CHAPTER 4

Biological Carcinogenesis: Theories and Models

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4.1 INTRODUCTION

All organisms are endowed with homeostatic mechanisms safeguarding the number of cells in a given tissue. Thus, cell replication and cell apoptosis are strongly regulated and coordinated, ensuring an equilibrium between these two processes. In cancer, the homeostatic mechanisms are deranged, cell replication is accelerated, apoptosis is decreased, and the cells acquire the capacity to infiltrate adjacent tissues and blood vessels and to metastasize (Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 2004). These changes, characterizing the malignant phenotype, are due to defects in a variety of oncogenes and oncosuppressor genes involved in the many steps of the carcinogenic process – for example replication, apoptosis, angiogenesis, cell–cell and cell–matrix interactions (Grana and Reddy, 1995; Weinberg, 1995; Hanahan and Folkman, 1996; Varner and Cherish, 1996; Christofori and Semb, 1999; Coussens and Werb, 1996). At least four or five gene mutations are required for the expression of the malignant phenotype. The gene defects encompass point mutations, deletions, insertions, amplifications and translocations, and result from the action of exogenous agents – physical, chemical and viral – and of endogenous metabolic products, such as reactive oxygen species. In addition, epigenetic events

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determine the transition from the normal to the malignant phenotype. The DNA repair machinery of the cell strives to repair the damage by means of stability (caretaker) genes, i.e. nucleotide mismatch repair genes, nucleotide-excision repair genes, base-excision repair genes and genes controlling processes involving large chromosomal segments, whose mutation leads to chromosomal instability and loss of heterozygosity. If the equilibrium between damage and repair shifts towards damage and enough deleterious mutations are accumulated, the cell takes the path of malignancy. The mutational concept of carcinogenesis predicts that tumors will have a monoclonal composition, which has been best demonstrated in colorectal tumors. Although the normal colon epithelium is polyclonal, arising from numerous stem cells, the tumors derived from these cells are monoclonal and are formed by clonal expansion of a cell provided with a growth advantage, due to a first somatic mutation of an oncogene or an oncosuppressor gene, over the other cells of the tissue. Subsequent somatic mutations will result in additional rounds of clonal expansion and thus in tumor progression.

To study the various steps of the carcinogenic process, experimental models are needed, in which the sequential events are amenable to detailed analysis. Information derived from the analysis of human tumors in various stages of carcinogenesis has also yielded important information but cannot cover all stages of this process. Both animal and cell models have been exploited to this end, each with its merits and its limitations (Balmain and Harris, 2000; Pories *et al.*, 1993; Herzig and Christofori, 2002; Bosland, 1992; Hann and Balmain, 2001; Mao and Balmain, 2003). The *in vivo* models are mostly cancers induced by carcinogens with or without parallel administration of promoter agents. Such models allow the reproducible isolation of all tumor stages (including normal tissue), which are then amenable to biochemical, genetic and pathological analysis and allow studies of the various steps of the carcinogenic process – initiation, promotion, progression and metastasis – in a defined time sequence.

Other models are based on the use of transgenic animals in which defined genes can be either introduced in specific cells and tissues using cell specific promoters, or knocked out (Pories *et al.*, 1991; McDorman and Wolf, 2002). Cell-based models are principally of two types. In one, cells or tissue are exposed to chemical carcinogens or to one or more oncogenes usually using retroviral vectors to induce cell proliferation, and the sequence of events follows in cell culture or after grafting of the cells to animals. The other type of cell model is based on the isolation and stable culture of cells from tumors in the various stages of the carcinogenic process, which enables correlation of molecular changes with morphological and biochemical phenotypes. Combinations of animal- and cell-based models for different tumor types have appeared, rendering vital information on the sequence of events along the carcinogenic pathway.

4.2 MODELS OF HUMAN CARCINOGENESIS

The establishment of human genetic models of various cancers has permitted the correlation of genetic, molecular and biochemical defects with pathology.

Furthermore, the possibility of obtaining surgical specimens or needle biopsies of tumors in various stages of the carcinogenic process, in combination with the development of sensitive molecular techniques, has provided important additional information. Four models of carcinogenesis will be briefly reviewed: those of prostate, colorectal, endometrial and skin cancer.

4.2.1 Prostate cancer

The earliest precancerous lesion which can be detected in the prostate is intraepithelial neoplasia (PIN), which from its early (initiated) condition can proceed to its more advanced form, with characteristics of infiltrating cancers, known also as *in situ* carcinoma (Coffey, 1993; Bostwick *et al.*, 1995). During this transition from premalignant initiated to malignant localized (*in situ*) and then to infiltrating carcinoma, characteristic changes in many parameters – molecular, biochemical and morphological – are observed which could be correlated etiologically to the carcinogenic process (Karp *et al.*, 1996). Although in half of the cases examined a genetic relationship between PIN lesions and cancer has been demonstrated, it seems that only a subset of PIN foci progresses to invasive prostate cancer. Furthermore, the analysis of PIN and contiguous foci of prostate cancer demonstrates the genetic and phenotypic heterogeneity among diverse PIN, cancer and metastatic lesions.

In apparently 'normal' cells, near the premalignant lesions, telomerase activity appears and glutathione thiotransferase activity is decreased, changes also observed in PIN lesions and carcinomas (Montironi *et al.*, 1999). As in other malignancies, prostate carcinogenesis represents a multistep process involving progression from small, low histologic grade tumors, to large, higher-grade metastasizing carcinomas. The introduction of rat and mouse models based on treatment of the animals with chemical carcinogens, sex hormones or a combination of both (Bosland, 1992) has yielded important information in this respect. Some of these induced animal tumors share a number of significant characteristics with human prostate cancer, with similar molecular and genetic alterations. Some of these models are low-incidence ones, adequate for study enhancement, whereas others are high-incidence models, better suited to the study of inhibition of carcinogenesis. Important information regarding molecular and biochemical changes during the carcinogenic process has also been gained from the study of clinical samples employing current microarray methodology, although no definitive proof has been provided to link specific genetic alterations with stages and grades of prostate cancer (Isaacs, 1995). The multicentric nature of the prostate carcinoma, its high variation in histological grade within the same prostate and the possible association of different pathways with different etiologies necessitate the introduction of multiple model systems of prostate carcinogenesis.

