A Genetic Approach to Cancer Epigenetics

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In over 20 years since the discovery of altered methylation in cancer, many epigenetic alterations have been found in human cancer, including global and specific gene hypomethylation, hypermethylation, altered chromatin marks, and loss of genomic imprinting. Cancer epigenetics has been limited by questions of cause and effect, since epigenetic changes can arise secondary to the cancer process and its associated widespread changes in gene expression. Furthermore, mutations in the DNA methylation machinery have not been observed in tumors, whereas they have been for chromatin modification. To address the issue of human cancer etiology, we have taken a genetic approach to cancer epigenetics. One line of investigation has been on the disorder Beckwith-Wiedemann syndrome (BWS). We have found that loss of imprinting (LOI) of the autocrine growth factor gene *IGF2* and of the untranslated antisense RNA *LIT1*, within the *K1LQT1* gene, account for most cases of BWS, and that cancer risk is specifically associated with LOI of *IGF2*. Wilms' tumors, both in BWS and in the general population, involve LOI leading to an expansion of nephrogenic precursor cells. We have also developed an animal model for the role of LOI of *IGF2* in cancer, showing that it cooperates with *Apc* mutations to increase cancer frequency, consistent with human data suggesting a severalfold increased cancer risk for this common epigenetic variant in the adult population. These data suggest that a major component of cancer risk involves epigenetic changes in normal cells that increase the probability of cancer after genetic mutation. They suggest a model of cancer prevention that involves the epigenetic analysis of normal cells for risk stratification and cancer prevention strategies.

Since its inception in 1983 with the discovery of altered DNA methylation of genes in human cancers (Feinberg and Vogelstein 1983), the field of cancer epigenetics has simultaneously become both more and less clear. It has become more clear that epigenetic changes lie at the heart of most tumor processes, and that without a disruption of normal epigenetic programs, tumors would neither arise nor progress (Jones and Baylin 2002; Feinberg and Tycko 2004). The field has become less clear, however, with the discovery of an increasing number of types of epigenetic alteration in tumors, as well as genes that are affected by these changes (Jones and Baylin 2002; Feinberg and Tycko 2004). Perhaps the most dominant genetic feature of any kind in human cancer is a global hypomethylation that appears to occur ubiquitously in tumors, both benign and malignant. That is not to say that every type of tumor shows this change, but neoplasms that have been examined systematically show a marked reduction in the level of DNA methylation (Gama-Sosa et al. 1983; Feinberg and Tycko 2004). This loss of methylation has important consequences. It can lead to increased expression of dominantly acting oncogenes, such as R-ras in gastrointestinal cancer (Nishigaki et al. 2005) and cyclinD2 and maspin in pancreatic cancer (Cho et al. 2000; Akiyama et al. 2003). This hypomethylation-associated gene activation received comparatively less attention than gene silencing until recently, but it is increasingly clear that these changes are as important as those associated with gene silencing. It can also lead to chromosomal instability and loss of heterozygosity (LOH), shown convincingly in mouse models (Gaudet et al. 2003; Yamada et al. 2005). A third epigenetic change is promoter hypermethylation associated with gene silencing. Most tumor suppressor genes that are inactivated by mutation also show epigenetic inactivation, even more

commonly than mutational inactivation with LOH of the wild-type allele (Jones and Baylin 2002). Until recently, it was believed that the methylation was the primary cause of this silencing, but that is not as clear at present. It is now apparent that chromatin changes in the promoters of genes, and possibly within the body of genes as well, may be ultimately responsible for this silencing (Sellers and Loda 2002; Lund and van Lohuizen 2004; Robertson 2005). These chromatin modifications include increased lysine 9 methylation of histones H3 and H4, loss of acetylation at the same residue, and many other specific chromatic modifications. The fact that chromatin changes may precede the methylation changes was indicated by a study in which a cell line engineered to lose DNA methylation by somatic cell knockout of DNA methyltransferases 1 and 3A nevertheless silenced p16 through chromatin modification (Bachman et al. 2003). That is not to understate the importance of hypermethylation, but it may be more important in stabilizing epigenetically silenced genes than their primary inactivation. A recent study showed that global changes in histones may parallel those found in DNA methylation. These global changes include loss of acetylated lysine 16 and trimethylation of lysine 20 of histone H4 (Fraga et al. 2005).

