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Review

Intracellular signalling and cancer: complex pathways lead to multiple targets

Helen S. Bell, Kevin M. Ryan *

Tumour Cell Death Laboratory, Beatson Institute for Cancer Research, Cancer Research UK Beatson Laboratories, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK

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Abstract

Normal cells proliferate, die and differentiate as and when they should for the proper functioning of any particular tissue type. These processes are governed by a complex series of intracellular pathways that have many internal checkpoints and safety nets. These ensuring a fine, but tight, balance on overall tissue growth and distribution. A series of key aberrations, resulting in the disruption of these intracellular pathways, can lead to the development of a malignancy. The nature of these alterations is often not only tumour-specific, but also different between individuals with the same tumour type. As a result, these pathways have to be carefully dissected and functionally assessed to identify valid targets with therapeutic potential in a wide range of tumour types.

Keywords: Oncogenes; Tumour suppressor genes; Cell signalling; Cancer progression; Therapy

1. Intracellular signalling and cancer

A common characteristic of tumour-related events involving intracellular signalling is the ability of a cell to grow and divide beyond its normal limits. This is in itself complex and involves the cell's ability to proliferate in the absence of growth promoting signals, the presence of growth inhibitory signals and the ability to couple cell division to cell growth. Upregulation and/or mutations that augment the function of oncogenes, or the loss or inactivation of tumour suppressor genes, can directly enhance this potential of the cell. However, tumour development would be much more common if just one mutational event in a gene controlling this process was allowed to pervade. As a result, there is a spectrum of checkpoint and repair mechanisms to counteract this possibility. If repair is not possible, cells invoke 'self-destruct' programmed cell death pathways to eradicate damaged cells which might otherwise go on to form a tumour. However, if the tumour is to succeed, it must find ways to evade these death mechanisms and this constitutes a second change in intracellular signalling that is common in tumour development. In addition, in the later stages of tumour development, there are a number of characteristics that need to be acquired, for example, the abilities to invade and to exist without normal stromal support, and the capacity to induce neovascularisation. Although these changes are considered mostly extracellular in nature, they also result from changes in intracellular events and add to the complex nature of signalling in malignant cells.

2. Regulation of cell division and growth

Oncogene activations can occur at many different points in the signalling cascades within cells. Constitutive activation of tyrosine kinase receptors, changes in

* Corresponding author. Tel.: +44 141 330 3655; fax: +44 141 942 6521.
E-mail address: k.ryan@beatson.gla.ac.uk (K.M. Ryan).

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second messenger systems and mutations in transcription factors that directly regulate cell division, cell growth and survival can all contribute to tumour development.

2.1. Receptor tyrosine kinase pathways

Changes in the regulation of receptor tyrosine kinases (RTKs) often cause the activation of non-linear pathways resulting in a massive upregulation of growth promoting genes (Fig. 1). RTKs bind to extracellular mitogenic growth factors and transduce these positive growth signals to intracellular second messengers, such as the mitogen activated protein (MAP) Kinases, phosphatidylinositol (PI3) Kinase and signal transducers and activators of transcription (JAK-STAT) signalling cascades [1]. Unlike normal cells, which tightly regulate extracellular ligand levels and receptor expression, cancer cells often acquire the ability to artificially manufacture extracellular growth factors and receptors. For example, epidermal growth factor (EGF) receptor overexpression, which is common in a number of cancers including brain and breast tumours [2,3], allows cancer cells to become hyper-responsive to ambient levels of growth factors that would not normally result in proliferation. In addition, constitutive activation of truncated EGFRs via somatic mutation can permanently activate these growth-promoting pathways without ligand engagement [4].