The data stemming from clinical and animal model studies correlating molecular and genetic data with stages of carcinogenesis are summarized in Figure 4.1. The intraepithelial lesions consist of dysplastic, replicating cells, whereas the cells of the basal stroma lose the capacity to multiply. These changes have been correlated to increased expression of the oncogenes *c-erbB-2*, *c-erbB-3* and *c-met* and inactivation of the oncosuppressor gene *mn23H1*, which in normal epithelia are

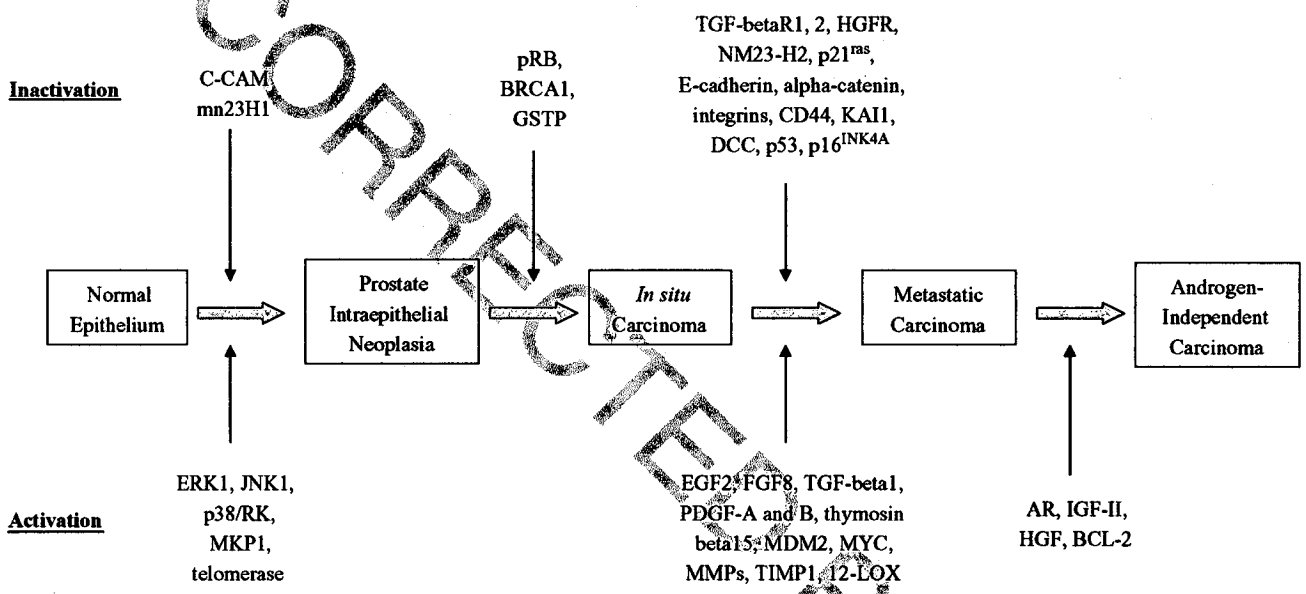

Figure 4.1 Gene abnormalities in prostate cancer. The diagram depicts in schematic form the known genes activated (mostly oncogenes) or inactivated (oncosuppressor genes) during the several stages of the carcinogenic process. These genes encode proteins functioning as growth factors (EGF2, FGF8, TGFβ1, PDGFA and B, IFGII, HGF) or growth factor receptors (TGFβR1 and 2, HGFR), metalloproteinases and their inhibitors (MMPs, TIMP1), cell adhesion (E-cadherin, alpha-catenin, integrins, KAI1) and signal transduction molecules (kinases and kinase inhibitors), cell-cycle regulators (pRB, p53, $p16^{INK4A}$), molecules involved in apoptosis (Bcl-2), telomerase, androgen receptor (AR) and enzymes involved in detoxification of carcinogens (GSTP).

expressed solely in the basal stroma cells. Furthermore, in 20 % of the lesions the expression of the *bcl-2* gene is deranged, leading to deregulation of apoptosis (Bonkhoff *et al.*, 1998). Chromosomal abnormalities are frequent in intraepithelial carcinoma (in 50 % of cases) and a similar percentage is observed also in infiltrating carcinoma (Emmert-Buck *et al.*, 1995). Ninety per cent of lesions show extensive methylation of deoxycytidine in the promoter region of the glutathione thiotransferase gene, leading to the lack of expression of the gene, apparently an early event in carcinogenesis (Lee *et al.*, 1994). In contrast to the total absence of telomerase in normal epithelium, 70 % of the precancerous lesions express the enzyme (Koeneman *et al.*, 1998).