Finally, an intriguing form of epigenetic change in cancer discovered about 10 years ago is loss of genomic imprinting. Imprinting refers to parent-of-origin-specific silencing of one allele of a gene. Many imprinted genes have been discovered, and most appear to be involved, at some level, in growth regulation. The prototype gene involved in LOI in cancer is *IGF2*, which encodes the insulin-like growth factor II protein. *IGF2* is normally expressed exclusively from the paternally inherited allele. LOI of *IGF2*, involving abnormal activation of the normally silent allele, was first found in about half of Wilms'

tumors of the kidney, the most common solid tumor of childhood (Ogawa et al. 1993; Rainier et al. 1993). This frequency is about tenfold that of WT1 mutations in Wilms' tumor. In particular, LOI occurs in tumors that have a histopathologic phenotype referred to as perilobar nephrogenic rest-like (Ravenel et al. 2001). Beckwith and colleagues identified this subgroup of tumors as those that arose from nephrogenic stem cells after their commitment to renal development and during fetal life (Beckwith et al. 1990). The overexpression of *IGF2* in these tumors is thought to lead to an increased proliferation of nephrogenic stem cells and their associated perilobar nephrogenic rests (Beckwith et al. 1990; Sharifah et al. 1995). These rests are in fact common in the population at the time of birth, as a study of stillborns showed a prevalence of approximately 1%, but disappear normally over time (Beckwith 1998). Since the discovery of LOI in Wilms' tumor, LOI has been observed in many other human tumors, including common cancers such as colon, lung, hepatocellular, ovarian, and bladder cancer (for review, see Feinberg 1999). Other genes also show LOI in cancer, including *PEG1/MEST* in lung cancer (Nakanishi et al. 2004), *p57^{KIP2}* in pancreatic cancer (Sato et al. 2005), *ARHI* in breast cancer (Yu et al. 1999), and *p73* in gastric cancer (Kang et al. 2000).

A GENETIC APPROACH TO HUMAN CANCER EPIGENETICS: BECKWITH-WIEDEMANN SYNDROME

Despite these many observations of epigenetic alterations in cancer, it has been difficult for the cancer epigenetics community to persuade the mainstream genetics community that epigenetics changes play a causal role in cancer. The counterarguments have been that epigenetic changes occur secondarily to widespread changes in gene expression in tumors, and/or that they simply reflect the underlying cell type from which tumors arise. Our laboratory has believed that a powerful approach to proving a mechanistic role for epigenetic changes in cancer is through conventional cancer genetics. Thus, we have endeavored to use both human and mouse genetics to build a strong argument for causality. Toward that goal, we borrowed a lesson from Albert Knudson, who pointed out several decades ago that by this study of rare cancer syndromes in the population, one may identify in the germ line genetic alterations that cause cancer which increase the risk of developing tumors many-fold. Knudson further argued that once one identified such changes, they might be present in somatically occurring common tumors, as well (Knudson 1987). We thus endeavored to identify a paradigm genetic syndrome that predisposes to cancer and involves an epigenetic mechanism. That syndrome was Beckwith-Wiedemann syndrome (BWS), a rare disorder that increases the risk of developing Wilms' tumor and other malignancies 850-fold. We thought that BWS might have an epigenetic basis, because it exhibits parent-of-origin-specific transmission, in particular through the mother (Mannens et al. 1994). At the time of our original studies of this disorder, no imprinted human genes had yet been identified, but they were highly suspected, based on stud-

