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Murine models of colorectal cancer: studying the role of oncogenic K-*ras*

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Abstract. Cancers of the intestine are amongst the most frequent tumors in the Western countries. They arise through the stepwise, progressive disruption of cellular signalling cascades which control cell proliferation, survival and differentiation. The proto-oncogene K-*ras* functions as an important molecular switch linking several of these signalling pathways. Activating mutations of K-*ras* are found in about 50% of colorectal cancers, but their contribution to tumor initiation and progression is still poorly understood. Murine models provide excellent opportunities to identify and define the roles of genes involved in cancer formation and growth in the digestive tract. In this review, I will discuss the biological properties of oncogenic K-*ras*, its influence on cell signalling and its role in colorectal tumorigenesis based on recently established murine models.

Key words. APC; colorectal cancer; K-*ras*; mouse model; oncogene; transgene.

Introduction: The Genetic Basis of Colorectal Cancer

The self-renewing adult intestinal epithelium with its distinct crypt-villus units is an intriguing model for many cellular processes such as cell differentiation, migration, adhesion, cell lineage allocation as well as for the control of cell number by proliferation and controlled cell death. In addition to these fundamental biological processes, outstanding interest arises from the important role of gastrointestinal neoplasia in human cancer. In fact, cancers of the colon and rectum are amongst the most frequent tumors in Europe and North America. More than 50% of the population develop a colorectal tumor by the age of 70, which progresses to malignancy in 10% of the cases [1]. The initiation and progression of cancer generally involve multiple steps of genetic alterations, including the loss of function of tumor suppressor genes and the activation of oncogenes [2, 3]. The cancer cells must acquire specific capabilities like limitless replicative potential, self-sufficiency in growth signals, escape from apoptosis, tissue invasion and metastasis [3]. This review will concentrate on K-*ras*, an oncogene with strong transforming potential that has been found to be misregulated in one-third of all human cancers and 50% of colorectal cancers [4]. Ras proteins are central components in many signal transduction cascades, and their malfunction has profound consequences for cells, leading ultimately to tumorigenic transformation. Murine models are excellent tools to test the implication of mutated genes in the formation and growth of digestive tumors. Understanding of the function of several tumor suppressor genes in the development of colorectal cancer has greatly benefited from mouse models in the case of *APC* (adenomatous polyposis coli) and the Wnt signalling cascade [5–7]. The role of the mismatch repair machinery in carcinogenesis has also been investigated intensively [8, 9]. Inactivation of the murine gene SMAD3, a component of the transforming growth factor (TGF)-β signalling pathway, resulted in colorectal cancer [10], whereas the tumorigenic effects of a deletion of the catalytic subunit of phosphoinositide 3-kinase are still under debate [11, 12]. However, modelling the role of oncogenes, in particular

K-*ras*^{V12G}, the most frequently mutated oncogene in colorectal cancer, has proven to be difficult [13–15]. Although activating K-ras mutations are associated with early-to-intermediate tumor stages, their tumorigenic effects are generally thought to develop only in the context of a preexisting *APC* gene mutation [16]. However, the long-standing view that the oncogenic effects of mutated K-*ras* depend upon previous *APC* mutations in digestive cancer has been challenged by several recent observations. In human tumors that arise in the small intestine, *ras* is frequently mutated, whereas *APC* mutations are very rare events [17]. A recent study on the mutational status of aberrant crypt foci (putative precursor lesions of colorectal cancer) demonstrated frequent K-*ras* mutations, but no evidence for mutations in *APC* or its downstream target β-catenin was obtained [18]. In sporadic colorectal carcinogenesis, as opposed to familial adenomatous polyposis (FAP), there seems to be a route where K-*ras* mutations occur mainly during the formation of aberrant crypt foci that then become adenomas where further mutations (in *APC*, for example) occur [18]. The question whether activated K-*ras* is able to induce intestinal tumors or whether it depends on previous mutations of the tumor suppressor gene *APC* is still open. In the light of these findings, new murine models could help to increase our knowledge of the in vivo consequences of activating K-*ras* mutations. Several laboratories have recently addressed these issues by generating new transgenic mouse models [19–22]. I will discuss the roles of normal and oncogenic Ras proteins in cellular signalling, and I will attempt to compare the contribution of K-*ras* mutations to tumorigenesis in the intestinal tract with respect to other signalling pathways that are known to play a major role in colorectal cancer, notably the *APC*/Wnt pathway.

