in MDF at frequencies similar to those of grossly identifiable tumors^{22, 26}.

Genetic Models in Other Rodents

The *Apc*^{*Pirc*/+} rat (*Pirc* rat) model was recently developed²⁸. Unlike the *Apc*^{*Min*/+} mouse model,²⁹ which was discovered based on its phenotype in an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen, *Pirc* rats were generated by screening the first-generation offspring of male rats exposed to ENU for truncating mutations in the *Apc* gene²⁸. A heterozygous point mutation at nucleotide 3409 of *Apc* resulting in a change at codon 1137, was identified in a single rat. The mutation is embryonic lethal in homozygotes; in the heterozygous state on the F344 genetic background, animals become moribund at about 11 months of age due to intestinal obstruction by large colonic tumors. Roughly equal numbers of tumors are found in the small intestine and colon. The colonic tumors in the *Pirc* rat are relatively large (often 1 cm or more), and animals of the F344 background live for relatively long periods of time, so they are better suited for longitudinal chemopreventative studies that involve non-invasive imaging or colonoscopy.

Tumors from *Pirc* rats of the F344 background examined by the workshop attendees were characterized as pedunculated adenomas; larger lesions had areas of high-grade dysplasia. Irregular infiltrative crypts indicative of invasive adenocarcinoma were present at the base of some of the lesions that developed in a more sensitive model (F1 generation of F344/Tac crossed with ACI/Hsd) after exposure to the inflammatory agent dextran sodium sulfate (DSS) (Figure 2B)^{28, 30}.

Mouse Models with Alterations in Genes Associated with Putative Stem Cell Markers

Models with alterations in genes associated with markers of stem cells included *Lrig1*^{*CreERT2*/CreERT2} and *Lrig1*^{*CreERT2*};*Apc*^{*fllox*/+} mice, contributed by Coffey and colleagues, and *Lgr5* models from Hans Clevers' laboratory. *Lrig1*, a negative regulator of ErbB, marks a population of stem cells near the intestinal crypt base that are less proliferative than *Lgr5* stem cells. By lineage labeling, single-labeled, long-lived, quiescent *Lrig1*-labeled cells are detected at the crypt base and are reactivated following irradiation damage. *Lrig1*^{*CreERT2*/+};*Apc*^{*fllox*/+} mice develop distal colonic adenomas with high-grade dysplasia that accumulate high levels of β -catenin in the nucleus. Functionally null *Lrig1*^{*CreERT2*/Cre-ERT2} mice develop duodenal adenomas with high penetrance³¹. The lesions were associated with an unusual plaque-like hyperplasia of Brunner's glands (Figure 2C). These tumors are predominantly low-grade adenomas, but foci of cribriform architecture that indicate high-grade dysplasia were found in larger lesions; superficial invasion was observed in 1 animal, characterized by infiltration of glands with fibrotic stroma at the base of the adenoma. These duodenal tumors were negative for nuclear β -catenin.

Mouse Models with Alterations in Multiple Pathways Implicated in Colorectal Cancer

Two models with alterations of Stat3 were reviewed, contributed by the laboratories of Elaine Lin and Florian Greten. *Stat3^{ΔIEC}* mice lacked a discernible phenotype and were largely protected from development of AOM-induced adenomas³². However, *gp130^{Y75F}* mice, when exposed to AOM and DSS, developed a greater number of colonic tumors—often larger than those in than wild-type mice; the example examined by the panel contained a pedunculated colonic adenoma with high-grade dysplasia (Figure 2D). The tumors in the *Stat3^{ΔIEC}*, although smaller and less numerous, were associated with more severe colitis. The Stat3-IKO mice contributed by the Lin laboratory, with inactivated Stat3 in myeloid and lymphoid cells, but not in epithelial cells, develop colitis with roughly 50% penetrance by 18 to 20 weeks of age³³. Grossly visible flat or plaque-like polyps developed in the cecum and proximal colon, similar to the dysplasia-associated lesions or masses that develop in patients with inflammatory bowel disease. Some of the lesions contained invasive adenocarcinomas that invaded the submucosa or muscularis propria. Development of adenomas and adenocarcinomas was found to require the intestinal microflora, in that antibiotics inhibited development of colitis and neoplastic lesions. Review of slides confirmed the presence of colitis with mucosal hyperplasia and displaced crypts representing herniation. A small focus of infiltrating crypts, most likely representing superficially invasive adenocarcinoma, was identified at the base of 1 of the lesions (Figure 3A).

