Chapter 2
Signal Transduction Pathways as Therapeutic Targets in Cancer Therapy

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Abstract Cancer is increasingly recognized as "miscommunication" disease, in which inter- and intracellular signals are aberrantly sent and/or received, resulting in the uncontrolled proliferation, survival, and invasiveness of the cancer cell. Indeed, many of the genetic and epigenetic aberrations, which underlie the process of neoplastic transformation and progression, ultimately impinge on the inappropriate activation/inactivation of intracellular signaling pathways. Such signaling cascades usually proceed from the cell surface, where growth factors interact with their specific receptors, to cytoplasmic signaling intermediates, where different signals are integrated and both positive and negative feedback circuitry are in place to ensure signal fidelity and transduction accuracy, to nuclear transcription factors/complexes, which ultimately lead to the transcription/translation of effector genes and proteins involved in specific cellular functions. While the signal may be inappropriately transduced at several, and usually multiple, levels, one interesting feature of aberrant cancer signaling is that cancer cells may become "addicted" to specific signals and hence exquisitely sensitive to their modulation. In this chapter we will describe the signaling process, highlighting the steps at which aberrant signal transduction may turn a normal cell into a cancer cell and the crucial points where aberrant signals can be modulated for therapeutic purposes. Finally, we will briefly touch upon relevant issues surrounding the clinical development of signal transduction inhibitors as anticancer agents.

Abbreviations

ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
AMPK	AMP-activated protein kinase
ASK1	apoptosis signal kinase 1
ATP	adenosine triphosphate

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BSC	best supportive care
Cdk	cyclin-dependent kinase(s)
CFC	cardio-facio-cutaneous syndrome
CML	chronic myelogenous leukemia
4EBP1	eukaryotic translation initiation factor 4E binding protein 1
EGFR	epidermal growth factor receptor
ERK	extracellular-signal-regulated kinase
FISH	fluorescence in situ hybridization
FLT3	Fms-like tyrosine kinase 3
GIST	gastrointestinal stromal tumor(s)
GSK3	glycogen synthase kinase 3
Hsp	heat-shock protein
IRS	Insulin receptor substrate
JNK	Jun N-terminal kinase
LAM	Lymphangioleiomyomatosis
MAPK	mitogen-activated protein kinase
MEK	MAPK and ERK kinase
MITF	microphthalmia transcription factor
MST-2	mammalian sterile 20-like kinase
mTOR(C)	mammalian target of rapamycin (complex)
NF1	neurofibromatosis 1
NSCLC	non-small cell lung cancer
PDGF	platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	pleckstrin homology domain
PI3K	phosphoinositide 3-kinase
PI3K	AKT (phosphatidylinositol-3 kinase–AKT)
PTEN	phosphatase and tensin homolog deleted on chromosome 10
Raptor	regulatory-associated protein of mTOR
Ras–Raf–MEK	(mitogen-activated and extracellular-signal-regulated kinase kinase)
Rheb	Ras homolog enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
RNAi	RNA interference
ROS	reactive oxygen species
RTK	receptor tyrosine kinase(s)
S6K1	ribosomal S6 kinase 1
SCLC	small cell lung cancer
STAT	signal transducer and activator of transcription
t-AML	therapy-induced AML
TGFa	transforming growth factor a
TK	protein tyrosine kinase(s)

TKI	tyrosine kinase inhibitor(s)
TNF	tumor necrosis factor
TSC	tuberous sclerosis complex

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2.1 Introduction

Transformation of a normal cell into a cancerous one, with its full-blown set of malignant properties, is, in most instances, a highly complex, multistep, genetic event [1]. During this multistep process, the genomes of incipient cancer cells acquire mutant alleles of proto-oncogenes, tumor suppressor genes, and other genes that control, directly or indirectly, cell proliferation, survival, and differentiation. On the basis of several lines of research, Hahn and Weinberg have hypothesized that the pathogenesis of human cancers is governed by a set of genetic and biochemical rules that apply to most, and perhaps all, types of human tumors [2]. These rules, in turn, reflect the operations of a few key intracellular regulatory circuits that operate in the majority of human cell types. Although we still do not fully understand the detailed operations of these regulatory circuits, experimental observations allow outlining the basic rules governing the neoplastic transformation of normal human cells. Part of this evolution in thinking about the origins of cancer comes from numerous observations indicating that most, if not all, cancer cells seem to share a common set of biologic attributes – essentially, changes in cell physiology – termed "acquired capabilities." These attributes include the ability of cancer cells to generate their own mitogenic signals, to resist exogenous growth-inhibitory signals, to evade apoptosis, to proliferate without limits (i.e., to undergo immortalization), to acquire vasculature

(i.e., to undergo angiogenesis), and in more advanced cancers, to invade and metastasize [2–4]. Such capabilities are usually acquired by cancer cells through complex genetic changes that accumulate throughout the cancer development process, usually spanning several decades: oncogenes may become upregulated by gains of chromosomes, gene amplification, translocations, and activating point mutations, and tumor suppressor genes may be inactivated by loss of whole chromosomes, gross deletions, intragenic deletions, and point mutations (as an example of the role of chromosomal aberrations in hematologic malignancies see Ref. [5]). However, the measured rate of mutation in normal human cells is so low that during the course of a person's lifetime, cancer cells could not acquire the full array of mutant alleles that are required to complete the progression to a highly neoplastic state, unless they acquired an additional attribute – genetic instability [6]. This calculation implies that the genomes of preneoplastic cells must become unstable for tumor progression to proceed to completion, even over a period of several decades. Indeed, even cursory examinations of human tumor cell genomes usually reveal instability at the level of either the DNA sequence or the karyotype – an observation that helps support the notion that increased mutability is essential for the development of many types of cancer in humans. Such increased mutability is acquired when the genes and proteins that ordinarily protect the genome by detecting and repairing damage in chromosomal DNA are inactivated. In addition, the cellular mechanisms (notably apoptosis) that usually eliminate cells with damaged DNA are often compromised in tumor cells; the result is the survival of a mutant cell and the possible outgrowth of a large population of its similarly mutated descendants [6–8].