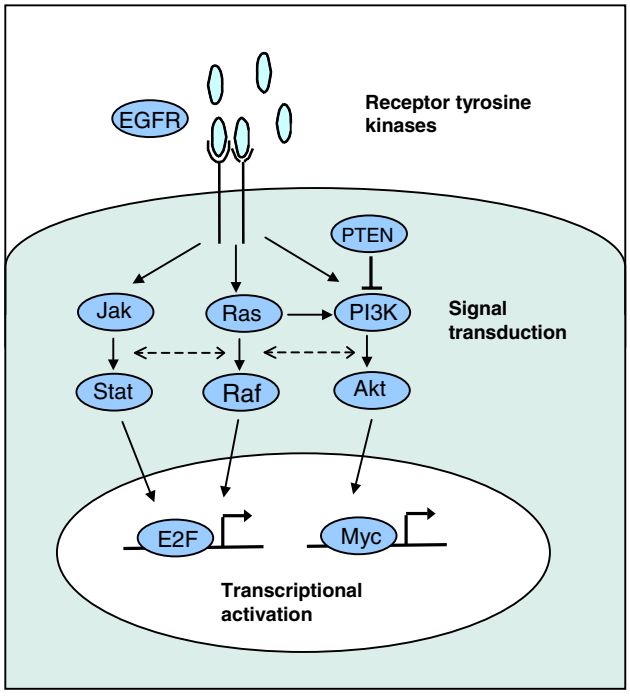

Fig. 1. Activation of second messenger systems from ligand/tyrosine kinase receptor engagement (e.g. epidermal growth factor receptor (EGFR)). Ras/Raf signalling pathways and Jak/STAT signalling can lead to increased E2F-mediated transcription and subsequent proliferation. Myc-mediated transcription can be activated by the phosphatidyl inositol 3-kinase (PI3-K) pathway leading to increases in cell size. Despite the delineation of linear signalling pathways, there is also cross-talk between the pathways as indicted by the dotted arrows.

Further downstream, some of the most commonly mutated genes in many cancer types (in nearly 30% of all cancers) involve the Ras family of small GTPases: Ha-Ras, Ki-Ras and N-ras [5]. Mutated Ras proteins can mimic growth-promoting guanidine triphosphate (GTP)-bound Ras leading to downstream activation of the MAPK cascade [6]. A similar effect can be generated by familial mutation of the tumour suppressor NF1 (Neurofibromatosis gene 1). NF1 is thought to act as a (*G*TPase-*A*ctivating *P*rotein) (GAP) protein. GAP proteins inactivate Ras by catalysing the hydrolysis of Ras-GTP to Ras-GDP. Therefore, inactivation of NF1 decreases this hydrolysis and potentiates sustained signalling from Ras [7]. Mutations in B-Raf – an immediate downstream effecter of Ras mitogenic function – have also been reported in tumours and often occur, although not exclusively, without additional mutations in Ras [8].

The JAK-STAT pathway also lies downstream of RTK signalling (Fig. 1). Large increases in pro-survival STAT3 expression have been associated with many cancer types. Although frequently thought to occur through constitutive activation of RTKs, activating mutants have also been identified for Abl, Fes and Jak kinases, all of which positively regulate STAT proteins [1,9]. Although these pathways are often defined independently, there is significant cross-talk between them. For example, JAK kinase function has been shown to be required for optimal activation of the Ras-MAP kinase pathway, as well as STAT signalling [10]. Therefore, JAK kinase deregulation can have growth-promoting effects distinct from STAT activation. There is also a wealth of evidence showing that tyrosine kinase signalling pathways, in addition to modulating Ras function, affect the Rho family GTPases: Rho, Rac and Cdc42. As well as roles in cell proliferation, these proteins are involved in cytoskeletal organisation and cell motility [11–13]. Taken together, these findings mean that alterations in specific genes can have global effects on the cell, aside from local activations via defined pathways.

Signalling also occurs from RTKs to the PI3-K/Akt pathway. This can occur directly from the RTK or via Ras (Fig. 1). In normal cells, Akt is kept in an inactive state by the effects of the PTEN tumour suppressor on phosphoinositides. Activation of Akt by either upstream events or loss of PTEN – a common event in many tumours – leads to a plethora of effects on survival and cell growth [14]. These include phosphorylation of the *m*ammalian *T*arget *O*f *R*apamycin (mTOR) protein and activation of c-Myc via the FOXO proteins [15,16].