In *Apc^{Min/+};Smad3^{-/-}* mice, activation of canonical Wnt signaling is combined with attenuation of transforming growth factor- β (TGF- β) signaling, resulting in large numbers of invasive adenocarcinomas in the distal colon by 60 days of age³⁴. Roughly 40% of mice develop rectal prolapse from the large distal tumors, with herniation of glandular epithelium in some lesions. Microscopically, the tumors examined by the panel showed the full spectrum of dysplastic changes, from typical adenomas to high-grade dysplasia to invasive adenocarcinomas with minimal desmoplastic reaction. The neoplasms arose in the background of colitis and mucosal hyperplasia (Figure 3B). Liver metastases were not reported³⁴. This model resembles human colorectal adenocarcinoma more closely than *Apc^{Min/+}* mice, in that most of the lesions are colonic instead of small intestinal.

Apc^{1638N/+};Villin-Cre;Tgfr2^{E2flx/E2flx} mice were produced with the goal of generating a mouse model replicating 2 events common in human colorectal cancer: *APC* mutation and TGFBR2 inactivation. These mice developed similar numbers of intestinal tumors compared with *Apc^{1638N/+}* mice. However, the tumors had more advanced histologic features, such as high-grade dysplasia and invasive adenocarcinoma, consistent with the hypothesis that Tgfr2 inactivation promotes the progression of *Apc*-initiated adenomas to adenocarcinoma³⁵. The invasive carcinomas that developed in these mice had histologic features often observed in sporadic colorectal cancers in humans, including infiltrating glands that contain abundant necrotic debris (Figure 3C).

Villin-Cre;LSL-K-ras^{G12D};Tgfr2^{E2flx/E2flx} mice were generated to assess the effects of introducing a mutant *K-ras* allele and inactivating TGF- β signaling. Activation of *K-ras* alone results in mucosal hyperplasia, with increased goblet cells and crypt length, and aberrant branching or villiform morphology in the distal colon—but no neoplastic lesions³⁶. Combined activation of *K-ras* with inactivation of Tgfr2 results in development of intestinal tumors in approximately 70% of mice by 22 weeks of age. The tumors are primarily invasive adenocarcinomas, and are evenly distributed between small and large intestine. Slides examined by the panel showed relatively low grade, but deeply invasive adenocarcinomas with mucinous features, arising out of relatively flat overlying mucosa without overt adenomatous or dysplastic change. About 15% of these mice developed

grossly visible metastases in regional lymph nodes or lung (Figure 3D). These metastases have morphologies typical of intestinal adenocarcinoma, including dirty necrosis in lung metastases, and were confirmed to have originated from tumors of the intestinal epithelium, based on detection of recombined *Tgfr2*.

Mouse Models with Metastasis

Conditional Knockout of *Apc* and Activation of *K-ras* with Adeno-Cre Recombinase

Mice with conditional knockout of *Apc* and activation of *K-ras* with adeno-cre recombinase following mechanical abrasion of colons were developed by Hung and colleagues. To create these mice, adenovirus-Cre recombinase was delivered following mechanical abrasion of the mucosa to induce recombination of the floxed *Apc* allele, as well as recombination of the *LSL-K-ras* allele, resulting in 1-copy of mutant (G12D) *K-ras* in the distal colonic epithelium³⁷. Because the tumors are restricted to the distal colon, tumor progression can be followed using serial colonoscopy. This model can therefore be used to test therapeutic agents. The entire adenoma–carcinoma sequence occurs in these mice, including spontaneous metastasis to liver in 20% of mice examined at 24 weeks after injection of adeno-Cre recombinase.

The panel examined the preinvasive lesions that develop in this model; microscopically, they resemble human colorectal adenomas and some contain areas of high-grade dysplasia. Most of the invasive carcinomas closely resembled human sporadic colorectal carcinomas (Figure 4A), but some were high-grade lesions with solid growth patterns, and areas of anaplastic spindle cell component were observed in liver metastases. Hepatic metastases (Figure 4B) expressed the transcriptional factor *Cdx2*, based on immunohistochemistry, indicating intestinal differentiation.