K-*ras* and the Ras family

The three different human *ras* genes (H-*ras*, N-*ras* and K-*ras*) all share a high degree of sequence identity [23]. The K-*ras* gene shows alternative splicing, producing the two protein isoforms K-ras4A and K-ras4B, which show differences in their type of membrane anchorage. The post-translational processing of Ras proteins involves farnesylation at a cysteine residue in the CAAX box (C = cysteine, A = aliphatic amino acid and X = methionine, serine, leucine or glutamine), which is localized at their C-terminus. After farnesylation, H-ras, N-ras and K-ras4A proteins are further modified by palmitoylation on cysteine residues close to the N-terminus. In the case of K-ras4B, where no cysteine residues are present, a cluster of positively charged lysine residues is thought to mediate binding to the plasma membrane. The proteins TC21, M-ras and R-ras, which are highly related to the classical members of the Ras family, may also have transforming activity [24–26]. The 21-kDa gene products of the Ras family are all able to cycle between an active GTP (guanosine triphosphate)-bound conformation and an inactive GDP (guanosine diphosphate)-bound form. Under normal conditions, quiescent cells have only 5% of their total Ras proteins in the GTP-bound active state, as compared with 50% upon mitogenic activation [27]. The same holds true for unstimulated murine cells, where GTP-Ras accounts for only 5–10% of the total Ras protein levels [28, 29]. Ras proteins are situated at the inner face of the plasma membrane where they play an important role as GTPase 'switches' in normal cellular signalling. They are activated by diverse extracellular signals, for example via growth factors such as epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), or cytokines such as interleukin-2, and pass the stimulating signal downstream via multiple effectors that can interact synergistically [30]. A typical example for the normal function of the GTP/GDP cycle of Ras is its activation by EGF [31]. Upon binding of its ligand, the epidermal growth factor receptor dimerizes and autophosphorylates tyrosine residues in the cytoplasmic domain of the receptor. Adaptor proteins such as Grb2 then bind to the phosphorylated tyrosine residues, and generate a link between the activated EGF receptor and guanosine nucleotide exchange factors (GEFs) like the protein SOS (son of sevenless) [31]. The recruitment of the otherwise cytosolic SOS to the plasma membrane allows it to bind to Ras. Upon binding to SOS, Ras proteins undergo a conformational change, allowing the exchange of GDP for GTP. Ras is now activated and can pass the signal to its own downstream effectors. Several proteins known as GTPase-activating proteins (GAPs) stimulate the otherwise low intrinsic GTPase activity of Ras, converting it back to the quiescent GDP-bound form. Oncogenic mutations of *ras* reduce the intrinsic GTPase activity of Ras proteins; furthermore, they abolish the down-regulation of Ras activity by GAPs [32]. Thus, oncogenic Ras proteins are locked in the active, GTP-bound state and are capable of constitutively activating their downstream effectors. Intriguingly, mutations in the *ras* genes are not randomly distributed between the different isoforms, since the vast majority of mutations occur in K-*ras*. The reason for this isoform specificity is still unknown. It has been speculated that there might be subtle, yet decisive, functional differences among the different Ras isoforms, or that the K-*ras* gene is simply more susceptible to genetic alterations [33]. Interestingly, it has been shown that a murine knockout of K-*ras*, but not of H-*ras* or N-*ras*, results in embryonic lethality [34], and K-*ras* is the prevalently expressed isoform in adult mouse intestine [35].This indicates that even though there may be considerable functional overlap between the Ras isoforms, K-*ras* might have a distinct and essential role.

Aberrant function of K-*ras* has long been known to contribute in a crucial way to human cancer development, and in 50% of all colorectal tumors, one finds mutated forms of K-*ras* [36]. Whereas K-*ras* seems to be the predominant Ras isoform in colon cancer, N-*ras* mutations are more frequently found in leukemia, and H-*ras* mutations seem to occur in bladder cancer [37]. It is conceivable that these differences arise from differential regulation of effector proteins. In a recent study, Raf-1 has been shown to be more efficiently activated by K-*ras* than by H-*ras* [38]. In contrast, H-*ras* seems to be a more potent activator of phosphoinositide (PI) 3-kinase [39].

Effectors of Ras function

Among the best-studied Ras effectors, there are the serine-threonine kinases of the Raf family (A-Raf, B-Raf and Raf-1/c-Raf) and their downstream target, the mitogen-activated protein kinase (MAPK) cascade; other important effectors are the PI 3-kinases [2, 40] (fig. 1). The MAPK cascade plays a major role in the mitogenic action of oncogenic Ras, and it involves the consecutive activation of three kinases. Once activated by Ras, Raf-1 phosphorylates MAPK kinase (MEK), which, in turn, phosphorylates the MAPK or extracellular signal-regulated kinase (MAPK/ERK) [41]. MAPK activation results in phosphorylation and activation of transcription factors such as c-Jun, c-Myc and c-Fos, causing enhanced transcription of genes that are associated with cell proliferation [40]. Like Ras itself, the G proteins Rho and Rac cycle between a GTP-bound and a GDP-bound state, and are regulated by GTPase-activating proteins and guanosine nucleotide exchange factors. Both play a role in the regulation of the actin cytoskeleton, and have been reported to be activated by oncogenic Ras [42–44]. Furthermore, Rac activates PI 4/PI 5 kinases, resulting in an increase in the concentration of PIP2 (phosphatidyl inositol 4,5-biphosphate), which has been implicated in activation of the microfilament system. Many actin-dependent processes, such as membrane ruffling, formation of focal adhesions, filopodia and stress fibers, are involved in the motile and invasive phenotype of tumor cells, and they can be all stimulated by oncogenic *ras* [43, 44]. PI 3-kinase produces mitogenic inositol lipids upon stimulation by Ras. One of these lipid products, PIP3 (phosphatidyl inositol 3,4,5-triphosphate), is able to activate the G protein Rac. However, the serine-threonine kinase Akt (or protein kinase B) constitutes the best-studied effector of PI 3-kinase. One of the downstream effects of oncogenic Ras is to suppress apoptosis; this effect is mediated by activation of Akt. Activated Akt phosphorylates caspase-9 [45] and Bad, a member of the Bcl-2 family [46]. Upon phosphorylation, these pro-apoptotic proteins are inhibited. Since apoptosis is tightly controlled in self-renewing tissues like the intestinal epithelium, one can easily imagine the importance of Ras mutations for the survival of neoplastic cells. In addition to its anti-apoptotic effect, Akt has been reported to mediate the effects of oncogenic Ras on cell cycle progression [47]. Other potential Ras effectors include the guanine nucleotide exchange factor RalGDS, whose role is still not completely clear [48], and the c-Jun N-terminal kinase JNK, that has been reported to be activated via Rac [49]. JNK is a part of the JNK/JUN kinase cascade, also called the stress-response pathway, which has been implicated in the induction of apoptosis.