An additional level of complexity is achieved by epigenetic control of gene expression programs that provide alternative and/or complementary routes to the gain of cancer's "acquired capabilities." Indeed, epigenetic alterations, which, by definition, comprise mitotically and meiotically heritable changes in gene expression that are not caused by changes in the primary DNA sequence, are increasingly being recognized for their roles in carcinogenesis [9, 10]. These epigenetic alterations may involve covalent modifications of amino acid residues in the histones around which the DNA is wrapped, and changes in the methylation status of cytosine bases (C) in the context of CpG dinucleotides within the DNA itself. Epigenetic alterations occur within a larger context of extensive alterations to chromatin in neoplastic cells in comparison with the normal cells from which they are derived. Although the molecular determinants that underlie these types of chromatin change in tumor cells are only beginning to be elucidated, the best understood component is the transcriptional repression of a growing list of tumor suppressor and candidate tumor suppressor genes. This suppression is associated with abnormal methylation of DNA at certain CpG islands that often lie in the promoter regions of these genes [11–13]. In addition, recent studies indicate that epigenetic alterations might initiate the expansion of pre-malignant cells during the early stages of tumorigenesis. During the earliest steps of development of principal tumor types, such as colon, lung, and prostate tumors, a subset of these pre-malignant cells undergo genetic alterations that allow them to mediate tumor progression and growth. The early epigenetic changes that occur in these cells might determine the subsequent genetic

changes and thereby foster progression of these clones. There has been increased effort to elucidate the molecular events in chromatin regulation that initiate and maintain epigenetic gene silencing in cancer cells as tumors progress. Clues are emerging as the entire field of chromatin regulation of gene expression patterns rapidly advances [9, 14–18]. A key concept is that, in order to effectively monitor and control human neoplasia, we might need to explore the cancer cell "epigenome" as completely as the mutations in the cancer cell genome. Another interesting point regarding the role of epigenetic changes in cancer initiation and progression is that, in contrast to genetic alterations, gene silencing by epigenetic modifications is potentially reversible. Indeed, treatment by agents that inhibit cytosine methylation and histone deacetylation can initiate chromatin decondensation, demethylation, and reestablishment of gene transcription. Accordingly, in the clinical setting, DNA methylation and histone modifications are very attractive targets for the development and implementation of new therapeutic approaches [19–22].

In order to allow cancer cell to develop the "acquired capabilities" typical of the transformed state, many of the genetic and epigenetic aberrations which underlie the process of neoplastic transformation and progression ultimately impinge on the inappropriate activation/inactivation of intracellular signaling pathways. Canonical signaling cascades usually proceed from the cell surface, where growth factors interact with their specific receptors, to cytoplasmic signaling intermediates, where different signals are integrated and both positive and negative feedback circuitry are in place to ensure signal fidelity and transduction accuracy, to nuclear transcription factors/complexes, that ultimately lead to the transcription/translation of effector genes and proteins involved in specific cellular functions. In many instances, canonical signaling pathways involved in cancer initiation and progression heavily rely on the activity of kinase enzymes, which transfer phosphate groups onto specific amino acid residues (e.g., tyrosine, serine, threonine) within regulatory and enzymatic proteins (protein kinases) or membrane-bound/intracellular lipids (lipid kinases), which act as important co-factors in signal transduction [23–25]. For example, protein tyrosine kinases (TK) are enzymes that catalyze the transfer of phosphate from adenosine triphosphate (ATP) to tyrosine residues in polypeptides. The human genome contains about 90 TK and 43 TK-like genes, the products of which regulate cellular proliferation, survival, differentiation, function, and motility. More than 25 years ago, TK were implicated as oncogenes in animal tumors induced by retroviruses and are now regarded as excellent targets for cancer chemotherapy [26].

In rare cases, such as in chronic myelogenous leukemia (CML), a single, "apical," genetic lesion (the t(9;22) chromosomal translocation that gives rise to the BCR-ABL fusion protein) can be identified that drives the activation of an array of diverse signaling pathways, including NF-kB, AKT, and STAT5 among others [27]. In such cases, pharmacological interference with the "causative" genetic alteration severely impairs the ability of transformed cells to proliferate and survive and dramatically alters the natural history of the disease, leading to arguably the most impressive "success story" in the field of cancer therapy over the past 20 years [28]. At the other end of the spectrum lies perhaps the deadliest of human cancers, pancreatic cancer, in which an average of 63 genetic alterations per case, the majority

of which were point mutations, were recently detected by comprehensive genetic analysis. These alterations defined a core set of 12 different cellular signaling pathways and processes that were each genetically altered in 67–100% of the tumors [29]. Although most of human cancers lie between these two extremes, a single genetic alteration necessary and sufficient to drive the array of phenotypic hallmarks of malignancy (as it is the case for the BCR-ABL fusion protein in CML) is the exception rather than the rule and the malignant behavior is usually driven by the accumulation of several genetic and epigenetic aberrations.

An additional level of complexity comes from the fact that our knowledge of signal transduction pathways has evolved, over the past 20 years, from the classical notion of "linear" signaling pathways, whereby a single receptor would transduce signals through specific "intermediates" to a limited number of final "effectors," to the much more complex vision of "signaling networks," in which every single component is closely intertwined with an array of different players, thereby creating an extremely complex scheme of vertical and parallel signaling pathways regulated by positive and negative feedback loops. In this context, even the most specific interference with a single signaling component may actually lead to unexpected, and sometimes "undesired" from a therapeutic perspective, functional outputs. Such new level of complexity obviously requires completely novel strategies to both pathway investigation (for example, the use of high throughput technologies and "omics" approaches) and interpretation of the results (the thriving science of "systems biology" applied to cancer biology and anticancer drug discovery) [30–33].

This may help explain why, in addition to a handful of success stories (such as the development of imatinib for the treatment of CML and GIST or that of trastuzumab for breast cancer), the clinical development of other compounds that specifically target protein kinases has been more troublesome, especially with regard to their combination with classical cytotoxic agents [34]. In addition to the inherent complexity of cancer signaling as a therapeutic target, these setbacks reflect a variety of other factors specifically related to the inadequacy of classical drug development paradigms when applied to "targeted" therapy, including a rush to get compounds into the clinic, a lack of validated biomarkers, insufficient characterization of patient populations appropriate for treatment, and oversight of pharmacodynamic and scheduling issues.

In this chapter we will describe the signaling process, highlighting the steps at which aberrant signal transduction may turn a normal cell into a cancer cell and the crucial points where aberrant signals can be modulated for therapeutic purposes. Finally, we will briefly touch upon relevant issues surrounding the clinical development of signal transduction inhibitors as anticancer agents.

2.2 Protein Tyrosine Kinases (TK) as Therapeutic Targets

TK are divided into two mains classes. Receptor TK are transmembrane proteins with a ligand-binding extracellular domain and a catalytic intracellular kinase domain, whereas nonreceptor TK lack transmembrane domains and are found in

the cytosol, the nucleus, and the inner surface of the plasma membrane [26]. The enzymatic activities of both types of TK are under tight control, so that nonproliferating cells have very low levels of tyrosyl phosphorylated proteins. The kinase domains of all TK have a bilobar structure, with an N-terminal lobe that binds ATP and magnesium, a C-terminal lobe containing an activation loop, and a cleft between the lobes to which polypeptide substrates bind. In the absence of ligand, receptor TK are unphosphorylated and monomeric, and the conformation of their kinase domains is inactive. In some receptor TK, the cytoplasmic juxtamembrane region further inhibits the enzyme by interacting with the kinase domain. Receptor TK become activated when the ligand binds to the extracellular domain, resulting in receptor oligomerization, disruption of the autoinhibitory juxtamembrane interaction, and autophosphorylation of a regulatory tyrosine within the activation loop of the kinase. These changes reorient critical amino acid residues, thereby increasing the catalytic activity of the enzyme. After activation, autophosphorylation generates binding sites for signaling proteins, recruiting them to the membrane, and activating multiple signaling pathways [35, 36].