2.2. The control of cell-cycle progression and cell growth

In contrast to the highly complex nature of second messenger systems, it is thought that nearly all mitogenic signalling converges on a small number of nuclear proteins that regulate cell-cycle progression. Most notable is the Retinoblastoma protein (pRb) which is inactivated in the vast majority of human cancers [17]. This protein inhibits cell cycle progression in resting cells, but when phosphorylated by cyclin/CDK (cyclin-dependent kinase) complexes, its inhibition of the E2F family (E2F1 to E2F7) of transcription factors is relieved, and activating E2Fs (E2F1, 2, and 3) then go on to invoke the expression of many target genes involved in traversing the G_1 to S-phase transition (Fig. 2) [18,19].

Constitutive activation of this pathway can occur via a variety of oncogenic lesions. Aside from increases in cyclin/CDK activity emanating from upstream lesions such as mutations in Ras, upregulation of cyclin D1 and increases in CDK4 and CDK6 levels have been detected in various tumour types. These include gliomas, sarcomas, breast carcinomas, as well as some lymphoid tumours [20,21]. The cyclin/CDK proteins are also subject to negative regulation by a group of proteins termed the cyclin-dependent kinase inhibitors (CDKIs) (reviewed extensively in [22]). The smaller CDKIs, p15 and p16, which bind to CDK4 and 6 preventing their association with D type cyclins, are frequently lost in many cancer types [23] (Fig. 2). Similarly, loss of p27, which inhibits CDK2-cyclin complexes, is a marker of poor prognosis in cancer patients [20,24]. More recently, aberrant transcriptional activation of the p16 inhibitor, Tal1, has been shown to be a frequent event in human T cell acute lymphoblastic leukaemia, again resulting in an increase in cyclin/CDK complex activity [25]. Further downstream, inactivation of Rb via the E7 protein of high-risk human papilloma viruses (HPVs) is also able to positively influence mitogenic signalling [26]. Although central to mitogenic signalling, mutations in E2F1 itself have rarely been observed in human tumours. Any that have never been shown to affect the activity of E2F1. E2F1 and E2F3 though have recently been found to be amplified in a variety of cancers and overexpression of E2F3 was shown to be an independent indicator of clinical outcome for prostate tumours [27–29].

Another protein that can influence the G1-S phase transition is c-Myc. Myc has been shown to be upregulated in a wide variety of human cancers. Together with its heterodimeric partner Max, Myc performs a vast array of functions which include stimulation of cyclin production, the degradation of p27 and p16, recruitment of histone acetylase complexes and activation of RNA polymerase III transcription (the polymerase responsible for transcribing a variety of small untranslated genes, e.g., those encoding the small ribosomal RNAs and tRNA) [30–32]. This latter function is reflective of the fact that, in contrast to E2F, Myc has a role in promoting cell growth; something which is essential for the propagation of cells with consistent size and therefore also an essential requirement for tumour development [33].

2.3. Dual roles for E2F1 and Myc

Deregulated mitogenic signalling usually results in the activation of certain checkpoints to ensure the cell