K-ras^{G12Dint};*Ink4a/Arf*^{-/-} mice contributed by Florian Greten and colleagues develop villiform hyperplasia of the colon, likened to serrated neoplasias in humans¹³, although serrated features on the submitted slide were not well developed. These mice develop intestinal spindle cell malignant tumors (Figure 4C) that require additional studies for characterization. A slide containing a lung nodule examined by the panel contained a papillary adenocarcinoma (Figure 4D) similar to those described in mice that express oncogenic forms of *K-ras*^{38, 39}; the panel could not confirm that this was a lung metastasis, based on morphology alone (evidence of recombination was not presented).

Villin-Cre;*LSL-K-ras*^{G12D};*Tgfr2*^{E2flx/E2flx} mice contributed by William Grady's laboratory develop lymph node and lung metastases³⁶.

Other Mouse Models

Mice with insertional mutations generated by use of a Sleeping Beauty system, with transposition confined to the GI tract, develop adenomas and carcinomas in the small and large intestines⁴⁰. Slides from these mice were examined by the panel and showed a variety of lesions, including duodenal adenomas, invasive mucinous adenocarcinomas arising in adenomas (Figure 5A), and undifferentiated round-cell neoplasms, likely representing hematopoietic malignancies. A similar screen conducted in *Apc*^{Min/+} mice resulted in 3-fold more polyps than in *Apc*^{Min/+} alone, but without evidence of metastatic lesions⁴¹. Results from a more extensive Sleeping Beauty screen of *Apc*^{Min/+} mice have been published recently⁴².

***Apc*^{Min/+};*Rab25*^{-/-}, and *Smad3*^{+/-};*Rab25*^{-/-} Mice**

As a member of the Rab11 family of small GTPases, Rab25 regulates vesicle trafficking and plasma membrane recycling. Rab25 is expressed in the GI epithelium, most abundantly in ileum and colon. Gene expression studies of human colorectal cancer samples found that expression of *RAB25* was decreased in carcinomas compared to the normal colonic mucosa, independent of tumor stage⁴³. *Apc*^{Min/+};*Rab25*^{-/-} mice have increased adenoma number and size, compared to *Apc*^{Min/+} mice⁴³. As described in our earlier review⁴³, *Smad3*^{-/-} mice develop proximal colonic cancers in a 129 background; *Smad3*^{+/-} mice rarely develop intestinal neoplasms but can develop benign hamartomas¹. When crossed with *Smad3*^{+/-} mice, 80% of *Smad3*^{+/-};*Rab25*^{-/-} mice develop the full spectrum of changes in the adenoma–carcinoma sequence, from dysplastic crypts to large invasive adenocarcinomas⁴³. The adenocarcinomas are relatively low in grade and many produce large amounts of mucin (Figure 5B), similar to lesions observed in *Smad3*^{+/-} mice. However, formation of invasive tumors is markedly accelerated in the *Smad3*^{+/-};*Rab25*^{-/-} mice.

The panel examined colon tissues from the *Villin-Cre*;*N-ras*^{LSL-G12D/+} and *Fabpl-Cre*;*Apc*^{CKO/+};*K-ras*^{LSL-G12D/+} mice and agreed that mutant *K-ras*, but not mutant *N-ras*, leads to mucosal hyperplasia in the colon. Colonic mucosa from *N-ras*^{G12D} mice appeared normal, whereas the mucosa was greatly thickened in mice with *K-ras*^{G12D}, with elongated crypts that contained enlarged goblet cells. Tumors developing in *Fabpl-Cre*;*Apc*^{CKO/+};*K-ras*^{LSL-G12D/+} mice had diffuse high-grade dysplasia with areas of intramucosal carcinoma (Figure 5C) in contrast to tumors that expressed wild-type *K-ras*, indicating that mutant *K-ras* accelerates the progression of adenomas that develop in mice with an *Apc* mutation.

A model of colorectal adenoma-carcinoma progression in which a *CDX2P-NLS Cre* transgene was used to inactivate *Apc* specifically in the colon was also examined⁴⁴. This model has the advantage of developing multiple colorectal tumors, with few small intestinal lesions. Most of the tumors are adenomas, as in typical *Apc*^{Min/+} models, but 17% of the mice develop carcinomas within 300 days. Slides examined by the panel showed typical *Apc*^{Min/+}-type adenomas in the colon, with early invasion at the base of 1 of the lesions (Figure 5D).

