Molecular foundations of cancer: New targets for intervention

Conceptual and practical advances in molecular medicine are changing our understanding of cancer pathogenesis. In time this should provide the opportunity to alter the natural history of many cancers

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Molecular medicine harnesses the genetic and biochemical determinants that govern the orderly progression of cellular physiology from cell birth to cell death. As such, it is the foundation of modern prevention, diagnosis and treatment. Cancer and certain formative pathways that regulate cell survival and determine how an individual cell or group of cells behaves in the context of a relevant external microenvironment illustrate the issues. Each of these pathways likely plays a pivotal role in tumorigenesis and provides a target for clinical intervention.

One of the most promising avenues comes from new developments in understanding cell cycle regulation. The molecular regulation of the cell cycle maintains homeostatic balance between cell growth, differentiation, survival and death. Two families of proteins that form complexes play key roles in this process as positive regulators (Table 1). They are the cyclins and cyclin-dependent kinases (cdks). The negative regulatory forces are provided by tumour suppressor proteins, notably those encoded by the tumour suppressor genes *p53* and *RB* (retinoblastoma), and a newly discovered family of proteins known as cdk inhibitors (Table 2). In their catalytically active state, cyclin/cdk complexes enable the cell to traverse the specific phases of the cell division cycle: the 'preparatory' or checkpoint phases (G1 and G2), DNA synthesis (S) and mitosis (M) (Figure). The first cyclins to be described and characterized are currently called the B cyclins, which were first isolated from marine invertebrates (clams, sea urchins) and then in the eggs of the amphibian *Xenopus laevis*[1,2]. B cyclins regulate the cell's movement through mitosis. Cyclin A facilitates progression through S phase in mammalian cells[1].

The G1 cyclins facilitate movement through the earliest phases of the cell cycle and across the G1/S boundary[3]. The G1 cyclins consist of at least three D cyclins and also cyclin E. The G1 cyclins and their cdk partners are rate-limiting with respect to progression through G1 into S phase in the normal setting[3-5]. The D family members are unusual among cyclins in that they are modulated by exogenous growth factors[6], for example, colony-stimulating factor (CSF)-1 induction and transforming growth factor (TGF)-β downregulation. The D cyclins abrogate the growth-inhibiting functions of retinoblastoma proteins (pRbs) by two mechanisms: direct binding to the critical pocket region of the retinoblastoma proteins (pRbs) and, in complex with various cdks, phosphorylation and thus **inactivation** of pRbs, thereby abrogating the growth-inhibitory, tumour suppressor functions of pRb proteins[7,8]. Cyclin E, on the other hand, resembles cyclins A and B in that its expression is cell cycle-dependent[4]. The cell cycle activity of cyclin E overlaps with that of D cyclins by extending from late G1 through the G1/S transition into S phase proper, suggesting that cyclin E acts to push cells through a critical checkpoint and thereby drive cell division[5].

ABBREVIATIONS

APC	adenomatous polyposis coli
AT	ataxia-telangiectasia
BRCA-1	breast cancer-1
Cdk	cyclin dependent kinase
CIP-1	cdk-interacting protein
CMV	cytomegalovirus
CSF	colony-stimulating factor
DCIS	ductal carcinoma in situ
EBV	Epstein-Barr virus
FAP	familial adenomatous polyposis
FGF	fibroblast growth factor
GADD	growth-arrest DNA damage-inducible
GST	glutathione-S-transferase
HNPCC	hereditary non-polyposis colorectal cancer
HVS	Herpesvirus saimiri
ICK	inhibitor of cyclin-dependent kinase
IFN	interferon
INK4	inhibitor of cdk4
KIP-1	kinase inhibitory protein
KS	Kaposi's sarcoma
MLH	mutL homologue
MSH	mutS homologue
MTS	multiple tumour suppressor
PCNA	proliferating cell nuclear antigen
PIN	prostatic intraepithelial neoplasia
PMS	postmeiotic segregation
RB	retinoblastoma
TGF	transforming growth factor
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau
WAF-1	wild-type p53-activating fragment-1

G1 cyclin dysregulation: A mechanism of tumorigenesis

The excessive, aberrant or temporally inappropriate expression of various cyclins during the cell cycle could have a pivotal role in the pathogenesis of diverse malignancies of epithelial, lymphoid and haematopoietic cells (Table 1). For instance, translocation and rearrangement with consequent overexpression of the cyclin D1 gene[9] typify certain intermediate lymphomas (especially centrocytic, or 'mantle cell'), whereas gene amplification at the D1 locus has been detected in roughly 15% of breast cancers and about one-third of oesophageal cancers, as well as bladder and non-small-cell lung cancers[10]. The cyclin D1 gene bears some

homology to a gene present in herpesvirus saimiri (HVS)[11], a DNA virus related to Epstein-Barr virus (EBV) that causes a lymphoproliferative disorder in non-human primates similar to that in humans[12]. Viral sequences with striking homology to HVS and EBV have been linked to AIDS-related Kaposi's sarcoma (KS) and suggest a new herpes virus as a potential etiologic factor[13]. Examination of human colon cancer has detected gene amplifications of cyclin D2 and E in individual tumour populations[14]. The cyclin D2 gene is located in a region characteristically disturbed in germ cell tumours[15] and amplified in chronic lymphocytic leukaemia[16]. Abnormalities of the cyclin D3 gene[17] are present in about 50% of retinoblastomas, lymphomas and acute lymphocytic leukaemias[10,17].

Recent studies have uncovered abnormalities in cyclin E gene expression in the vast majority of breast tumours[18,19]. In particular, amplification of the cyclin E gene and altered expression of cyclin E protein have been detected in all human breast cancer lines examined, frequently as the sole cyclin-related abnormality, suggesting that dysregulation of cyclin E expression could induce hyperproliferation and eventual malignant transformation[18]. The alterations in cyclin E expression correlate directly and quantitatively with tumour aggressiveness as determined by histologic grade and invasiveness. The alterations appear to be relegated to tumour cells and are not present in adjacent normal tissues, thus representing a true tumour cell-associated abnormality that discriminates malignancy from non-neoplastic states. Dysregulation of cyclin E expression appears to be a remarkably consistent molecular marker of breast epithelial cell malignant transformation, more so than other markers. Alterations in cyclin E might occur at a formative time during the carcinogenesis process and, as such, their detection might provide a biomarker for early 'premalignant' lesions that could identify individuals at high risk who, in turn, might benefit from chemosuppression (perhaps tamoxifen) or other preventive measures.

In addition to breast cancer, the constitutive production of multiple isoforms of cyclin E due to gene amplification or in some cases deletional mutations is detectable in many malignancies including aggressive prolymphocytic leukaemias and lung, stomach, kidney, prostate and colon cancers and, as such, may represent a general mechanism for tumour initiation and/or promotion[18,19]. The ability to detect aberrations in the expression of one or more cyclins or the loss of cdk inhibition could provide clinically germane information regarding diagnosis and prognosis. The abnormal cyclin mRNAs and proteins may also present novel molecular targets for therapy and prevention interventions, including vaccine development.