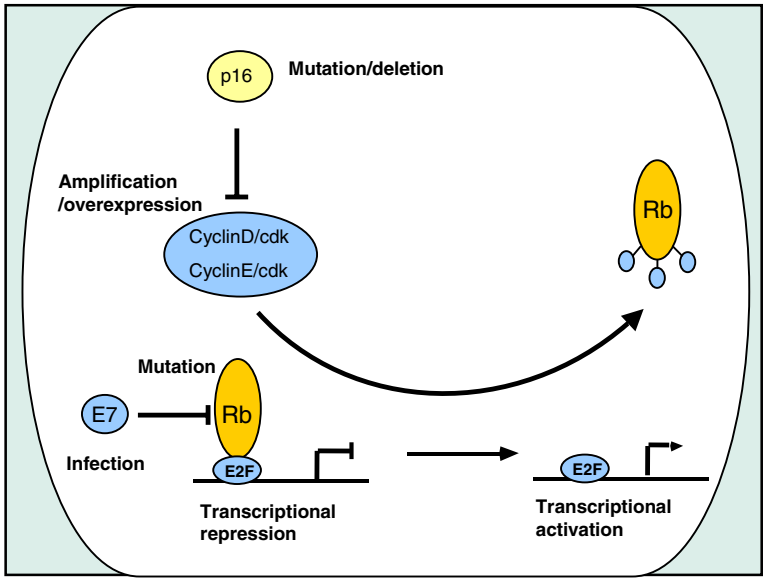

Fig. 2. In the G_0/G_1 stage of the cell cycle, Rb binds to E2F, repressing E2F target genes. Activated Cyclin/CDK complexes phosphorylate Rb allowing its release from E2F. E2F-mediated transcription then allows the cell to pass from G1 into the S phase of the cell cycle. p16 mutation, Cyclin/cdk upregulation and infection with human papilloma virus (HPV) E7 can all lead to constitutive activation of E2F.

does not proliferate out of control. For this reason, in normal cells, as well as stimulating growth-promoting signals, many oncogenes stimulate 'safety nets', in case of abnormal growth or proliferation. For example, both E2F1 and Myc can induce ARF [34,35] (the *A*lternative *R*eading *F*rame of the INK4a locus which also encodes the CDKI, p16) which is indirectly able to stabilise the tumour suppressor, p53. However, to succeed, many tumours have defective cell cycle control AND aberrations in cell death signalling pathways resulting in loss of this safety net.

3. Inhibition of cell death pathways

3.1. Inactivation of p53

p53 is the most frequently mutated gene in human cancer and it is widely accepted that, with mutation of *p53* itself being one mechanism, inactivation of *p53* function occurs in the majority, if not all tumours. *p53* exists at low levels in normal cells, but when stabilised and activated by various forms of cellular stress, it then mediates its tumour suppressor effects [36]. DNA damage has been shown to stimulate phosphorylation of the N-terminal domain of p53 via the kinases Chk1, Chk2 and Ataxia Telangiectasia Mutated protein (ATM)/Ataxia Telangiectasia and Rad3-related protein (ATR) [37–39]. It is reported that these phosphorylation events can disrupt the interaction between p53 and its negative regulator, Mdm2, which normally results in the ubiquitin-mediated degradation of p53 [40,41] (Fig. 3). Oncogene activation can also inhibit Mdm2-mediated degradation of p53 by upregulation of ARF. This ultimately also results in the inhibition of Mdm2 and stabilisation of p53. In normal cells, these two pathways ensure that a set of anti-proliferative genes are activated when a cell is either damaged or where there is evidence of uncontrolled proliferation through oncogene activation.

Many responses in cells have been reported following activation of p53, but it is the ability to induce cell-cycle arrest and apoptosis that are considered the most important for tumour suppression (Fig. 3). In certain cell types following cellular stress, p53 can induce a growth arrest through the activation of targets genes such as *p21* and *14-3-3-3σ* [42,43]. In other types, or perhaps where the cellular and/or environmental context is different, p53 can activate other target genes for example, *Bax* [44], p53-upregulated modulator of apoptosis (PUMA) [45,46] and *NOXA* [47] and/or can invoke transactivation-independent mechanisms to induce programmed cell death [48–51]. Although a last resort for the individual cell, this serves as a safety mechanism for the whole organism causing the eradication of cells which have replicated in adverse conditions, and if mutated, might otherwise go on to form tumour. Moreover, as mentioned earlier, many tumour cells have lost the ability to effectively control cell-cycle progression due to the deregulation of E2F. It is therefore considered that the selective pressure to lose p53 during tumour development is often because of its ability to induce programmed cell death. This is reflected in the fact that a number of tumour-derived mutants of p53 have been described which have lost apoptotic function, but which are equivalent to wild-type in their capacity to induce growth arrest [52,53]. However, no mutants have been found, with reciprocal characteristics.