Recommendations for Immunohistochemistry

Diagnostic pathology and research studies to characterize human tumors rely on immunohistochemistry (IHC) to identify and localize proteins of interest, or that are characteristic of a particular tumor. In rodent models, immunohistochemical analyses using antibodies that label paraffin-embedded tissues are important for morphologic characterization of tissues and tumors. IHC is invaluable for assessing subtle changes in number, localization, or morphology of differentiated cell types in the intestine, such as goblet cells, Paneth cells (restricted to small intestine and proximal large intestine), enteroendocrine cells, tuft cells and M cells. IHC is also important for identifying differentiation along these lines in neoplasms. Rodents with disruptions in specific genes can be used to determine antibody specificity and cross-reactivity. Methods for IHC, including tissue fixation and epitope retrieval, are widely available and have been described by Igor Mikaelian et al.⁴⁵.

Criteria for Antibody Validation

Although IHC is widely used to assess animal models of intestinal neoplasia, the stringency of antibody validation varies widely. Human diagnostic applications are often challenging because of pre-analytical and analytical factors that affect results on formalin-fixed, paraffin-embedded tissues. These factors can be minimized in animal models by following

standardized protocols that limit variations in time to fixation, differences in fixatives used, fixation period, and tissue processing. The use of mouse monoclonal antibodies on mouse tissue presents special challenges, which can often be overcome by use of mouse-on-mouse methods or systems such as the universal animal detection system⁴⁶. A compendium of useful antibodies for animal studies is maintained by The Jackson Laboratory at <http://tumor.informatics.jax.org/html/antibodies.html>. Web sites such as Antibodypedia could also be useful in helping researchers choose commercially available antibodies appropriate for specific applications⁴⁷.

The panel endorsed the general approach to antibody validation recommended in a recent review by Jennifer Bordeaux et al.⁴⁸ and used in David Rimm's laboratory. In brief, new antibodies should be tested by immunoblot analysis for reactivity with cell line lysates or tissue homogenates; positive and negative cell lines or tissues should each be tested. The pattern of labeling should then be evaluated to make sure it is localized and consistent with published descriptions of the biology of the antibody target. Protein expression and localization determined by IHC should be compared to results from immunoblot analyses performed with the same tissue samples or cell lines. Assays must then be repeated to determine reproducibility. Tests for cross-reactivity are now more rigorous, because there are better negative controls due to the creation of many knockout animals.

Metastatic Models and Recommendations for Validation

A long-sought goal of mouse intestine researchers is a model that recapitulates the full spectrum of disease progression in humans. Such a model would not only develop invasive primary carcinomas of the intestine with high penetrance, but also distant metastases. The slides and associated papers from mouse models of metastatic colon cancer reviewed by the Histopathology Consensus Workshop attendees^{13, 36, 37} can be viewed at <http://mmgint.org>. The descriptions of these models included various strategies to confirm that putative metastatic lesions are derived from primary GI tumors. A lively discussion ensued regarding the degree of validation necessary to instill confidence that a given metastatic lesion did indeed arise from a given primary tumor.

The consensus of the panel is that detailed morphologic and molecular evaluation of putative metastatic lesions in rodent models of GI cancer is essential to validate models of metastasis. Many genetically engineered rodent models of neoplasia have a propensity to develop neoplasms in multiple organs. Many, if not most, of these tumors are carcinomas. In some instances, these non-enteric tumors arise as primary tumors in common sites of metastasis such as the lung and the liver, whereas, in other instances, the non-enteric tumors have the potential to metastasize to these same organs. For example, Moritz Bannenke et al. reported detecting metastatic GI and primary lung lesions side-by-side¹³. Furthermore, many of the background strains of mice used to create models of human cancer have spontaneous neoplastic disease, including pulmonary adenomas and carcinomas³⁹.

Since many carcinomas are similar in histologic appearance, it can be difficult to determine the tissue of origin of metastatic lesions based on this feature. Lineage marking techniques, such as expression of green fluorescent protein (GFP) by cells of the GI tract or transplanted GI tumors, could be used to determine whether distant metastases arose from these cells. However, when it is not possible to incorporate lineage markers into the experimental design, immunohistochemical analyses can provide information about the tissue of origin. Surgical pathologists have published many findings that can be used to select antibodies to distinguish between human metastatic and primary tumors. Because of the scarcity of animal models of metastatic disease, less information is available, but the same principles apply.