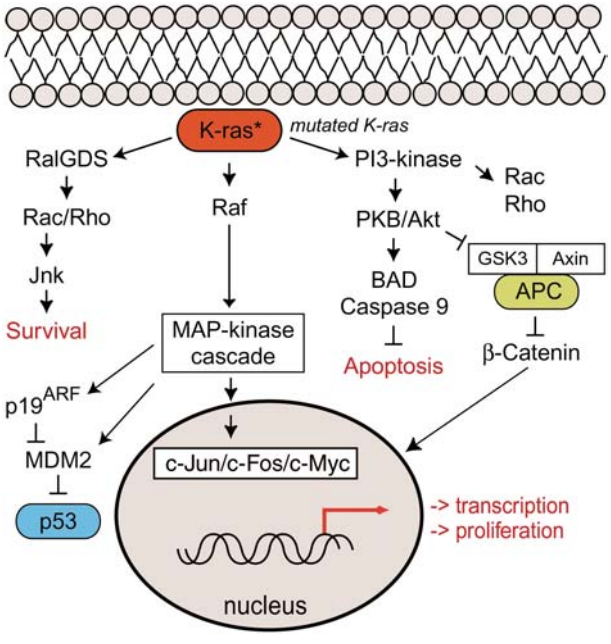

Figure 1. Schematic representation of the signalling cascades downstream of *K-ras*. Oncogenic K-*ras* is a potent activator of several downstream effectors, the most prominent being the MAP-kinase cascade, the GTPases Rac/Rho and the PI 3-kinase. Activation of these pathways leads to suppressed apoptosis and transcriptional activation, resulting in increased cellular proliferation. There are several interconnections with other signalling cascades, such as the Wnt/APC pathway, and the tumor suppressor *p53*.

Ras mutations in human cancers and their role for tumorigenesis

The Ras signalling cascade constitutes a major pathway which has been demonstrated to be misregulated in 30% of all human tumors [4]. In fact, *ras* genes are the most frequently mutated oncogenes that can be detected in human tumors. However, the frequency of the mutations is tissue- and tumor-type dependent: whereas *ras* mutations are present in almost all pancreatic adenocarcinomas and in about 50% of colorectal cancers, they are very rare in breast and ovarian cancers [2, 16, 36, 50]. Point mutations

are found at a 'hot spot' at codon 12 of the *ras* coding sequence [4], and they all abolish the GAP-induced GTP hydrolysis of Ras. This means that a single point mutation leads to a Ras protein that is constitutively activated. The prognostic importance of *ras* mutations in human colon cancer is still a matter of debate. One study failed to find a correlation between *ras* status and clinical prognosis [51]. However, other reports indicate that K-*ras* mutations are associated with poor prognosis and aggressive tumor behavior [52, 53]. Normal, nonmutated Ras proteins are activated in response to extracellular stimuli that control growth and differentation. In contrast, oncogenic Ras proteins are active in the absence of external stimuli, and their sustained signalling capacity presents a selective advantage for tumor cells by increasing cell proliferation and cellular survival, or by changing their adhesive properties. It has to be kept in mind, though, that the effects of Ras activation depend on the cell type and the tissue context. An in vitro example for the strikingly divergent biological effects of Ras is the observation that activated Ras induces proliferation in fibroblasts, whereas it causes growth arrest and differentiation in the neuronal-like cell line PC12 [54]. Ras proteins have also been implicated in control of the cell cycle, and multiple Ras-effector pathways converge in increased cell cycle progression. A prominent example is the regulation of the protein cyclin D1, a component of the cell cycle machinery, through activation of the MAPK pathway [55, 56]. Increased cellular levels of cyclin D1 are thought to promote cells through the G1 checkpoint, leading to cellular proliferation. In fibroblasts, strong sustained activation of MAPK/ERK results in the expression of cyclin D1, but both integrin-dependent signalling and *ras*-mediated growth-factor stimulation are required for this effect [57]. The importance of cell-type specificity was demonstrated in a study that examined the effect of oncogenic Ras on cyclin D1 in mammary tumors [58]. While cyclin D1 was required for Ras to predispose mammary epithelium to tumorigenesis, it was dispensable for Ras-induced skin tumors [58]. Ras proteins not only stimulate proliferation by promoting cell cycle progression, but they are also implicated in the inhibition of apoptosis. Reduced apoptosis is an important factor for tumorigenesis, since it helps tumor cells to escape elimination. This effect has been reported to be mediated by activation of the *ras* effector protein Akt/PKB [45, 46]. Ras has also been shown to protect mammary epithelial cells from apoptosis by downregulation of the Fas receptor [59]. Furthermore, it has been reported that oncogenic Ras plays a role in angiogenesis and the metastatic process, and the development and maintenance of solid tumors requires sustained expression of H-ras [60]. The earliest effects of inhibiting Ras expression in melanocytes was the destruction of the tumor vasculature, and it has been proposed that Ras expression in tumor cells elicits non-cell-autonomous responses in the vasculature [60]. Transformation of cells with oncogenic H-*ras* has been shown to increase the expression of metalloproteinases, which are key factors in invasive processes and tumor metastasis [61]. Metastatic tumors often express lower levels of the extracellular matrix component thrombospondin, and the expression of thrombospondin has been shown to be negatively regulated by oncogenic K-*ras* [62]. In another study, K-*ras* was found to disturb the processing and expression of adhesion molecules in MDCK cells, resulting in a loss of cell-cell and cell-matrix adhesion [63, 64]. Altering the adhesive properties of epithelial cells is thought to play an important role in invasion and metastasis of colorectal tumors. There is also increasing evidence for cross-talk between integrin-mediated signalling and Ras-mediated pathways [65]. Ras-dependent hyperactivation of the Raf/MAPK pathway cooperates with TGF-β-receptor signalling; it is required for epithelial-mesenchymal transition in vitro, and was associated with tumor as well as metastasis formation in nude mice [66]. Ras-activated PI 3-kinase signalling, on the other hand, caused cell scattering and and was found to be responsible for protection from TGF-β-induced apoptosis [66].