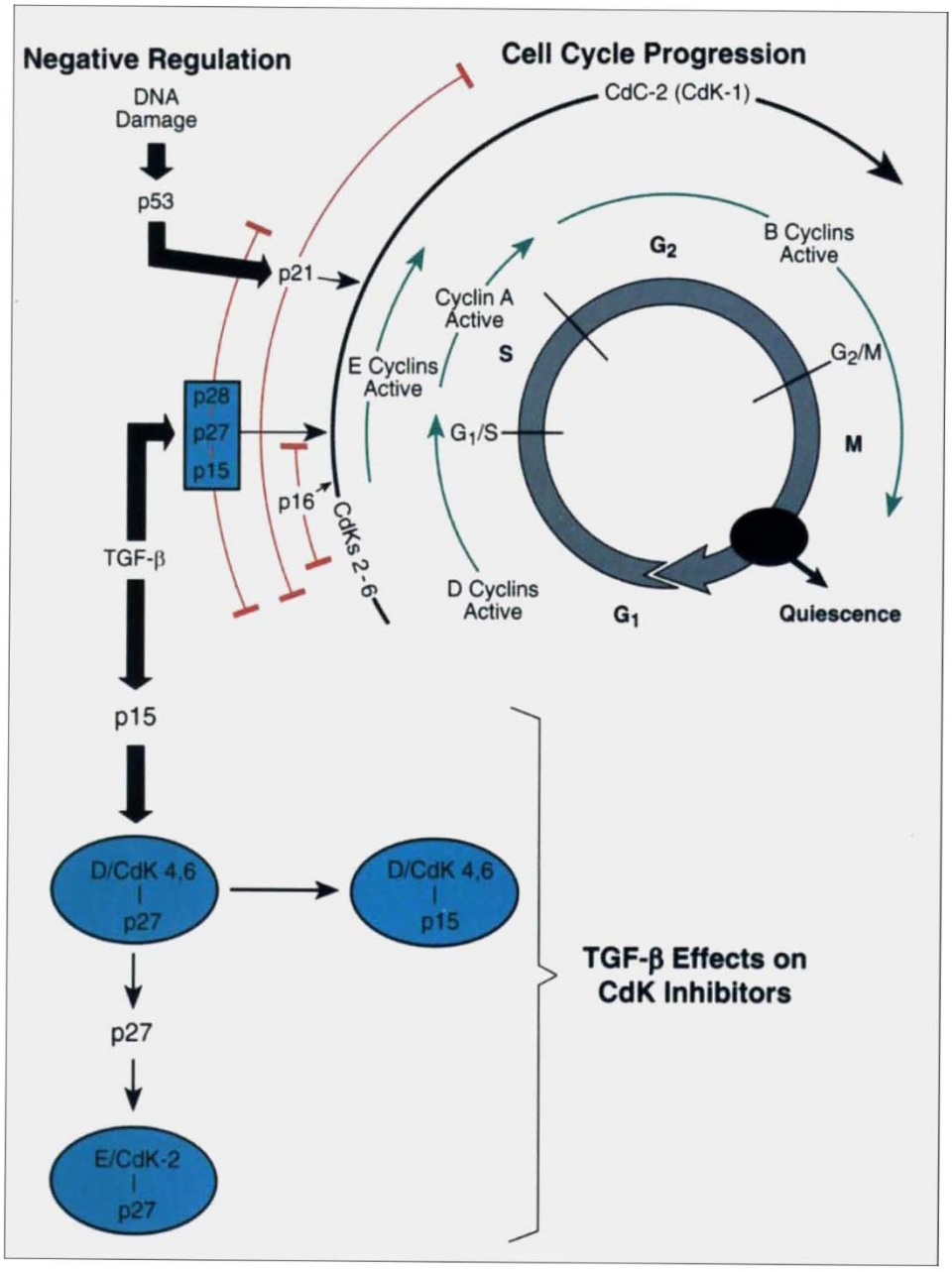
Negative regulatory mechanisms

In order to maintain homeostasis, cells must possess negative regulatory mechanisms to balance the growth-promoting forces that drive cellular proliferation. Many groups of investigators have uncovered the existence of several distinctive cdk inhibitors, linked to p53, exogenous growth inhibitors and pRb. These inhibitory proteins target the net activities of a broad array of cdks and cyclin/cdk complexes (Table 2, figure).

The p53 protein is a DNA-binding, cell cycle-regulating transcription factor that sits at the crossroads of critical pathways governing orderly cell division and the balance between cell survival and cell death[20,21]. On the cellular level, losses, deletions and/or mutations of *p53* play a crucial role in the pathogenesis of a strikingly large number of malignancies[21]. Such genetic aberrations can be etiologically linked to familial syndromes — notably the devastating cancer-prone Li-Fraumeni syndrome[22] and, most recently, certain families with hereditary early-onset breast cancer or breast–ovarian cancer syndrome[23]. They can also occur as a consequence of exposure to various types of exogenous carcinogens. Some environmental agents may be directly mutagenic[20] or, as is the case with viruses, may act on the *p53* gene products post-transcriptionally or post-translationally to modify the conformation and DNA binding capabilities of the p53 protein[24] This process has recently been implicated in coronary artery disease as well as in viral carcinogenesis[25]. Cytomegalovirus (CMV) produces a protein, IE84, that binds to and inactivates p53 in smooth muscle cells and consequently could play a central role in the development of restenosis and reocclusion of coronary arteries following angioplasty[25]. It is perhaps not far-fetched to think that these observations could have substantial impact in the field of cardiology.

The p53 protein has a checkpoint function and plays an integral role in the repair of DNA damage induced by ionizing radiation[26]. The elucidation of this critical activity has been made possible in part through the study of ataxia-telangiectasia (AT), an inherited multisystem disorder rising from mutations in a recessive gene. The syndrome is characterized on both the molecular and clinical levels by hypersensitivity to radiation-induced DNA damage and defective repair of such damage[27]. Indeed, p53 sits in the center of a signal transduction pathway that links radiation-induced activation of the normal version of the *AT* gene with the eventual expression of specific growth-arrest DNA damage-inducible (GADD) gene products[28]. The GADD45 protein prolongs G1 and thereby permits the cell to repair the DNA damage before entering S phase.