3.2. Other ways to inactivate p53: Mdm2, ARF, HPV E6 and beyond

The incidence of *p53* mutations in all human cancers is approximately 50% and therefore other mechanisms must exist to evade p53-mediated tumour suppression in tumours containing wild-type p53 (Fig. 3). Overexpression and/or upregulation of Mdm2 is one other mechanism by which this can occur and elevated levels of Mdm2 have been found in approximately 8% of human cancers [54]. Tumours containing *mdm2* amplification and p53 mutations are rare suggesting Mdm2 is the primary regulator of *p53* stability [55]. A variety of Mdm2 splice variants have also been identified, some of which contain transforming functions *in vivo* even in the absence of p53, indicating that the oncogenic effects of Mdm2 may involve more than its ability to inactivate p53 [56,57].

The E6 protein from high-risk HPVs acts in a similar way to Mdm2, by binding to and targeting p53 for ubiquitin-mediated proteolysis [58]. Nearly all cervical cancers are infected with HPV. Moreover, *p53* mutations although found in cervical cancer, are rare in HPV-associated cervical cancer, suggesting that, like Mdm2 upregulation, degradation of p53 by E6 can completely substitute for p53 mutation in human cancer [59].

Another alteration which can destabilise p53 is loss of the Mdm2 regulator, ARF (Fig. 3). ARF and the CDKI, p16, are encoded by the same gene such that both proteins can both be lost through a single mutation. However, the two transcripts encoding the proteins are controlled by different promoter sequences [60]. As a result, methylation of the ARF promoter can silence ARF expression separately from the expression of p16. Downregulation of ARF in this manner is a frequent event in colorectal cancers making the tumours permissive for the presence of activated oncogenes even in the context of wild-type p53. However, unlike *mdm2* gene amplification, *ARF* mutations do occur in tumours harbouring p53 mutations [61]. Indicating, as mentioned earlier, that other signals can activate *p53* in an ARF-independent manner, or that ARF has p53-independent tumour suppressive effects [62,63].

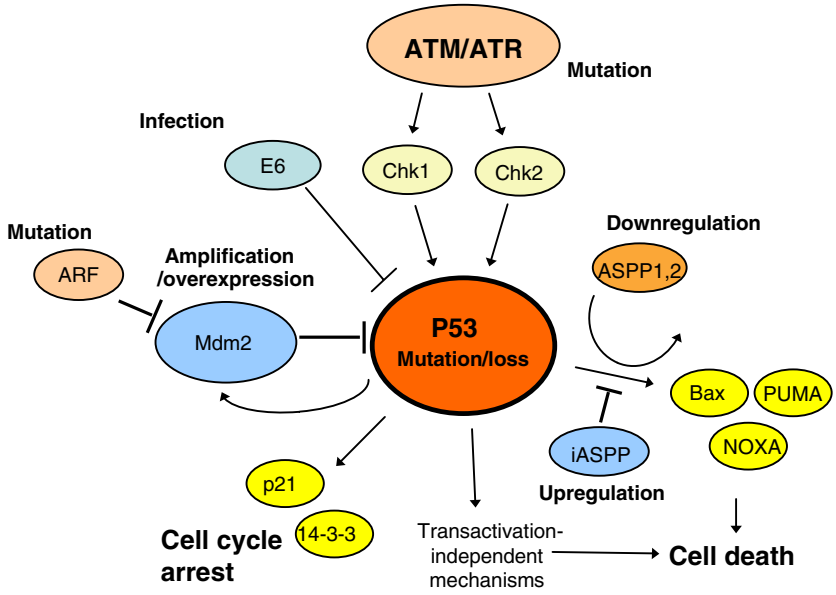

Fig. 3. Following DNA damage, ATM/ATR and Chk kinases activate p53 leading to activation of p53 target genes, such as *p21* and *14-3-3σ* which activate cell-cycle arrest, or genes such as *Bax*, *PUMA* or *NOXA* which results in apoptosis. In addition to activation of target genes, p53 can also invoke transcription-independent mechanisms to induce cell death. *p53*, *ATM* and *ARF* mutations, amplification of Mdm2, altered ASPP expression and infection with HPV E6 can all lead to impeded p53-mediated tumour suppression.