Other signalling pathways involved in colorectal cancer and their cross-talk with Ras

About 15% of colorectal carcinomas arise due to a genetic predisposition in affected families [67, 68], and these familial forms often serve as a paradigm for colorectal cancer. Dominantly inherited germline mutations in the gene *APC* have been shown to be responsible for the familial adenomatous polyposis syndrome (*FAP*) [69–71]. This inherited predisposition results in the formation of multiple colonic adenomatous polyps with a high propensity to develop colon carcinoma. The carcinoma cells have been found to express COOH-terminally truncated, nonfunctional forms of the APC protein [72]. Mutations of *APC* are not only present in inherited forms of cancer predisposition, they have also been found in the much more common 'sporadic' or nonfamilial form of colorectal cancers, which represent the vast majority of all cases [73]. In sporadic colorectal cancer, *APC* mutations have been reported to occur in over 60% of the cases [74–77]. It has been shown that in human as well as in mouse colon tumor development, inactivation of both alleles of the *APC* gene occurs already at the earliest stages that are observable [78, 79]. Based on these results, it has been proposed that loss of the wild-type allele of *APC* is the earliest genetic change during tumor development, and this loss is sufficient for progression to the adenoma stage. Intriguingly, APC is ubiquitously expressed in many different cell types, but it seems to play an important role in tumorigenesis mainly in the colon or rectum,

and rarely in other organs. Kinzler and Vogelstein thus proposed a special role for *APC* in colonic epithelial cells as 'gatekeeper' for cellular proliferation [1]. A mutation of the gatekeeper leads to an imbalance of cell division over cell death. However, the role of *APC* in the initiation of colorectal cancer has been questioned by recent studies on the mutational status of colonic aberrant crypt foci (ACF). ACF are regarded as early precursor lesions of colorectal cancer, but it appears that only a small fraction of all ACF progress to polyps, and even fewer to malignant tumors. Two main types of ACF have been described for human colon mucosa: nondysplastic ACF that are associated with *ras* mutations, and dysplastic ACF that are associated with *APC* mutations. Dysplastic ACF have been suggested to be more likely to progress to adenoma [78]. Alternatively, nondysplastic ACF have been proposed to progress through the advent of adenomatous transformation [80]. A recent study found frequent K-*ras* mutations in ACF (in 80% of nondysplastic, and in over 60% of dysplastic ACF), but no evidence for *APC* or β-catenin mutations was obtained [18]. This observation indicates that *APC* may not be indispensable for the initiation of colorectal carcinogensis.