Because of the need to accurately determine the tissue of origin of putative metastatic lesions in rodent models of intestinal cancer, we have assembled a recommended panel of immunohistochemical markers that can be used to distinguish some of the most common epithelial neoplasms (Supplemental Table 2). A panel of several positive and negative markers of tumors of the proposed tissue of origin, as well as metastasis site-specific markers (for example, lung markers for pulmonary metastases), should be used in every study. Neoplastic cells tend to lose expression of tissue-specific markers and gain expression of atypical markers, so there is always some degree of uncertainty in assigning cell type of origin. However, despite the imperfections of IHC, it is the best available tool to determine the tissue of origin, and is essential to experimental studies of metastatic disease in animal models.

The broad impact of animal studies necessitates the highest possible degree of confidence in the accurate attribution of putative metastatic lesions. Over time, the list of suggested markers will surely be refined, expanded, and improved. However, we feel that the field has an immediate need to define and adopt a best practice standard for assessing metastatic lesions, to allow the community to evaluate research findings with confidence. We are aware that best practice is a moving target that changes as our understanding of cancer pathogenesis improves.

Stem Cell Markers

Intestinal epithelial stem cells are defined by their ability to self-renew and to give rise to all differentiated epithelial cell types. Although a number of surface molecules have been proposed as markers of intestinal stem cells, definitive evidence requires lineage tracing, wherein a single intestinal cell within a crypt is marked in such a way that it and all its progeny are labeled⁴⁹. If a stem cell is marked, it divides and gives rise to more stem cells, along with all the differentiated lineages within that crypt; adjacent crypts could also be marked if crypt fission occurs. Lineage tracing can demonstrate the number of progeny from the founder cell, their location, and their differentiation status⁴⁹.

By this stringent metric, Lgr5⁵⁰, Bmi-1⁵¹, Tert⁵², Hopx⁵³, and Lrig1³¹ qualify as markers of intestinal stem cells (Supplemental Table 3). Of these, Lgr5 is the best characterized; it is present on rapidly dividing cells at the base of small intestinal and colonic crypts. Statistical modeling predicts these proliferative Lgr5⁺ stem cells divide symmetrically^{54, 55}. It should be noted, however, that symmetrical cell division does not mean symmetrical cell fate. Nor do these modeling results preclude the existence of a population of quiescent stem cells. In fact, recent evidence indicates that Lgr5⁺ stem cells are dispensable for small intestinal crypt homeostasis; upon ablation of Lgr5 cells, crypt architecture is maintained and Bmi-1⁺ stem cells are able to substitute and give rise to Lgr5⁺ cells⁵⁶.

In addition, the lines between quiescent stem cells and differentiated epithelia may not be as fixed as once thought, as discussed in a recent review from Hans Clevers and colleagues⁵⁷. Along these lines, Doug Winton and colleagues have recently shown that quiescent, label-retaining cells within the small intestine are committed to a secretory cell fate, yet are able to revert to a stem cell state. Surprisingly, a subset of these cells express Lgr5, which was thought to be expressed only by actively proliferating, intestinal stem cells⁵⁸. Ultimately, it is likely that functional heterogeneity exists among intestinal stem cell populations; a central question is whether there is 1 population that expresses different markers within a continuum of stem cell potential, or whether they form distinct populations or subpopulations. For example, long-term lineage tracing experiments of Bmi-1⁺ cells showed that they had similar kinetics to Lgr5⁺ cells⁵¹, and sorted Lgr5-EGFP^{hi} cells had the highest levels of *Bmi-1*^{59, 60}. Lgr5 and Bmi1 could therefore mark overlapping populations,

possibly with different potentials. It will be interesting to determine their relationships with the other markers mentioned.

In the highly dynamic and rapidly self-renewing intestinal epithelium, it has been suggested that only stem cells have a sufficient lifespan to be the cell from which cancer develops. By this reasoning, if a marked population of cells is engineered to undergo an initiating event and tumor formation ensues, then the original population is a stem cell population. Lgr5⁺ intestinal stem cells give rise to full-blown small and large intestinal tumors upon simultaneous removal of both *Apc* alleles, whereas transit-amplifying or differentiated cells do not have this ability⁶¹. Of note, Lgr5⁺ stem cells that have lost 1 copy of *Apc* do not form tumors⁶¹. However, removal of only 1 copy of *Apc* from Lgr1⁺ intestinal stem cells does result in small and large intestinal tumors upon stochastic loss of the second *Apc* allele^{31, 62}. Together, these results show that intestinal tumors develop upon induction of an initiating event in stem cell populations.