The nonreceptor TK, typified by c-ABL, are maintained in an inactive state by cellular inhibitor proteins and lipids and through intramolecular autoinhibition. Nonreceptor TK are activated by diverse intracellular signals through dissociation of inhibitors, by recruitment to transmembrane receptors (causing oligomerization and autophosphorylation), and through trans-phosphorylation by other kinases. TK signaling is then terminated in part through the action of tyrosine phosphatases that hydrolyze tyrosyl phosphates and by the induction of inhibitory molecules [26, 37].

Given the multiple levels of regulation of TK, it is not surprising that TK are dysregulated in cancer cells in several ways. A common mechanism of TK activation in hematologic cancers is the fusion of a receptor or nonreceptor TK with a partner protein, usually as a consequence of a balanced chromosomal translocation. A frequent feature of the partner protein is a domain that causes constitutive oligomerization of the TK in the absence of ligand-binding or physiologic-activating signals, thereby promoting autophosphorylation and activation. A primary example of this mechanism is BCR-ABL, the nonreceptor fusion TK in CML, in which a tetramerization domain in BCR overcomes autoinhibition of ABL catalytic activity through oligomerization and autophosphorylation. With some receptor TK, absence of the juxtamembrane inhibitory domain in the fusion protein contributes to activation. A second important mechanism of TK dysregulation is a mutation that disrupts autoregulation of the kinase. Mutations in the Fms-like tyrosine kinase 3 (FLT3) receptor in acute myeloid leukemia (AML) render this TK active in the absence of ligand; in another example, small deletions and point mutations in the kinase domain of epidermal growth factor receptor (EGFR) in a subset of non-small-cell lung cancers (NSCLC) increase the sensitivity of the receptor to its ligand and alter receptor signaling (see below). A third mechanism of TK dysregulation is increased or aberrant expression of a receptor TK, its ligand, or both. Examples include overexpression of the receptor TK ERBB2 (HER-2/neu) in breast cancer and overexpression of a mutant form of platelet-derived growth factor (PDGF),

a receptor TK ligand, in dermatofibrosarcoma protuberans with t(11;17). Lastly, increased TK activity can result from a decrease in factors that limit TK activity, such as impaired tyrosine phosphatase activity or decreased expression of TK inhibitor proteins [38–41]. Aberrant TK activation can increase the survival, proliferation, and cytotoxic drug resistance of malignant cells, and in tumors it can increase angiogenesis, invasiveness, and metastatic potential.

TK can be inhibited pharmacologically through multiple mechanisms. The idea behind much of anti-TK drug discovery is to find small molecules that directly inhibit the catalytic activity of the kinase by interfering with the binding of ATP or substrates. Other anti-TK drugs may inhibit activation of fusion TK by blocking their dimerization. Antibodies against receptor TK or their ligands interrupt TK signaling through neutralization of ligand, blockade of ligand binding, receptor internalization, and perhaps antibody-mediated cytotoxicity. The stability of some TK is regulated by binding to heat-shock proteins (e.g., heat-shock protein 90 [Hsp90]), and inhibitors of Hsp90 can disrupt the binding of client proteins such as BCR-ABL and HER-2, causing their degradation. An important advantage of TK-directed therapy is that it is possible to perform pharmacodynamic studies that correlate inhibition of the targeted TK in cancer cells (or surrogate tissues) with clinical responses to the drug [42, 43].

2.2.1 RTK as Therapeutic Targets: The Paradigm of EGFR Mutations in NSCLC

The ErbB family is a member of the receptor tyrosine kinase (RTK) super-family of cell surface receptors, which serve as mediators of cell signaling by extracellular growth factors. Members of the ErbB family, such as EGFR (also known as ERBB1 or HER1), ERBB2 (also known as HER2), ERBB3 (also known as HER3), and ERBB4 (also known as HER4), have received much attention, given their strong association with malignant proliferation [44] (see also Chapters 12 and 13). Increased levels of *EGFR* gene expression are observed in cancers of the head and neck, ovary, cervix, bladder, esophagus, stomach, brain, breast, endometrium, colon, and lung, and frequently seem to confer an adverse prognosis [45, 46, 39]. Extending previous observations of almost two decades ago, recent retrospective analyses have reported EGFR overexpression in 62% of NSCLC cases and its expression is correlated with a poor prognosis. In some cases, genomic analyses documented the amplification of chromosomal region 7p12, where the *EGFR* gene is located [47, 46]. In addition to EGFR overexpression, its cognate ligands, epidermal growth factor (EGF), and transforming growth factor-α (TGFα) are also frequently expressed in NSCLCs and can establish autocrine loops that lead to receptor hyperactivity. The disruption of these autocrine loops is the primary rationale for antibody-based EGFR-targeted therapeutics [48, 49]. Various strategies involving small molecule inhibitors have also been developed to target EGFR and/or its family members, and these are in various stages of clinical testing. As mentioned

earlier in this chapter, development of small molecule drugs that specifically target the tyrosine kinase activity of EGFR (EGFR-TKIs), such as gefitinib (Iressa; AstraZeneca) and erlotinib (Tarceva; OSI Pharmaceuticals, Genentech), has actually led to the identification of a set of acquired alterations of the EGFR, which, in turn, render NSCLC cells dependent on its activity and exquisitely sensitive to its inhibition in a clinical context (see below). Gefitinib and erlotinib received fast-track approval from the US Food and Drug Administration (FDA) in 2003 and 2004, respectively, for patients with advanced NSCLC who had failed to respond to conventional chemotherapy. Both drugs are reversible inhibitors of the EGFR kinase, designed to act as competitive inhibitors of ATP binding at the active site of the EGFR kinase. Early NSCLC clinical trials were modestly encouraging, with partial responses observed in approximately 10% of treated patients. Most responses were seen in East Asians, females, or non-smoking patients. These patients had a high frequency of adenocarcinoma with bronchioloalveolar features, and many showed a dramatic and lasting response to second- or third-line gefitinib or erlotinib monotherapy [50, 51]. The sequencing of the *EGFR* gene in tumor samples from these responders showed somatic gain-of-function mutations [52–54]. The observation that sensitivity to gefitinib and erlotinib correlated very strongly with such newly discovered class of somatic activating mutations in the EGFR kinase domain explained the unique subset of drug–responsive cases; indeed, in unselected NSCLC samples, *EGFR* mutations are present in ~10% of cases in North America and Western Europe, but in ~30–50% of cases in individuals of East Asian descent, and are associated with most (over 50%) adenocarcinomas with bronchioloalveolar features that arise in non-smokers [55, 56, 39].

