Histopathology 2008, **53**, 1–19. DOI: 10.1111/j.1365-2559.2007.02937.x

REVIEW

General morphological and biological features of neoplasms: integration of molecular findings

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(2008) *Histopathology* **53**, 1–19

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This review highlights the importance of morphology–molecular correlations for a proper implementation of new markers. It covers both general aspects of tumorigenesis (which are normally omitted in papers analysing molecular pathways) and the general mechanisms for the acquired capabilities of neoplasms. The mechanisms are also supported by appropriate diagrams for each acquired capability that include overlooked features such as mobilization of cellular resources and changes in chromatin, transcription and epigenetics; fully accepted oncogenes and tumour suppressor genes are highlighted, while the pathways are also presented as activating or inactivating with appropriate colour coding. Finally, the concepts and mechanisms presented enable us to understand the basic requirements for the appropriate implementation of molecular tests in clinical practice. In summary, the basic findings are presented to serve as a bridge to clinical applications. The current definition of neoplasm is descriptive and difficult to apply routinely. Biologically, neoplasms develop through acquisition of capabilities that involve tumour cell aspects and modified microenvironment interactions, resulting in unrestricted growth due to a stepwise accumulation of cooperative genetic alterations that affect key molecular pathways. The correlation of these molecular aspects with morphological changes is essential for better understanding of essential concepts as early neoplasms/precancerous lesions, progression/dedifferentiation, and intratumour heterogeneity. The acquired capabilities include self-maintained replication (cell cycle dysregulation), extended cell survival (cell cycle arrest, apoptosis dysregulation, and replicative lifespan), genetic instability (chromosomal and microsatellite), changes of chromatin, transcription and epigenetics, mobilization of cellular resources, and modified microenvironment interactions (tumour cells, stromal cells, extracellular, endothelium). The acquired capabilities defining neoplasms are the hallmarks of cancer, but they also comprise useful tools to improve diagnosis and prognosis, as well as potential therapeutic targets. The application of these concepts in oncological pathology leads to consideration of the molecular test requirements (Molecular Test Score System) for reliable implementation; these requirements should cover biological effects, molecular pathway, biological validation, and technical validation. Sensible application of molecular markers in tumour pathology always needs solid morphological support.

Keywords: cell kinetics, differentiation, genetic instability, molecular pathology, neoplasia, pathogenesis, progression, tumour heterogeneity, tumour microenvironment

Abbreviations: AIF, apoptosis-inducing factor; ALT, alternative lengthening of telomeres; APE, apurinic/apyrimidinic endonuclease; BRCA, Breast cancer; CAD, caspase-activated DNase; CDK, cyclin-dependent kinase; DFF, DNA fragmentation factor; EndoG, endonuclease G; HDAC, histone deacetylase; HIF, hypoxia inducible factor;

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The protocol used in the study was approved by the Hospital Research Board and Ethical Committees and complied with their requirements.

LOH, loss of heterozygosity; MAP, mitogen-activated protein; MBD, methyl CpG-binding domain; Mcm, mini-chromosome maintenance complex; MMP, matrix metalloproteinase; MSI, microsatellite instability; MTA, metastasis-associated protein; mTOR, mammalian target of rapamycin, NAD, nicotinamide adenine dinucleotide; NF, nuclear factor; ORC, origin recognition complex; PARP, polyADP-ribose polymerase; PAX, paired box; PDK1, 3-phosphoinositide-dependent protein kinase-1; PIP3, phosphatidylinositol 3,4,5-triphosphate; PPAR, peroxisome proliferator-activated receptor; PTC, patched; PTEN, phosphatase and tensin homologue; RAS, rat sarcoma; Rb, retinoblastoma; RET, rearranged during transfection; ROS, reactive oxygen species; TGF, transforming growth factor; TMM, telomere maintenance mechanism; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor

Introduction

Histopathology remains the gold standard of tumour diagnosis, but it needs strong links to all new developments such as molecular markers. Proper morphological evaluation has been demonstrated to be invaluable for both establishing concepts and helping with technical issues, especially in dealing with the heterogeneous components in neoplasms; the application of these principles would allow more reliable comparison of results and implementation of meaningful concepts. Any sustained progress in this area should refer to the morphological changes for verification and validation. Hanahan and Weinberg's article on tumorigenesis is one with the greatest degree of impact,[1] but does not pay much attention to molecular–morphological correlation in neoplasms. Nevertheless, key oncological features need careful morphological correlation when new markers are introduced in the diagnosis and management of patients. In tumour pathology new markers have been incorporated at the tissue level to help defining subtypes, but there is an increased demand for molecular tests on tissue sections (*in situ* techniques) and on solid support (techniques after extraction). Proper application of these techniques requires sensible selection of markers that should be based on concepts of tumour biology (both general and tissue specific).

General aspects of tumorigenesis

A general definition of neoplasm, such as 'cellular disease characterized by abnormal growth regulatory mechanisms', is descriptive and difficult to apply routinely, working definitions being required. The introduction of new markers has improved diagnostic precision, but can potentially result in big changes in prevalence and uncertainties for particular lesions. The current World Health Organization classifications of tumours incorporate new developments based on pathology and genetics, the leading criteria still being morphological; in this context, molecular findings complement the histological evaluation without replacing it. Additionally, any new definition should be validated against the accepted standard (specificity/sensitivity), should improve patient management, and should provide a biological meaning for its application. The first requirement is normally met on the initial design, and the second would be expected in any successful implementation. The third criterion is more difficult to apply, but any new definition should be biologically meaningful and would incorporate core elements in tumour biology (in particular, genetic and kinetic correlates).[2–6] These elements need to be included in a score system. Examples include the paired box (*PAX*) 8/peroxisome proliferator-activated receptor (*PPAR*) γ fusion gene described in follicular thyroid carcinomas and adenomas, and rearranged during transfection (*RET*)/patched (*PTC*) fusion genes reported in papillary thyroid carcinomas and Hashimoto's thyroiditis.

Biologically, neoplasms develop through acquisition of capabilities that involve tumour cell aspects and microenvironment interactions, as explained in more detail below (General molecular mechanisms).[1] The unrestricted growth observed in neoplasms is generally due to a stepwise accumulation of cooperative genetic alterations in oncogenes and tumour-suppressor genes, the number being more important than the order of changes;[7] the evidence available suggest that five to seven genetic alterations are required for clinically detectable tumours, correlating with morphological progression in some locations. These capabilities are not equally relevant at different stages during tumorigenesis, as highlighted by careful morphological evaluations. The markers should be selected considering the capability to test and the marker role during tumour initiation and promotion. Tumour promotion markers would be more relevantly assessed during progression, which needs to be defined on clear clinical and morphological grounds. These aspects are relevant for three essential concepts: early neoplasms/precancerous lesions, progression/dedifferentiation, and

intratumour heterogeneity (considering tumour cell segregation and heterotypic biology/landscaper effect).

EARLY NEOPLASMS AND PRECANCEROUS LESIONS

These two concepts are closely related to tumour initiation, have been developed for epithelial neoplasms and corroborate the concept of multistep tumorigenesis and accumulation of cooperative genetic abnormalities ('gatekeeper' and 'caretaker' pathways).[7] The paradigm is the concept of intraepithelial neoplasm/malignancy, it being more difficult to extrapolate the concept to non-epithelial lesions. These lesions would be meaningful when they are present in structures with anatomical boundaries and the cells do not recirculate/migrate in physiological conditions, regardless of lesion size. Regarding anatomical considerations, the limiting structure is the basement membrane, not the tumour capsule.

Although some genetic alterations are described as neoplasm-specific, the presence of a single genetic alteration cannot be considered diagnostic of malignancy, even for early stages. These problems preclude establishing reliable diagnoses of follicular carcinomas *in situ* for encapsulated neoplasms carrying *PAX8/PPARγ* fusion genes, even for lesions that initially carry molecular changes reported in malignancy. The opposite situation is equally important: histologically confirmed intraepithelial lesions are considered precursors, but they can accumulate genetic alterations and show kinetic features of malignancies, as reported for C-cell hyperplasias in multiple endocrine neoplasia 2A.[8,9] Non-random genetic alterations can also be used to test clonal expansions and the clonal evolution of neoplasms, especially analysing hypervariable DNA regions from patients heterozygous for a given marker. These tests rely basically on the demonstration of loss of heterozygosity (LOH) resulting from either hemizygosity (non-random interstitial DNA deletions) or homozygosity of mutant alleles observed in neoplasms. LOH analyses identify clonal expansions of a tumour cell population and point to monoclonal proliferation when multiple and consistent LOH is demonstrated (high fractional allelic loss).[4] Applied appropriately, these tools can establish the clonal evolution of tumour cell populations (tumour heterogeneity) and differentiate field transformation from metastatic tumour growths in synchronic and histologically identical neoplasms.