The p53 protein also binds to and activates the transcription of a gene[29] that encodes a cdk inhibitor known as p21WAF-1/CIP-1 (refs 29,30). p21 binds to multiple cdks and blocks the activation of cyclin/cdk complexes, thus impeding their ability to phosphorylate target substrates, notably pRb proteins[31–33]. Like the AT-p53-GADD pathway, the p53-p21-pRb pathway is operative in fibroblasts and perhaps other cell types as well[28,30,31]. The loss of *p53* expression in fibroblasts is accompanied by a loss of p21 from G1 cyclin/cdk complexes, demonstrating that p21 is regulated by p53, as is the induction of the GADD45 protein following radiation-based DNA damage (ref. 31). Given the high incidence of *p53* gene and p53 protein abnormalities associated with a broadly diverse group of malignancies, it is reasonable to speculate that the p21-based mechanism of cell cycle control could be involved in the pathogenesis of many types of cancers. In this regard, p21 has been isolated from a human brain tumour cell and, through gene transfection experiments, shown to inhibit growth of human brain, lung and colon tumour cell cultures[29]. Thus, p21 is likely a central mediator of p53-induced cell cycle arrest in the G1 phase and stands as a pivotal negative cell cycle regulator that links the tumour suppressor activities of p53 and pRb through a cyclin/cdk-mediated pathway. Furthermore, p21 presents a



Movement through the cell cycle is driven by positive regulators (green lines) consisting of cyclins complexed with cyclin dependent kinases (cdks). In contrast, cell cycle progression is retarded by negative regulators (red lines) consisting of diverse cdk inhibitors: p21, which induced by p53 in response to DNA damage and binds to multiple cdks; p16, the product of the MTS1 (multiple tumour suppressor-1) gene located on chromosome 9p21, which specifically binds to cdk4 and inhibits cyclinD/cdk4 activity; and three cdk inhibitors whose net activities are enhanced by transforming growth factor (TGF)-b — p15 (the product of MTS2, located adjacent to MTS1), p27 and p28. TGF-b induces the production of p15, which displaces and replaces p27 from its complexes with cyclin D/cdk4 and cyclin D/cdk6. The liberated p27 is now free to form complexes with and to inhibit the activity of cyclin E/cdk2.

point of convergence through which tumour-initiating and tumour-promoting agents could disrupt the orderly process of cell cycle progression.

The p53-mediated response to DNA damage can culminate in at least two disparate outcomes with regard to cell survival: G1/S arrest or apoptosis. What determines the specific path that the cell travels after incurring DNA damage is not yet clear, and it is likely that other factors in signalling activities help to determine which path the cell takes. In this regard, proliferating cell nuclear antigen (PCNA) interacts with both p21 and GADD45 and could serve as a 'switching mechanism'. PCNA drives DNA replication[34] and also plays a pivotal role in DNA repair[35]. Dysregulation of PCNA expression occurs in a variety of malignancies — head and neck, breast, colon and brain cancer and certain leukaemias — and serves as a marker of tumour aggressiveness[36–38]. It appears that the p53 protein mediates the cell's response to DNA damage through two pathways, both of which intersect with PCNA and modulate PCNA's DNA replication and DNA repair functions.

Although p21 may represent a p53-induced cellular response to DNA damage or oxidative stress, other cdk inhibitors — $p27^{KIP-1}$ (kinase inhibitory protein)[39,40] and $p28^{ICK}$ (inhibitor of cyclin-dependent kinases)[41,42] — are induced by different factors, namely, cell–cell contact[39], TGF-β (refs 39,42) and the G1-arresting anti-hypercholesterolemic agent lovastatin[41]. TGF-β (a molecule with complex and sometimes opposing functions in divergent settings) is an important growth inhibitor for both normal and malignant epithelial cells, in particular breast, prostate, lung and skin, and may mediate some of the antitumour activities of the retinoids (vitamin A derivatives) and the anti-oestrogen tamoxifen[43]. Another cdk inhibitor, $p16^{INK4}$ (inhibitor of cdk4), specifically binds to cdk4 and thus blocks the cycle-propelling activities

of cyclin D/cdk4 complexes[44,45]. The gene encoding p16, the so-called multiple tumour suppressor-1 (*MTS1*) gene, has been found to be structurally abnormal in diverse primary and cultured tumour cells — melanoma, lymphocytic leukaemias and cancers of the lung, breast, ovary, brain, bone, skin and bladder[44,46]. The *MTS2* gene is adjacent to *MTS1* and encodes $p15^{INK4B}$, which also binds directly to cdk4 and cdk6, in competition with the D cyclins[47]. p15 also resembles p27 in that the net expression of both cdk inhibitors are increased by TGF-β and both mediate TGF-β-induced cell cycle arrest[47,48]. TGF-β appears to directly induce p15 expression[49]; p15, in turn, displaces p27 from cyclin D/cdk complexes, thus making the liberated p27 available to bind to cyclin E/cdk2 complexes[48,49] (Table 2, Figure).

In summary, each of the cdk inhibitors described to date provides a crucial mechanism by which diverse agents — DNA damage, oxidative stress, cell–cell contact, TGF-β and lovastatin — prevent pRb phosphorylate and exert cell cycle suppression. The modulation of net G1 cyclin activity, as mediated by the induction of cdk inhibitors, may serve as a point of convergence through which diverse agents (for example, those that induce TGF-β) may exert their antitumour effects (Figure). The search for small molecules that alter these pathways could provide new opportunities for rational drug design. This principle may provide a central molecular target for both therapy and prevention for a broad spectrum of epithelial malignancies (for example, breast, prostate, lung, brain) and lymphohaematopoietic tumours (lymphomas, lymphoid leukaemias) where G1 cyclins play an integral role in the emergence and perpetuation of the malignant clone.

Genetics of cancer

The ability to identify and characterize the structure and function of cancer-related genes is fundamental to every discipline of basic and clinical cancer investigation, from understanding tumorigenesis and tumour suppression to the detection and quantification of an individual's risk for developing a particular cancer to monitoring the efficacy of a therapeutic or preventive intervention. This entire field, in its modern sense, is the outgrowth of recombinant DNA technology. At this point, it is conceivable that we will be able to uncover a major gene that, when structurally and functionally altered by either a heritable defect (loss or mutation) or an exogenous transforming agent, is responsible for the initiation of (or predisposition to) the process of carcinogenesis in a specific cell type for virtually every organ system (Table 3). Yet, the ability to diagnose a particular predisposition to cancer may carry with it a number of practical considerations, especially in the setting where effective non-invasive and non-toxic interventions are not available. We are often in uncharted waters. Genetic counseling may or may not be feasible depending on the genes involved and the individual circumstances of the patient. In the long run, science will answer our questions and point the way to specific prevention and treatment. In the short run, life will be very complicated.