EGFR kinase domain mutations target four exons (18–21), which encode part of the tyrosine kinase domain (the entire kinase domain is encoded by exons 18–24) and are clustered around the ATP-binding pocket of the enzyme. The most prevalent of *EGFR* kinase domain mutations, accounting for 45% of *EGFR* mutations in NSCLC, are in-frame deletions of exon 19, nested around the LREA string of amino acids located between residues 747–750 of the EGFR polypeptide. Another recurrent mutation is the L858R substitution in exon 21, within the activation loop of *EGFR*, which comprises approximately 40–45% of *EGFR* mutations. Nucleotide substitutions in exon 18 (for example, G719C or G719S) account for another 5% of *EGFR* mutations, as do in-frame insertions in exon 20 [39]. The most noteworthy, clinically relevant mutation in exon 20 is T790M, which is detected in 50% of the cases as a second site mutation associated with acquired gefitinib and erlotinib resistance [57, 58]. Recently, D761Y, a T790M-like secondary mutation in exon 19 of *EGFR* (at the border of exon 19 and exon 20), was also reported to be associated with resistance to gefitinib and erlotinib in NSCLC cells that contain the L858R-*EGFR* mutation [59, 60].

Although the inclusion of most of these sensitizing mutations is based on their occurrence in drug responders, increased biochemical and cellular activity of these mutations has been documented in some cases. Indeed, in addition to providing a genetic marker for a highly EGFR-TKI-responsive subset of NSCLCs, this correlation has also highlighted the crucial importance of mutationally activated

kinases as anticancer drug targets. Consistent with their purported role in the etiology of NSCLC, recent studies have shown that exon 19 deletions that involve the LREA motif, L858R, G719S and ins 770(NPG)-mutated EGFR proteins are oncogenic in both cell culture and transgenic mouse studies. These mutations also increase the kinase activity of EGFR, leading to the hyperactivation of downstream pro-survival pathways, and consequently confer oncogenic properties on EGFR [61–65]. Kinase domain mutations in *EGFR* are generally referred to as activating mutations, as they seem to result in the increased kinase activity of the receptor. However, this does not imply that these mutated EGFRs are necessarily constitutively or fully active, as their degree of ligand independence might be a function of the experimental context. These partially activated mutant EGFRs can be rendered fully ligand independent, and therefore constitutively active, by second site substitutions in *EGFR*, such as the T790M mutation in exon 20. In vitro biochemical studies using purified recombinant wild-type and mutant (L858R and $\triangle$E746-A750) EGFR cytoplasmic domains have shown that mutants have increased K*cat* values and an increased K*m* for ATP. Moreover, as has been observed in cell-based studies, the mutants show an increased sensitivity to inhibition by erlotinib (reduced K*i*) in these in vitro kinase assays. The reduced ATP affinity seen with mutant kinases most probably accounts for their increased sensitivity to the selective EGFR-TKIs, which compete with ATP for binding to the catalytic site (reviewed in [39]). Another study, in which the phage-display method was used to examine the interaction of a large panel of kinases with selective inhibitors, concluded that EGFR mutations, including $\triangle$E746-A750, do not themselves affect the affinity for gefitinib and erlotinib [66].

For unknown reasons, *EGFR* kinase domain mutations seem to be restricted to a subset of NSCLC, although very rare mutations have also been reported in SCLC, cholangiocarcinoma, ovarian, colorectal, head and neck, esophageal and pancreatic cancers [67–71]. Although *EGFR* mutations were present in most cases of NSCLC that were identified by virtue of their dramatic clinical response to TKIs, controversy has surrounded the predictive value of *EGFR* mutations in unselected patients. Approximately 10–20% of patients who do show a partial response to EGFR-TKIs do not have identifiable *EGFR* mutations, indicating that *EGFR* mutations are not the sole determinants of TKI response. Other molecular abnormalities, including the amplification of wild-type *EGFR* or alterations in other ErbB family members have been detected, although it is unclear whether they account for most clinically responsive cases that lack *EGFR* mutations. In particular, the amplification of *EGFR* has been difficult to interpret by itself, because gene copy number alterations that affect both mutant and wild-type *EGFR* alleles have not been distinguished in most studies. In addition, inter-study variability stemming from the different techniques used to measure copy number, including quantitative PCR (qPCR), which provides a "global" copy number assessment, and fluorescence in situ hybridization (FISH), which evaluates copy number at the single cell level, have yielded divergent results, possibly owing to the use of different threshold measurements and the distinction between specific amplification of the EGFR locus versus more general alterations in gene copy numbers linked to aneuploidy. Significantly, *EGFR* kinase mutations

seem to be highly correlated with clinical characteristics that are predictors of TKI-responsive disease, whereas *EGFR* gene amplification, as measured by qPCR, seems to be more common in smoking-associated cancers and does not show the same predilection toward distinct ethnic background and tumor histology (reviewed in [39]).

Recent results, however, suggest that sensitivity to EGFR-TKIs is not simply recapitulated by expressing the mutant constructs in transfected cells, pointing to the importance of cellular context in conferring dependency on the EGFR pathway. Furthermore, caution should be exercised in interpreting in vitro data using NSCLC cell lines as surrogates for clinical responses. NSCLC cell lines show varying degrees of sensitivity to these inhibitors, ranging from hypersensitive (IC_{50} in the low nM) to sensitive (IC_{50} in the high nM) to extremely insensitive (IC_{50} in the high μM). The hypersensitive cell lines NCI-H3255 and PC9 harbor the EGFR tyrosine kinase domain mutations L858R and $\triangle$E746-A750, respectively. Insensitive cell lines such as NCI-H1975 and NCI-H1650, although harboring the same kinase domain mutations (L858R and $\triangle$E746-A750), have additional changes such as T790M (NCI-H1975), phosphatase, and tensin homolog (PTEN) loss (NCI-H1650) or *KRAS* mutations in NCI-H460 cells [72, 73]. Although these cell lines have been used extensively, conclusions derived from such in vitro systems should be interpreted with caution in view of the off-target effects seen with these inhibitors, especially at supra-physiological concentrations, in excess of 1 and 2.5 μM for gefitinib and erlotinib, respectively. The in vitro concentrations used in tissue culture roughly correlate to the plasma concentrations of these drugs in patients treated with the standard doses of these agents (250 mg a day of gefitinib and 150 mg a day of erlotinib), and have been used by researchers as a useful threshold to distinguish sensitive from insensitive and/or resistant cell lines. In vitro studies with NSCLC cell lines have highlighted the fact that gefitinib- and erlotinib-sensitizing mutations invariably hyperactivate the EGFR signaling pathway and promote EGFR-mediated anti-apoptotic and pro-survival signals through the Ras–Raf–MEK (mitogen-activated and extracellular-signal-regulated kinase kinase), ERK1 and ERK2 (extracellular-signal-regulated kinase 1 and 2), PI3K–AKT (phosphatidylinositol-3 kinase–AKT), and STAT3 and STAT5 (signal transducer and activator of transcription proteins 3 and 5) pathways such that cancer cells might become dependent on a functional EGFR for their survival. Interestingly, these are the same pathways that are activated after ligand engagement and are inhibited by gefitinib, including the ERK pathway involved in cell proliferation and the pro-survival AKT pathway. The obvious implication is that shutting off EGFR with specific kinase inhibitors, antibodies, or RNA interference would extinguish these proliferative and survival signals on which the tumor cell is dependent, therefore resulting in tumor cell death. Normal cells (or non-EGFR-dependent tumor cells that do not respond to gefitinib or erlotinib) remain unaffected, as their pro-survival signals are either driven by other genes or can be compensated for by other RTK in the event of EGFR inhibition. This is consistent with the observation that gefitinib and erlotinib response in sensitive cells results in the downregulation of ERK, AKT, and STAT3 and STAT5, whereas a