The multiple and profound implications of *APC* in cellular signalling are reflected by the fact that its gene product is a large multifunctional protein (300 kDa), that has the ability to bind to many diverse ligands [1, 81]. The interaction of the APC protein with β-catenin and its ability to regulate the β-catenin concentration in the cytoplasm deserve special interest, since β-catenin (the homologue of armadillo in *Drosophila*) plays important roles in cadherin-dependent cell adhesion as well as in signal transduction of the Wnt/Wingless pathway [82, 83]. In vivo studies on transgenic mice showed that truncated forms of β-catenin are able to promote tumorigenesis: expression of an NH_2-terminally truncated β-catenin resulted in a mitogenic response in intestinal crypt epithelial cells; the cell proliferation was increased by a factor of 4 [84]. In the absence of a Wnt signal, phosphorylation of β-catenin binding sites in APC by glycogen synthase kinase-3 (GSK-3) promotes the APC/β-catenin association, which ultimatively leads to rapid reduction of the cytoplasmic pool of β-catenin by its degradation through the ubiquitin-proteasome pathway. Upon stimulation of the Wnt pathway, the stability of β-catenin is enhanced. The increase in β-catenin concentration leads to its migration to the nucleus where it binds to and activates members of the TCF/LEF-1 (T cell factor/lymphocyte enhancing factor) family of transcription factors, which subsequently activate transcription of target genes like the oncogene c-myc [85], cyclin D1 [86] and the metalloprotease matrilysin [87]. Apart from transcriptional regulation in the nucleus, both microtubules and the actin cytoskeleton are downstream targets of Wnt signalling through APC [88]. The mutational inactivation of APC or mutations of β-catenin itself, both of which have frequently been observed in human colon cancers, lead to the accumulation of cytoplasmic β-catenin. Recent findings indicate that both oncogenic Ras and the tumor suppressor *p53* do not act in a completely separate fashion from the Wnt/APC pathway. In fact, there is significant cross-talk between the different signalling cascades, and there are several interconnections [89]. The protein Mdm2, one of the major regulators of *p53*, as well as its inhibitor $p19^{ARF}$ have been shown to be a target of the Ras/Raf/MAP kinase pathway [90]. The protein glycogen synthase kinase 3 is not only a component of the Wnt/Wingless pathway, but also a substrate of Ras-activated PKB that is capable of downregulating its activity [2]. The Myc protein is stabilized by Ras [91], and the transcription of *myc* is induced by the β-catenin pathway [85]. Moreover, oncogenic β-catenin induces the accumulation and activation of the *p53* protein [92]. In contrast, the putative modulation of TGF-β-receptor signalling by Ras is still under debate. It was reported that Ras attenuates SMAD2/3 signalling [93], but other groups failed to observe Ras-dependent inhibition of SMAD2/3 [94].

In addition to the constitutive activation of the Wnt signalling pathway, genomic instability is a hallmark of human colorectal cancer. This instability can be present in the form of minisatellite instability (MIN) or chromosome instability (CIN). MIN is found in a subgroup of digestive cancers, and it is caused by defects in DNA mismatch repair genes, which are also the underlying cause in the hereditary non-polyposis colorectal cancer (HNPCC) [95–97]. However, most cancers that demonstrate extensive MIN were found to result from somatic inactivation of mismatch repair genes like hMLH1 rather than from an inherited germline mutation [98]. Recent results suggest that the DNA mismatch repair genes are also involved in the control of cell cycle arrest or apoptosis after DNA damage [99]. The molecular mechanism underlying CIN, the more frequent form of genomic instability, has just begun to emerge. CIN tumors exhibit a defect in chromosome segregation, which manifests as both numerical and structural chromosomal alterations. However, several recent studies indicate that APC dysfunction not only deregulates the Wnt signalling cascade, but can also give rise to chromosomal instability [100–102]. It is known that the APC protein binds to EB1 and microtubules, and APC has been found to localize at the mitotic spindle; furthermore, the expression of truncated APC in embryonic stem cells caused chromosomal instability [100]. Moreover, in CIN cells APC is often mutated, whereas spindle checkpoint genes are not [102]. Other somatically acquired mutations are likely to cooperate with mutations in the *APC* gene to elicit CIN during tumor progression. Interestingly, oncogenic Ras has been shown to induce genomic instability via the MAPK pathway in a cell culture model [103].

Mouse models (I): the utility of mice

The role of oncogenic Ras proteins is typically studied by overexpressing activated *ras* with powerful artificial promoters in cultured cells. These approaches have greatly increased our knowledge concerning the basic biological functions of *ras*. However, subtle effects and differences could be obscured by saturating levels of Ras protein. Furthermore, transferring the information gained from cell culture experiments to the complex process of tumor development in the intestinal tract is not a trivial issue. Therefore, in vivo systems would be highly desirable to recapitulate the effects of mutated Ras in a tissue context, involving physiological levels of expression. Since mice have the same tissues and organ systems as humans, and they are amenable to precise genetic modifications, they have become a versatile experimental system that has extensively contributed to the field of colorectal cancer. The technical advances in homologous recombination and transgenic technology have allowed the creation of various preclinical models for cancer development (reviewed in [104, 105]). Transgenic mice provide excellent opportunities to dissect the involvement of genes of interest in intestinal carcinogenesis. In addition, the influence of environmental factors such as diet composition on the susceptibility for cancer can be studied in mutant mice. Finally, successful mouse models could serve as valuable tools for the development of novel diagnostic and therapeutic approaches. There are several promising reports for successful therapeutic tests of anti-Ras compounds that interfere with the posttranslational modification of Ras proteins by the enzyme farnesyl-protein transferase [106, 107]. These compounds reverse many of the malignant phenotypes of Ras-transformed cells in vitro. Farnesyl-transferase inhibitors have been tested on murine models carrying the human oncogenic K-ras^{V12G} under the control of the mouse mammary tumor virus enhancer/promoter [106]. Treatment of the transgenic mice caused inhibition of tumor growth in the absence of systemic toxicity. These results demonstrate the utility of transgenic mice for testing potential anticancer agents, and it would be of great interest to expand these approaches to the field of colorectal cancer. Several attempts have been made so far to obtain murine models to study the development of colorectal cancer [13]. The first mouse model that was described to contain a mutation in the *Apc* gene (a nonsense mutation at codon 850 that was obtained serendipitously in a mutagenesis screen) was designated multiple intestinal neoplasia (*Apc^{min}* [5]). These mice can develop more than 100 adenomas in the small intestine, but this phenotype greatly depends on the genetic background. Many of the mutations in the human *APC* gene occur in the last and largest exon. This type of mutation is more closely mimicked by a transgenic mouse named *Apc^{1638N}* [6], where a neomycin expression cassette was inserted in reverse orientation at codon 1638 of the murine *Apc* gene. Heterozygotes of this phenotype develop aberrant crypt foci, polyps and about three to five adenomas or carcinomas of the small intestine. In the homozygous state, however, both of the *Apc^{min}* or *Apc^{1638N}* alleles lead to early embryonic lethality. Several transgenic and knockout mice have been created to study the role of the mismatch repair machinery in carcinogenesis, and it was shown that a dysfunctional mismatch repair contributes to the formation of tumors [9, 108]. Inactivation of the murine gene SMAD3, a mediator of TGF-β signalling, as well as a knockout of the catalytic subunit of PI 3-kinase have been reported to result in colorectal cancer [10,11]. A combination of mutations in mismatch repair genes with mutated alleles of *Apc* resulted in a dramatic decrease of viability and accelerated tumorigenesis [109, 110].