In 70 % of prostate cancers, amplification of certain genes as well as loss of heterozygosity (LOH) is noted, correlated to the aggressive behavior of the tumor (Sandberg, 1992; Bova *et al.*, 1993) (Figure 4.1). Most defects are localized on chromosomes 7 and 8, but also on chromosomes 2q, 5q, 6q, 9p, 10p, 10q, 13q, 15q, 16q, 17p, 18q, 20q and Y. The chromosome regions lost could harbor oncosuppressor genes (Gao *et al.*, 1993, 1995, 1997; Dong *et al.*, 1995), whereas gene amplification probably involves oncogenes. Thus, the *hpc 1* gene is localized in the genetic locus 1q24-25, the oncosuppressor genes *Rb1*, *cdh1* and *DCC* in 13q, 16q, 17q and 18q, whereas gene *p53* and gene *hic-1* (hypermethylated in cancer) encoding a Zn-finger transcription factor are found in locus 17p13. An important role in prostate carcinogenesis is ascribed to the *Krev-1* oncosuppressor gene (Burney *et al.*, 1994), whereas *n33* and *mx11* seem to play a secondary role in this process. Gene amplification involves the oncogene *c-myc*, localized in locus 8q24. Microsatellite instability is observed in one-third of carcinomas, particularly in those with high Gleason scores and in advanced stages. It seems that the early stages of carcinogenesis are correlated with oncosuppressor gene inactivation, whereas later on activation of oncogenes is also observed, particularly of regions 8q, 7q, Xq and 18q. In one-third of hormone-resistant cancer cases, the androgen receptor gene is amplified.

Several proteins are increasingly expressed, correlated to carcinogenesis, acting through signal transduction mechanisms involving, among others, *src* and *ras*. These are EGF, TGF-α, c-erbB2, FGF7, FGF8, IGF-II, the IGF-1 receptor and TGF-β1, whereas TGF-β1 and TGF-β2 receptor expression is progressively decreased in aggressive carcinomas (Scher *et al.*, 1995; Kaltz-Wittmer *et al.*, 2000).

During the advanced, metastatic stage an increased mutation and amplification rate of the androgen receptor gene is observed, rendering the receptor sensitive to other steroid hormones and increasing its sensitivity to androgens, respectively. In advanced cases of prostate cancer low levels of expression of the NGF receptor are observed. Expression of IL-6 and IL-6R is also correlated to aggressive behavior of prostate cancer.

Loss of expression of the *E-cadherin* gene, located on chromosome 16q22, alpha-catenin and integrins, as well as of KAI1, encoding a transmembrane glycoprotein, is coupled with progressive disease (Morton *et al.*, 1993; Umbas *et al.*, 1994) and is linked to acquisition of a metastatic phenotype, as substantiated in a transgenic model of mouse carcinogenesis.

4.2.2 Colorectal cancer

One of the best-studied genetic models of human carcinogenesis is that of colorectal cancer. The 1990 model proposed by Fearon and Vogelstein has been the paradigm for the genetic alterations involved in the development of colorectal carcinoma (Fearon and Vogelstein, 1990). The change of the normal epithelium into a malignant and metastatic cell proceeds, in the majority of cases, through intermediary adenoma stages. A series of genetic alterations involving oncogenes and oncosuppressor genes occur; some of the most significant are depicted in Figure 4.2 (Alitalo *et al.*, 1983, 1984; Ashton-Rickardt *et al.*, 1989; Baker *et al.*, 1989; Bos *et al.*, 1987; Calabretta *et al.*, 1985; D'Emilia *et al.*, 1989).

Although multiple stages of adenomas may exist in the process of adenoma progression to the malignant phenotype, three discrete stages of adenoma formation are shown in Figure 4.2. Colorectal cancer is thought to be initiated by the inactivation of the adenomatous polyposis coli (APC) gene. A further important somatic mutation in the appearance of colorectal carcinomas is the *K-ras* gene mutation found in 50% of these tumors and in adenomas larger than 1 cm in size. Adenomas bearing *ras* gene mutations may be more likely to progress than adenomas without mutation. Hyperactive mutant *Ras* has been known to induce cellular proliferation. Recently, however, other effects of mutant *Ras* have been reported. Active *Ras* can phosphorylate pro-caspase-9, thereby inhibiting cytochrome-c-induced apoptosis (Cardone *et al.*, 1998). In mice, oncogenic *Ras* has been shown to cause cell-cycle arrest due to up-regulation of both tumor suppressors of the IKN4a-ARF locus, $p19^{ARF}$ and $p16^{INK4a}$, which in turn activate *p53* and *Rb*, respectively (Palmero *et al.*, 1998).

Allelic loss of chromosome 5p, harboring the APC locus has been observed in 20–25% of colorectal carcinomas. APC has a role in the Wnt signalling pathway, acting as a partner molecule of beta-catenin, which is degraded and inactivated through binding to APC. The mutated and truncated APC product is unable to bind and titrate beta-catenin, so that Wnt signalling in an APC mutated cell becomes deranged (Ilyas and Tomlinson, 1997). Another molecular partner of beta-catenin is conductin, the mammalian homologue of axin, which seems to be involved in proper conduction of the complex formation of APC and beta-catenin (Behrens *et al.*, 1998). Furthermore, one of the genes inappropriately activated in a deranged Wnt signalling system turned out to be *c-myc* (He *et al.*, 1998).

Loss of specific chromosomal regions involving one of the two parental chromosomes (allele loss, LOH) occurs frequently in colorectal tumors and is interpreted as evidence that these regions contain tumor suppressor genes. In more than 75% of these tumors, a large portion of chromosome 17p, which contains the *p53* gene, is lost. This event is rarely observed in adenomas. In addition, mutations resulting in amino acid substitution in the *p53* gene product of the remaining *p53* allele are frequently found in colorectal carcinomas and render the p53 protein ineffective as tumor suppressor (Knudson, 1985).