There is another cautionary note, and it has to do with actively maintaining a broad perspective on the potential relevance of a specific cancer-predisposing gene. We should be cautious about naming genes and unthinkingly acting on incomplete, or self-referential nomenclatures. Thus, the genes commonly linked to hereditary non-polyposis colorectal cancer (HNPCC) also clearly confer a heightened predisposition to endometrial, gastrointestinal and genitourinary cancers[50]. What is called the breast cancer-1 (*BRCA-1*) gene is linked to prostate and colon cancers as well as breast and, of course, it is powerfully linked to ovarian cancers[51]. In each of these cases, the 'disease-related' genes may be discovered initially in the setting of one particular cancer. The linkage with one cancer, however, does not preclude an equal or perhaps even tighter linkage to other malignancies. The potential spectrum of linkages to a variety of cancers unfolds over time as the gene in question is examined in greater numbers of kindreds and in sporadic settings as well, giving rise to a fuller picture of gene expression and variation in cancer predisposition.

Progress in colorectal cancer genetics. In less than a decade, sequential studies of the molecular genetics of colorectal cancer have provided crucial experimental evidence to support the 'multistep hypothesis' of carcinogenesis[52]. It is through these studies, involving careful genetic linkage analyses, that colorectal cancer has become a paradigm for inherited malignancies that arise as a result of the loss of particular alleles of tumour suppressor genes. The first such studies led to the molecular dissection of familial adenomatous polyposis (FAP), an autosomal dominantly inherited disorder that affects about 1 in 5,000 Americans and is characterized by the emergence of multifocal 'premalignant' colonic adenomas that acquire multiple genetic abnormalities and undergo malignant degeneration[53]. The adenomatous polyposis coli (*APC*) gene is mutated in germline cells from individuals with FAP and in somatic tumour cells from non-FAP individuals with colon cancer[51]. *APC* is thus critical to colon carcinogenesis in both the inherited and acquired settings[54,55]. Most recently, there is the dramatic discovery of a completely new type of gene — a 'master' or 'mutator' gene, whose protein product oversees the process of DNA replication and maintains its fidelity by recognizing the occurrence of mismatched nucleotides or recombination errors arising during the process and coordinating the repair of such replication errors[56–61]. This mechanism, initially uncovered through the study of bacterial genetics and found to be evolutionarily conserved from bacteria through human cells[57,58], has forged a new concept in our understanding of the means by which genomic integrity is actively preserved or, in the case of susceptibilities to certain cancers, destabilized.

Last year, several groups of researchers uncovered the genetic defect associated with HNPCC, namely, the presence of microsatellite alterations (or variations in length of short repetitive DNA sequences) throughout the genome associated with abnormalities of chromosome 2p16 (refs 56–61). Although only 1% of all colon cancers are linked to FAP, HNPCC accounts for roughly 10–15% of overall colon cancer incidence[53]. The HNPCC lesion is also linked to a number of seemingly disparate cancers occurring within affected families — notably endometrial, stomach, bladder, ovarian and possibly breast cancers[50,56,62].

A total of four genes, each of which has its evolutionary roots in the MutHLS DNA repair pathway in bacteria, act in concert as 'proofreaders' of DNA to detect and repair any mismatched DNA base pairs that occur during DNA

replication[63]. The *MSH-2* gene encodes a DNA-binding protein that targets mismatched nucleotides for eventual excisional repair[56–59,63]. *MSH-2* is mutated in 60% of patients with HNPCC and is responsible for the so-called replication error phenotype that is characterized on the genomic level by microsatellite alterations and is linked on the clinical level to the HNPCC predisposition[60]. Interestingly, cells from individuals with only one *MSH-2* allele affected and the other remaining intact are phenotypically normal with regard to overall DNA replication and recombination, a behaviour not unlike that seen for the more classical tumour suppressor genes where functional loss of the normal allele is necessary for full dysregulation to ensue — in other words, the gene acts in a recessive manner on the cellular level, independent of the mode of inheritance. The *MLH-1* gene is mutated in 30% of patients with HNPCC and appears to act in concert with *MSH-2*, actively excising the mismatched pair identified by the *MSH-2*-encoded protein, and is associated with the same clinical phenotype[64,65]. Most recently, the third and fourth components of this repair complex, namely, additional MutL homologues called *PMS-1* and *PMS-2* ('postmeiotic segregation') have been uncovered[66].

These evolutionarily conserved genes and their encoded proteins work together by forming a complex with the erroneous base pairs and with each other[63], cooperating within the same pathway to identify and remove mismatched nucleotides or recombination errors arising during DNA replication and thereby to prevent genomic instability. Lesions in one or more of these genes permit DNA replications errors throughout the genome, thus conferring genetic instability. Together, mutations in *MSH-2* and *MLH-1* account for more than 90% of all hereditary colon cancers, and may affect as many as 1 in 200 individuals[53,56,58,64]. It is tempting to speculate that these or related genes might have a causative role in the emergence of some non-familial sporadic cancers as well. However, recent studies in sporadic colorectal cancers demonstrate that germline mutations in the mismatch repair genes are uncommon and that microsatellite alterations in cultured tumour cells can occur in the absence of germline lesions, suggesting that lesions in genes other than *MSH-2*, *MLH-1*, *PMS-1* and *PMS-2* may be involved in permitting replication errors in the non-familial setting[67].

The clinical differences between FAP and HNPCC reflect differences on the molecular genetic level. In FAP, the defective *APC* gene is present in the germline DNA of every cell, thus conferring upon every cell the potential to become malignant[54,55,68]. Individuals with FAP form large numbers of 'premalignant' adenomas throughout the colon, which then go on to become full-blown colorectal cancers. In HNPCC, however, the *APC* gene *per se* is initially intact and thus there is not an affected stem cell population *per primum*; rather, *APC* gene lesions arise only as the aftermath of *MSH-2* or *MLH-1* allelic losses[56,60]. Thus, in contradistinction to FAP, the clinical picture is one of a single cancer (most often in the proximal colon) arising from a single transformed cell rather than multiple cancers arising from multiple abnormal stem cells.

One prospective outgrowth of these dramatic discoveries is the development and implementation of a rapid diagnostic test to screen high-risk individuals with positive family histories for the presence or absence of mutations in *MSH-2* and *MLH-1*. Such a rapid and sensitive test has already been developed for FAP, using genomic material derived from peripheral blood lymphocytes to identify APC proteins synthesized *in vitro* and endogenous allele-specific *APC* gene expression[68]. These novel assays detect the truncated APC protein and the allelic reduction in *APC* gene transcription that characterize FAP. The combined use of these assays identifies almost 90% of people affected by FAP[68]. Indeed, the ability to detect *MSH-2* and/or *MLH-1* aberrations would lead to the identification of individuals at high risk for colon and other cancers and could have far-reaching implications for genetic screening aimed at detection and therapeutic intervention of early lesions prior to invasion and dissemination. In addition to colon cancer, the HNPCC syndrome is linked to a propensity to develop endometrial cancer[50,62]. One area where screening for *MSH-2* and *MLH-1* might play an important role right now is in the Breast Cancer Prevention Trial, where tamoxifen — the synthetic hormone which acts as an oestrogen antagonist (specifically in breast cells) or agonist (for example, in endometrium, bone, liver, vasculature) depending on the tissue in question — is being tested for its ability to prevent breast cancer in certain high-risk women[69]. One potential complication of