NEOPLASTIC PROGRESSION AND DEDIFFERENTIATION

Neoplastic transformation evolves over a period of time and involves the phenotypic progression of tumour cells along with the interaction of the initiated cell with its microenvironment. The elucidation of the steps of cancer progression (Figure 1) relates to the acquisition of invasive capability in intraepithelial lesions and metastatic potential in invasive malignancies and is of utmost importance in the differential diagnosis of neoplasms and in the establishment of more efficient therapeutic regimens. This functional characterization of the particular stage of tumour will certainly allow for better diagnosis, staging, prognostication and treatment of cancers. Tumour cell metabolic activation also determines the degree of differentiation and needs to be carefully coupled with kinetic features; any imbalance in the ribosomal activation/kinetics will result in apoptosis activation (see General molecular mechanisms). Changes in gene expression are common findings in neoplasms, due to general and capability-specific factors. On one hand, general mobilization of resources will result in activation of the cellular machinery needed for transcription (with corresponding chromatin changes) and translation, which is regulated by mammalian target of rapamycin) and eIF4E pathways, as well as for ribosomal activation. On the other hand, capability-specific markers activate transcription factors that modified the gene expression. These two collaborative aspects explain gene expression modifications that can be powerful markers of neoplastic transformation and have been proposed as diagnostic criteria for a molecular classification of neoplasms.[10–15]

Histopathological examination of solid tumours frequently reveals pronounced tumour cell heterogeneity, often demonstrating substantial diversity within a given tumour. The molecular mechanisms underlying the phenotypic heterogeneity are very complex, with genetic, epigenetic and environmental components, such as shortage of oxygen. Hypoxia greatly influences cellular phenotypes by altering the expression of specific genes, makes the tumours more aggressive, and is an important contributor to intra- and intertumour cell diversity, as revealed by the pronounced but non-uniform expression of hypoxia-driven genes in solid tumours.[16] Hypoxic tumour cells lose their differentiated gene expression patterns and develop stem cell-like, immature or dedifferentiated phenotypes.[17] Not only will hypoxia-induced dedifferentiation contribute to tumour heterogeneity, but it could also be one mechanism behind increased aggressiveness of hypoxic tumours. Intratumoral hypoxia is an independent indicator of poor patient outcome, and increasing evidence supports a role for hypoxia in the development of metastatic disease.[16]

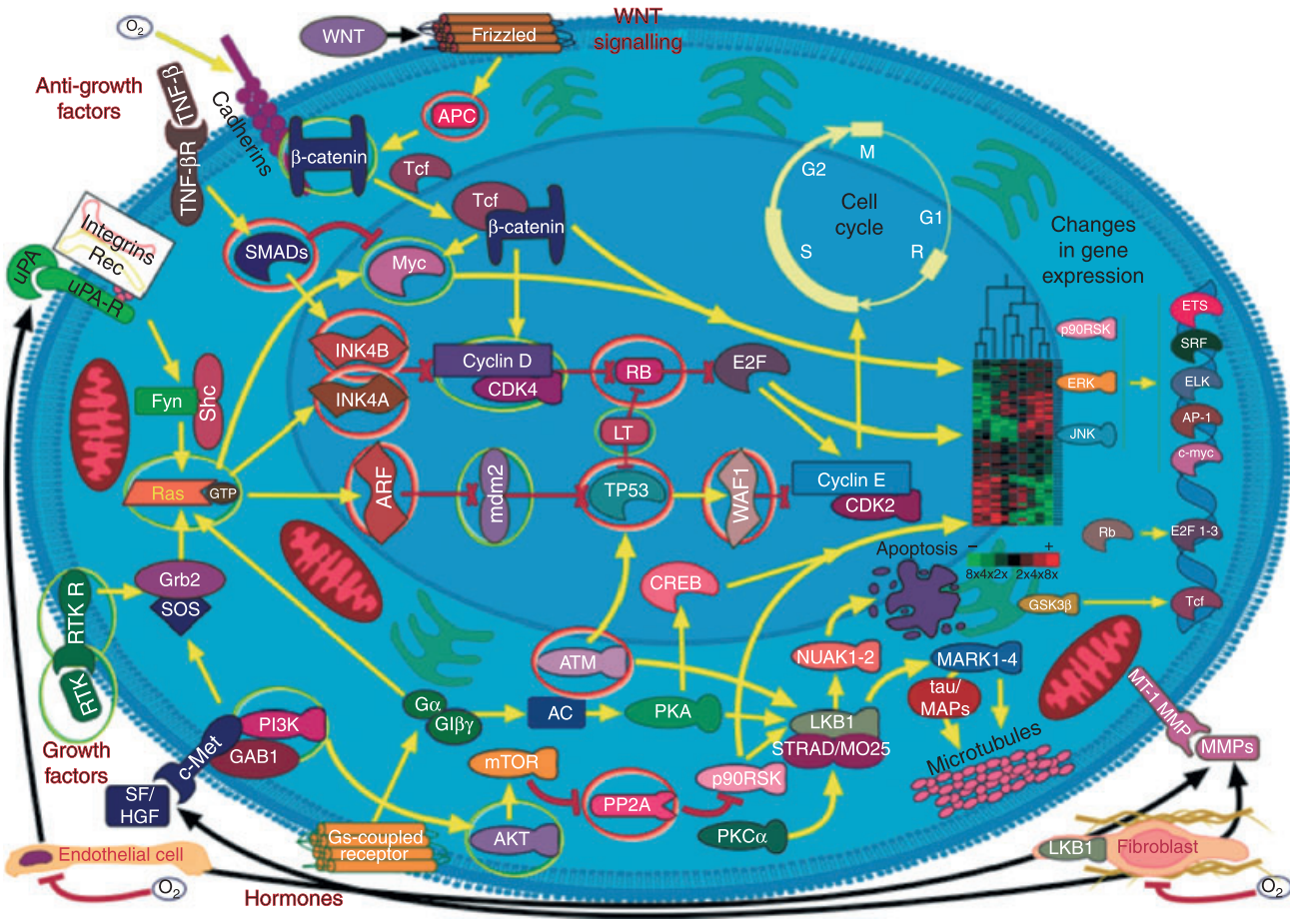

Figure 1. Molecular pathways involved in tumour cell progression.

Studies suggest that the acquisition of the metastatic phenotype is not simply the result of dysregulated signal transduction pathways, but instead is achieved through a stepwise selection process driven by hypoxia.[18,19] Hypoxia facilitates disruption of tissue integrity through repression of E-cadherin expression, with concomitant gain of N-cadherin expression, which allows cells to escape anoikis. Through up-regulation of urokinase-type plasminogen activator receptor expression, hypoxia enhances proteolytic activity at the invasive front and alters the interactions between integrins and components of the extracellular matrix, thereby enabling cellular invasion through the basement membrane and the underlying stroma. Cell motility is increased through hypoxia-induced hepatocyte growth factor–MET receptor signalling, resulting in cell migration towards the blood or lymphatic microcirculation. Hypoxia-induced vascular endothelial growth factor (VEGF) activity also plays a critical role in the dynamic tumour–stromal interactions required for the subsequent stages of metastasis.

Metastasis, the final step in malignancy, is the result of selected aspects of the complex tumour-progression process. Tumour cell dissemination is the prerequisite of metastasis and is correlated with loss of epithelial differentiation and the acquisition of a migratory phenotype, a hallmark of malignant tumour progression.[17,20,21] A stepwise, irreversible accumulation of genetic alterations is considered to be the responsible driving force, but, strikingly, metastases of most carcinomas recapitulate the organization of their primary tumours. The loss of epithelial characteristics (i.e. lack of intercellular adhesion molecules) results in breakdown of epithelial-cell homeostasis, correlates with the acquisition of a migratory phenotype and leads to progression.[22] This epithelial to mesenchymal transition is considered to be a crucial event in malignancy.[21] The important steps that enable metastasis are reversible,

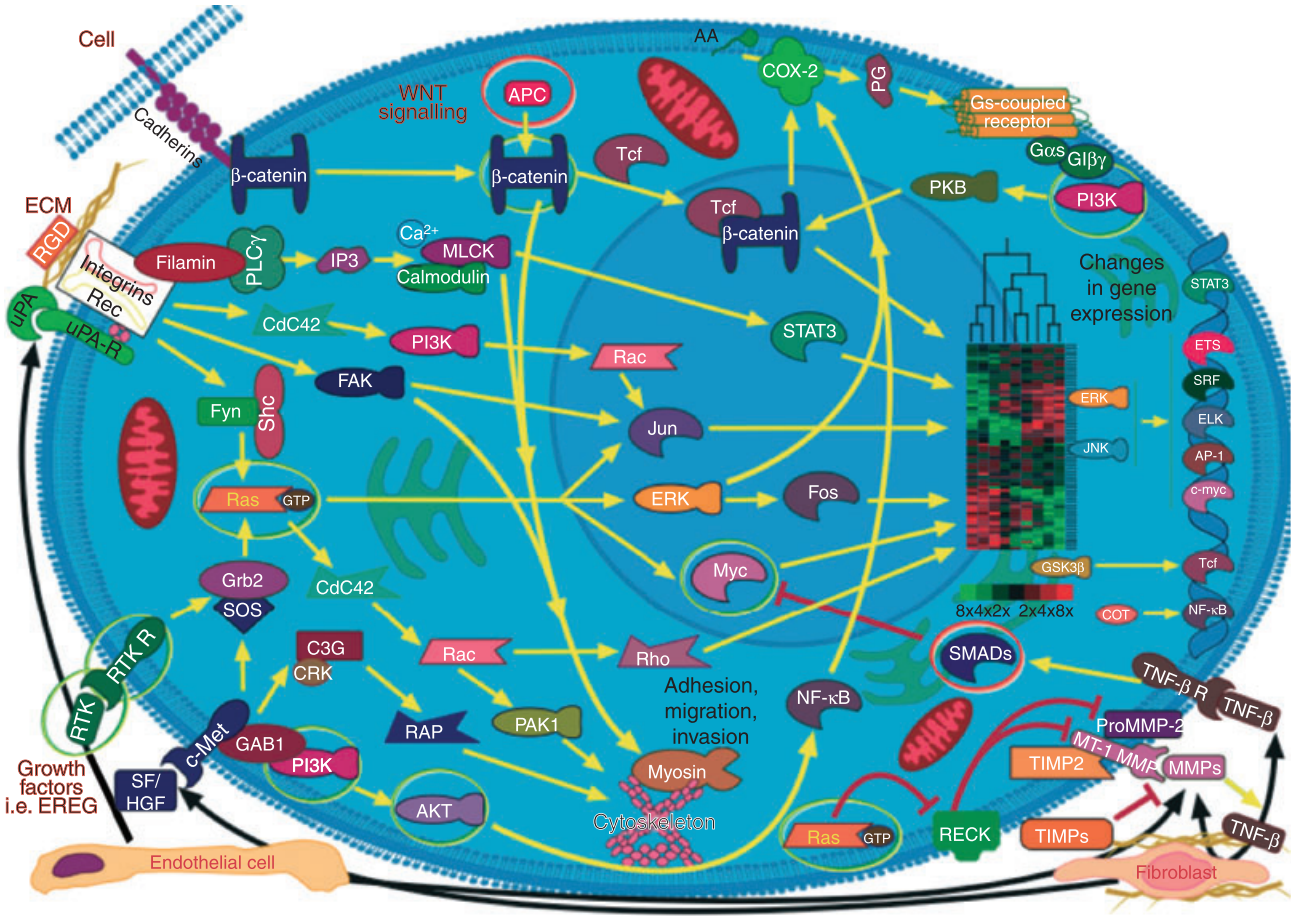

Figure 2. Molecular pathways involved in microenvironment interactions (intercellular, and stromal, neoangiogenesis, and invasion).