similar downregulation is not evident in insensitive or resistant cells. Although these pro-survival signaling pathways are probably controlled by many RTK outputs in normal cells, their dependency on mutated and/or activated EGFR in some NSCLC tumors and cell lines bears the hallmark of oncogene addiction (see below).

2.3 Cytoplasmic Signaling Intermediates

2.3.1 The Ras/Raf/MAPK Pathway

The mitogen-activated protein kinase (MAPK) module is a key integration point along the signal transduction cascade that links diverse extracellular stimuli to proliferation, differentiation, and survival [74]. Approximately 20 years of intensive study have led to a quite detailed molecular dissection of this pathway, which has now grown to include five different MAPK subfamilies [ERK-1/2, c-Jun-N-terminal kinase (JNK)-1/2/3, p38 $\alpha/\beta2/\gamma/\delta$, ERK-3/4, and ERK-5], with distinct molecular and functional features [75, 76]. While certain subfamilies, such as the p38 family, are becoming therapeutic targets in inflammatory and degenerative diseases, the MAPK cascade that proceeds from Ras to ERK-1/2 (the main mitogenic pathway initiated by peptide growth factors) is starting to emerge as a prime target for the molecular therapy of different types of human cancer [77–83]. Not surprisingly, this MAPK pathway is indeed aberrantly activated in many human tumors as a result of genetic and epigenetic changes, resulting in increased proliferation and resistance to apoptotic stimuli [79, 81, 82]. The core MAPK signaling module consists of three protein kinases that are sequentially activated by a phosphorylation cascade: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. Fourteen MAPKKKs, 7 MAPKKs, and 12 MAPKs have been so far identified in mammalian cells [75, 84–86]. The bottleneck of signaling through MAPK cascades is recognition and regulation of MAPK by their activating kinases and inactivating phosphatases, where the high degree of specificity characteristic of these signaling modules is achieved. MAPKK, the least numerous in the MAPK module, is the point of convergence of multiple MAPKKK, but exhibit a high specificity toward their respective MAPK substrates, allowing for little or no cross talk between the different families at the MAPK level. These enzymes have the unique ability to phosphorylate their substrates on both Thr and Tyr residues, thereby belonging to a small family of dual specificity kinases [87]. Dual phosphorylation, which is required for full MAPK activation, takes place on Thr and Tyr residues of the – Thr-X-Tyr – sequence present in the activation (or T) loop of MAPKs. The length of the T loop and the identity of the amino acid separating the activating residues differ between individual MAPK and could play a role in the efficiency of substrate phosphorylation; however, they are not essential for the selective recognition of individual MAPK by their respective MAPKK [77]. Inactivation of MAPKs can similarly be achieved by different families of phosphatases that dephosphorylate the activating Thr, the activating Tyr, or both (dual specificity phosphatases). Selectivity

of Tyr and dual specificity phosphatases for individual MAPKs is starting to emerge as another mechanism to achieve specificity in the inactivation of different MAPK modules, in a manner similar to that operating in MAPKK-mediated MAPK activation [88]. In addition to recognition through the active site of regulating kinases and phosphatases, specificity in the activation/inactivation of individual MAPK modules appears to require conserved sequences, distinct from the phospho-acceptor residues, which can be responsible for enhancing the efficiency of substrate phosphorylation and for providing specificity. Some of these sequences, named docking domains (D-domains), are found in MAPK regulatory proteins including MAPKK, MAPK phosphatases, and scaffold proteins, as well as in many transcription factors and other MAPK substrates [89, 90, 77]. These regulatory proteins interact through their D-domains with the same stretch of negatively charged residues of MAPK, thereby directing the level of activation of MAPKs, the phosphorylation of their substrates, and in some cases their subcellular localization. The putative sequence of MAPKs that binds D-domains of regulatory proteins and substrates has been recently identified as a conserved C-terminal common docking motif outside the catalytic domain of ERK, p38, and JNK. Overall, the interaction of MAPKs with their regulatory and effector proteins critically contributes to the spatio-temporal regulation of the intensity, duration, and fidelity of the signal that is transduced through these modules. This tight control of the dynamics of MAPK signaling is a key parameter in setting the multiple biological responses that can be achieved upon growth factor stimulation [89–91, 77].

Among the different MAPK modules thus far identified in eukaryotes, the Raf/MEK/ERK cascade was the first MAPK module to be identified in mammalian cells and is the most extensively studied. This signaling module is activated by several extracellular stimuli that converge on the small G-protein Ras and plays a pivotal role in the control of cell proliferation, differentiation, and survival in response to the engagement of receptor Tyr kinases, G protein-coupled receptors, and integrins [74, 92]. Activated Ras recruits the MAPKKK Raf to the plasma membrane in a necessary, but not sufficient, step of a complex activation process, allowing the mitogenic signal to proceed through the MEK/ERK module [93]. Among Raf family members, B-Raf displays the highest affinity for MEK-1/2 and is the most efficient MEK kinase, but its expression is more restricted as compared to the ubiquitous expression of Raf-1 [94–96]. MEK-1/2 belong to a small family of dual specificity kinases and catalyze the phosphorylation of ERK-1/2 on both Ser/Thr and Tyr residues, allowing their full activation [97, 87, 98]. ERK-1/2, initially identified as the kinases responsible for the Ser/Thr phosphorylation and activation of the ribosomal protein S6 kinases p70^{s6k} and p90^{rsk}, was cloned in 1990 [99] and has been subsequently shown to regulate the expression and function of a wide array of cytoplasmic and nuclear proteins (particularly transcription factors), through transcriptional and non-transcriptional mechanisms [100, 101]. The pivotal role played by the Raf/MEK/ERK module in the physiological regulation of many cellular processes, such as growth, proliferation, differentiation, survival, motility, and angiogenesis, provides the conceptual framework to understand the oncogenic potential of deranged signaling through this MAPK module. Many