Table 1 Cell cycle regulators: Cyclins

Cyclin	Associated Cdk inhibitor	Peak activity phase of cell cycle	Regulation of expression	Potential spectrum of malignancies
D	Cdk 2–6	G1	Growth factors (e.g., CSF-1)	
1				Lymphoma, breast,oesophagus parathyroid, bladder, lung
2				Colon, testicular,chronic, lymphocytic, leukaemia
3				Retinoblastoma, lymphoma, acute lymphocytic leukaemia
E	Cdk2	Late G1 - Early S	Cycle-dependent	Breast, prolymphocytic, leukaemia,lung, stomach, kidney, prostate, colon
A	Cdk1[cdc-2], Cdk-2	Mid S - G2	Cycle-dependent	Liver
B	Cdk1[cdc-2]	G2 - M	Cycle-dependent	Breast?

long-term tamoxifen administration is a heightened risk for endometrial cancer due to tamoxifen's oestrogenic activity in endometrial tissue69. The detection of *MSH-2* and/or *MLH-1* might connote an increased risk for endometrial cancer which, in turn, might dictate more intensive uterine monitoring or strategies to decrease the impact of tamoxifen on the uterus. The presence of such mutations could theoretically also have relevance for women receiving postmenopausal oestrogen replacement therapy. These are topics for further research.

The concept of microsatellite instability may also apply to other tumours by mechanisms that are not well understood. Along these lines, a recent study demonstrates the use of microsatellite alterations or deletions, namely, one or more tri- and tetranucleotide repeat sequences occurring on nine different chromosomes, as clonal markers for the rapid detection of head and neck, lung and bladder cancers in roughly 25% of all cases studied[70]. The ability to identify exceedingly tiny clonal populations in urine and sputum samples suggests that this technology could be used for rapid screening and early detection of premalignant clones. Moreover, the ability to detect small numbers of such genetically altered tumour cells in surgical margins implies that this technology could be used perioperatively for highly sensitive tumour staging and rapid determination of the need for further surgical, radiotherapeutic and/or systemic intervention as well.

The serial genetic studies of colon cancer have placed the *APC* tumour suppressor gene in a crucial and early role in the carcinogenesis pathway for colorectal cancer arising in both the familial and sporadic settings[52,55]. The APC protein, unlike p53 or pRb, is localized to the cytoskeleton and binds to cytoplasmic proteins known as β-catenins[71]. Catenins contribute to the formation of adherens junctions, which mediate intercellular adhesion and 'contact inhibition' between epithelial cells. The adherens junction is also comprised of cell surface adhesion molecules known as cadherins. Mutant APC proteins are unable to bind to β-catenin, a defect that disrupts the normal interaction between catenins and cadherins[71] and causes loss of normal intercellular adhesion and growth inhibition. It is likely that APC is a vanguard for a whole class of tumour suppressors, and especially metastasis suppressors, which act at the level of the cytoskeleton and cell surface to modulate the flow of information along specific intracellular signalling 'highways'. In turn, genes and proteins of this class may be prominent molecular targets for therapy and prevention of tumour cell invasion and dissemination. At a minimum, these genes might help us identify populations for special screening with flexible sigmoidoscopy or colonoscopy.

BRCA-1 and BRCA-2: Unexpected findings. The long-awaited identification of the breast cancer-1' gene (*BRCA-1*)[72–74] and the discovery of a second gene (*BRCA-2*)[75] have important implications for our understanding of breast and ovarian cancer inheritance and for prostate and colon cancers as well[51]. *BRCA-1* may account for 70–80% of familial ovarian cancers and, together with *BRCA-2*, may account for a similar majority of all familial breast cancers[76]. *BRCA-1* can be inherited from either the mother or the father, the latter generally unaffected by breast cancer[77]. Taken together, inherited mutations in *BRCA-1* and *BRCA-2* appear to be responsible for roughly 5–10% of all breast cancers, suggesting that inherited mutations in either gene could affect 1 in 200 to 1 in 400 women[76]. Although the two genes account for approximately equal frequencies of familial breast cancers in women, they exhibit some striking differences. For instance, *BRCA-2* (but not *BRCA-1*) confers a heightened risk of breast cancer in males but is not associated with ovarian cancer predisposition[75], and inherited *BRCA-1* mutations are linked to a three-fold increased risk of prostate cancer and a fourfold increased risk of colon cancer[51], although much more research is neces-

Table 2 Cell cycle regulators: Inhibitors of cyclin-dependent kinases (cdks)

CDK inhibitor	Location/Gene	Mechanism	Cell Source (initial isolation)	Potential diseases
$p16^{INK4}$	9p21[*MTS1*]	Binds to cdk4 inhibits cyclin D/ cdk4 activity	Fibroblasts, HeLa	Melanoma, ALL, bladder, head, neck lung, breast, ovary oesophagus, pancreas
$p15^{INK4}$	9p21[*MTS2*] Induced by TGF-β	Binds and sequesters cdk4 and cdk6 Releases bound $p27^{KIP}$ from cyclin D/cdk4 & 6 for binding to complex	Human keratinocytes	Melanoma, T-ALL, head & neck, lung
p21	6p21 Induced by p53 following damage	Binds to G1 and S cdks (including cdc-2) Inhibits cyclin/cdk complexes involving cyclins D, E, A	Fibroblasts, brain	Brain, lung, colon, leukaemias
$p27^{KIP}$ $p28^{ICK}$	? Induced by cell–cell contact TGF-β, Lovastatin	Binds to pre-formed G1 and early S cyclin/ cdk complex involving cyclins D, E, A, and cdks 2,4	Mink lung epithelium Human breast epithelium HeLa	?

sary to pin these relationships down. The tight linkage between mutations in these genes and the occurrence of familial breast and ovarian cancers is reminiscent of situation in familial colon cancers and renal cell cancers. However, unlike the association of both familial and sporadic colon cancers with *APC* mutations or the association of both familial and sporadic kidney cancers with mutations in the von Hippel-Lindau (VHL) gene, the mutations detected in *BRCA-1*-related familial breast cancers do not seem to be recapitulated in the majority of sporadic breast cancers studied thus far[73,76]. It is likely that additional breast cancer-related genes will be uncovered.

The *BRCA-1* gene poses special problems in terms of its use for wide genetic screening to detect heritable risk for breast and ovarian cancers. The gene is extremely large, spanning more than 100 kilobases[73,74], and to date more than 40 distinctive mutations have been identified among affected families[78,79]. Most of these mutations are frameshift or nonsense mutations that result in a truncated protein product, but missense mutations in the gene segment encoding the zinc-finger motif at the amino terminus of the BRCA-1 protein also confer increased breast cancer risk[78].