Mouse models (II): studying the role of Ras

Whereas the understanding of the function of the tumor suppressor gene *APC* has taken great profit from transgenic mice, the role of oncogenic K-*ras* in digestive cancer has only recently been recapitulated [22]. Earlier attempts to recapitulate the role of oncogenic Ras in transgenic models had only limited success. Transgenic expression of K-ras^{V12G} in postmitotic villus enterocytes under control of the FABP promoter caused intestinal dysplasia, but the authors did not observe any neoplasms [14, 15]. Expression of the transgene in postmitotic enterocytes was apparently not sufficient to induce cancer. This finding was explained by the short residence time of migrating enterocytes on the villus, which does not allow clonal expansion. In a recent publication, Johnson et al. demonstrated an elegant mouse model for Ras-dependent carcinogenesis that is based upon spontaneous recombination events in the whole animal [19]. The mutant K-*ras* is generated at random by recombination within the allele, and this randomness is thought to recapitulate the sporadic occurrence of K-*ras* mutations. Whereas the transgenic mice failed to develop intestinal tumors, they showed a high predisposition for lung cancer and other tumor types. According to the authors, this may be due either to tissue-specific differences in the frequency of recombination events, or to the relative order of *ras* gene mutations in the course of tumorigenesis. In other words, the sporadic activation by homologous recombination may be very rare in intestinal epithelia, or oncogenic *ras* may be activated, but it is unable to induce tumors in the intestine. However, tumorigenesis is a rare event even in the villin-K-ras^{V12G} transgenic animals, which express oncogenic Ras throughout the intestinal epithelium and develop intestinal neoplasms [22]. The number of cells that express oncogenic Ras in the model de-

scribed by Johnson et al. is certainly strongly reduced as compared with the villin-K-ras^{V12G} transgenic model. Thus, one can assume that the probability of acquiring additional somatic mutations, based on results from the villin-K-ras^{V12G} model, might simply be too low to induce tumorigenesis in the model developed by Johnson et al. The authors also produced a double mutant that carried both the mutated K-*ras* allele and the *Apc^{min}* mutation [19]. In the double-mutant animals, the tumor type and frequency found in the *Apc^{min}* animals did not change. However, it is not clear whether K-*ras* was really activated in the intestinal tumors that had formed in these animals [111].

Several groups have created additional transgenic Ras models based upon the Cre/loxP system, an increasingly important tool to unravel the function of genes of interest with a high degree of experimental control [112]. Two recent transgenic models for pulmonary tumorigenesis utilized conditionally activatable K-*ras*, and the activation was carried out with a recombinant adenovirus expressing Cre [20, 113]. A recombinant adenovirus was used to deliver Cre in a colon cancer model involving conditional mutant *Apc* alleles [114]. A different kind of approach was used for the creation of the rasH2 mouse, a transgenic line that contains several copies of oncogenic H-*ras* together with the promoter/enhancer region of this gene [115]. The transgene is ubiquitously expressed, and the tumor spectrum includes lung adenocarcinomas and other cancer types, but no intestinal neoplasms. This lack of intestinal lesions might be explained by the preferential role of K-*ras*, but not H-*ras*, in digestive cancer. A conditional K-*ras* knockin was recently reported by the laboratory of M. Barbacid [21]. An oncogenic, Cre-activatable allele of K-*ras* has been introduced in the endogenous murine K-*ras* locus, together with a β-galactosidase reporter gene [21]. In the group of S. Robine, a 9-kb regulatory region of the mouse villin gene was used to drive the selective expression of oncogenic K-*ras*^{V12G} in intestinal epithelial cells [22]. The villin promoter has been shown to target stable and homogeneous expression of transgenes along the crypt-villus axis, in differentiated enterocytes as well as in the immature, undifferentiated cells of the crypt [116, 117]. It seems crucial to target expression of the transgene in the rapidly proliferating undifferentiated cell population, as has been shown in a study focusing on the Wnt pathway [118]. These cells remain anchored in the crypt during the normal renewal process of the intestinal mucosa; in addition, they undergo several cycles of cell division. These two features are necessary to accumulate the somatic mutations that are a prerequisite for tumorigenesis. The transgenic K-ras^{V12G} was expressed at relatively low levels (12% of endogenous Ras) in the mucosa of transgenic animals [22]. The physiological expression level of the transgene in this model may explain the apparent absence of unwanted phenotypic side effects, apart from tumorigenesis. The continuous presence of oncogenic GTP-Ras in the intestinal epithelium led to constitutive activation of the MAP kinases ERK1/2 in the intestinal mucosa [22]. Similarily, in human colon carcinomas, the MAPK activity was reported to be significantly higher than in normal mucosa [119]. The GTPase Rac was also found to be significantly activated in the mucosa of villin-K-ras^{V12G} transgenic animals, as compared with wild-type mice [unpublished observations].