and therefore cannot be explained solely by irreversible genetic alterations, indicating the existence of a dynamic component to human tumour progression, in particular a regulatory role for the tumour microenvironment (Figure 2).[23] Tumours are morphological and functionally heterogeneous and segregate into tumour cells with different capabilities: individual tumours show distinct sub-areas of proliferation and cell-cycle arrest, differentiation, cell adhesion and dissemination, some of them determined by topography (see below). VEGF promotes angiogenesis and lymphangiogenesis in the primary tumour, providing the necessary routes for dissemination. VEGF-induced changes in vascular integrity and permeability promote both intravasation and extravasation, whereas VEGF-induced angiogenesis in the secondary tissue is essential for cell proliferation and establishment of metastatic lesions. Through regulation of these critical molecular targets, hypoxia promotes each step of the metastatic cascade and selects tumour cell populations that are able to escape the unfavourable microenvironment of the primary tumour.

INTRATUMOUR HETEROGENEITY AND TUMOUR CELL SEGREGATION

Intratumour heterogeneity comprises both tumour cells and heterotypic components (immune/inflammatory cells, myofibroblasts, endothelial cells and extracellular matrix). Intratumour heterogeneity is assumed to occur randomly, but some factors such as topography control the segregation of tumour cells within neoplasms.[24–29] The topographic intratumour heterogeneity suggests differential selection of tumour cells, but can also be expression of either selective clonal evolution or a simple passive by-product of genetic instability. The differential kinetic profile by topographic compartments has been related to lower cell turnover and apoptosis down-regulation in deep/peripheral compartments, resulting in accumulation

of genetic alterations and segregation of tumour cells with differential genetic backgrounds as demonstrated in adrenal gland, colon and bladder. This process has been linked with mismatch repair protein down-regulation and it is unlikely to be related with hypoxia, which is more pronounced in central compartments. However, the coexistence of genetic alterations supports a key role in tumorigenesis, the topographic heterogeneity resulting from the accumulation of genetic damage. This concept is central and supports multiple sampling to assess reliably the genetic abnormalities of neoplasms.

The heterotypic biology of neoplasms is an essential element in understanding tumour growth. The underlying defect (clonal genetic alteration) may reside in stromal and not tumour cells, as reported in juvenile polyposis syndrome and ulcerative colitis hamartomatous polyps.[30] This finding suggests that, at least initially, the stromal cells are the neoplastic cells, whereas secreting factors drive the epithelial proliferation, and might thus eventually also be responsible for the induction of epithelial malignancy. This bystander role, mutations inducing stromal abnormalities that in turn induce epithelial neoplasia, has been called a landscaper effect: the microenvironment surrounding epithelial cells as a major determinant of the disturbed epithelial architecture, differentiation and proliferation (Figures 1 and 2).

General molecular mechanisms in tumorigenesis

The main tumorigenesis molecular pathways must be evaluated according to the acquired capabilities: self-maintained replication (cell-cycle dysregulation), extended cell survival (cell-cycle arrest, apoptosis dysregulation, and replicative lifespan), genetic instability (chromosomal and microsatellite), changes of chromatin, transcription and epigenetics, mobilization of cellular resources, and modified microenvironment interactions (tumour cells, stromal cells, extracellular, endothelium). All these aspects must finally be integrated in the mechanisms of tumour initiation (including clonality) and progression (see above). Knowledge of these pathways in each acquired capability is also essential to plan any sensible molecular evaluation of neoplasms: it will allow marker selection based on biological features and it will allow precise selection of surrogate/secondary markers to validate the results. In addition, some pathways are mutually exclusive [i.e. rat sarcoma (*RAS*) and *B-RAF* mutations or epidermal growth factor receptor (EGFR) and RAS analyses] and have to be evaluated simultaneously.

SELF-MAINTAINED PROLIFERATION (FIGURE 3)

The cell-cycle transition from G_1 to S phase is a key regulatory point in the cell cycle. The G_1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first 'gap' phase (G_1) into the DNA synthesis phase (S). Two main cyclin-dependent kinases (CDK) complexes, CDK4/6–cyclin D and CDK2–cyclin E, and the transcription complex that includes retinoblastoma (Rb) and E2F are pivotal in controlling this checkpoint.[31–33] During G_1 phase, the Rb–HDAC repressor complex binds to the E2F-DP1 transcription factors, inhibiting downstream transcription.[34] Phosphorylation of Rb by CDK4/6 and CDK2 dissociates the Rb–repressor complex, permitting transcription of S-phase genes encoding for proteins that amplify the G_1 to S phase switch and that are required for DNA replication. Many different stimuli exert checkpoint control, including transforming growth factor (TGF)-β, DNA damage, contact inhibition, replicative senescence, and growth factor withdrawal. The first four act by inducing members of the INK4 or Kip/Cip families of cell cycle kinase inhibitors.[31,32] TGFβ additionally inhibits the transcription of Cdc25A, a phosphatase that activates CDKs.[35,36] Growth factor withdrawal activates GSK3β, which phosphorylates cyclin D, leading to its rapid ubiquitylation and proteosomal degradation. Ubiquitylation, nuclear export and degradation are mechanisms commonly used to rapidly reduce the concentration of cell-cycle control proteins.

Other pathways acting through Ras, Rac and Rho also regulate the G_1 to S transition.[37,38] Ras regulates cyclin D1 expression to affect the G_1 to S transition. Transforming forms of Ras or Raf induce cyclin D1 expression and cause early entry into S phase. Signalling from Ras to Raf to MEK to ERKs induces cyclin D1 expression, allowing cyclin D1 to complex with Cdk4 and Cdk6 and phosphorylate Rb. Rac-1 and PAK appear to induce cyclin D1 expression and induce G_1 to S transition primarily through activation of nuclear factor (NF)-κB to activate the cyclin D1 promoter. Rho activates cdk2 and also inhibits p21 and p27 to induce cyclin D1 and stimulate G_1 to S transition. Rho represses p21 expression to block p21 induction by Ras and to allow Ras-induced progression from G_1 to S. Cells that lack p21 do not require Rho for Ras to induce cell cycle progression from G_1 to S phase. The cooperative action of Ras, Rac and Rho to induce cyclin D1 expression is a key component of oncogenic transformation.

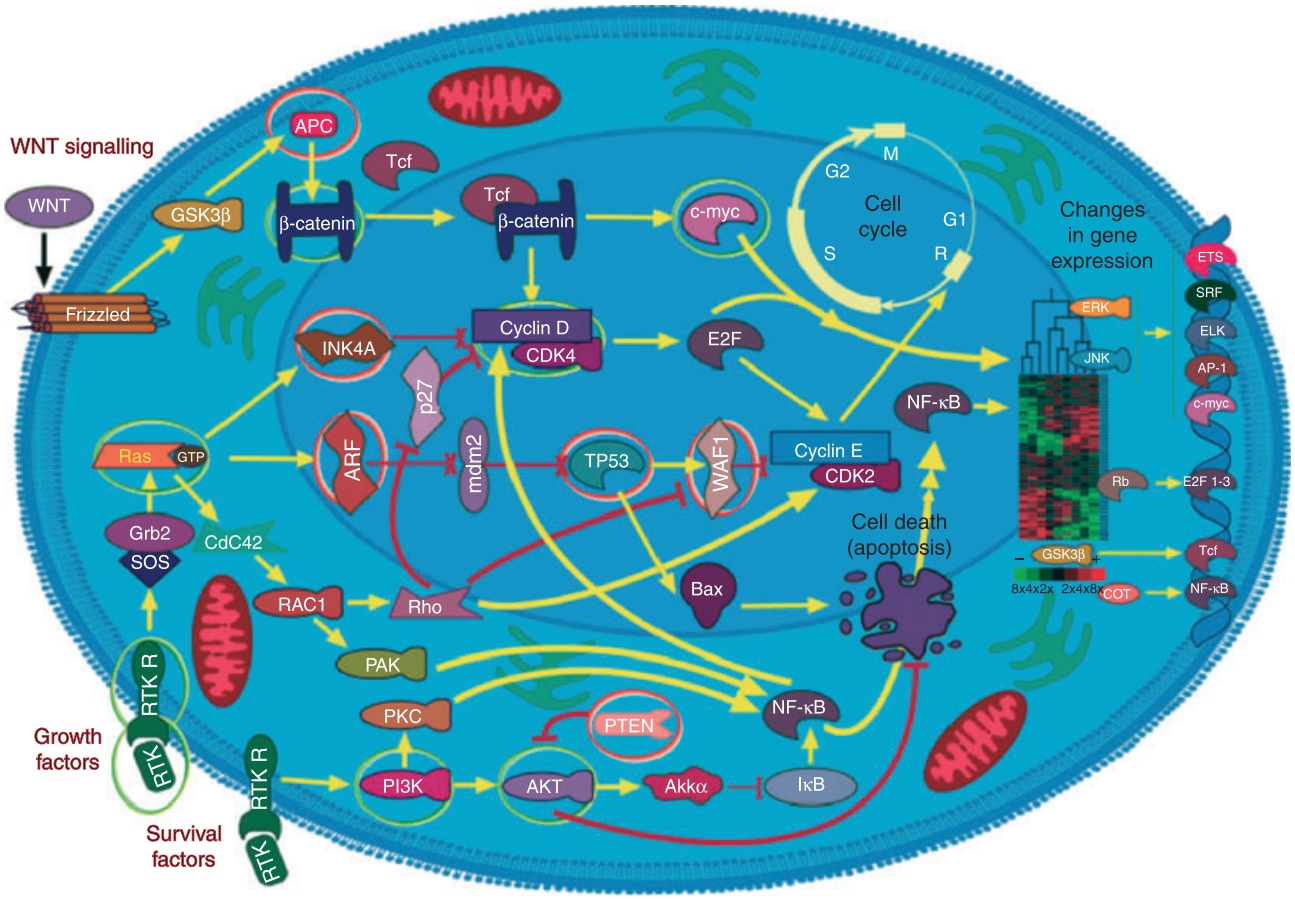

Figure 3. Molecular pathways involved in the tumour cell acquired capability of self-maintained proliferation.