ies of the neurological disorders Prader-Willi syndrome and Angelman syndrome, both of which show parental-origin-specific chromosomal alterations. The distinguishing features of BWS are prenatal overgrowth; organomegaly, particularly affecting the liver, kidney, pancreas, and adrenal gland; pancreatic islet cell hyperplasia with hyperinsulinemia and neonatal hypoglycemia; macroglossia (large tongue); midline abdominal wall defects independent of the organomegaly; and dysmorphic features of the face, notably ear pits or creases (DeBaun and Feinberg 2004). These children are at increased risk of developing so-called embryonal tumors that appear to involve an expansion of progenitor cells during prenatal development, such as in the kidney leading to Wilms' tumor, in the liver leading to hepatoblastoma, and in the muscle leading to rhabdomyosarcoma (DeBaun and Feinberg 2004). We initially mapped BWS in two large families to chromosomal band 11p15 by genetic linkage analysis (Ping et al. 1989). At about the same time, Tilghman and others showed that the *H19* gene is imprinted and maps to a syntenic location in the mouse (Bartolomei et al. 1991), and Efstratiadis demonstrated that the *IGF2* gene in mice is imprinted (DeChiara et al. 1991). We and other workers found that *IGF2* and *H19* are imprinted in humans, and that *IGF2* undergoes LOI in Wilms' tumor and in BWS (Zhang and Tycko 1992; Ogawa et al. 1993; Ohlsson et al. 1993; Rainier et al. 1993; Weksberg et al. 1993). Over the next several years, we identified a domain of imprinted genes spread out over approximately one megabase of 11p15, and including a number of growth-related genes, such as *p57^{KIP2}*, a cyclin-dependent kinase inhibitor normally expressed only from the maternal allele (for review, see Feinberg 1999). This gene is also rarely mutated in BWS (Hatada et al. 1996). *p57^{KIP2}* is part of an imprinted subdomain of genes including *TSSC3*, *TSSC5*, and *K_vLQT1*, a voltage-gated potassium channel gene (for review, see Feinberg 1999). We found that within the *K_vLQT1* gene, which is expressed from the maternal allele, lies a paternally expressed antisense transcript, encoding an untranslated RNA that appears to regulate the expression of other genes within this subdomain (Lee et al. 1999b). We termed this gene *LIT1*, for Long Intronic Transcript 1. *IGF2* and *H19* lie within a separate imprinted subdomain approximately 500 kb telomeric to the group just described (Lee et al. 1999a). *IGF2* and *H19* appear to be independently regulated, and the two subdomains are separated by the non-imprinted genes *TSSC4* and *TSSC6* (Lee et al. 1999a).

The mechanism of BWS turns out to be heterogeneous. 15% of patients demonstrate LOI of *IGF2*, just as in sporadically occurring Wilms' tumors in the general population. Approximately 40% of cases involve LOI of the *LIT1* gene, leading apparently to epigenetic silencing of *p57^{KIP2}* and possibly other genes within this subdomain. In addition, approximately 5% of patients show mutations of *p57^{KIP2}*. Approximately 1% of patients show balanced or unbalanced chromosomal rearrangements leading to relative duplication of genes on 11p15. Approximately 10% of patients show uniparental disomy with duplication of the paternal alleles throughout both imprinted subdomains of 11p15 (for review, see Beckwith 1998; DeBaun and Feinberg 2004).

To sort out the complexities of the genetics of BWS, we have collaborated over many years with Michael DeBaun and colleagues at Washington University of St. Louis. He has developed a registry of BWS patients that is population-based; i.e., not biased toward malignancy through referrals from oncologists. In a study of 192 BWS patients within the registry, we performed the first epigenotype/phenotype study for any human disorder, asking whether any of the features of BWS were specifically associated with any of the genetic alterations observed in this disorder. The results of that study demonstrated that BWS is a contiguous gene disorder, and that the cancer phenotype is specifically associated with LOI of *IGF2* and associated hypermethylation of *H19* and silencing of *H19* (DeBaun et al. 2002). This represented compelling genetic evidence for a causal role of epigenetic changes in human cancer. Thus, not only is an epigenetic change, namely LOI of *IGF2*, associated with increased cancer risk in this disorder, but it is this particular change rather than alterations of *LIT1*, *p57^{KIP2}*, or other genes that is associated with that risk. Alterations in these other genes are associated with the midline abdominal wall defects and generalized overgrowth in this disorder (DeBaun et al. 2002).