The second most common region of allelic loss in colorectal tumors is chromosome 18q, lost in more than 70% of the carcinomas and in 50% of the late

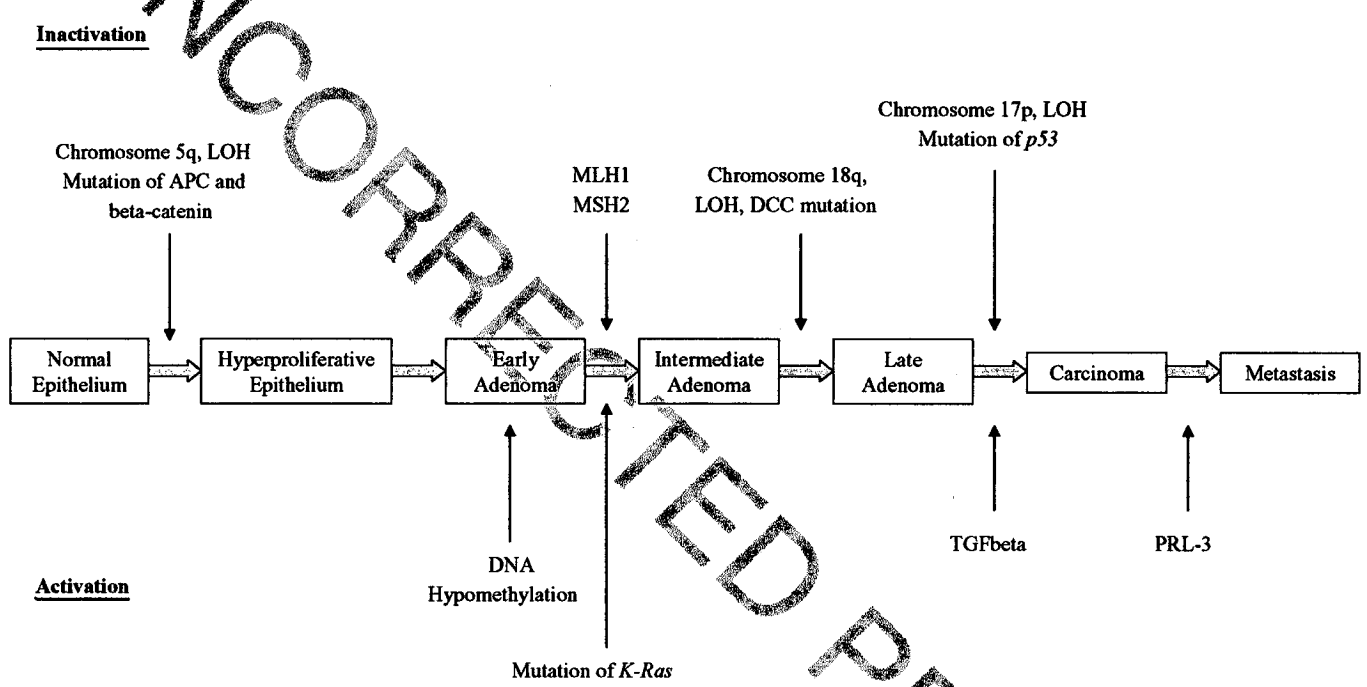

Figure 4.2 The Fearon–Vogelstein genetic model for colorectal carcinogenesis (38, modified). Colorectal cancer is thought to be initiated by inactivation of the *APC* tumor suppressor gene in a colon epithelial cell localized in crypts. In 85 % of cases the *APC* gene is mutated, while the beta-catenin gene is mutated in 50 % of cases. Cells become dysplastic and accumulate. Further mutations (*K-ras* and *DCC*) lead to formation of large polyps, whereas TGF-beta and *p53* gene mutations lead to the cancer phenotype. Chromosomal instability (CIN) seems to be an early event and accounts for LOH. Thirteen per cent of sporadic colon cancers show microsatellite instability (MIN), due to mutation in the mismatch repair enzymes MLH1 and MSH2. Mutations of the *PRL-3* (protein tyrosine phosphatase) gene are found in metastatic tumors.

adenomas, harboring the *DCC* (deleted in colorectal cancer) gene, encoding a transmembrane receptor for netrins, which shows strong homology to the adhesion family molecules affecting cell–cell and cell–extracellular matrix interactions (Mehlen and Fearon, 2004). Although *DCC* was originally though to be the oncosuppressor gene involved in 18q deletions, other tumor suppressors were found in this locus, including *SMAD-4*, one of the components involved in the TGFbeta signalling pathway. *SMAD-4* mutations have been observed in 6–30 % of colorectal carcinomas (Thiagalingam *et al.*, 1996). The *SMAD-2* gene, coding for another component of the TGFbeta signalling pathway, is positioned close to *SMAD-4* and can be induced by Ras (Eppert *et al.*, 1996). Since TGFbeta singalling normally results in cell-cycle inhibition and cellular differentiation, it thus appears that defects in this pathway may play an important role in tumorigenesis. Wnt and TGFbeta-signalling pathways converge on the $p27^{Kip1}$ molecule, whose release from the cyclin E-cdk2 complex is induced by *c-myc* and inhibited by TGFbeta. It, therefore, appears that when the Wnt pathway is deranged resulting in upregulation of *c-myc* and the TGFbeta pathway is disrupted due to a *SMAD-4* mutation, a synergistic effect on the cell cycle ensues (Arends, 2000). This may be a crucial effect in colorectal tumorigenesis. Indeed, increased degradation of $p27^{Kip1}$ has been reported in aggressive colorectal cancer (Loda *et al.*, 1997).

Many other chromosomal losses, in addition to those of chromosomes 5p, 17p and 18q, are detected in the colorectal carcinomas, involving chromosomes 1q, 4p, 6p, 6q, 8p, 9p and 22q. Such losses could either have no specific effect on the phenotype, arising coincidentally with the other genetic alterations, or could contain many suppressor genes present throughout the genome.

Another somatic alteration in colon carcinogenesis is the loss of DNA methyl groups. One-third of the DNA regions studied, even of small adenomas, have lost methyl groups present in normal DNA of colonic mucosa. This epigenetic change could contribute to the instability of the tumor cell genome and change the rate at which genetic alterations, such as allelic losses, occur. Some specific DNA regions, however, could be hypermethylated (Polyak and Higgins, 2001). Although the molecular and genetic defects usually occur at characteristic stages of tumor progression (as shown in Figure 4.2), the progressive accumulation of the defects is more important than the order of their occurrence.