TP53 is a transcription factor whose activity is regulated by phosphorylation.[39–41] The function of p53 is to keep the cell from progressing through the cell cycle if there is damage to DNA present. It may do this in multiple ways, from holding the cell at a checkpoint until repairs can be made to causing the cell to enter apoptosis if the damage cannot be repaired. The critical role of p53 is evidenced by the fact that it is mutated in a very large fraction of tumours from nearly all sources.

Phosphatase and tensin homologue (PTEN) is a tumour suppressor gene capable of dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP3), the product of phosphatidylinositol 3-kinase. Many of the cancer-related mutations have been mapped to the phosphatase catalytic domain, and it has been suggested that the phosphatase activity of PTEN is required for its tumour suppressor function.[42–44] The activation of PKB/AKT is regulated in a complex manner via phosphorylation of AKT by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and integrin-linked kinase (ILK), respectively.[45] Inactivation of PTEN will constitutively activate the PKB/AKT pathway. In addition to its role in regulating the PI3–K/AKT cell survival pathway, PTEN also inhibits growth factor-induced Shc phosphorylation and suppresses the mitogen-activated protein (MAP) kinase signalling pathway.

EXTENDED CELL SURVIVAL (FIGURE 4)

This capability is due to cell-cycle arrest (which is opposed to the capability above), apoptosis dysregulation, and replicative lifespan.

Apoptosis dysregulation

Apoptosis can be triggered by many different stimuli that result in activation of caspase signalling pathways from extracellular [i.e. tumour necrosis factor (TNF) or FAS pathway] and intracellular (mitochondria) signals. There are also signal pathways (i.e. AKT pathway) regulating these mechanisms.[45]

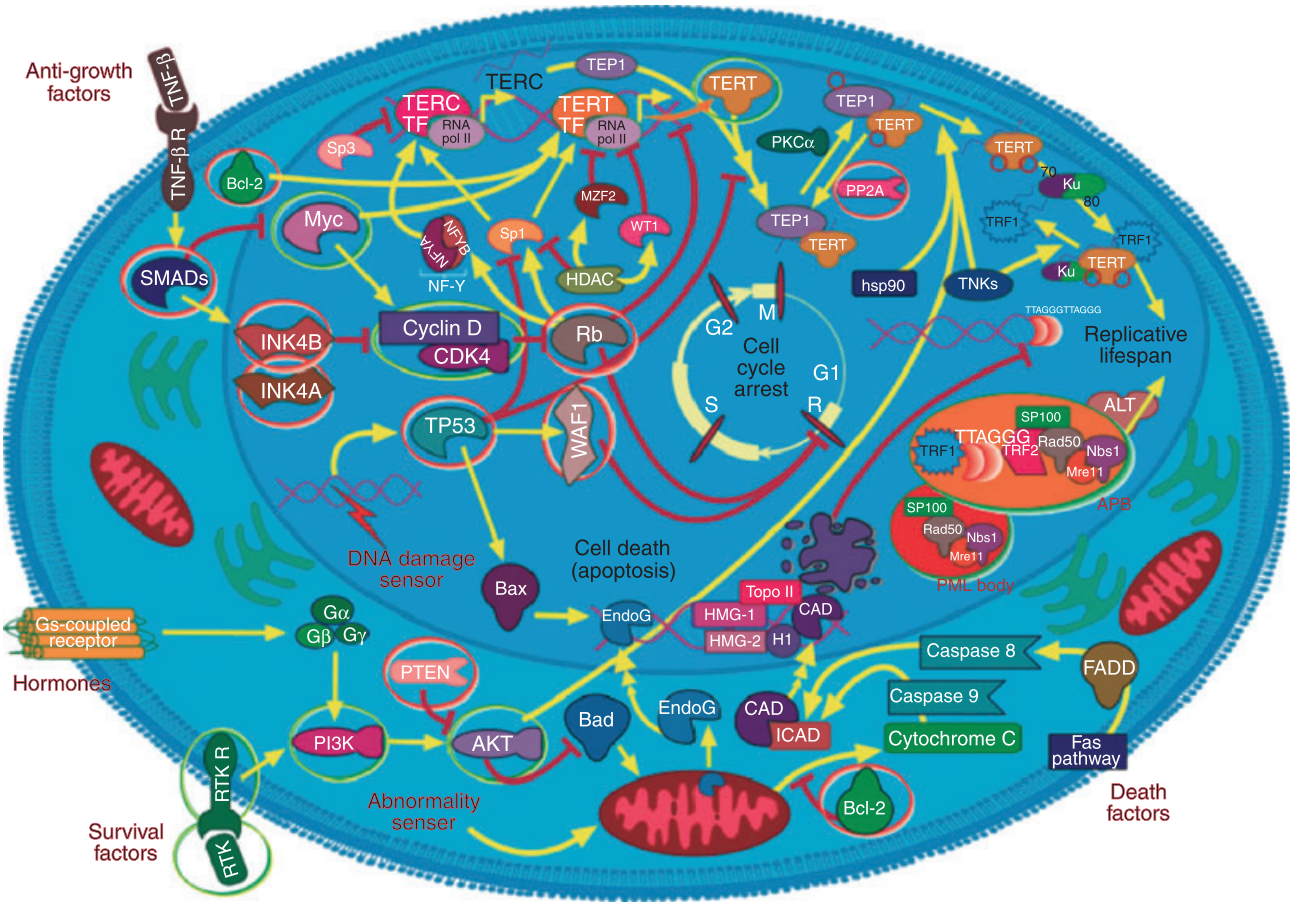

Figure 4. Molecular pathways involved in the tumour cell acquired capability of extended cell survival.

TNFR1 is the receptor for TNF-α and will also bind TNF-β.[46,47] Upon binding TNF-α, a TNFR1+ cell is triggered to undergo apoptosis by activating the proteolytic caspase cascade that results in the degradation of many critical cellular proteins, and by activating Bid, a Bcl-2 family member, which activates mitochondria-mediated apoptosis.[48] Mitochondria participate in apoptotic signalling pathways through the release of mitochondrial proteins [cytochrome c, Apaf-1, Smac/DIABLO, or apoptosis-inducing factor (AIF)] into the cytoplasm.[49–51] Cytochrome c activates the protease Apaf-1, which then activates caspase-9 and the rest of its pathway. Smac/DIABLO inhibits IAP proteins that normally interact with caspase-9 to inhibit apoptosis. AIF released into the cytoplasm induces a non-caspase-dependent apoptosis. Apoptosis regulation by Bcl-2 family proteins occurs as family member complexes enter the mitochondrial membrane, regulating the release of cytochrome c and other proteins. Activated Bax, a Bcl-2 family member, localizes to the mitochondrial membrane and increase its permeability, whereas Bcl-2 and Bcl-xL prevent pore formation, blocking apoptosis.

PIP3 conveys signals to the cytoplasm that activate the kinase PDK1, which in turn activates the kinase AKT, also known as protein kinase B.[45] Proteins phosphorylated by activated AKT promote cell survival: phosphorylation of Iκ-B kinase leads to activation of the transcription factor NF-κB to oppose apoptosis; phosphorylation of Bad blocks anti-apoptotic activity to promote cell survival; phosphorylation of caspase 9 or forkhead transcription factors block their apoptosis induction. AKT promotes cell survival and opposes apoptosis by a variety of routes.[52]

The characteristic cellular response of apoptosis is the internucleosomal fragmentation of the nuclear genome to create a DNA ladder pattern, due to activation of multiple nucleases.[53] One nuclease involved in apoptosis is DNA fragmentation factor (DFF), a caspase-activated DNase (CAD).[54–56] DFF/CAD is activated through cleavage of its associated inhibitor ICAD by caspase proteases during apoptosis.

DFF/CAD interacts with chromatin components such as topo II and histone H1 to condense chromatin structure and perhaps recruit CAD to chromatin. Another apoptosis-activated protease is endonuclease G (EndoG). EndoG is encoded in the nuclear genome, but is localized to mitochondria in normal cells.[57,58] EndoG may play a role in the replication of the mitochondrial genome, as well as in apoptosis. Apoptotic signalling causes the release of EndoG from mitochondria. Mitochondria are involved in apoptotic signalling in other ways as well, through the release of cytochrome c induced by Bid to activate the caspase protease cascade. These pathways are independent, since the EndoG pathway still occurs in cells lacking DFF.

Replicative lifespan

Telomeres, which define the ends of chromosomes, consist of short, tandemly repeated DNA sequences loosely conserved in eukaryotes. Human telomeres consist of many kilobases of (TTAGGG)*n* together with various associated proteins. Small amounts of these terminal sequences are lost from the chromosome tips during each S phase because of incomplete DNA replication, but *de novo* addition of TTAGGG repeats by the enzyme telomerase compensates for this loss. Many human cells progressively lose terminal bases with each cell division, a loss that correlates with the apparent absence of telomerase in these cells. There has been considerable interest in the possible relationship between human telomeres and cellular senescence, immortalization, and cancer.[59–61] The activation of a telomere maintenance mechanism (TMM) is indispensable for cellular immortalization, a hallmark of human cancer.