Considerable progress has also been made in understanding the mechanism of LOI in BWS and Wilms' and other tumors, through basic research in mouse models, as well as human genetic studies. Imprinting of *IGF2* and *H19* are coregulated by a differentially methylated region (DMR) located several kilobases upstream of the *H19* gene and between *IGF2* and *H19* (for review, see Bartolomei and Tilghman 1997; Ohlsson 2004). In mouse, the DMR consists of four binding sites for the insulator protein CTCF, and in humans, the DMR consists of seven binding sites for CTCF. The DMR is normally methylated on the paternal allele and unmethylated on the maternal allele, allowing allele-specific binding of CTCF to the maternal allele. This binding of CTCF insulates the *IGF2* promoter from a shared enhancer downstream of *H19*. Thus, normally, the maternal *H19* allele is active and the paternal *IGF2* allele is active. There is a consequent single dose of *IGF2* protein expression from the paternal allele, as well as a single dose of *H19* expression from the maternal allele (Bartolomei and Tilghman 1997; Ohlsson 2004). The role of *H19* in growth regulation is unclear, as Tycko and colleagues showed that it has a growth-inhibitory effect on tumor cells in vitro (Hao et al. 1993), but Tilghman and colleagues were able to replace the body of the *H19* gene with a luciferase construct, leading to no phenotype within the resulting mice (Jones et al. 1998). In tumors or BWS patients with LOI, the *H19* DMR becomes aberrantly methylated on the maternal allele (Moulton et al. 1994; Steenman et al. 1994). CTCF does not bind to the aberrantly methylated maternal allele, and the enhancer has access to both maternal and paternal *IGF2* alleles, doubling the mRNA and protein levels of *IGF2*, and extinguishing *H19* expression (Ravenel 2001; Ohlsson et al. 2004). Thus, there is a double dose of this growth-promoting factor and loss of a potential growth-inhibitory role of *H19* in these cells.

The study of Wilms' tumor has also provided insight into the mechanism by which LOI increases tumor risk in

BWS patients and promotes cancer development in sporadically occurring Wilms' tumors. LOI appears to increase the number of nephrogenic progenitor cells leading to perilobar nephrogenic rests. Abrogation of WT1 expression, in contrast, impairs the development of nephrogenic progenitor cells, leading to an overgrowth of mesonephric blastemal cells, referred to by the pathologist as intralobar nephrogenic rests. This hypothesis is supported by the fact that the perilobar nephrogenic rests themselves show LOI of *IGF2* (Ravenel et al. 2001). Thus, one can hypothesize a model in which epigenetic changes play a role similar to genetic changes in the Knudson hypothesis. The original Knudson model simply states that there are two events that lead to tumors, one occurring rarely and one occurring commonly. Although they may be allelic in the case of tumor suppressor genes, they need not be so. That is because a common and rare event best fit the epidemiology of tumor incidence in familial and sporadic tumors of a given type, without reference to a specific gene. We would therefore argue that LOI represents the common change, albeit an epigenetic one, and that the second event is a more rarely occurring genetic event. In the case of sporadically occurring Wilms' tumors, LOI leads to the presence of perilobar nephrogenic rests (Feinberg and Tycko 2004). Although this is not common, it is far more common than mutational mechanisms in cancer, and may involve as many as 1% of newborn children. In the case of BWS, all of the cells of the kidney would have undergone this epigenetic change, and therefore, the chance of a subsequent genetic mutation affecting a predisposed epigenetically altered precursor cell is much greater than in the general population (Fig. 1) (Feinberg and Tycko 2004).

LOI AND COMMON CANCER RISK

BWS has thus served as a paradigm for understanding both the causal link between epigenetic changes in human cancer and the mechanism for those changes. Nevertheless, it is important to determine whether similar epigenetic changes occur in common human malignancies and contribute significantly to cancer in the general population. Toward that end, about 7 years ago we began to study human colorectal cancer, the original tumor type in which altered DNA methylation was discovered. We found that LOI occurs in approximately one-third of human colon cancers (Cui et al. 1998). What distinguished LOI in colon cancer from other tumor types such as ovarian or bladder cancer was the fact that when LOI was present in the tumor, it was also present in the matched normal colonic mucosa from the same patients. These changes were common even in noncancer patients, occurring in approximately 10% of colon samples (Cui et al. 1998). The fact that LOI was enriched about threefold in cancer patients suggests that either LOI occurs in normal tissue of some individuals and increases the chance of developing cancer, or that it is a normal, perhaps age-dependent, change coincidentally associated with cancer. To distinguish between these possibilities, we performed an epidemiologic study of patients who were undergoing colonoscopic exam in a gastroenterology clinic setting.