Certainly, genetic alterations or epigenetic changes that confer stem-like characteristics to otherwise differentiated cells could impart tumor-forming potential⁶³. Studies of either *Apc*, *β-catenin*, and/or *K-ras* manipulation within progenitor and differentiated intestinal epithelial compartments have demonstrated this potential^{13, 44, 64–69}. At present, we cannot be certain which cell populations promote tumorigenesis. Moreover, the relationship between normal intestinal stem cells and cancer stem cells—if they exist—is uncertain⁷⁰. Arnout Schepers et al. recently provided evidence for the presence of stem cell activity within intestinal adenomas⁶². Using lineage retracing, they demonstrated that Lgr5 marks a subpopulation of multi-potent adenoma cells that mediate clonal growth of established intestinal adenomas—fulfilling at least 2 of the criteria for cancer stem cells. These Lgr5⁺ cells, which account for about 5%–10% of cells in the adenoma, generated additional Lgr5⁺ cells, as well as all other epithelial cell types found within the adenoma. Although these findings provide evidence for tumor stem cells in mice, many questions remain about the ultimate potential of these types of cells and how relevant they are to human colorectal cancer formation. Further studies are also needed to understand more fully how the proliferative and functional status of a stem cell population relates to its tumor-forming potential.

Summary

Since the initial Mouse Models of Intestinal Neoplasia Workshop in 2000, considerable progress has been made in developing rodent models of colorectal cancer, and the terminology recommended by the group has largely been adopted by the scientific community. Newer models with multiple genetic alterations develop intestinal tumors that closely resemble their human counterparts in morphology, location, and behavior. These models, as well as those that develop local and distant metastasis, are important advances. Models derived from stem cell populations offer new tools for studying proliferative and quiescent stem cells and their roles in intestinal neoplasia. The creation and ongoing curation of a virtual slide box (<http://mmgint.org>) offers an opportunity to share histologic findings and have them assessed, community-wide. Rodent models remain an invaluable tool for elucidating molecular pathways, identifying novel biomarkers, and devising innovative strategies to prevent and treat intestinal cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

Submitted on behalf of the participants in the 2010 Mouse Models of Human Intestinal Cancer Pathology Workshop:

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Figure 1.

A) Intramucosal carcinoma in human colorectal adenocarcinomas (arrow) is characterized by infiltration of the lamina propria by tumor cells, without extension into deeper layers of the bowel wall. B) Traditional human serrated adenoma (right), with epithelial infoldings and ectopic crypt formation, compared to tubular normal crypts (left). C) Small adenoma that developed in a mouse following exposure to AOM. It has densely packed neoplastic crypts with low-grade dysplastic changes. D. Invasive adenocarcinoma that developed in a mouse following exposure to AOM, with extension through full thickness of intestinal wall.

Figure 2.

A) Mucin-depleted focus characterized by loss of alcian blue-positive goblet cells (alcian blue) and low-grade dysplastic changes. B) Intestinal neoplasms that form in *Apc*^{Pirc/+} rats are often bulky, pedunculated lesions. Although herniation is present in the stalk of the adenoma, focal invasion of muscularis mucosae is also observed (arrow). C) *Lrig1*^{CreERT2/CreERT2} mice develop intestinal adenomas that are predominantly low grade and overly plaque-like hyperplasia of Brunner's glands. D) *gp130*^{Y75F} mice exposed to AOM and DSS develop pedunculated colonic adenomas. The solid growth pattern in this adenoma indicates high-grade dysplasia.

Figure 3.

A) The lesions that develop in Stat3-IKO mice are primary low grade in appearance, but infiltrating irregular crypts, which indicate superficial invasion, were seen in the slides examined by the panel (arrow). B) Colonic adenomas in *Apc*^{Min/+}; *Smad3*^{-/-} mice arise in a background of colitis and mucosal hyperplasia. This example shows a complex growth pattern that indicates high-grade dysplasia. C) *Apc*^{1638N/wt}; *Villin-Cre*; *Tgfbr2*^{E2flx/E2flx} mice develop high-grade adenocarcinomas with prominent intraluminal dirty necrosis, similar to human colorectal adenocarcinomas. D) Lung metastases that form in *Villin-Cre*; *LSL-K-ras*^{G12D}; *Tgfbr2*^{E2flx/E2flx} mice are similar to the primary intestinal tumors of these mice.