Interestingly, no activation of Akt/PKB was observable in the villin-K-ras^{V12G} transgenic animals. However, the tissue-specific role of Akt in Ras-mediated transformation of epithelial cells is still under debate. The expression of oncogenic H-Ras in cultured rat intestinal epithelial cells has been reported to result in the PI3-kinase-dependent activation of Akt/PKB [120]. Another study showed that PI3-kinase/Akt signalling was neither necessary nor sufficient for K-*ras*-mediated transformation in the same cell type [121]. The lack of activation of Akt/PKB and the unchanged expression of the death suppressor Bcl-2, a downstream target of MAPK cascade [122], suggest that the regulation of apoptosis might not be affected in the normal intestinal mucosa of transgenic animals, at least not through these two key players. The villin-K-ras^{V12G} transgenic animals developed two to three tumors per animal with a relatively late onset (at an age of over 6 months) [22]. Adenomas and invasive adenocarcinomas were found to be present mainly in the duodenum and jejunum, thus in the small intestine. ACF were frequently found in the large intestine, while no malignant lesions developed in the colon or rectum. In the human disease, most cancers arise in the large intestine [73]. However, several other mouse models, like the *Apc^{1638N}* or *Apc^{min}* mice, also seem to develop tumors more easily in the small intestine than in the colon or rectum [5, 6]. Tumor analysis by flow cytometry showed that a majority of the lesions were hyperproliferative. However, hyperproliferation was not found in the normal mucosa of transgenic animals, even though oncogenic K-*ras* was expressed. In line with this observation, activation of Ras was reported to promote progression throughout G_1, but not S phase, in premalignant lesions [123]. This finding suggests that the constitutive activation of the MAPK cascade was not sufficient to induce hyperproliferation. Additional, spontaneous genetic alterations are most likely required to overcome the tightly controlled system of cell number homeostasis in the mucosa. Moreover, it is generally assumed that K-*ras* mutations develop in the context of previously inactivated *APC* [16, 36, 124]. Surprisingly, no evidence for invalidation of the *Apc* gene has been obtained in the tumors from villin-K-ras^{V12G} animals [22]. Murine intestinal tumors in the absence of mutations in *Apc* or β-catenin have also been reported for a recent mouse model that investigates the role of the TGF-β pathway [125].

Whereas inactivation of *Apc* seemed not to be required for tumorigenesis in the villin-K-ras^{V12G} transgenic animals, about 40% of the lesions displayed an altered *p53* state. Accordingly, spontaneous mutation of *p53* is a frequent but non-exclusive feature in human colorectal cancer. Inactivation of *p53* could shift the balance of cell number homeostasis by favouring proliferation and inhibiting apoptosis or cell cycle arrest, thus conveying a selective growth advantage for mutated cells. Interestingly, inactivation of *p53* seems not to be required in the case of *Apc*-induced tumorigenesis in mice, since *p53* mutations are very rare in the case of several murine models that recapitulate the role of inactivated *Apc* (fig. 2). Neither in the *Apc^{1638N}*, nor in the *Apc^{min}* mouse, have spontaneous *p53* alterations been detected [126, 127]. Moreover, combinations of *Apc^{min}* or *Apc^{1638N}* mice with knockout mutants for *p53* did not lead to increased tumor formation in the gastrointestinal tract [128, 129]. The results obtained with the villin-K-ras^{V12G} mice underline the notion that the pathway to colon cancer is not necessarily a single road that depends solely on invalidation of *Apc*. Furthermore, they indicate that inactivation of the tumor suppressor gene *p53* seems to cooperate with K-*ras* in the progression of colorectal tumors. Since the villin promoter is active during murine development [130, 131], the transgenic mice may express the oncogenic K-*ras* already in the embryonal stage. Therefore, it would be desirable to induce oncogenic mutations only in adult epithelial cells. This could be achieved in the future by inducible systems that take use of the Cre-LoxP system, similar to existing murine models [20, 21, 113].