Microsatellite instability is frequently seen in colon cancer tissue from patients with hereditary non-polyposis colorectal cancer (HNPCC), which is caused by a germline mutation of one the mismatch repair genes. HNPCC-associated cancer exhibits microsatellite instability. Germline mutations of each of the six known mismatch repair genes have been identified in HNPCC kindreds. Mutations are most commonly seen in the *hMSH2* gene, found on chromosome 2p, or in the *hMLH1* gene, found on chromosome 3p. Mutations of *hPMS1*, *hPMS2*, *hMSH3* and *hMSH6* account for few reported cases (Calvert and Frucht, 2002). Persons with germline mutations of a mismatch repair gene typically have high microsatellite instability, although the *hMSH6* mutation can be associated with low microsatellite instability (Parc *et al.*, 2000). Although 10–15 % of cases of sporadic colon cancer can exhibit microsatellite instability, it is usually of the low type (Boland *et al.*, 1998).

4.2.3 Endometrial cancer

The major known gene alterations during carcinogenesis of another well-studied cancer, endometrioid adenocarcinoma, are depicted in Figure 4.3 (Caduff *et al.*, 1997; Boyd and Risinger, 1991; Terakawa *et al.*, 1997).

Two different clinicopathological types of endometrial cancer can be distinguished: the estrogen-related or endometrioid type (type I) and the non-estrogen-related or non-endometrioid type (mainly papillary serous or clear cell carcinomas) (type II). Type I is a carcinoma of endometrioid type and low cellular grade, expressing estrogen and progesterone receptors, frequently preceded by endometrial hyperplasia and having a good prognosis. Type II endometrial cancers without associated hyperplasia are negative for estrogen and progesterone receptors and are characterized by high cellular grade and poor prognosis. Recent advances in the molecular genetics of endometrial cancer have shown that the molecular changes involved in its development differ in estrogen-dependent type I and non-estrogen-dependent type II. Type I carcinomas frequently show mutations of DNA mismatch repair genes (*MLH1*, *MSH2*, *MSH6*), *PTEN*, *k-ras* and beta-catenin genes, whereas type II malignancies are characterized by aneuploidy, *p53* mutations and her2/neu amplification. This dualistic model of type I and II endometrial cancers is not applicable in some cases, which show overlapping features. Mutations of the steroid receptor genes have not been linked with a distinct type of endometrial carcinoma (Oehler *et al.*, 2003).

Germline mutations in one of several identified DNA mismatch repair genes, most commonly in *MLH1*, *MSH2* or *MSH6*, are observed in approximately 60–80 % of patients with the HNPCC syndrome. Female carriers of such mutations have a 42 % risk of endometrial cancer by the age of 70 years (Dunlop *et al.*, 1997). Microsatellite instability, a characteristic of HNPCC, occurs also in 15–25 % of sporadic endometrial cancers, although it is very uncommon in uterine serous carcinomas (Tashiro *et al.*, 1997). MLH1 promoter hypermethylation has also been described in microsatellite instability-negative endometrial neoplasia coexisting with microsatellite instability-positive endometrioid endometrial cancers, suggesting that *MLH1* promoter hypermethylation occurs in the transition between hyperplasia and carcinoma. Furthermore, additional mismatch repair genes are secondary mutated, which accelerates genomic instability and the accumulation of additional genetic changes of oncogenes and tumor suppressor genes involved in early carcinogenesis (Inoue, 2001).

K-ras mutations have been identified in 11–31 % of endometrial carcinomas. They are more frequent in endometrioid carcinomas and more common in mucinous subtypes, but almost absent in papillary serous and clear cell carcinomas (Lagarda *et al.*, 2001). *K-ras* mutations have also been demonstrated in about 15 % of endometrial hyperplasias, with a frequency similar to that seen in endometrial cancers. Thus a role of *K-ras* in the early steps of carcinogenesis seems very likely (Sasaki *et al.*, 1993).

Between 10 % and 30 % of all endometrial carcinomas and up to 80 % of uterine serous papillary malignancies show HER2/neu overexpression. As overexpression