Figure 4.

A) In *Apc^{flox}; LSL-K-ras^{G12D}* mice, adeno-cre administration and mechanical abrasion of colon leads to formation of invasive adenocarcinomas in the distal colon; these are morphologically similar to human colorectal carcinomas. B) Liver metastases that develop in *Apc^{flox}; LSL-K-ras^{G12D}* mice are high-grade carcinomas with a solid and glandular pattern of growth, similar to the primary tumors. C) The *K-ras^{G12D}; Ink4a; Arf^{-/-}* mice develop unusual spindle-cell intestinal tumors; infiltration around non-neoplastic crypts is shown. D) Lung lesions in the slides from *K-ras^{G12D}; Ink4a; Arf^{-/-}* mice were examined by workshop attendees; they contained a papillary adenocarcinoma that differed histologically from the primary tumor.

Figure 5.

A) Use of Sleeping Beauty transposon mutagenesis in *Apc*^{+/+} and *Apc*^{Min/+} mice resulted in formation of mucinous adenocarcinomas that deeply invaded the intestinal wall. B) *Smad3*^{+/-}; *Rab25*^{-/-} mice develop rectal adenocarcinomas with mucinous features and deep invasion of intestinal wall and adjacent structures. C) *Fabp1*^{Cre}; *Apc*^{flox/+}; *K-ras*^{LSL-G12D/+} mice develop adenomas with diffuse high-grade dysplasia and areas of intramucosal adenocarcinoma. D) *CDX2P*^{Cre}; *Apc*^{flox/+} mice develop multiple colorectal adenomas. Approximately 17% of mice develop invasive adenocarcinomas, which are shown here, invading the submucosa.