Molecular genetics of prostate cancer. The molecular dissection of prostate cancer is beginning to uncover the complement of genetic components that determine the process of carcinogenesis in prostate epithelial cells. Presently, it is estimated that 5–10% of all prostate cancer and more than 40% of early onset prostate cancer (men under the age of 55) is familial, with Mendelian inheritance patterns[80]. This pattern is strikingly similar to the situation for breast cancer. Indeed, men with two or three first-degree relatives with prostate cancer have a 5- or 11-fold increased risk, respectively, of developing prostate cancer[80]. And as noted above, there is an apparent familial linkage between the propensity for early prostate cancer development in men and early onset familial breast cancer in women associated with inherited mutations in *BRCA-1*[51].

Recent studies have uncovered deletions in chromosome 8p22 in up to 70% of all prostate cancers, occurring both early and late in the course of the disease[81]. The detection of homozygous deletion of this gene segment in a nodal metastasis and the ability of chromosome 8 transfection to suppress the metastatic process in a rat prostate cancer model supports the hypothesis that a gene or genes in this chromosomal region may encode a tumour suppressor that plays a fundamental role in regulating normal prostate epithelial cell growth.

The activity of specific intracellular metabolic enzymes in the net processing of potential carcinogens is likely to play a role in the promotion or suppression of carcinogenesis in many tissues. Such enzymes include the cytochrome P-450 family, which promotes metabolic activation of procarcinogens and formation of carcinogen-DNA adducts, and the glutathione-*S*-transferase (GST) family, which detoxify and inactivate carcinogens. A recent study demonstrates a striking defect in the expression of the gene encoding GSTs of the *pi* class (the *GSTP1* gene) in the vast majority of malignant prostatic tissues, including prostatic intraepithelial neoplasia (PIN), but not in normal prostate epithelium or in tissues exhibiting benign hyperplasia[82]. The loss of *GSTP1* gene expression is directly linked to hypermethylation of cytidine nucleotides in the promoter region of the gene. The presence of this lesion at the PIN stage implies that the detection of *GSTP1* hypermethylation may serve as an important tool for early detection, risk identification and perhaps monitoring of the effects of a preventive intervention. In addition, the ability to augment the net expression of *GSTP1* through GST-inducing agents or methylation-reversing agents could form the basis for a targetted chemoprevention strategy.

Along these lines, aberrant expression of tyrosine kinase receptor proteins of the epidermal growth factor receptor (EGFR) family — $p185^{erbB-2}$ and $p160^{erbB-3}$ — occurs with strikingly high frequency in PIN and in prostatic adenocarcinoma, both primary and metastatic lesions[83]. As with *GSTP1*, the alterations in production and intracellular distribution may serve as biomarkers of early transformation that could be useful in identifying individuals who might benefit from targetted intervention and as 'surrogate endpoints' to monitor the outcome of such interventions on a molecular level. Moreover, these aberrantly expressed tyrosine kinase receptor molecules might themselves present relevant targets for direct therapeutic inhibition in localized or invasive cancers.

As discussed in the context of the *APC* gene and colon carcinogenesis, intercellular adhesion and thus intercellular biochemical communication are crucial determinants of so-called contact inhibition that, at least in theory, prevents tumour cell invasion and dissemination. Aberrations in the molecular effectors of these processes — catenins and cadherins — have now been detected and characterized in prostate cancer[84,85]. In particular, the loss of expression of the E-cadherin gene has been coupled with progressive disease[85]. The continued molecular dissection of the intercellular adhesion process and attendant signal transduction pathways may provide novel biomarkers for the detection of invasive-metastatic potential, thus identifying patients who might benefit from therapeutic intervention. Additionally, the molecules themselves present templates on which to design agents that could augment their production and/or replace their function.

VHL gene: A precedent-setting tumour suppressor. Elegant genetic linkage studies of affected families have shown that alterations in the structure and function of the VHL gene are responsible for familial kidney cancer[86]. The VHL gene encodes a tumour suppressor protein, much like the other genes linked with familial cancers — *RB* in retinoblastoma, *p53* in the Li-Fraumeni syndrome, *MTS1* (and perhaps *MTS2*) in familial melanoma, *APC* in FAP and presumably *BRCA-1* in early onset breast–ovarian cancer. In the familial setting, VHL gene abnormalities are present in the germ line of affected individuals, and the kidney cancer cells show VHL gene loss of heterozygosity. VHL mutations are also found in sporadic kidney cancers. Indeed, VHL mutations have been detected in roughly 67% of renal cell cancers[87]. However, 'sporadic' mutations occur in a different part of the gene from the familial mutations, suggesting that certain gene segments may be especially susceptible to mutagenic interactions with environmental carcinogens[87]. In addition, an increase in DNA methylation at the VHL gene locus can be detected in an additional 20% of the kidney cancers studied to date[88]. This hypermethylation, in turn, inhibits the transcription of the

VHL gene — a phenomenon that translates into a loss of VHL protein synthesis and a consequent loss of VHL-based tumour suppressor activity. This mechanism is the same as the one detected for *GSTP1* inactivation, discussed above[82]. The *in vitro* exposure of patient-derived hypermethylated kidney cancer cells to 5-aza-2′-deoxycytidine results in reversal of VHL hypermethylation, re-expression of the VHL gene and protein, and restoration of normal growth controls[88]. In cases where VHL gene hypermethylation is detected, clinical testing of agents capable that modulate methylation status in an attempt to reinstate VHL gene expression present an exciting direction of investigation for both therapy and prevention.

All told, roughly 85% of all kidney cancers — familial and sporadic — are accompanied by some abnormality of the VHL gene locus, thus providing definitive evidence that the VHL gene is indeed the 'kidney cancer gene'. Nonetheless, a new familial entity has been discovered that is clinically and genetically distinct from the familial VHL-related syndrome — namely, an unusual Hereditary Papillary Renal Cell Carcinoma (different from the more usual 'clear cell' kidney cancers, described above) involving multiple, bilateral tumours[89]. Further dissection of the unique molecular-clinical correlates of this familial syndrome and the potential extrapolation to non-familial cancers is in progress.

Angiogenesis: A target for therapy and prevention

Tumour dissemination, or metastasis, involves several distinct and complementary components, including the ability of tumour cells to penetrate and traverse basement membranes and establish self-sustaining tumour foci in diverse organ systems. To this end, the development and proliferation of new blood vessels, or angiogenesis, is central to several aspects of overall tumour survival: nourishment and growth of the primary tumour, the tumour cells' progressive ability to leave the primary site, and the establishment and expansion of such 'secondary' tumour deposits. In general, patients do not die from their primary tumours; they die from secondary, metastatic deposits.