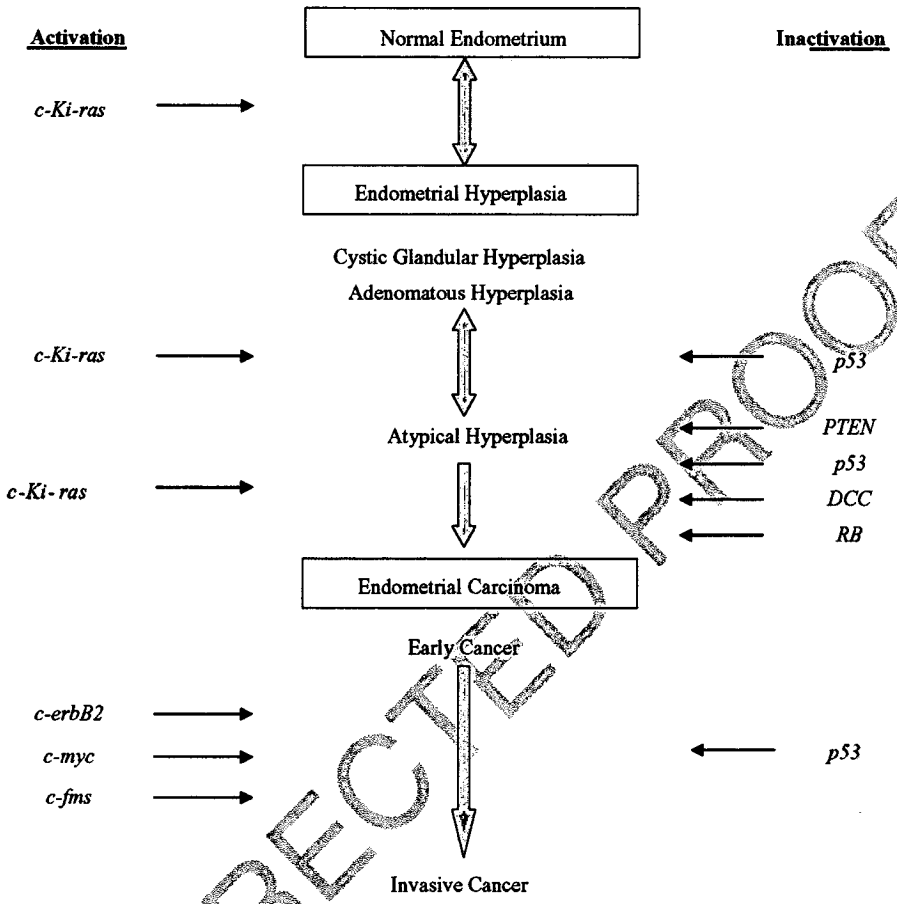

Figure 4.3 A genetic model for endometrial carcinogenesis. Type I carcinomas frequently show mutations of DNA mismatch repair genes (*MLH1*, *MSH2*, *MSH6*), *PTEN*, *K-ras* and beta-catenin genes, whereas type II malignancies are characterized by aneuploidy, *p53* mutations and *her2/neu* amplification.

of HER2/neu is also found in up to 15 % of normal and hyperplastic endometrial samples (Santin *et al.*, 2002), this may play a role in the early development of some endometrial cancers (Rasty *et al.*, 1998).

PTEN is a tumor suppressor gene encoding a phosphatase with homology to tensin. Loss of heterozygosity at the *PTEN* locus of 10q23.3 occurs in about 40 % of endometrial cancers (Matias-Guiu *et al.*, 2001). *PTEN* is also the most frequently mutated tumor suppressor gene in endometrial cancer (in 37–61% of these cancers), particularly in type I malignancies (Risinger *et al.*, 1997). *PTEN* mutations are found in up to 55 % of endometrial hyperplasias, but also in histologically normal-appearing endometrium exposed to estrogen (Mutter *et al.*, 1992). In addition,

identical *PTEN* mutations occur in hyperplasias coexisting with microsatellite instability-positive endometrial cancers (Levine *et al.*, 1998). Inactivation of *PTEN* could therefore represent one of the earliest events in the multistep progression of endometrial carcinogenesis.

Mutations of the *p53* gene and accumulation of the p53 protein are detected in up to 90 % of serous papillary carcinomas, but only in about 20 % of endometrioid malignancies. Mutations are most common in high-grade tumors and are rarely found in endometrial hyperplasias. This finding suggests that *p53* mutations in endometrioid carcinomas are closely related to dedifferentiation and occur relatively late in tumor development. In contrast, the majority (78 %) of endometrial intraepithelial carcinomas, the putative precursor of serous carcinomas, have *p53* mutations, supporting a role of *p53* alterations in the early carcinogenesis of serous malignancies (Lax *et al.*, 2000).

The frequency of beta-catenin mutations in endometrial carcinomas ranges from 13 % to 50 %. However, stabilization of beta-catenin leading to accumulation in the cytoplasm and/or nucleus was also observed in endometrial carcinomas lacking mutations. This finding suggests that alterations in other genes of the beta-catenin/Wnt pathway might be responsible for the stabilization of Wnt in these tumors (Fukuchi *et al.*, 1998).

As in the previously described cancer cases, the accumulation of the gene defects rather than the order of their appearance seems to be more important in carcinogenesis. Additional genetic changes, depicted in Figure 4.3, are correlated to the metastatic potential of the cancer cells.

4.3 THE MULTISTAGE MOUSE SKIN CARCINOGENESIS MODEL

Mouse skin has provided a paradigm for studies of multistage chemical carcinogenesis in epithelial cells. The chemical carcinogenesis regimen applied to mouse skin is the two-stage induction, which involves the administration of a single dose of the polycyclic aromatic hydrocarbon 7,12-dimethyl-benz[α]anthracene (DMBA), followed by weekly applications of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which has the role of carcinogenesis promoter. This treatment results in the development of numerous benign papillomas, some of which progress to malignant squamous cell carcinomas 20–40 weeks after the first exposure to carcinogens. Because of the problems associated with studying biological mechanisms of carcinogenesis using *in vivo* tumour material, a series of cell lines has been developed in Allan Balmain's laboratory. They represent the development of the three distinct stages of mouse skin carcinogenesis – initiation, promotion and progression – thus covering the full spectrum of mouse skin carcinogenesis.

The mouse skin carcinogenesis model constitutes mainly of the following cell lines: the *C5N* immortalized, non-tumorigenic keratinocyte line derived form a Balb/c mouse (Kulesz-Martin *et al.*, 1983); the *P1* and *P6* benign papilloma cell lines, derived from a DMBA/TPA treated spretus X CBA F1 hybrid mouse (Haddow *et al.*, 1991); and the *B9* squamous cell line and the *A5* highly anaplastic,