Figure 1. Human genetics of Beckwith-Wiedemann syndrome provides causal support for epigenetics in cancer etiology. (A) Loss of imprinting (LOI, dark nuclei) has been shown to arise sporadically as a somatic mosaic epigenetic alteration, because LOI has been found in parenchymal kidney tissue of patients with Wilms' tumor, as well as in premalignant nephrogenic rests (pink circles). (B) Alternatively, LOI can arise in the germ line or very early in development in BWS, causing nephromegaly (overgrowth of the whole kidney). In both cases, overgrowth is caused by a double dose of *IGF2* expression and possibly silencing of *H19*. (C) A second, presumably genetic, event can then lead to Wilms' tumor formation (red circles). (D) This will be more common in those with BWS, so multiple tumors arise. (Reprinted, with permission, from Feinberg and Tycko 2004 [Nature Reviews Cancer © Macmillan Magazines Ltd.; www.nature.com/reviews].)

Over 400 patients were enrolled in the study, of whom 172 were informative for a polymorphism within *IGF2*, allowing us to determine the imprinting status. All of these patients were examined at the level of imprinting in peripheral blood lymphocytes and at multiple locations within the normal colonic mucosa, and they also received a complete assessment of the presence or absence of tumors at the time of examination, as well as a past history of cancer and a history of cancer among first-degree relatives. Surprisingly, the adjusted odds ratio for LOI was 5.15 for those patients who had a positive family history for colorectal cancer among first-degree relatives. Similarly, the adjusted odds ratio for LOI was 4.72 among those patients who had past or present colonic tumors, and this number stratified for the type of tumors with an adjusted odds ratio of 3.26 for adenomas and 21.7 for colorectal cancers

(Cui et al. 2003). It should be noted that among the many genetic mutations that have been identified in the germ line of cancer patients, none occurs commonly. For example, the *BRCA1* mutation occurs in fewer than 1 in 1000 individuals. All of the mutations that increased the risk of developing colorectal cancer, combined, account for only approximately 3% of colorectal cancers in the general population in the United States. Thus, a genetic change that occurs as commonly as in 5–10% of the population, if it increases cancer risk modestly, e.g., two- to threefold, would account for a significant population-attributable risk in the general population. Moreover, the fact that these changes are epigenetic suggests that one might be able to intervene to reverse the abnormality or at least to prevent the consequences of cancer developing late in these patients. Since the original publication of this finding, the results have been confirmed by two other groups. The first examined a series of women with adenomas and found a 5.2 odds ratio for adenomas among patients with LOI. The second study was done on a community-based population that included minority patients (Woodson et al. 2004; M. Cruz-Correa et al., in prep.).

A MOUSE GENETIC MODEL OF HUMAN CANCER EPIGENETICS

More recently, we have developed an animal model to test the hypothesis that LOI of *IGF2* increases the risk of developing intestinal tumors. There have been animal models generated with LOI of *IGF2*, by deleting or mutating the *H19* DMR and/or the associated *H19* gene. Mice that inherit these deletions from their mothers lack the inhibition of maternal *IGF2* expression and show LOI. These mice, although showing biallelic *IGF2* expression, have not exhibited an increased frequency of tumors (Leighton et al. 1995; Ripoche et al. 1997). However, mice with this deletion crossed with a *p57^{KIP2}* knockout have shown changes consistent with BWS, such as adrenocytomegaly, although the latter mice are perinatal lethals and, therefore, tumors have not been observed (Caspary et al. 1999).