In addition to perturbations that attenuate the ability to induce p53, mutations have also been found in tumours that affect the ability of p53 to induce an apoptotic response. The recently discovered apoptotic stimulating protein of p53 (ASPP) family of proteins act as gene-specific co-activators of p53's pro-apoptotic target genes (Fig. 3). ASPP1 and ASPP2, which enhance the apoptotic function of p53, have been shown to be downregulated in human breast carcinomas and in human leukaemic cell lines expressing wild-type p53, but not mutant p53 [64,65]. In addition, the inhibitory member of the family, iASPP, was found to be upregulated in the same tumour types [65,66].

p53 activity can also be affected by its family members, p63 and p73, which have been shown to be required for p53-induced death [67]. Unlike p53, p63 and p73 are not frequently found to be mutated in cancer. However, natural dominant-negative isoforms, named ΔNp63 and ΔNp73, that do not contain N-terminal transactivation domains have been found to be overexpressed in a variety of epithelial cancers [68]. Although these isoforms cannot heterodimerise with p53 they can bind to p53 responsive promoters and prevent p53-, p63- and p73-mediated transcription, thereby impeding the apoptotic response of p53 [69].

4. Enhancement of angiogenesis

In addition to increased proliferative potential and the ability to evade pro-death signalling, tumour cells need to provide an ideal environment in which to survive. Hypoxia-inducible factor 1α (HIF-1α) is a major factor in creating this environment. HIF-1α is normally induced in response to hypoxic and metabolic stress and can activate a wide variety of genes responsible for angiogenesis, e.g., Vascular endothelial growth factor (VEGF) and glucose metabolism, e.g., *GLUT1* [70]. HIF-1α has been shown to be upregulated in many human cancers such as brain, breast and cervix and is closely correlated with patient mortality [71].

HIF-1α upregulation can arise 'naturally' due to the hypoxic nature of the intratumoural environment [72] (Fig. 4). In addition, a vast array of genetic alterations can increase this activity, these include aberrant PI3-K [73], MAPK [74] and Src signalling [75], as well as loss of p53 function [76]. One well described alteration involves the tumour suppressor Von Hippel-Lindau (VHL). Under aerobic conditions, HIF-1α is hydroxylated at specific residues by the prolyl hydroxylase-domain protein (PHD). This results in the binding of the VHL protein to HIF-1α, resulting in the ubiquitin-mediated degradation of HIF-1α (Fig. 4). In low levels of oxygen or in the presence of mutant VHL, HIF-1α levels increase leading to HIF-1α-mediated transcription [77,78]. VHL loss is common in clear-cell renal carcinomas (RCCs) and cerebellar haemangiomas [79].

5. Increasing motility, invasion and metastasis

The ability to invade and metastasise is a feature of many malignancies. An example of alterations that can aid this process is through the proto-oncogene Src. Src