invasive spindle cell line, both isolated from the same primary tumour from a multiple DMBA/TPA treated spretus X CBA F1 hybrid mouse (Burns *et al.*, 1991). Furthermore, the *CarB* highly anaplastic, invasive spindle cell line, derived from a DMBA/TPA treated NIH mouse (Fusenig *et al.*, 1978), the *PDV* squamous cell line derived from a DMBA treated epidermal cell culture from a newborn mouse, and the *PDVC57* squamous cell line developed in one out of eight sites of injection of PDV cells in an adult syngeneic C57B1 mouse (Fusenig *et al.*, 1985). Although the *A5* spindle cell line was isolated from the same primary tumour as the *B9* cell line, it has different morphological and growth properties. The *C5N*, *P1*, *P6* and *B9* cells have a typical epithelial morphology, being cuboidal in shape, and are characterized by a cobblestone pattern of growth, while the *A5* and *CarB* cell lines show a fibroblastic morphology. The *C5N*, *P1*, *P6* and *B9* cell lines have a typical pattern of keratin and E-cadherin expression, whereas the *A5* cell line has an altered cytoskeleton and fails to express E-cadherin. The other spindle cell line, the CarB, does not express E-cadherin, but expresses vimentin. Compared with *PDV* cells, the *PDVC57* cell line has a more heterogeneous morphology, is characterized by an increased number of giant cells and is more tumorigenic when reinjected into adult syngeneic mice. *PDVC57* cells are eight times as invasive and secrete twice as much type IV collagenase compared to *PDV* cells, and are also more chemotactic.

The multistage mouse skin carcinogenesis model, although an artificial one, is an ideal system to study the timing of qualitative and quantitative alterations which take place during the different stages of chemical carcinogenesis, allowing analysis of the events that lead to the transition from the stage of initiation to the stage of promotion and finally to the progression of carcinogenesis. The following passage summarizes the main alterations observed in signal transduction molecules in the mouse carcinogenesis cell lines.

The *H-ras* mutations have a causal role in the initiation stage of carcinogenesis. Papillomas and carcinomas initiated with different carcinogens exhibit distinct spectra of point mutations in the *H-ras* gene (Quintanilla *et al.*, 1986). Furthermore, *H-ras* plays an important role in more advanced stages of carcinogenesis, in which the mutant allele is further duplicated and amplified. The squamous and spindle cell lines differ in the ratio of wild-type to mutant *H-ras* alleles: The *B9* cell line carries two wild-type and four mutant allels, the *A5* cells have 1 wild-type and 2 mutant alleles, the *CarB* cell line carries two mutant alleles and the ratio of wild-type to mutant *H-ras* alleles in *PDV* and *PDVC57* cell lines is 2:1 and 1:2, respectively.

Ras-mediated tumorigenesis depends on signalling pathways that act preferentially through cyclin D1, whose mRNA and protein levels are generally higher in mouse skin carcinomas than in papillomas. Cyclin D1 deficiency also results in up to an 80 % decrease in the development of squamous tumours generated through two-stage chemical carcinogenesis. Cyclin D1 participates, therefore, in the stage of promotion of carcinogenesis (Robles *et al.*, 1998).

Ras activates members of the JNK group of MAPKs, which are the major mediators of c-Jun and ATF-2 terminal phosphorylation, as well as the Raf/MEK/ERK branch of the MAPK pathway. The content of JNK1 and JNK2 isoforms, as well as JNK activity, is increased in the malignant mouse skin cell lines, with JNK2

elevated to a lesser extent than JNK1 in the spindle cell lines *A5* and *CarB*. The ERK1/2 isoforms are preferentially activated in advanced tumor stages, since phosphorylated ERK1 and ERK2 are elevated in the *A5* and *CarB* spindle cells, compared with the *P1* and *B9* epithelial cell lines. Studies in the *PDV:PDVC57* cell line pair revealed increased ERK1/2 phosphorylation in the *PDVC57* cells, which are more aggressive than the *PDV* cell line. This finding suggests that the biological characteristics of the squamous phenotype may depend on the activation of ERK1/2 (Katsanakis *et al.*, 2002).

The c-Jun and ATF-2 AP-1 transcription factor family members are likely to be involved in the progression of carcinogenesis in mouse epidermis. Increased levels of total and phosphorylated c-Jun are detected in the malignant cell lines, with maximum levels observed in the *A5* and *CarB* spindle cell lines. High levels of Fra-2, hyperphosphorylated Fra-1 and total and phosphorylated ATF-2 also characterize the malignant phenotypes. This increase probably takes place due to Ras protein overexpression, also observed in these cells. These changes in expression and post-translational modification of the AP-1 family members result in enhanced AP-1 DNA activity at the collagenase I TRE and Jun2 TRE in the metastatic cell lines *A5* and *CarB*. The major AP-1 components pariticipating in the AP-1/DNA binding complex are c-Jun and ATF-2 (Zoumpourlis *et al.*, 2000).

Increased serum response factor (SRF) protein levels and SRF DNA binding activity to the c-*fos* serum response element are observed in the mouse skin spindle cell lines. Furthermore, both total and active RhoA levels are significantly higher in *A5* than in *B9* cells. Transfection experiments with active and dominant negative forms of RhoA have shown that SRF overexpression has an important role in spindle phenotype formation and RhoA signalling regulates DNA binding activity of SRF (Psichari *et al.*, 2002).

A5 spindle cells, which are characterized by increased amounts of mutant H-ras protein, do not express any Tiam-1 (T lymphoma invasion and metastasis gene) protein, in contrast to *P1* cells, which express high levels of Tiam-1. Moreover, loss of Tiam-1 protein in *A5* cells is accompanied by a strong reduction in Rac basal activity. Tiam-1 function appears, therefore, to be essential for the initiation and promotion of Ras-induced skin tumours, but histological and biochemical data suggest that a subsequent loss of Tiam-1 increases the rate of malignant conversion of benign tumours (Malliri *et al.*, 2002).

High levels of the matrix metalloproteinase MMP-9 (which is regulated by the AP-1 and ets transcription factors) have been demonstrated in the invasive *A5* and *CarB* spindle cell lines, whereas MMP-2 levels are independent of tumorigenic and invasive cell properties (Papathoma *et al.*, 2001).