We reasoned that LOI of *IGF2* might not act alone but in conjunction with genetic changes as postulated by the modified epigenetic Knudson model described above. We therefore tested this idea by generating doubly heterozygous mice. The mothers in these matings had a deletion or mutation of the *H19* DMR. The fathers in these matings are the Min mouse with a mutation in the adenomatous polyposis coli gene (*APC*), which causes intestinal tumors, and involving the same gene that undergoes gatekeeper mutations in virtually all human colon adenomas and carcinomas. The results of this mating led to four possible genotypes. First are mice with mutations in both genes; i.e., showing a predisposition to tumors based on the mutation in *APC*, as well as LOI of *IGF2* due to the loss of the *H19* gene. A second genotype is the Min mutation without LOI. The third and fourth possibilities are with and without LOI but without the Min mutation. These mice would not be expected to develop tumors but might still show pathological changes of interest. A large number of mice were generated over several years, with a total of 140 mice

with the Min mutation, 81 of these with the wild-type *H19* gene, and 59 with the deleted *H19* and consequent LOI. We found that in the normal colon as well as in the tumors themselves, there was a doubling of *IGF2*, similar to what we have seen in other human tumors exhibiting LOI (Sakatani et al. 2005). We confirmed that this doubling of *IGF2* mRNA was associated with a doubling of the *IGF2* protein as well. Thus, although there had been other models of *IGF2* expression that provided compelling evidence for a role of this growth factor in tumor progression (Christofori et al. 1994), a model showing physiological doubling of *IGF2* as one sees with LOI had not previously been generated. The results on tumorigenesis were dramatic. Depending on the method used to score tumors, there was an increase of 1.9- to 2.5-fold in the numbers of tumors in doubly heterozygous mice compared to those with the Min mutation but normal imprinting. This was true in both the intestine and the colon, although the number of colon tumors is much smaller in the Min mouse for reasons that are unknown (Sakatani et al. 2005). These data in and of themselves provide genetic confirmation of our human studies and prove that LOI in normal tissue increases the risk of developing cancer in the intestine.

These mice also provided insight into the mechanism for this increased tumor risk. The ratio of macroadenomas to microadenomas was the same in mice with and without LOI that had the APC mutation. Thus, there was no difference in the rate of progression of tumors once they arose, but there was an increase in tumor initiation in the LOI mice, consistent with the idea that LOI precedes the development of intestinal tumors and in some way increases the risk of developing cancer once an APC mutation has taken place (Sakatani et al. 2005). Histopathologic examination of the intestines of mice with LOI versus mice without LOI revealed that the progenitor cell compartment of the intestine, i.e., the crypts, were enlarged approximately 50%. Furthermore, staining with a variety of histochemical markers for progenitor cells showed a dramatic increase in the number of progenitor cells within the intestinal epithelia of LOI mice, regardless of whether these were mice that also had the APC mutation. Furthermore, this increased presence of progenitor cell markers was also seen in the compartment that should represent relatively differentiated epithelia, namely the villi, and there was a concomitant retreat of differentiated markers within the villi away from the crypt–villus boundary (Sakatani et al. 2005). We also found that within the pancreas, the β -islet cells were hyperplastic in the doubly heterozygous mice. There was also a relative increase in staining in these cells for progenitor cell markers within the double heterozygotes (Sakatani et al. 2005). Taken as a whole, these data support the stem cell hypothesis of cancer, that the source of malignancy in the intestine is an undifferentiated progenitor cell. We would add to this stem cell model the idea that the LOI increases the progenitor cell pool and/or shifts the epigenetic program toward an earlier developmental state. This change in progenitor cells increases the probability of cancer after a genetic mutational insult, in this case, mutation of the APC gene, whether that is in the mouse model or in the development of human colon tumors. This view is also con-

sistent with our earlier studies showing that in BWS there is an overrepresentation of progenitor cells within the renal epithelium. We hypothesize that a primary role of IGF2 in embryogenesis is the careful titration of the progenitor cell pool size. Thus, an overexpression of IGF2 would lead to an increased number of progenitor cells and overall shift toward a less differentiated state, increasing the probability of malignancy, whether in BWS or human colorectal cancer (Fig. 2). These changes might not occur prenatally, but in the case of the colon, might occur postnatally as well, given that this is a highly regenerative tissue source in a state of dynamic equilibrium. An alternative explanation for our data was suggested by George Klein (Klein 2005), who argued that LOI might represent the process of tumor cell promotion first suggested by Berenblum in the 1960s (Berenblum 1962). By this view, LOI could fulfill the dedifferentiation component of tumorigenesis, an appealing idea, since both epigenetic changes and tumor promotion are potentially reversible.