cellular oncogenes, such as growth factor receptors and Ras, indeed, critically rely on activation of the Raf/MEK/ERK pathway to induce the transformed phenotype. In addition, members of this MAPK cascade, such as Raf-1, B-Raf, and Mos, have been themselves identified as cellular oncogenes [81, 82]. Germ line *MEK* mutations have been demonstrated in patients with cardio-facio-cutaneous (CFC) syndrome, a complex developmental disorder involving the heart, face, and skin [102], with currently unknown potential for predisposition to cancer; more recently, somatic activating mutations in exon 2 of the *MEK1* gene have been reported in an ovarian cancer cell line [103] and in two patients with lung adenocarcinoma [104]. Although the oncogenic nature of such mutations remains to be demonstrated, it is well established that both MEK and ERK proteins can efficiently transform mammalian cells to a neoplastic phenotype when expressed in constitutively active forms [105–107] and that disruption of their activation by pharmacological inhibitors severely impairs the transforming ability of many upstream-acting cellular oncogenes [108–111]. As a result, constitutive MEK/ERK activation is detected in a significant proportion of a variety of human tumors, including breast, kidney, colon, pancreatic, thyroid, and lung cancers, as well as glioblastomas, and has recently emerged as a potential target for anticancer therapies [79, 82].

Ras and its downstream effectors may actually have paradoxically opposite effects in the regulation of cell cycle progression. Indeed, Ras ability to alter the expression of many cell cycle-regulating molecules, including p16 ^{Ink4a}, p15 ^{Ink4b}, and p21 ^{Cip1}, and can lead to premature cell cycle arrest at the G1 phase and subsequent senescence, in a Raf/MEK/ERK-dependent fashion [112, 113, 81]. On the other hand, overexpression of activated Raf proteins is associated with such divergent responses as cell growth, cell cycle arrest, or even apoptosis [96, 81]. The fate of the cells depends on the level and isoform of Raf kinase expressed. Ectopic overexpression of Raf proteins is associated with cell proliferation in cells including hematopoietic cells; erythroid progenitor cells; and A10 smooth muscle cells [80, 81]. However, overexpression of activated Raf proteins is associated with cell cycle arrest in rat Schwann cells, mouse PC12 cells, human promyelocytic leukemia HL-60 cells, small cell lung cancer cell lines, prostate cancer LNCaP cells, and some hematopoietic cells (reviewed in [81]). Depending on the Raf isoform, overexpression of Raf can lead to cell proliferation (A-Raf or Raf-1) or cell growth arrest (B-Raf) in NIH-3T3 fibroblast and FDC-P1 hematopoietic cells. It is not clear why overexpression of the Raf gene can lead to such conflicting results, but it has been suggested that the opposite outcomes may be determined by the amount or activity of the particular Raf oncoprotein [81]. NIH-3T3 cells have been transfected with the three different Raf genes. The introduced A-Raf molecule was able to upregulate the expression of cyclin D1, cyclin E, Cdk2, and Cdk4 and downregulate the expression of Cdk inhibitor p27Kip1 [114]. These changes induced the cells to pass through G1 phase and enter S phase. However, in B-Raf- and Raf-1-transfected NIH-3T3 cells, there was also a significant induction of p21Cip1, which led to G1 arrest. Using cytokine-dependent FDC-P1 hematopoietic cells transfected with conditionally active mutant *Raf-1*, A-Raf, and B-Raf genes as a model, it has been demonstrated that moderate Raf activation, such as that induced by A-Raf and Raf-1,

leads to cell proliferation, which was associated with the induction of cyclin expression and Cdk activity. However, ectopic expression of the much more potent B-Raf leads to apoptosis [112, 114]. An alternative explanation for the diverse proliferative results obtained with the three Raf genes is the different biological effects of *A-Raf*, *B-Raf*, and Raf-1. The individual functions of these three different Raf proteins are not fully understood. Even though it has been shown that all three Raf proteins are activated by oncogenic Ras, target the same downstream molecules, i.e., MEK1 and MEK2, and use the same adaptor proteins for conformational stabilization, different biological and biochemical properties have been reported and their functions are not always compensatable [115, 96, 81]. Moreover, targeted disruption of individual *raf* genes in the mouse has demonstrated that their functions are not fully redundant, since null mutations for each gene result in distinct phenotypes, and has confirmed that B-Raf is the major MEK activator in vivo [116].

The Raf/MEK/ERK cascade and Raf itself also have diverse effects on key molecules involved in the prevention of apoptosis. The Raf/MEK/ERK pathway can phosphorylate Bad on S112, thereby leading to its inactivation and subsequent sequestration by 14-3-3 proteins [117]. This, in turn, allows Bcl-2 to form homodimers and an anti-apoptotic response is generated. In addition to BAD, the Raf/MEK/ERK cascade can also lead to the phosphorylation of the anti-apoptotic Mcl-1 and the pro-apoptotic Bim proteins. In particular, phosphorylation of Bim results in its disassociation from Bcl-2, Bcl-XL, and Mcl-1 and Bim becomes ubiquitinated and targeted to the proteasome. This allows Bcl-2, Bcl-XL, and Mcl-1 to bind Bax and prevent Bax activation and the formation of Bax:Bax homodimers. Thus apoptosis is inhibited [118–120, 117]. ERK phosphorylation of Bim on S69 can result in ubiquitination of Bim and subsequent proteosomal degradation [121]. In contrast, phosphorylation of Bim at S65 by JNK can result in apoptosis due to stimulation of Bax:Bax interactions. JNK also phosphorylates 14-3-3 family members, which allow translocation of Bax from the cytosol to the mitochondria membrane where it can promote apoptosis (reviewed in [81]). More controversially, Bcl-2 can also be phosphorylated by the Raf/MEK/ERK cascade on certain residues in the loop region, which has been associated with enhanced anti-apoptotic activity [122, 123]. Recently, it has been shown that the Raf/MEK/ERK cascade can phosphorylate caspase 9 on residue T125, which contributes to the inactivation of this protein [124]. Interestingly, both Bad and caspase 9 are also phosphorylated by the AKT pathway [125] indicating that the Raf/MEK/ERK and PI3K/AKT pathways can cross talk and result in the prevention of apoptosis (see below). As noted earlier, Raf-1 has MEK- and ERK-independent functions at the mitochondrial membrane by phosphorylating Bad, which results in its disassociation from the mitochondrial membrane [94]. Recently Raf-1 was shown to interact with mammalian sterile 20-like kinase (MST-2) and prevent its dimerization and activation [126]. MST-2 is a kinase, which is activated by pro-apoptotic agents such as staurosporine and Fas ligand. Raf-1 but not B-Raf binds MST-2. Depletion of MST-2 from Raf-1–/– cells abrogated sensitivity to apoptosis. Overexpression of MST-2 increased sensitivity to apoptosis. It was proposed that Raf-1 might control MST-2 by sequestering it into an inactive complex. This complex of Raf-1:MST-2 is independent of MEK

and downstream ERK. Raf-1 can also interact with the ASK1 to inhibit apoptosis [127]. ASK1 is a general mediator of apoptosis and it is induced in response to a variety of cytotoxic stresses including TNF, Fas, and ROS. ASK1 appears to be involved in the activation of the JNK and p38 MAP kinases. This is another example of MEK/ERK-independent interactions of Raf-1.