It has been suggested that *p53* alterations arise before the transition from squamous to spindle phenotype. In a study carried out on chemically induced mouse skin tumours, LOH at the *p53* locus is detected in approximately one-third of carcinomas, but not in papillomas. Furthermore, no loss of heterozygosity is detected in *PDV* and *PDVC57* cell lines. Moreover, the mutant p53 protein is present in the primary carcinoma which gives rise to *B9* and *A5* cell lines, but not in *CarB* cells (Burns *et al.*, 1991).

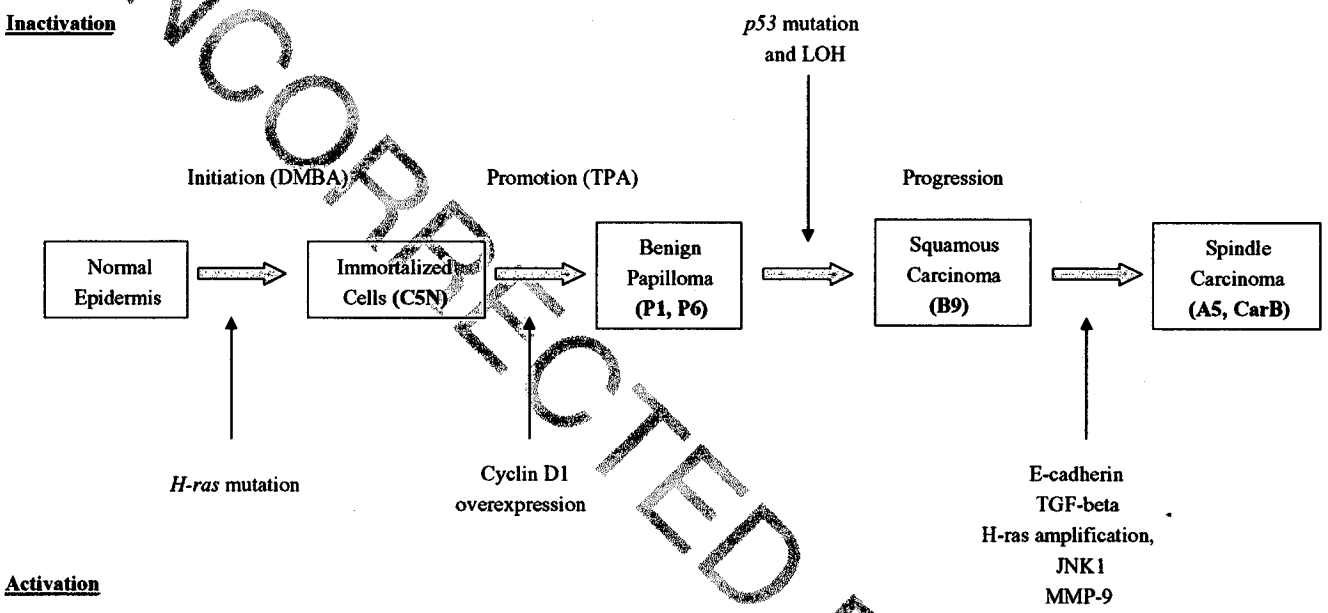

Figure 4.4 Gene abnormalities in the multistage mouse skin carcinogenesis model generated by application of a two-stage induction chemical carcinogenesis regimen, which involves the administration of a single dose of DMBA, followed by weekly applications of TPA, which has the role of carcinogenesis promoter. This treatment results in the development of numerous benign papillomas, some of which progress to malignant squamous cell carcinomas 20–40 weeks after the first exposure to carcinogens. *H-ras* is a critical target of chemical carcinogens and has a crucial role in initiation of carcinogenesis. Further mutations in *H-ras* target genes, as well as in oncosuppressor genes, guide the transition to advanced stages of carcinogenesis.

The immortalized, benign and malignant cell lines comprising the mouse skin carcinogenesis system have proved to be an ideal model for the study of multistage carcinogenesis, easy to manipulate and handle, and have been valuable tools in investigations that succeeded in correlating specific genetic alterations with specific stages of carcinogenesis (Figure 4.4). These observations were verified by *in vivo* experiments in knock-out and transgenic mice. These data could serve as a background for the identification of genes having a critical role in stage-to-stage transition in human multistage cancers.

4.4 EPILOGUE

Much of the data concerning the sequential genetic changes leading to the transformation of a normal cell into a cancer cell with metastasizing potential has been derived from the development of animal and animal cell models, although the analysis of surgically removed tumors or tissue biopsies has also contributed valuable information.

Objections have been raised concerning the value of animal models due to the discrepancies between human and rodent carcinogenesis (Balmain and Harris, 2000; Hann and Balmain, 2001). However, there is a high degree of genetic and biological similarity between development of cancer in human and rodent systems (Zoumpourlis *et al.*, 2003). Mice develop tumors in the same tissues as humans and with similar histopathological course, and the genetic events in humans are mostly also observed in rodents, with a similar stepwise progression from benign to malignant stages. Rodents have a short life span and develop tumors quite rapidly and rodent cells can be – in contrast to human cells – easily immortalized (Zoumpourlis *et al.*, 2003), which could be due to differences in telomerase activity and repair of chromosome ends (Rhyu, 1995). Independent of their possible shortcomings, the animal models serve the purpose of following the carcinogenic process from carcinogen exposure and genetic alterations afflicted, to the biological response and the malignant phenotype. The development of a series of animal and animal cell carcinogenesis models, taking into account the spectacular advances in molecular techniques – microarrays and proteomics – permitting the simultaneous analysis of thousands of genes and proteins, heralds important advances in our understanding of carcinogenesis, with significant impact on cancer risk assessment, tumor prevention, diagnosis, prognosis and therapy.