Angiogenesis is driven by diverse fibroblast growth factors (FGFs) and other cytokines produced by tumour cells and/or surrounding supportive network of stromal cells and extracellular matrix[90–92]. The FGFs cause striking blood vessel proliferation and promote regeneration of several disparate epithelial and endothelial tissues, haematopoietic and other mesenchymal cell precursors. Indeed, recent studies involving the in vivo transfer of the gene encoding acidic FGF (or FGF-1) into arterial cells have demonstrated the direct, local induction intimal hyperplasia and, specifically, new capillary formation (that is, neovascularization) by FGF-1 (ref. 93). The proliferative effects of the FGFs may be balanced by the antiproliferative actions of other cytokines, for example, the α- and β-interferons (IFNs)[91]. Other heparin-binding angiogenic cytokines include interleukin-8 (IL-8), an inflammatory molecule produced by monocytes[94], and vascular endothelial growth factor (VEGF), a distinctive heparin-binding glycoprotein that binds exclusively to endothelial cells, shares some structural homology with platelet-derived growth factor (PDGF) and, like bFGF, directly promotes endothelial cell proliferation *in vitro* and *in vivo*[95,96]. VEGF is also known as vascular permeability factor (VPF), based on its ability to induce vascular 'leakiness' as well as endothelial cell proliferation[96]. This permeability may play a crucial role in the local fluid extravasation that accompanies certain tumours, notably glioblastoma and ovarian cancer (see below). Most recently, VEGF has been linked to the proliferative retinopathy responsible for blindness in diabetics and premature infants[97]. VEGF and/or its receptor could be a target for novel anticancer therapeutics.

In addition to stromal sources, certain tumour cells produce large quantities of active bFGF or other angiogenesis factors, a finding that can be correlated with the detection of clinically significant tumour-associated neovascularization. This situation is dramatically exemplified by AIDS-related KS, a complex malignancy of endothelial (or possibly mesenchymal) origin[98]. KS is characterized by brisk angiogenesis that is driven in both autocrine and paracrine fashion by the exuberant production of growth-promoting, inflammatory and angiogenic cytokines, in particular bFGF[99] and perhaps a KS-related FGF (K-FGF)[100]. Such pathogenically

Table 3 Selected suppressor genes in human malignancies

Gene	Location	Selected diseases
RB	13p14	Retinoblastoma, osteosarcoma, lymphoid leukaemias lung, breast, ovarian, prostate, bladder
p53	17p13	Li-Fraumeni syndrome, breast, prostate, lung, colon bladder, liver, lymphomas/leukaemias, brain, adrenal
WT1	11p13–15	Wilm's tumor
NF-1	17q11.2	Neurofibromatosis, peripheral neurofibromas,
	NF-2	22q Acoustic neuroma, central schwannoma, meningioma, breast, colon
VHL	3p26	Renal cell
APC	5q21	Colon (FAP)
MSH-2	2p16	Colon (HNPCC, proximal colon), endometrial, stomach
MLH-1	3p21.3–23	Pancreas, bladder, ovarian, breast
PMS 1	2q31–33	
PMS 2	7p22	
MTS1(p16)	9p21	Familial melanoma, glioblastoma, T-ALL, mesothelioma
MTS2(p15)		NSCLC, bladder
BRCA-1	17q21	Early-onset breast, ovarian, prostate, colon
BRCA-2	13q	Early-onset breast, (male breast cancer)

important FGF-induced perturbations are not relegated to AIDS-KS, however. For instance, melanoma cells express high levels of bFGF, which in turn acts in an autocrine fashion to drive melanoma cell proliferation and in a paracrine fashion to promote endothelial proliferation and blood vessel development[101]. Glioblastoma cells produce both bFGF and VEGF (refs 95,96), and certain leukaemias and lymphomas are pathogenically linked to bFGF and IL-8 through an autocrine loop or through paracrine mechanisms (with the angiogenesis factors produced by bone marrow stromal cells)[102].

A clinically relevant, pathophysiologic link between tumour activity and bFGF has been established for a wide array of malignancies, using urinary levels of bFGF as a surrogate marker of systemic bFGF production[103,104]. Strikingly high levels have been detected in bladder, ovarian and breast cancers, certain sarcomas, active lymphomas and acute lymphocytic leukaemia[104]. Measurement of urinary bFGF may provide a non-invasive means to discriminate locally active and disseminated tumour on a semiquantitative basis, monitor therapeutic efficacy and predict clinical outcome for at least some tumour types.

The correlation of active microvessel disease with metastasis and shortened survival has been documented for breast cancer, independent of nodal status, and other cancers as well (for example, prostate, lung, head and neck)[91,105,106]. Now, using an immunostaining technology with an antibody directed against platelet-endothelial cell adhesion molecule to optimize microvessel detection, investigators have defined angiogenesis as one of the most important independent determinants of disease-free and overall survival in node-negative breast cancer[105]. Angiogenesis may be a prognostic determinant of progression in pre-invasive breast cancers as well. Recent observations demonstrate the association of high stromal microvessel density with aggressive comedo-type ductal carcinoma *in situ* (DCIS)[107], and substantiate the hypothesis that angiogenesis is fundamental to the transition from DCIS to invasive breast cancer.

Angiogenesis is also a normal physiologic mechanism and a crucial part of the overall repair response to tissue injury, specifically wound healing. A classic example is ovulation which causes a 'tear' in ovarian tissue. This injury elicits the production of various angiogenic growth factors which, in turn, promote neovascularization. A similar phenomenon occurs in the uterus during endometrial growth. Not surprisingly, both normal and malignant ovarian tissues are rich in multiple angiogenesis factors. Both normal and malignant ovarian epithelial cells constitutively express VEGF, in particular the two lower molecular weight, extracellularly secreted isoforms (VEGF 121 and 165)[108]. The expression of VEGF by normal ovarian tissue implies that VEGF is present from the earliest stages of tumorigenesis and may have a formative role in the clinically significant vascular permeability and resultant ascites that characterizes ovarian cancer. The constant production of VEGF by both normal and transformed cells and the chronic exposure of the malignant clone to VEGF may also relate to the high percentage of women who present with disseminated disease: only 23% of women with ovarian cancer have Stage I disease at the time of initial diagnosis (vs. 53% for breast cancer), while 46% present with widely disseminated disease (vs. 7% for breast cancer)[109].