Amplification of ras proto-oncogenes and activating mutations that lead to the expression of constitutively active Ras proteins are observed in approximately 30% of human cancers [128]. *B-Raf* has been reported to be mutated in approximately 7% of all cancers [129]. However, it was recently shown that *B-Raf* is frequently mutated in certain types of cancer, especially melanoma (27–70%), papillary thyroid cancer (36–53%), colorectal (5–22%), and ovarian cancer (30%) [130, 129]. The most common *B-Raf* mutation is a change at nucleotide 600, which converts a valine to a glutamic acid (V600E). This *B-Raf* mutation accounts for over 90% of the *B-Raf* mutations found in melanoma and thyroid cancer. In some cells, *B-Raf* mutations are believed to be initiating events but not sufficient for full-blown neoplastic transformation. Moreover, there appears to be cases where certain *B-Raf* mutations (V600E) and *Ras* mutations are not permitted in the transformation process as they might result in hyperactivation of Raf/MEK/ERK signaling and expression, which leads to cell cycle arrest [130]. In contrast, there are other situations, which depend on the particular *B-Raf* mutation and require both *B-Raf* and *Ras* mutations for transformation. The *B-Raf* mutations in these cases result in weaker levels of B-Raf activity [130, 131]. The reasons for mutation at *B-Raf* and not *Raf-1* or *A-Raf* in certain cancer, such as melanoma, are not entirely clear. Based on the mechanism of activation of B-Raf, it may be easier to select for B-Raf than either Raf-1 or A-Raf mutations. It has been recently proposed that the structure of B-Raf, Raf-1, and A-Raf may dictate the ability of activating mutations to occur at these molecules, which can permit the selection of oncogenic forms [129, 132]. These predictions have arisen from determining the crystal structure of B-Raf. Like many enzymes, B-Raf is proposed to have small and large lobes, which are separated by a catalytic cleft. The structural and catalytic domains of B-Raf and the importance of the size and positioning of the small lobe may be critical in its ability to be stabilized by certain activating mutations. In contrast, the precise substitutions in A-Raf and Raf-1 are not predicted to result in small lobe stabilization thus preventing the selection of mutations at *A-Raf* and *Raf-1*, which would result in activated oncogenes [132]. Recent studies also indicate that mutated alleles of *Raf-1* are present in therapy-induced acute myelogenous leukemia (t-AML) [133], arising after chemotherapy treatment for breast cancer. The mutated *Raf-1* genes detected were transmitted in the germ line, thus they are not a spontaneous mutation in the leukemia but may be associated with the susceptibility to induction of t-AML in breast cancer patients. Most interestingly from a therapeutic perspective, *BRAF* mutations may constitute the Achilles' heel of malignant melanoma, as well as of other malignancy, since *BRAF*-mutated tumors appear to be exquisitely sensitive to clinically available MEK inhibitors, when compared with wild-type cells and cells harboring various *RAS* mutations [134]. From a molecular standpoint, data from Garnett et al. [135] indicate that, even though a small fraction of *BRAF*

mutations generates an enzyme that is impaired in its ability to activate the downstream MEK/ERK cascade, kinase-impaired mutants also work through the mitogenic cascade culminating in ERK activation. The mechanism is rescue of kinase-impaired mutant *BRAF* by wild-type *CRAF* through a process that involves 14-3-3-mediated hetero-oligomerization and transactivation [135, 136]. Finally, it has been reported that a high frequency of acute myeloid leukemias (AML) and acute lymphocytic leukemias (ALL) displays constitutive activation of the Raf/MEK/ERK pathway in absence of any obvious genetic mutation [137, 138, 109–111]. While there may be some unidentified mutation at one component of the pathway or a phosphatase, which regulates the activity of the pathway, the genetic nature of constitutive activation of the Raf/MEK/ERK pathway is unknown. Elevated expression of ERK in AMLs and ALLs is associated with a poor prognosis and Raf, and potentially MEK inhibitors, may prove useful in the treatment of a large percentage of AML and ALL [109–111].

2.3.2 The PI3K/AKT/mTOR Pathway

Phosphoinositide 3-kinases (PI3K) are a family of proteins involved in the regulation of cell growth, metabolism, proliferation, glucose homeostasis, and vesicle trafficking [139]. Most of the members of this family are bound to regulatory subunits, which determine the signals modulating its activity. There are three members in the family [140]: class I PI3K, which is divided into IA and IB, is activated by RTK (PI3K1A) and G-protein-coupled receptors (GPCR, PI3K1B). Class IA and IB PI3K have different regulatory subunits, p85a/p85b/p55 for IA and p101/p84/p87PIKAP for IB. This class is characterized for generating primarily PI-3,4,5-P3 (PIP3) [141]. PI3K class II utilizes PI-3-P in vitro to generate PI-3,4-P2 and can also produce PI-3-P from PI. This class does not require a regulatory subunit to function and comprises three different isoforms (α, β, and γ) that diverge in the N terminus and present different domains within the C terminus. Class II PI3K is involved in membrane trafficking and receptor internalization and can be activated in response to RTK, integrins, and cytokine receptors [139]. Class III PI3K (Vps34), which was first identified in the budding yeast, is involved in vesicle trafficking and cross talks with class I PI3K through the regulation of mTORC1 signaling (see below). Class I PI3K is the most studied among the three members of the family [142].

The phosphatase and tensin homolog deleted on chromosome #10 (PTEN) was originally discovered as a candidate tumor suppressor mutated and lost in various cancers [143, 144]. Several lines of evidence soon highlighted PTEN as a lipid phosphatase hydrolyzing phosphates in position 3′ from phosphoinositides [145]. The major function of PTEN is the buffering of PI3K signaling; yet recent studies point to additional novel, lipid phosphatase-independent functions that may contribute to its tumor suppressive activity. The loss and mutation of PTEN in various cancers lead to hyperactive PI3K signaling. For example, PTEN is commonly mutated in

its phosphatase domain [146]; and in glioblastoma, mutations that impair its proper membrane localization might result in deficient tumor suppressive activity [147]. It is therefore clear that PTEN is a main player in the regulation of PI3K signaling and perturbations in its levels or function can dramatically impact on this pathway [142].