In summary, LOI is a common epigenetic variant in the population, at least in adults. This population variation in epigenetic marks may be due to variable stringency in the maintenance of these marks that may itself be genetically determined. That would explain why we see an association of LOI with cancer in first-degree relatives. In any case, this abnormality appears to represent the first common molecular marker for cancer risk in the general population. Many challenges lie ahead, both in the application of this information to reducing cancer mortality and in cancer epigenetics as a whole. In particular, for an LOI cancer risk test, given that this is epigenetic and also of unclear timing during the human life span, it will be important to study this change serially in individuals who have the abnormality. Furthermore, it will be important to analyze samples that were collected archivally, through nested case/control studies, in order to determine whether LOI is a predictive marker for cancer that occurs eventually in a given individual who was sampled before the tumor arose. Finally, LOI testing is very different from conventional cancer testing. The culture of cancer testing is oriented toward early cancer detection rather than cancer risk assessment. Some type of intervention to ameliorate the risk would be indicated, even though that intervention might be unnecessary in a large number of the patients who were so treated. Still, such an approach has been the most effective one in reducing cardiovascular mortality, and there has been little progress in reducing cancer mortality since the war on cancer was declared in the 1970s. This approach also raises important ethical considerations, because the nature of epigenetic testing is so unfamiliar that it will be difficult to explain to patients in a way that they can understand so as to provide the type of consent that is necessary for long-term prospective studies.

CONCLUSIONS

The field of cancer epigenetics as a whole is now entering its maturity. One of the great challenges will be to identify those changes that occur in cancer prior to the earliest recognizable pathological alterations, even before benign tumors can be detected. It is important to bear this

Figure 2. Mouse genetic model of colorectal cancer provides causal support for epigenetics in cancer etiology. Min mice with a mutation in the *Apc* gene (leading to intestinal tumors) with or without LOI were generated by crossing male Min mice with female mice harboring a deletion in the *H19* gene and differentially methylated region needed for normal *IGF2* silencing. In LOI (-) mice, the progenitor compartment is shown in yellow within the crypts, and the differentiated compartment in the villi in brown, with the Paneth cells in white. In LOI (+) mice, the progenitor compartment is larger and also altered epigenetically (orange color). Finally, adenomas (red) arise from this altered epithelium but are capable of differentiation given their progenitor cell origin. (Reprinted, with permission, from Feinberg 1999 [© American Association for Cancer Research].)

in mind as experiments are contemplated for a human cancer genome project. As initially conceived, that project called for the comparison of the genetic sequence in pairs of normal tissue and tumor tissue from the same individuals in a relatively large number of tumor types. However, if important epigenetic changes occur in normal tissue even before benign neoplasms arise, then we must compare the epigenetic state in the normal tissue of patients who have developed tumors, to comparable material obtained from control individuals who have not developed tumors. Will this be a technically daunting task, given the large number of potential epigenetic changes that occur throughout the genome? I think not, because the technology for epigenetic analysis genome-wide is advancing very rapidly. For example, it has become possible in other organisms to analyze whole chromosomes for epigenetic alteration using restriction enzymes that cut approximately half of methylated DNA sites, such as *McrBC*, and then prepare probes from this DNA, compare that to DNA that has not been so digested, and hybridize to arrays of sequence over the genomic region of interest. Although this might not provide a level of resolution at the single-nucleotide level, it might nevertheless provide a very strong indication about epigenetic changes and where they might occur, without our preconceptions interfering with data analysis. I am most hopeful that this new field of epigenomics will be of practical value in identifying patients at risk of cancer and in reducing cancer mortality, while also providing the next wave of mechanistic understanding of the basic biology of cancer.

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