Thrombospondin and angiostatin: Angiogenesis inhibitors. Although independent on a clinical level, there is evidence to substantiate the notion that abnormal net *p53* expression is coupled in some way to angiogenesis, again making our old friend *p53* a convergence point for diverse mechanisms that promote tumour growth and survival. In particular, the p53 protein regulates the production of thrombospondin, a naturally occurring inhibitor of endothelial cell proliferation, by inducing thrombospondin gene transcription[110]. Thrombospondins are large, multifunctional, heparin-binding glycoproteins that compete with the FGFs for binding to cellular or extracellular matrix elements. Li-Fraumeni fibroblasts that lose their wild-type (normal) *p53* allele *in vitro* become highly tumorigenic when injected into nude mice; this tumorigenicity can be directly linked to a downregulation of thrombospondin synthesis and a consequent brisk induction of angiogenesis by the abnormal clone. The reinstatement of normal p53 activity by *p53* gene transfection restores thrombospondin production which, in turn, blocks the angiogenic and tumorigenic activity of Li-Fraumeni fibroblasts[110]. Measurements of local thrombospondin production by tumour cells, particularly in the context of p53 expression by local tumour cell cohorts, could provide important correlates with observed variations in microvessel density within a tumour mass, as well as between tumours from different individuals, and thus serve as a marker by which to measure disease progression and therapeutic efficacy.

The observation that removal of a primary tumour stimulates the growth of metastatic lesions has led to the discovery of a tumour-associated inhibitor of angiogenesis. This inhibitor, angiostatin, is a plasminogen fragment that inhibits endothelial cell proliferation *in vitro* and, when administered systemically, blocks neovascularization and growth of metastases *in vivo* in a mouse model of Lewis lung carcinoma[111]. Angiostatin, like thrombospondin, represents an endogenous angiogenesis inhibitor that could theoretically be exploited for the purposes of therapy and prevention of metastatic disease. This would constitute a kind of secondary prevention. Agents that augment the endogenous production of either angiostatin or thrombospondin or possibly the exogenous administration of these inhibitors (including synthetic analogues and active fragments) could, in theory, provide a provocative antiangiogenesis approach, with implications in oncology, ophthalmology and many other fields.

Antiangiogenesis: Targetting the endothelial cell. Inhibition of angiogenesis may be a central molecular mechanism on which disparate antitumour agents converge. The molecular components of angiogenesis present exciting therapeutic options. Antiangiogenesis approaches that are currently being tested in the clinical arena are directed toward angiogenesis-promoting growth factors and their receptors on tumour cells or endothelial cells, for example, IFN-α (refs 91,112), the heparin-binding agent suramin[113] and analogues of the angioinhibin fumagillin (a by product of the fungus *Aspergillus fumigatus*)[114], to name a few.

More recently, the distinctive endothelial cell surface molecules that may discriminate tumour-associated vasculature from normal blood vessels are becoming important targets for intervention. For example, endoglin[115]

and endosialin[116] are two recently identified endothelial cell surface antigens that appear to be preferentially expressed on proliferating vascular cells and might be exploited for the tumour-targetted delivery of cytotoxic drugs, monoclonal antibodies, radionuclides or toxins. Integrins present another class of cell surface target. Integrins are a family of cell surface adhesion molecules that are expressed by a variety of normal and malignant cells. One particular member of the integrin family, integrin $a_v b_3$, is expressed specifically on actively cycling endothelial cells[117]. Antagonists to this integrin, either a monoclonal antibody or an targetted cyclic peptide, effectively abrogate the angiogenesis induced by human tumour fragments (including lung, larynx, pancreas and melanoma) in *in vitro* and *in vivo* model systems[118]. The integrin antagonists promote programmed cell death (apoptosis) in actively proliferating endothelial cells by interrupting integrin-based transmembrane signalling pathways[118]. Integrin $a_v b_3$ could be an important tumour-specific molecular target for inhibition, particularly since the expression of this cell surface molecule is relegated to newly developing blood vessels. Integrin $a_v b_3$ antagonists might afford a high therapeutic index, sparing normal vascular cells that are already differentiated and thus not in active cell cycle[117,118].

An exciting new direction in monoclonal antibody research involves a two-pronged attack using immunotoxin constructs: one aimed at destroying the tumour vasculature and the other aimed at those tumour cells that survive independent of an intact vasculature, namely cells living on the 'edge' (the outer rim of the tumour mass) and unvascularized micrometastases[115,119]. This concept unifies multiple disciplines: tumor pathophysiology (including the influence of the microenvironment), immunology and radiobiology. The potential for broad clinical application depends on several factors. The first factor is the identification of endothelial cell antigens that are distinctively expressed on tumour-associated vessels, thereby discriminating tumour from normal vasculature for both diagnostic and therapeutic purposes. Additional challenges that must be addressed in order to design curative immunotoxin-based therapy include the development of representative animal models that can test all human components of tumour cell–vascular cell interactions and the development of systems to scan the vasculature with radionuclides in a way that could shed light on the potential therapeutic specificity and efficacy of the radioimmunoconjugate under study. The preclinical and clinical development of such approaches stands to have major impact on the diverse malignancies such as breast, prostate and brain cancers, melanomas, lymphomas and certain leukaemias where tumour-linked neoangiogenesis plays a pivotal role in tumour survival and dissemination.

The ability to modulate the expression of genes that influence vascular cell proliferation could have potential impact on vascular proliferative disorders of diverse etiologies, including atherosclerosis and restenosis following balloon angioplasty. Two such genes are c-*myb* and *RB*, both of which are expressed in vascular smooth muscle cells and both of which may serve as targets for gene-directed therapies. To this end, antisense oligonucleotides targetting *myb* mRNAs severely inhibited smooth muscle cell proliferation *in vitro* and inhibited smooth muscle cell accumulation in rat carotid arteries that had been subjected to balloon angioplasty[120]. More recently, in a gene therapy approach, a replication-defective adenovirus encoding a non-phosphorylatable and, therefore, constitutively active form of pRb blocked the proliferation of transfected cultured rat aortic smooth muscle cells in response to angiogenic growth factors and, following *in vivo* gene transfer at sites of balloon angioplasty, inhibited the occurrence of smooth muscle cell proliferation, intimal hyperplasia and restenosis in both carotid and femoral arteries[121].

Summary

This brief and highly selective overview glimpses a few of the conceptual and practical advances in molecular medicine that are changing our understanding of disease pathogenesis. By so doing, these discoveries are continuing to open new clinical avenues that, with continued refinement, promise to move us closer to the goal of eradication of death and suffering from cancer. And yet, with this knowledge, we also face new challenges — perhaps the most ironic is emerging from the stunning technologic advances that hold the realistic promise of being able to pinpoint individual cancer risk with astonishing precision and accuracy. Ultimately, the fulfillment of this promise will be the successful modification of that risk through rationally designed interventions. At the present time, however, our abilities to accurately identify and, more important, to modify such risk for the majority of malignancies are just beginning to unfold. In some cases we will identify risk without being able to offer satisfactory interventions. And so we are in the unenviable position of having molecular knowledge which may not confer a clinical benefit. However, as we steadily accrue more knowledge, we will have the opportunity to change the natural history of many cancers from destructive and fatal to treatable, preventable and, perhaps in some cases, completely curable. But we must continue to temper our optimism with the realization that cancer is presenting one of the most fundamental challenges we face in the world of molecular medicine.

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