Telomerase is a ribonucleoprotein complex, which *in vitro* recognizes a single-stranded G-rich telomere primer and adds multiple telomeric repeats to its 3-prime end by using an RNA template. Telomerase may also have a role in *de novo* formation of telomeres. Telomerase has been identified in actively dividing cell types. The active reverse transcriptase component has been identified in the TERT protein. The presence of this factor determines the availability of the telomerase function.[62] The TERT protein has a high turnover rate and its expression is regulated by factors that promote growth (c-MYC, v-k-ras, Bcl-2 and E6) and inhibiting factors (RB and p53) that promote cell death or that block cell division. It appears that the regulation of active telomerase has many levels and can be inhibited by TEP1 not releasing TERT or by TRF1 which binds the end repeats and prevents access to the chromosome ends.[63] Additional modulation is due to phosphorylation by PKC and AKT or dephosphorylation by PP2A.

Some human cancers with complex karyotypes, such as specific subtypes of soft tissue sarcomas, astrocytic brain tumours and osteosarcomas, use an alternative lengthening of telomeres (ALT) mechanism as their TMM.[64] Some ALT cells have atypical features, suggesting the possibility that there is more than one ALT mechanism. ALT cells are characterized by specific minisatellite instability with stable microsatellites and by high rates of telomeric recombinational exchange. In ALT cells, asymmetrical chromosome segregation and unequal telomeric exchange contribute to telomere length maintenance. In at least some ALT cells, TMM requires the integrity of the MRN (MRE11-RAD50-NBS1) recombination complex and is efficiently repressed by its sequestration. Microsatellite instability (MSI) often results in disruption of MRN, so ALT may usually be incompatible with MSI. We suggest that ALT in human tumours is a dysregulated version of an aspect of normal mammalian telomere homeostasis, which may be a vestige of the TMM used by ancient eukaryotes.

GENETIC INSTABILITY — DNA DAMAGE AND REPAIR (FIGURE 5)

The accumulation of genetic alterations is the result of the balance between DNA damage and repair. The main damaging signals are dysfunctional telomeres, replication stress, and the reactive oxygen species (ROS, in particular H_2O_2), which finally target proteasomes, kinases, phosphatases, cytoskeleton, histones, transcription factors, telomerase and DNA. The DNA damage is variable and ranges from base/nucleotide abnormalities, mismatch, single- and double-strand breaks. These changes will be detected by sensor mechanisms that through kinases and adaptors activate the effector mechanisms, which restore genomic integrity.[65,66] The cellular response to the genetic damage will be apoptosis (when the damage cannot be repaired), cell-cycle arrest (to provide time for repair) and changes in gene expression. One of the most important consequences of this damage/repair disbalance is tumour genomic instability,[40] which has been normally classified in chromosomal and microsatellite instability.[67]

As regards the signalling pathway, one of the most important systems is the G_2/M DNA damage checkpoint that prevents the cell from entering mitosis (M phase) if the genome is damaged. The Cdc2-cyclin B kinase is pivotal in regulating this transition. During G_2 phase, Cdc2 is maintained in an inactive state by

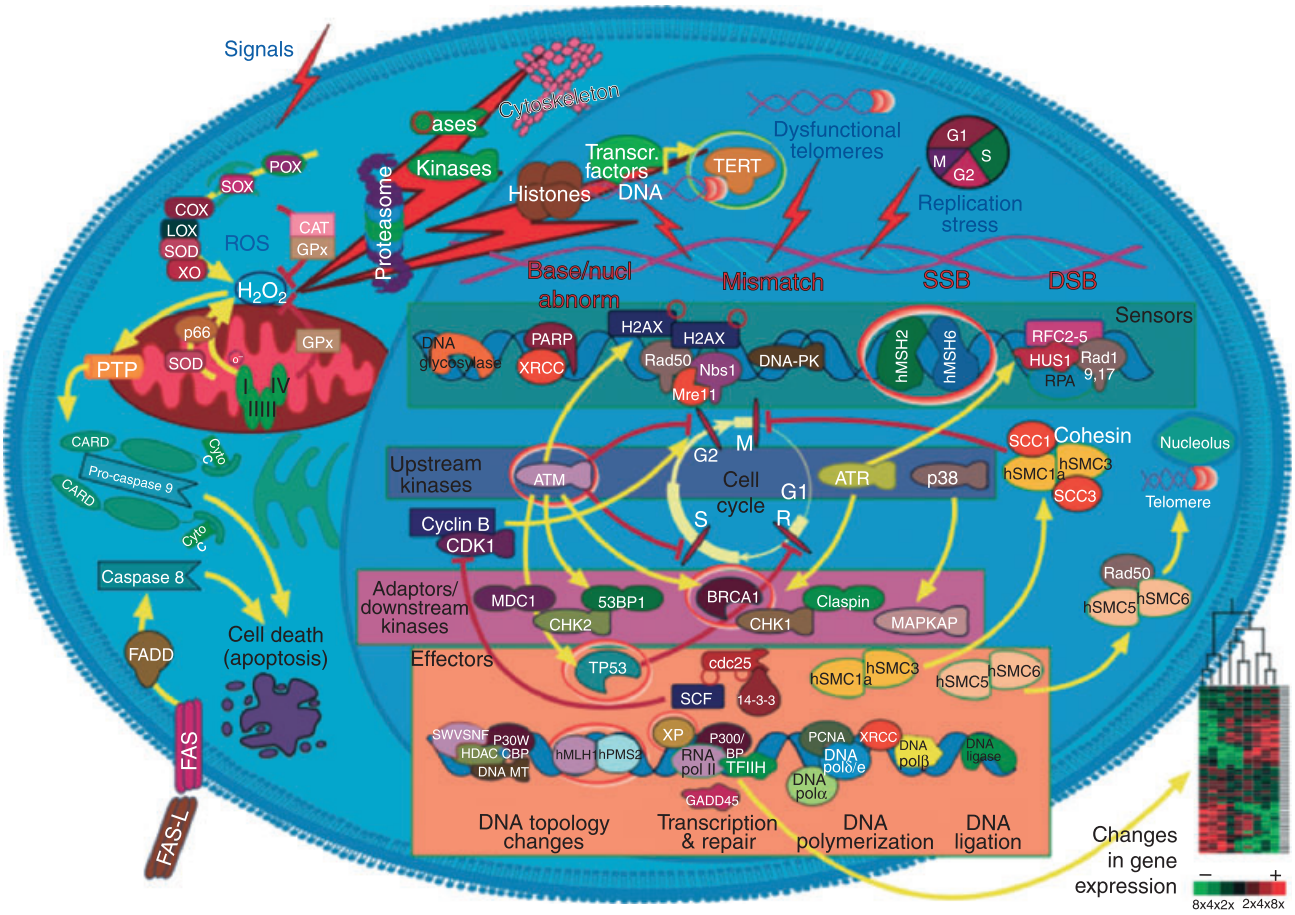

Figure 5. Molecular pathways involved in the tumour cell acquired capability of genetic instability – DNA damage and repair.

the kinases Wee1 and Mt1. As cells approach M phase, the phosphatase Cdc25 is activated, perhaps by the polo-kinase Pik1. Cdc25 then activates Cdc2, establishing a feedback amplification loop that efficiently drives the cell into mitosis. DNA damage activates the DNA-PK/ATM/ATR kinases, initiating two parallel cascades that inactivate Cdc2-cyclin B.[68,69] The first cascade rapidly inhibits progression into mitosis: the CHK kinases phosphorylate and inactivate Cdc25, which can no longer activate Cdc2. The second cascade is slower. Phosphorylation of p53 dissociates it from MDM2, activating its DNA binding activity. Acetylation by p300/PCAF further activates its transcriptional activity. The genes that are turned on by p53 constitute effectors of this second cascade. They include 14-3-3s, which binds to the phosphorylated Cdc2-cyclin B kinase and exports it from the nucleus; GADD45, which apparently binds to and dissociates the Cdc2-cyclin B kinase; and p21Cip1, an inhibitor of a subset of the cyclin-dependent kinases including Cdc2 (CDK1).

Breast cancer (BRCA) 1 and BRCA2 are involved in the cellular response to DNA damage, including blocking cell cycle progression and inducing DNA repair to preserve the integrity of the genome during cell division.[70] BRCA1 and BRCA2 induce double-stranded repair of breaks using homologous recombination, in part through activation of RAD51. BRCA1 acts as a ubiquitin ligase targeting the protein FancD2 that activates checkpoint control, integrating the ATM response to ionizing radiation and the Fanconi anaemia (FA) response to cross-linking agents such as mitomycin C. One member of the FA complex has recently been identified as BRCA2. Another related factor involved in the response of cells to DNA damage is the kinase ATM. Like ATM, ATR serves as a checkpoint kinase that halts cell-cycle progression and induces DNA repair when DNA is damaged. Loss of ATR results in a loss of checkpoint control in response to DNA damage, leading to cell death, and deletion of the *ATR* gene in mice is embryonic lethal. ATRIP is a protein that interacts with ATR and is a substrate for its kinase activity. ATRIP is

required for ATR function, and removal of ATRIP also leads to loss of checkpoint control of the cell cycle. ATR and ATM kinase targets include repair enzymes such as Rad51, and the checkpoint kinases Chk1 and Chk2, as well as BRCA1 and BRCA2.

MODIFICATIONS IN CHROMATIN, TRANSCRIPTION AND EPIGENETIC CHANGES (FIGURE 6)

Histone and non-histone proteins package the eukaryotic genome to form chromatin, which is compacted in nucleosomal arrays. Their assembly into higher-order chromatin structures creates a highly restrictive environment for nuclear processes that require access to DNA, resulting in the repression of transcription, replication and repair.[41] As counterbalance, a variety of ATP-dependent chromatin remodelling factors facilitate the interaction of proteins such as replication and transcription factors with nucleosomal DNA. ATP-dependent chromatin remodelling complexes are characterized by the presence of an ATPase subunit from SNF2-like family of the DEAD/H (SF2) DNA-stimulated ATPases. The highly conservative hSWI/SNF multisubunit complexes contain hBRM or BRG1 ATPases, which alter the histone–DNA contacts, enabling access of general transcription factors to promoter regions.[71] Remodelling complexes are targeted to promoters via interactions with sequence-specific transcription factors.