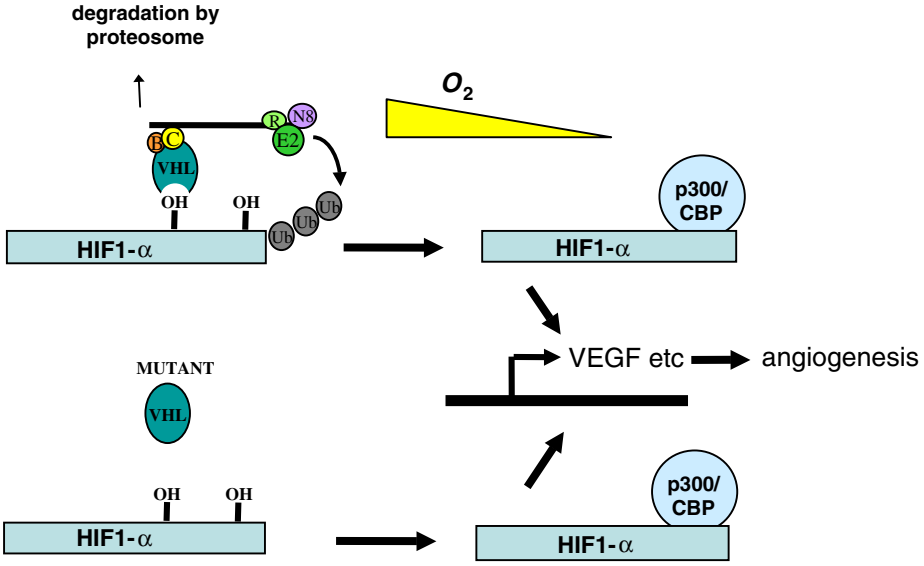

Fig. 4. Under normoxic conditions, HIF-1α is hydroxylated by the prolyl hydroxylase-domain protein (PHD). This results in the binding of Von-Hippel-Lindau (VHL) to hypoxia-inducible factor 1α (HIF-1α) resulting in the recognition of the complex by E3 ubiquitin ligase. When oxygen levels decrease, HIF-1α becomes stable and activates downstream target genes, such as Vascular endothelial growth factor (VEGF). Similarly, in the presence of mutated VHL, HIF-1α is stabilised in the presence or absence of oxygen.

is a non-receptor tyrosine kinase that is localised to cell-matrix adhesions and transduces integrin signalling. Src plays an important role in tumour cell invasion, in particular through its interaction with the focal adhesion kinase (FAK) [80]. Following integrin engagement, Src interacts with FAK at cell-matrix adhesions, this creates docking sites for proteins, such as paxillin, vincullin and talin, which connect integrins to the actin cytoskeleton [81]. This eventually results in the creation of membrane ruffles and invadapodia which are prominent in some cancer cells, particularly those exhibiting an invasive phenotype [82]. In such cancers, expression of Src and FAK have been shown to be elevated and knock-down of FAK can lead to the reversion of cancer cells to a less invasive phenotype [83]. In addition, Src can also deregulate E-cadherin [84] and promote expression of the matrix metalloproteases [85], both of which can promote tumour cell invasion.

Highly invasive phenotypes have also been attributed to alterations in the activity of small GTPases. Levels of Rac, Cdc42 and Rho, have been shown to be altered in various cancers, where Rac is required for the formation of lamellipodia at the leading edge of migrating cells [86,87]. In contrast, signalling from Rho can lead to a different kind of 'bleb-associated' motility via its downstream effectors, the ROCK kinases [88]. Regulation of cell polarity, and the direction of movement is controlled by Cdc42 [89].

Pathways that influence invasion and metastasis are closely interlinked with pathways that regulate cell growth and proliferation. For example, ERK-MAPK signalling can regulate Rac activity via the activation of Fra-1, a member of the Fos family [90]. Rac, in turn, has been shown to be required for Ras transformation [91]. Furthermore, links between genes responsible for invasion have been found with other tumorigenic factors. For example, activation of STAT3 by Src/FAK leads to increased expression of the pro-angiogenic VEGF [92].

6. Therapeutic advances

The complex nature of these interactions continues to be a challenge for the cancer biologist. Proper dissection and analysis of all of these signalling systems holds the key for elucidating which target molecules are the most credible for the design of successful therapeutics. However, a wide variety of molecules have been developed that target many of the pathways discussed in this review.