Concluding Remarks

Although activating K-*ras* mutations are associated with early to intermediate tumor stages in the human disease, their transforming potential is thought to develop only in the context of a preexisting inactivation of the tumor suppressor gene *Apc* [16]. The unexpected finding that Ras was able to induce intestinal tumors in the absence of *Apc* mutations in the villin-K-ras^{V12G} transgenic model incites the hypothesis that Ras contributes not only to tumor progression, as has previously been suggested, but also to tumor initiation [22]. According to Fearon and Vogelstein, mutated K-*ras* may either be the initiating event in some colorectal tumors, or depend upon previous *APC* mutations to exert its oncogenic effects [16]. Even though *APC* mutations are present in virtually all cases of FAP, this is not the case for sporadic colorectal cancer, where *APC* is mutated in about 60% of the cases [74–77]. Furthermore, several recent studies show that a subgroup of human digestive cancer carries mutations in K-*ras*, but not in *APC*. In human primary carcinomas that arise in the small intestine, *ras* is frequently mutated (53% of the cases), *p53* to a lesser extent (27%), whereas *APC* mutations occur at a very low frequency (6%) [17]. Analysis of the mutational status of colonic ACF could help to clarify which genetic alterations initiate the tumorigenic process since ACF are regarded as early precursor lesions of colorectal cancer. A recent study found highly frequent K-*ras* mutations in ACF, but no evidence for *APC* or β-catenin mutations could be obtained [18]. Taken together, these results indicate that there seems to be an 'alternative

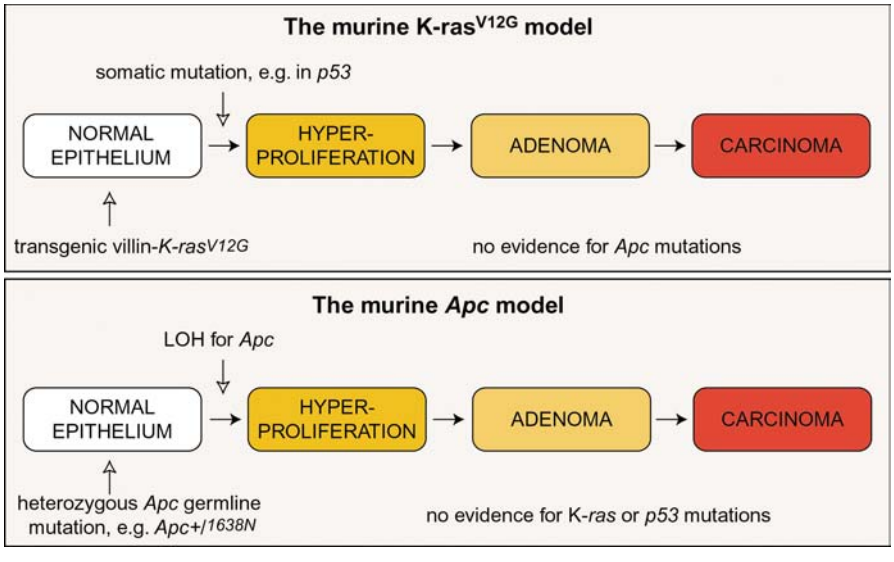

Figure 2. The murine villin-K-ras^{V12G} model of intestinal tumorigenesis compared with *Apc* models. Transgenic villin-K-ras^{V12G} animals express oncogenic K-ras in the intestinal epithelium; spontaneous, somatic genetic alterations (e.g. inactivation of *p53*) are required for tumorigenesis. Murine models that recapitulate the role of *Apc* (Apc^{min}, Apc^{1638N}) have demonstrated that a somatic inactivation of the wild-type allele of Apc is sufficient to induce tumorigenesis, even in the absence of further mutations in other oncogenes or tumor suppressor genes.

road' to sporadic colorectal cancer where K-*ras* mutations occur during the formation of precursor lesions, which then progress to adenomas [18]. In fact, a significant proportion of colorectal cancer may not be initiated by mutation of *APC*, as is generally supposed, but by other mechanisms, for example activating Ras mutations. In contrast to human colorectal cancer, where at least five genetic alterations have been calculated to be necessary for tumorigenesis [132], the number of mutations required for tumorigenesis in the murine models seems relatively small. Inactivation of both alleles of *Apc*, or a combination of oncogenic K-*ras* and inactivation of *p53*, are able to induce tumorigenesis (fig. 2). The murine models presented in this review have greatly helped to increase our knowledge of the formation of intestinal cancer, to identify and dissect several routes that can lead to colorectal cancer. One of the great remaining challenges is to model the features of advanced human cancer, including the metastatic process [133]. Metastasis formation is a rare event in the Apc^{1638N} model, and no metastasis has been detected in the villin-K-ras^{V12G} mouse [6, 22]. While tumorigenesis in humans can take several decades to progress to the metastatic stage, the relatively short lifespan of transgenic animals may preclude the acquisition of mutations that contribute to invasion and metastasis. This limitation could be overcome by intercrossing the villin-K-ras^{V12G} transgenic mice with other genetically modified mouse lines, which would increase the similarity of the model system to human sporadic colorectal cancer with its multiple genetic changes, and might increase the likelihood for metastasis formation. Furthermore, the cross-talk mechanism between oncogenes and tumor suppressor genes could be tested in vivo.

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