The chromatin packaging of the genome is dynamic, changing with transcriptional regulation and with the cell cycle, whereas the nuclear matrix network provides structure and regulates chromatin condensation. Chromatin is condensed for chromosome segregation during mitosis, whereas chromatin is more open for transcription. Regulated interactions of matrix proteins, DNA and other factors in different cell-cycle phases alter the structure and function of chromatin.

Transcription factors that can interact with the repressive chromatin structure and remodel the

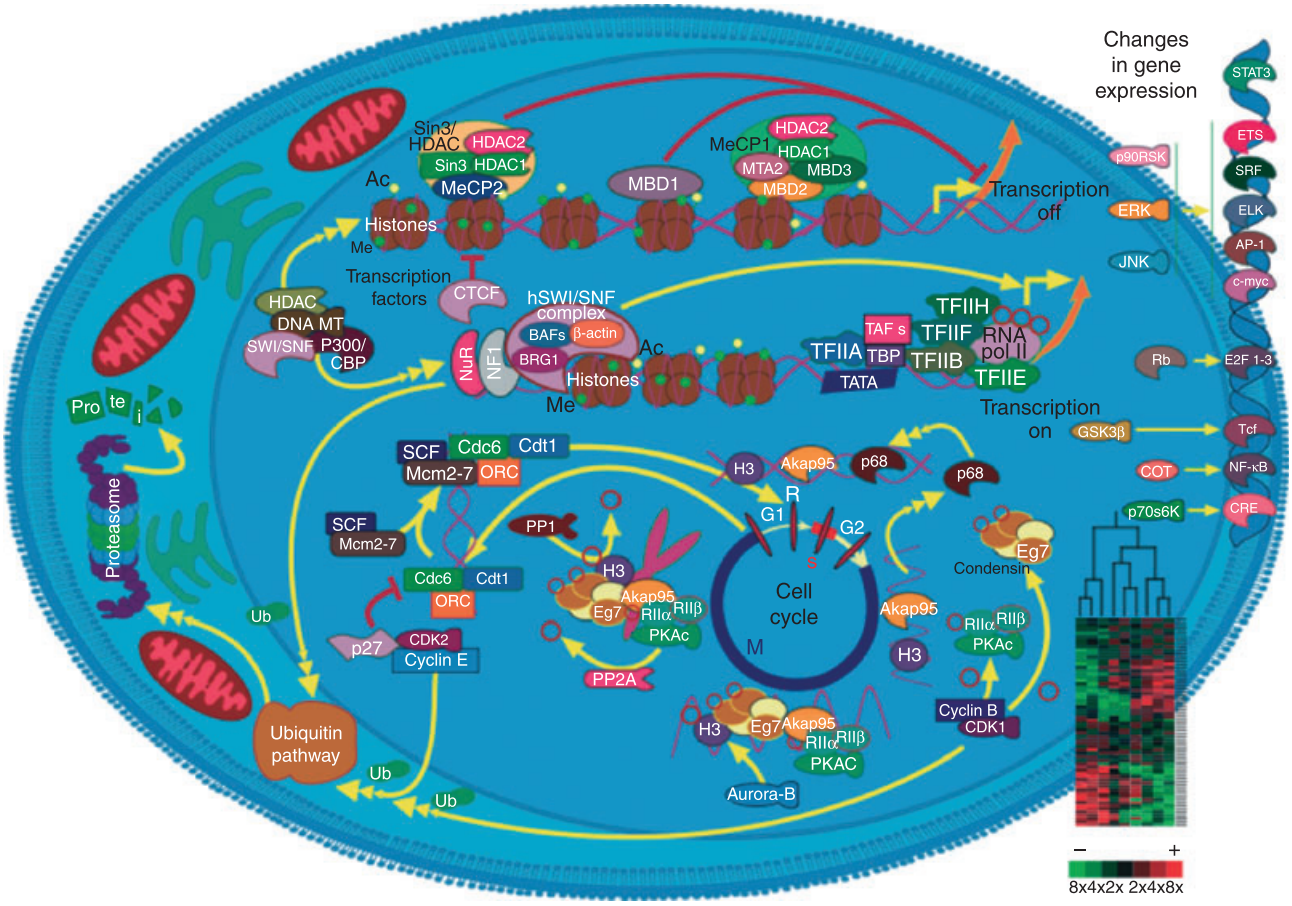

Figure 6. Molecular pathways involved in the tumour cell acquired capability of modifications of chromatin, transcription and epigenetic changes.

chromatin to allow other transcription factors to bind. In addition to nuclear receptors, transcription activators as c-Myc, HSF1, or EBNA2 have been found to recruit SWI/SNF to specific promoters. Nuclear receptors recruit an ATP-dependent remodelling hSWI/SNF complex (BRG1–BAF) to remodel the chromatin.[71] The remodelling process converts the closed conformation of the promoter to an open one without altering the nucleosomal positioning. The remodelling of the promoter permits NF1 binding and the assembly of a transcription initiation complex.

Initiation of DNA replication in eukaryotes is a highly conserved, multi-step process (replication licensing) designed to restrict initiation events to once per replication origin per S phase. Its control has been uncovered by the discovery of CDKs as master regulators of the cell cycle and the initiator proteins of DNA replication, such as the origin recognition complex (ORC), Cdc6/18, Cdt1 and the mini-chromosome maintenance complex (Mcm). The proteins and the sequence of events involved in this process are conserved throughout the eukaryotic kingdom. First, the ORC comprising six proteins binds to replication origins in the chromosomal DNA. At the end of mitosis, ORC, Cdc6/18 and Cdt1 assist the binding of Mcm proteins 2–7 to chromatin, and chromatin becomes licensed for replication. The activated Mcm complex functions as a replicating helicase and moves along with the replication fork to bring the origins to the unlicensed state. The cycling of CDK activity in the cell cycle regulates the two states of replication origins, the licensed state in G_1 phase and the unlicensed state for the rest of the cell cycle. The restriction on licensing is relieved when CDK falls off at the completion of mitosis to allow a new round of replication.

Nuclear matrix associated proteins such as AKAP95 bind to PKA (cAMP-dependent protein kinase) through a PKA RII regulatory subunit, an interaction that requires PKA phosphorylation by Cdk1. PKA activity and cAMP are reduced during entry into mitosis, but recruited PKA to condensed chromosomes is essential to maintain the condensed state.[72] Other proteins recruited during mitotic chromatin condensation such as Eg7 form part of a multiprotein condensin complex, recruiting another key component of mitotic chromatin condensation.[73] Modification of the core histones through phosphorylation regulates chromatin condensation. Histone H3 interacts with the condensin complex and is phosphorylated during mitosis. Histone H3 phosphorylation by Aurora-2 induces chromatin condensation, and dephosphorylation by PP1 promotes chromatin decondensation for re-entry into interphase. Nuclear matrix-associated proteins may play a role during the regulation of chromatin structure for transcription during interphase also. The interaction with the p68 RNA helicase recruits this enzyme to the nuclear matrix during interphase.[74] Other nuclear RNA helicases interact with transcription factors and cofactors, suggesting that the p68 RNA helicase also may regulate interactions of transcription complexes.

EPIGENETICS

Tumorigenesis is known to be a multistep process that accumulates defects in various cancer genes. Epigenetic modifications, most importantly DNA methylation events, are frequently involved in transcriptional changes in both tumour suppressor genes and oncogenes.[75] DNA methylation of gene promoter regions (generally at CpG dinucleotides) is generally correlated with gene silencing due to two underlying mechanisms.[41] First, binding of transcription factors or enhancer blocking elements, such as CTCF, may be inhibited by DNA methylation. The second and more general mechanism involves proteins that detect methylated DNA through methyl CpG-binding domains (MBDs). These proteins mediate recruitment of repressor complexes that include histone deacetylases (HDACs). HDACs remove acetyl groups from lysine residues of histones H3 and H4, which results in condensation of chromatin and thus limits access of transcription factors to promoter regions of genes localized nearby. Co-repressor complexes specifically bind methylated DNA, and copurify with the Sin3A/HDAC corepressor complex. The two main protein complexes share four polypeptides (HDAC1, HDAC2, RbAp46 and RbAp48) and contain unique polypeptides (Sin3A, SAP30 and SAP18 in the Sin3 complex, and Mi2, metastasis-associated protein (MTA) 1, MTA2 and MBD3 in the NuRD complex). The NuRD complex possesses nucleosome-remodelling activity because of the presence of Mi2, a member of the SWI2/SNF2 helicase/ATPase family.[76] This complex preferentially binds, remodels and deacetylates methylated nucleosomes. MTA1 or MTA2 expression levels are elevated in metastatic cancer cells; MTA2 modulates the enzymatic activity of the histone deacetylase core complex.[77,78]

Transcription repression is also related to nuclear receptors (such as RXR and RAR). Once retinoic acid binds the receptors, a receptor conformational change causes the dissociation of the corepressors and the binding of coactivators with histone acetylase activity. Following ligand binding by the heterodimer, the receptors and proteins in the basal transcription

machinery (such as TBP and TAF135) are degraded by the proteasome.

MOBILIZATION OF CELLULAR RESOURCES (FIGURES 7 AND 8)

Neoplastic transformation requires adequate machinery for protein synthesis and the provision of energy for the active process of signalling and control of the acquired cell capabilities. In this sense, protein synthesis–ribosomes (Figure 7) and mitochondria (Figure 8) are going to play a central role in neoplastic transformation.