Tyrosine kinase inhibitors are one subset of compounds that have reached the most advanced stage of development. One of the most successful is Imatinib mesylate (STI-571, Gleevec) [93]. Imatinib reversibly competes with adenosine triphosphate (ATP) for binding to the kinase domain of platelet-derived growth factor (PDGFR), c-kit and Abl tyrosine kinase. The compound was tested on patients with Philadelphia chromosome (Bcr-Abl) translocations. 70% of patients treated with Imatinib showed no evidence of the Phi+ cells following treatment. However, Imatinib-resistant cells subsequently expand, therefore further developments must be made to target these cells [94]. Other

developments have been made targeting the HER2/Neu transmembrane tyrosine kinase. Impressive levels of regression have been observed in breast cancer patients with high HER2 activity following treatment with a humanised HER2 antibody (trastuzumab/Herceptin) [95]. In addition, inhibitors designed to specifically inhibit EGFR such as gefitinib (Iressa) have also been developed. The effectiveness of these agents, however, when combined with standard chemotherapy in large-scale randomized phase III trials is questionable [96].

Further downstream, various Ras and Raf inhibitors are currently being tested at the Phase I level [97,98]. BAY 43-9006, which blocks MEK phosphorylation by Raf-1 is currently being tested against metastatic cancer having been shown to be effective in cell and animal assays [98]. CDK inhibitors are also being tested in the clinic. UCN-01 and flavopiridol which inhibit CDKs 1, 2 and 4 have shown some activity in trials against melanoma, sarcoma and lymphoma [99]. Drugs designed to limit the invasive nature of some cancers are also entering pre-clinical/Phase I trials. These include numerous compounds designed to target the kinase domain of Src [100]. If these toxicity studies pass the test, hopefully these therapies will be even more effective when used in combination with current chemotherapeutic strategies.

Some of the most promising potential therapies may lie with targeting pro-apoptotic pathways. Although gene therapy based on the reintroduction of wild-type p53 to mutant cells is not realistic due to size restrictions, other small molecules have been designed that can reactivate mutant p53 to induce apoptosis. One such molecule, p53 reactivation and induction of massive apoptosis, can restore sequence-specific binding of a variety of p53 mutants, such as 273H and 175H. This leads to induction of pro-apoptotic signalling and subsequent apoptosis *in vitro* [101]. Another series of small molecules, termed Nutlins, have been designed that bind to the p53 binding cleft of Mdm2. By inhibiting the wtp53/Mdm2 interaction, Nutlins are able to induce p53 dependent apoptosis specifically in cancer cells [102]. A further series of compounds have been recently developed that target IAPs, a family of anti-apoptotic proteins that inhibit the caspases. Treatment of a variety of tumour cell lines with IAP antagonists has been shown to cause sensitization to apoptosis from the death receptors, tumour necrosis factor-related apoptotis-inducing ligand (TRAIL) and tumour necrosis factor (TNFα), or chemotherapeutic drugs [103,104]. Compounds that bind to and inhibit Bcl-2, a pro-survival member of the Bcl family of proteins, have also been shown to induce apoptosis in tumour cells [105]. Hopefully, many of these small molecule inhibitors/antagonists will soon be translated into the clinic where their true efficacy will become apparent.

7. Concluding remarks

The sheer complexity of the pathways that are altered in human cancer means that the design of therapies becomes equally complex. It is possible that dozens of drugs may have to be administered to cancer patients to both bypass/reactivate cell death signalling pathways and deactivate proliferative, angiogenic and metastatic signalling pathways. Different alterations are present in different cancer types and the progression of each cancer is often unique to each patient. It is therefore likely that each combinational therapy has to be tailor-made to the individual patient. In addition, prospective therapies have to be tumour cell-specific and easy to administer. Although a daunting prospect, the vast amount of research currently being undertaken to dissect oncogenic pathways, design drug delivery systems and develop good prognostic markers allows us to be cautiously optimistic.

Conflict of interest statement

None declared.

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