Animal Models of Intestinal Cancer Reviewed at the Workshop

Table 1

Model	MGI Allele	Contributor	Strain	Tumor location	Tumor type	# tumors/mouse	Age when analyzed	Other lesions	Metastasis	Reference
gp130Y757F(AOM/DSS)	I16st ^{tm1Em}	Ernst	C57BL/6	LI	AD & ACA	8	3 mos. after challenge	Gastric ACA	No	Bollrath, 2009 ³²
MDF in colon of DMH- exposed rats	N/A	Caderni	F344	Distal LI	MicroAD	7 MDF/LI (DMH dosage: 300 mg/kg)	3 mos. after DMH	No	No	Caderni 2003; Femia 2008 ^{21, 23}
Lrig1 ^{CreERT2} ;CreERT2	Lrig1 ^{tm1.1(Gw/ERT2)Rq}	Coffey	129/SV X C57BL/6	Duodenum (atop Brunner's gland)	AD & ACA	1	6 mos.	NR	No	Powell 2012 ³¹
Lrig1 ^{CreERT2} ;Apc ^{fllox/+}	Apc ^{tm2.1Cip} Lrig1 ^{tm1.1(Gw/ERT2)Rq}	Coffey	129/SVX C57BL/6	SI & LI	AD	40 (SD); 12 (distal LI)	3–4 mos. after tamoxifen	NR	No	Powell 2012 ³¹
Sleeping Beauty transposon mutagenesis in Apc ^{Min/+} and WT mice	Apc ^{Min}	Cormier	C57BL/6	SI & LI	AD & ACA	In WT screen, 3 tumors in SI and 1 tumor in LI; in Apc ^{Min} screen 350 in SI and 15 in LI	In WT screen, 10–12 mos.; in Apc ^{Min} screen, 3 mos.	Thymic lymphomas, intestinal myeloid leukemias, liver adenomas	No	Starr 2009; Starr 2011 ^{40, 41}
Apc ^{Pirc/+}	N/A	Dove	F344XNTac	SI & LI	AD & ACA	Male, 22 in SI and 14 in LI; Female, 4 in SI and 7 in LI	7–13 mos.	Jaw osteomas, benign epidermoid cysts	No	Amos- Langraf 2007 ²⁸
Apc ^{Pirc/+}	N/A	Dove	F344/Tac X ACI/Hsd	Predominantly LI (some SI)	AD & ACA	Male, 13 in SI and 26 in LI; Female, 1.5 in SI and 8 in LI	5–6 mos.	NR	NR	Irving 2011 ³⁰
CDX2p ^{Cre} ;Apc ^{fllox/+}	Apc ^{tm.1Tno/+} Tg(CDX2-cre)101Erf	Featon	C57BL6X SJL/J	Predominantly LI	AD & ACA	11	6 mos.	NR	No	Hinoi 2007 ⁴⁴
Apc ^{Min/+} ;Rab25 ^{-/-}	Apc ^{Min/+} Rab25 ^{tm1Irigo}	Goldenring	C57BL/6	SI & LI	AD	175 (SD); 4.7 (LI)	4 mos.	NR	No	Nam 2010 ⁴³
Smad3 ^{+/-} ;Rab25 ^{-/-}	Rab25 ^{tm1Irigo} Smad3 ^{tm1Par}	Goldenring	129/J	LI	AD & ACA	5.75	10 mos.	Squamous carcinoma of the vagina	No	Nam 2010 ⁴³
Apc ^{1638N} ;Villin ^{Cre} ; Tgfb ² ^{fllox/flox}	Apc ^{tm1Rak} Tg(Vil-cre)997Gum Tgfb ² ^{tm1.2Hlm}	Grady	C57BL/6	Intestine (not specified)	AD & ACA	5	12 mos.	NR	No	Munoz 2006 ³⁵
Villin ^{Cre} ;LSL- K-ras ^{G12D/+} ; Tgfb ² ^{fllox/flox}	K-ras ^{tm4Tyj} Tg(Vil-cre)997 Tgfb ² ^{tm1.2Hlm}	Grady	C57BL/6	SI & LI	AD & ACA	2.4	6 mos.	NR	Lymph nodes & lung	Trobridge 2009 ³⁶
LSL- K-ras ^{G12D/+} ;Ink4a;Arf ^{-/-}	Cdkn2a ^{tm1Rdp} K-ras ^{tm4Tyj}	Greten	129X C57BL/6	Proximal LI	Serrated lesions & malignant	NR	12 mos.	NR	Reportedly, lung	Bennecke 2010 ¹³

Model	MGI Allele	Contributor	Strain	Tumor location	Tumor type	# tumors/mouse	Age when analyzed	Other lesions	Metastasis	Reference
					spindle cell tumors					
Villin-Cre;LSL-N-ras ^{G12D/+}	N-ras ^{m1Tyj} Tg(Vil-cre)20Syr	Haigis	C57BL/6		No phenotype		4 to 6 mos.			Haigis 2008 ⁶⁵
Fabp1-Cre;Apc ^{flox/+} ;LSL- K-ras ^{G12D/+}	K-ras ^{m4Tyj/+} Tg(Fabp1- cre)1Jig	Haigis	C57BL/6	LJ	AD & ACA	NR	4 to 6 mos.	LJ mucosal hyperplasia in K-ras ^{G12D/+} mice	No	Haigis 2008 ⁶⁵
Apc ^{flox/+} ;LSL-K-ras ^{G12D/+} ; Adeno-cre after colonic abrasion	Apc ^{m2Rak} K-ras ^{m4Tyj}	Hung	C57BL/6	Distal LJ	AD & ACA	3.6	1 to >6 mos.	Liver metastases	Liver	Hung 2010; ³⁷ Jackson 2001
Apc ^{Min/+} ;Smad3 ^{-/-}	Apc ^{Min} Smad3 ^{tm1Par}	Laird	129/SV	Distal LJ	AD & ACA	15	2 mos.	NR	No	Sodir 2006 ³⁴
Csf1r-iCre ^{+/-} ;Stat3 ^{flox/flox} (Stat3-IKO)	Tg(Csf1r- iCre)jwp+/- Stat3 ^{tm1Div}	Lin	FVBX C57BL/6	LJ	Hyperplasia & dysplasia & ACA	NR	2 to 10 mos.	Colitis	No	Deng 2010 ³³

Definitions:

- AD - adenoma
- ACA - adenocarcinoma
- SI - small intestine
- LJ - large intestine
- WT - wild-type
- NR - not reported
- IKO - inducible knockout