Protein synthesis–ribosomes

mTOR and MAP kinase signalling pathways modulate the phosphorylation of translation factors, and the association of RNA-binding proteins with specific mRNAs.[79,80] These effects contribute both to overall control of protein synthesis and to modulation of the translation or stability of specific mRNAs. eIF4E plays an important role in human cancers, being expressed at high levels in many cancers, which correlate positively with tumour aggressiveness. eIF4E represses apoptosis and promotes the translation of the mRNAs for proteins with roles in cell-cycle progression (e.g. cyclin D1), cell transformation (e.g. ornithine decarboxylase), tumour vascularization (e.g. VEGF) or metastasis [e.g. matrix metalloproteinase (MMP)-9]. eIF4E increases the cytoplasmic levels of the cyclin D1mRNA, apparently involving a feature in the 3′-UTR of the cyclin D1 mRNA, rather than the cap-binding function of eIF4E, which is thought to allow eIF4E to promote transport of the cyclin D1 mRNA from the nucleus to the cytoplasm. Such a function would require eIF4E to spend some time in the nucleus, and indeed a proportion of the total cellular eIF4E is found in the nuclear fraction.

The existence of the S6K-based negative-feedback loop means that mTOR blocking will probably lead to enhanced PI(3)K–AKT activation in some tumours. Depending on other mutations found in the tumour,

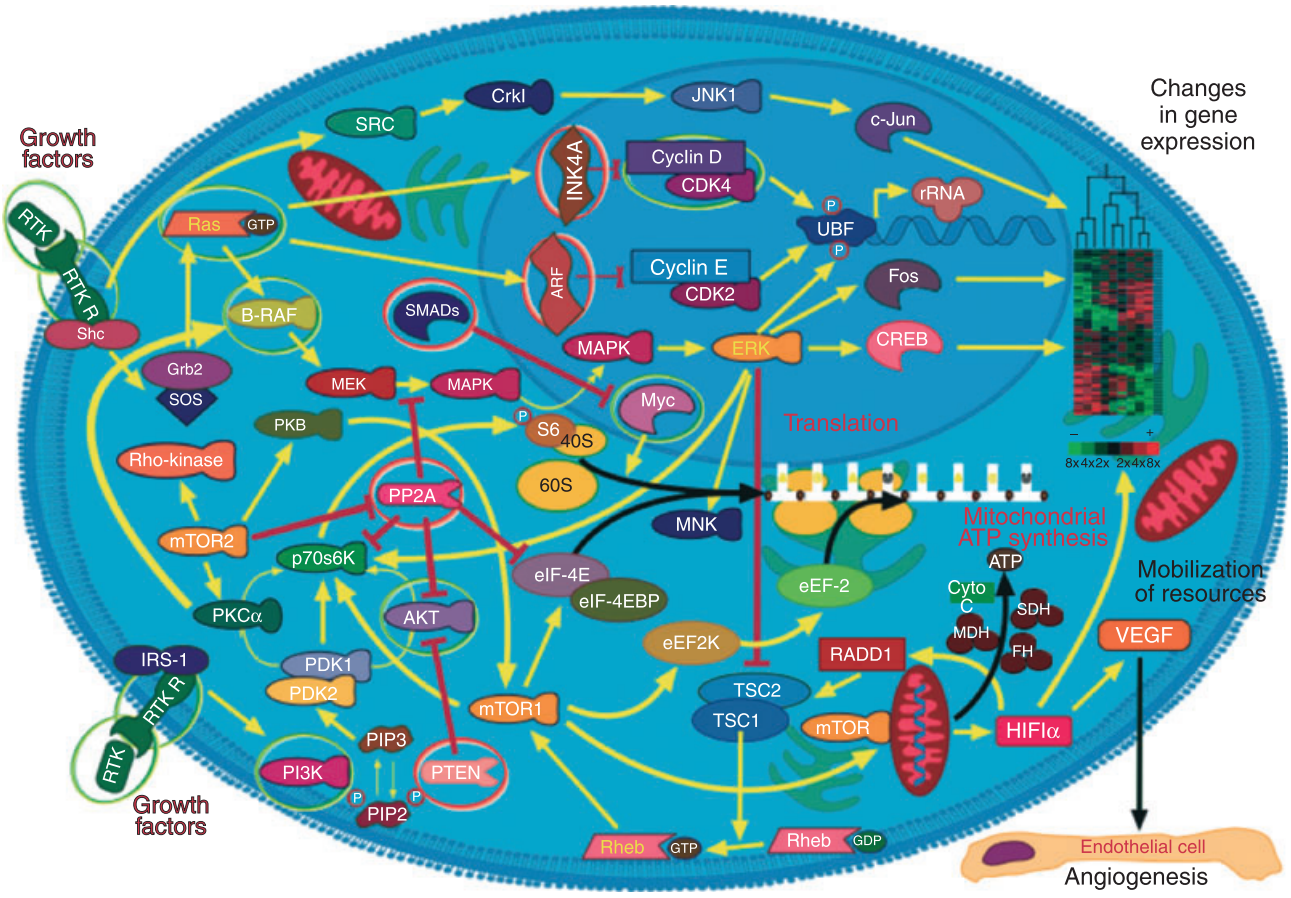

Figure 7. Molecular pathways involved in the tumour cell acquired capability of mobilization of cellular resources – ribosomes (protein synthesis).

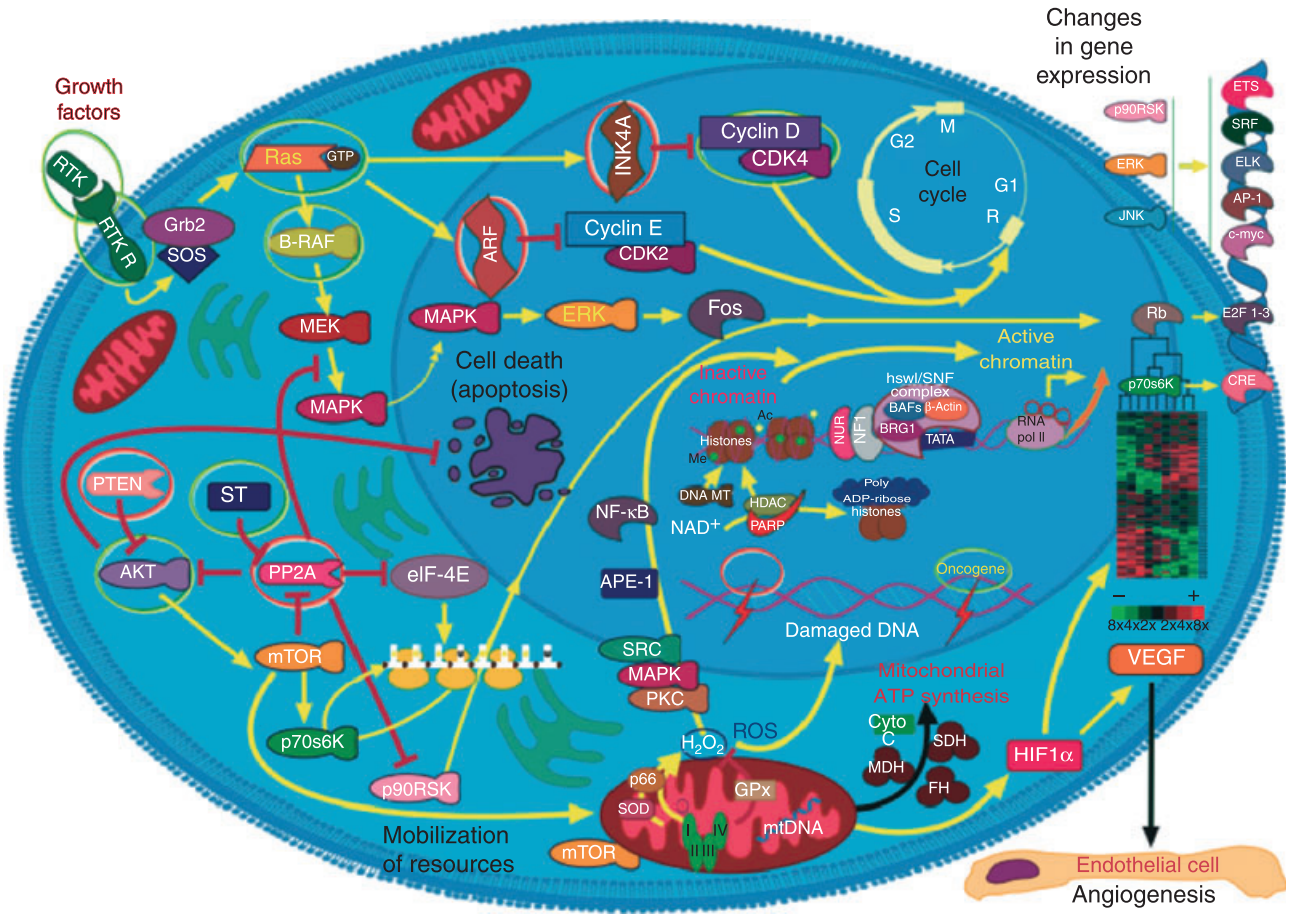

Figure 8. Molecular pathways involved in the tumour cell acquired capability of mobilization of cellular resources – mitochondria (ATP synthesis, apoptosis, reactive oxygen species and damage).

this hyperactivation of PI(3)K–AKT signalling could make the tumour more aggressive. The critical function of the mTORC1 complex required for cell growth is activation of eIF4E. Because eIF4E transcription is controlled by multiple signalling inputs, resistance to rapamycin might develop by a variety of paths that will depend on the genetic background of the tumour.

A critical target for tumour cell growth and survival is the activation of eIF4E and hypoxia inducible factor (HIF). Tumours with initiating mutations in RTKs, Ras or Raf have multiple routes to signal to eIF4E and HIF. In contrast, tumours with initiating lesions in PI(3)K or more direct regulators of mTOR (such as LKB1 and TSC) do not have alternative routes to activate eIF4E and HIF. Hence, these tumours show greater response to rapamycin. Similarly, the expression and use of specific adaptor proteins that enhance certain arms of pathway signalling will dictate the therapeutic response.

More broadly, identifying the primary mechanisms by which the PI(3)K pathway is activated in a given tumour should facilitate the choice of potential interventions. For tumours bearing *Ras* mutations, defining the critical downstream effector in each given tissue context is essential. In some settings where Raf has been assumed to be critical, its effectors must be evaluated: *Raf* mutations are common and mutually exclusive with *N-ras* mutations or loss of PTEN. So in these tissues testing these critical effectors may be critical in tumorigenesis. Conversely, there is also clear evidence for both synergy and redundancy between Raf and PI(3)K-mediated signalling on specific biochemical effectors. Activation of mTORC1 activity can be mediated by distinct phosphorylation events on tuberin by ERK and RSK, as well as by AKT. The cell-death effector BAD is also inhibited by both of these pathways. In addition, given the close similarity of the RSK and AKT kinase domains, it is possible that both can phosphorylate the same sites on some target proteins, such as GSK-3 or even tuberin. The availability of eIF4E controlled by signalling through mTOR provides one probable

link between mTOR signalling and tumorigenesis/cell proliferation.[81,82]

ATP synthesis–mitochondria

Mutations in nuclear DNA or mtDNA OXPHOS genes that impede electron flow increase mitochondrial ROS production.[83–87] The resulting H_2O_2, which is relatively stable, diffuses out of the mitochondrion, through the cytosol and into the nucleus, activating normally inactive genes and inducing nuclear DNA mutations. The resulting nuclear DNA damage activates the nuclear DNA repair systems, including the polyADP-ribose polymerase (PARP). The activated PARP degrades the nuclear nicotinamide adenine dinucleotide (NAD)+ in the process of adding poly ADP-ribose chains to histones and other nuclear proteins.

The degradation of the nuclear NAD^+, together with the high NADH/NAD+ ratio, inactivates histone deacetylase, and nuclear transcription is repressed by the deacetylation of histones and activated by histone acetylation. Therefore, histone acetylation turns on the transcription of normally inactive genes. In post-mitotic tissues, histone acetylation permits the activation of the genes that regulate cell replication and differentiation, the proto-oncogenes. Nuclear H_2O_2 can mutate proto-oncogenes, converting them into functional oncogenes. Moreover, increased cytosolic and nuclear H_2O_2 activates a variety of cellular signal transduction factors, including NF-κB, apurinic/apyrimidinic endonuclease (APE)-1, *Fos*, *Jun* and tyrosine kinases. This drives the cell into replication. Consequently, mutations in mitochondrial genes that inhibit electron flow result in chronically increased mitochondrial ROS production, which can act as both a tumour initiator (mutation of proto-oncogenes) and tumour promoter (activation of transcription and replication).[85,88,89]

Gene defects in certain nuclear DNA-encoded mitochondrial genes have been directly linked to some hereditary cancers.[83–87] Mutations in mitochondrial fumarate hydratase have been associated with uterine leiomyomas and renal cell carcinomas, and mutations in three of the four nuclear DNA-encoded subunits of complex II have been linked to paragangliomas: SDHD, SDHC and SDHB subunit. In addition, mutations in the SDHB subunit are also associated with pheochromocytoma and early-onset renal cell carcinoma. The striking difference in clinical effects of SDHA subunit mutations versus SDHB, C and D mutations strongly implicates mitochondrial ROS production in the aetiology of cancer. Transformation of certain tumours with the MnSOD cDNA can reverse the malignant phenotype, and a cluster of three mutations in the MnSOD gene promoter region that alter AP-2 binding and promoter efficiency is found in a number of tumours. Moreover, ROS production in association with the inactivation of *p16ink4a* has been hypothesized to be one of the two main mechanisms for tumorigenesis; the other is p53 deficiency.

Mitochondrial DNA mutations that inhibit OXPHOS and impede electron flow should increase ROS production and contribute to cancer.[83–87] Mitochondrial ROS could contribute to neoplastic transformation, both as a tumour initiator by causing nuclear DNA mutations in proto-oncogenes and tumour-suppressor genes, and as a tumour promoter through driving cellular proliferation. At low levels, ROS has been found to be an active mitogen, thought to act though interaction with various kinases (Src kinase, protein kinase C, MAPK, and receptor tyrosine kinases), as well as with different transcription factors (Fos, Jun, NF-κB). Furthermore, the dual-function APE-1 functions not only in the DNA base excision pathway but also in the redox regulation of the transcription factors Fos, Jun, NF-κB, PAX, HIF-1α and p53.

Moreover, the role of mitochondrial defects in the pathophysiology of cancer would appear to be the generation of increased ROS, which acts as both a nuclear DNA mutagen and cellular mitogen. Since mitochondrial mutations that increase ROS would result in the accumulation within the cell of unoxidized NADH and pyruvate, the excess NADH and pyruvate would then be converted to lactate by lactate dehydrogenase, also described as aerobic-glycolysis. Mitochondrial damage is the result of nuclear or mitochondrial DNA damage that affects the electron transport chain and ATP production, which express morphologically as mitochondrial hyperplasia (oncocytic changes/metaplasia).[90–92] In tumour pathology, the timing between the neoplastic-inducing damage and the mitochondrial damage is important. Mitochondrial damage preceding neoplastic-inducing damage will result in oncocytic neoplasms, whereas the opposite scenario gives neoplasms with oncocytic differentiation (normally focal).

MODIFIED MICROENVIRONMENT INTERACTIONS – INVASION AND ANGIOGENESIS (FIGURES 1 AND 2)

Cellular transformation is accompanied by many cellular changes, including loss of the differentiated cell morphology and invasion of the extracellular matrix. These processes are dependent on cellular and stromal interactions and on extracellular matrix degradation.[20]

Cellular interactions responsible for cell–cell adhesion also can communicate signals, often involving interactions with cytoskeletal elements, to produce

changes in cell motility, migration, proliferation and shape.[21,93,94] The cadherins are cell-surface adhesion molecules that help form tight junctions between cells, such as formation of epithelial cell layers. In addition to mediating adhesion with other cells, cadherins transduce signals into cells through interactions with the catenins.[23] Catenins probably affect actin cytoskeletal function through interactions with proteins such as actinin and vinculin. Catenins also probably trigger changes in cell shape and motility with signals through the Rho small GTPases. Paxillin acts as an adaptor protein between proteins involved in adhesion signalling such as FAK and src and cytoskeletal elements. In addition to signals created by adhesion molecules to alter cellular responses, other signalling pathways can alter adhesion through components of the focal adhesion complex.

Extracellular matrix interactions depend on integrins, cell-surface receptors that mediate intracellular signals to control cellular shape, motility and progression through the cell cycle.[95,96] Integrins do not themselves possess a kinase domain or enzymatic activity, but rely on association with other signalling molecules to transmit signals. Interactions between the extracellular matrix and the actin cytoskeleton commonly take place at focal adhesions on the cell surface that contain localized concentrations of integrins, signalling molecules and cytoskeletal elements. Talin forms a direct interaction with the integrin cytoplasmic domain, and interacts with cytoskeletal elements (actin) and signalling factors. Paxillin and Crk-associated substrate (CAS) also localize in focal adhesions and may serve as a scaffold for other integrin signalling components such as FAK and src. Interaction of FAK, CAS and src may be required for integrin regulation of cell-cycle progression. The CrkL adaptor protein may regulate downstream integrin signalling. Growth factor signalling pathways and the caveolin receptor exhibit important crosstalk with integrin receptors in cellular responses such as activation of map kinase, proliferation and motility. PTEN also interact with FAK, a key molecule implicated in integrin signalling pathways, and it directly dephosphorylates tyrosine-phosphorylated FAK. PTEN down-regulation of p130CAS through FAK results in inhibition of cell migration and spreading.

Extracellular matrix degradation is a key component of tumour cell invasion into surrounding tissues. MMPs are a class of proteases secreted by tumour cells, degrading extracellular matrix proteins and allowing metastasis.[97,98] RECK is a membrane-anchored inhibitor of MMPs, inhibiting MMP-2, MMP-9 and MT1-MMP.[99] The processing of MMPs to their active form occurs at the plasma membrane, making the localization of RECK at the membrane a key to its potent activity as an inhibitor of MMP activity.[100] Soluble secreted MMP inhibitors have also been identified, TIMPs, which appear to be less active at inhibiting MMPs and even perhaps to be essential for MMP maturation. The inhibition of MMPs inhibits tissue invasion, metastasis and tumour angiogenesis, and is essential for angiogenesis, inflammation and normal development. RECK expression is inhibited by ras, suggesting one component by which ras induces transformation. High levels of RECK expression in tumours is correlated with cancer patient survival, and overexpression of RECK may offer a therapeutic strategy for the control of cancer.

Conclusions

The application of these concepts in oncological pathology leads to consideration of the molecular test requirements (Molecular Test Score System) for a reliable implementation, which covers biological effects (1–3), molecular pathway (4, 5), biological validation (6–8) and technical validation (9, 10).

1 As tumours are the result of multiple and cooperative genetic abnormalities, tests should be assessing more than one acquired capability.

2 Genetic targets are more likely to be useful if they are involved in more than one acquired capability.

3 Selected targets must be testing both initiation (including tests for clonality and fractional allelic loss) and promotion/progression of tumours in a given location.

4 Molecular pathways are redundant and overlap in several markers that will potentially be the most informative.

5 Molecular markers can be pleiotropic and their effects will be observed in different pathways (biological consequences).

6 The effects of each marker/target must be evaluated in view of the pathway (both upstream and downstream markers), including the appropriate surrogate markers to validate the expected results.

7 Markers from mutually exclusive pathways must be tested simultaneously for consistency of results.

8 Molecular tumour assessment must be based on the selection of sensible markers, single tests being generally insufficient.

9 Tumours must be tested using samples from at least two areas that should be covering any topographic heterogeneity demonstrated in neoplasms.

10 Samples should be run in duplicate to check consistency of results and must include positive, negative and sensitivity controls.

Competing interests

None to declare.

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