Upon 3′ phosphorylation of PI-4,5-P2 by PI3K, proteins containing pleckstrin homology (PH) and PH-like domains are recruited to the plasma membrane, thereby transmitting the signal elicited by PI3K activation [139]. One of the best-characterized members of this group of proteins is the pro-survival AKT kinase. AKT contains a PH domain; upon PIP3 production it becomes anchored to the membrane, where another phosphoinositide-binding protein, PDK1 (3-phosphoinositide-dependent protein kinase 1 [148]), and a recently discovered protein complex, mTORC2 [149], phosphorylate and activate the kinase. Activated AKT mediates several of the well-described PI3K responses, mainly growth, metabolism, survival, and glucose homeostasis [150]. Therefore, the PI3K–AKT axis is considered the canonical PI3K signaling. In addition, PI3K leads to the modulation of other pathways that are of great importance for the described function of this kinase. AKT phosphorylates up to 100 substrates thereby modulating a variety of cellular functions. First, AKT signaling exerts a strong anti-apoptotic effect through the phosphorylation and inhibition of key pro-apoptotic proteins, such as BAD, MDM2, and members of the Forkhead family (reviewed in [142]). Second, AKT activates cell proliferation by inactivating p27 [151] and inhibiting glycogen synthase kinase 3 (GSK3)-mediated Myc and cyclin D1 inhibition [152]. Third, this kinase regulates a subset of proteins involved in growth, metabolism, and angiogenesis. AKT phosphorylates and inactivates GSK3β, increases glucose transporter Glut4 translocation to plasma membrane by blocking AS160, and, through FOXO inactivation, inhibits phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [142]. All these actions converge in an increased glucose catabolism rate. Additionally, AKT is one of the main regulators of a complex involved in protein translation and ribosome biogenesis; this is mTORC1, which is composed of the protein kinase mTOR and a series of interactors [153].

In response to growth factors and nutrients mTORC1 (mammalian target of rapamycin complex 1) regulates cell growth by modulating many processes, including protein synthesis, ribosome biogenesis, and autophagy [142, 154]. mTORC1 is a heterotrimeric protein kinase that consists of the mTOR catalytic subunit and two associated proteins, raptor (regulatory-associated protein of mTOR) and mLST8 (also known as GβL). The molecular mechanisms that regulate mTORC1 kinase activity are still poorly understood, but it is increasingly clear that many if not most cancer-promoting lesions activate the mTORC1 pathway. Most dramatically, the TSC1 (tuberous sclerosis 1, also known as harmartin)–TSC2 (also known as tuberin) tumor suppressor complex, the inactivation of which causes the tumor-prone syndrome tuberous sclerosis complex (TSC) and the related disease lymphangioleiomyomatosis (LAM), has emerged as a key negative regulator of mTORC1 [155, 156]. The TSC1–TSC2 heterodimer is a GTPase-activating protein for Rheb (Ras homolog enriched in brain), a GTP-binding protein that activates

mTORC1, most probably by binding to it. TSC1–TSC2 and Rheb also have important roles in the activation of mTORC1 that occurs when cells lose the PTEN, NF1 (neurofibromatosis 1), LKB1 (also known as serine–threonine kinase 11), or p53 tumor suppressors (reviewed in [157], 154). In all cases, inactivation of the tumor suppressor triggers a pathway that eventually leads to inhibition of TSC1–TSC2. For example, as discussed above, the loss of PTEN activates AKT, which then directly phosphorylates and inhibits TSC1–TSC2, whereas the loss of LKB1 suppresses AMPK (AMP-activated protein kinase), which normally mediates an activating phosphorylation of TSC1–TSC2. The mTORC1 pathway regulates growth through downstream effectors, such as the regulators of translation 4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) and S6K1 (ribosomal S6 kinase 1) [157, 154]. In addition to its role in promoting protein synthesis, S6K1 represses the PI3K–AKT pathway by inhibiting IRS1 (insulin receptor substrate 1) and IRS2 expression (reviewed in [154], [142]). Therefore, an active mTORC1 pathway can suppress PI3K–AKT signaling, helping to explain the non-aggressive nature of the tumors that are found in TSC [158, 159]. The opposite is also true: inhibition of mTORC1 activates PI3K–AKT signaling and the activation of PI3K–AKT that is caused by mTORC1 inhibitors might significantly diminish the anti-tumor activity of such molecules. Mammalian TORC2 also contains mTOR and mLST8 but, instead of raptor, it contains two proteins, rictor (rapamycin-insensitive companion of mTOR) and mSin1 (also known as mitogen-activated protein kinase-associated protein 1), that are not part of mTORC1. This second mTOR-containing complex is less understood than mTORC1 but recent work indicates that it should be considered part of the PI3K–AKT pathway as it directly phosphorylates AKT [160, 149] on one of the two sites that are necessary for AKT activation in response to growth factor signaling. This finding makes mTORC2 a key part of the pathway that activates AKT and, like PDK1 and PI3K, a potential drug target for cancers in which there is AKT deregulation. The AKT-activating function of mTORC2 sets up the intriguing situation in which mTOR, as part of two distinct complexes, is potentially both "upstream" and "downstream" of itself. Mammalian TORC2 has other functions besides activating AKT, such as regulating the cytoskeleton [161, 162], but the implications for cancer of these roles are still unknown. Mammalian TOR was discovered in the early 1990s in studies into the mechanism of action of rapamycin (also known as sirolimus), which is a macrolide that was originally found as an antifungal agent and was later recognized as having immunosuppressive and anti-cancer properties. Even today, exactly how rapamycin perturbs mTOR function is not completely understood. The complex of rapamycin with its intracellular receptor FKBP12 binds directly to mTORC1 and, at least in vitro, suppresses mTORC1-mediated phosphorylation of the substrates S6K1 and 4EBP1. Rapamycin also weakens the interaction between mTOR and raptor [163], which is a component of mTORC1 that can recruit substrates to the mTOR kinase domain [164]. It is not known if mTORC1 has functions that depend on its kinase activity but are not sensitive to rapamycin, so it is still unclear if a molecule that directly inhibited the mTORC1 kinase domain would have different biological effects to those of rapamycin. A rapamycin analog, CCI-779 (also known as temsirolimus), has

recently been approved for the treatment of renal cell cancer and mantle cell lymphoma and two other, RAD001 (also known as everolimus) and AP23573, are currently in clinical development for anticancer use in humans. These molecules inhibit mTORC1 through the same mechanism of action as rapamycin, but have different pharmacokinetic and solubility properties that increase their desirability for clinical use. In contrast to mTORC1, FKBP12-rapamycin cannot bind directly to mTORC2 [161, 162], suggesting that the effects of rapamycin on cellular signaling are due to inhibition of mTORC1. A potentially important wrinkle in this seemingly closed story has recently emerged [165]. It turns out that prolonged treatment with rapamycin – clearly a situation that is relevant to its use in patients – perturbs mTORC2 assembly and, in about 20% of cancer cell lines, the drop in intact mTORC2 levels is sufficient to strongly inhibit AKT signaling. The binding of FKBP12-rapamycin to mTOR seems to block the subsequent binding of the mTORC2-specific components rictor and mSin1 [166, 165] but it is unknown why in certain cell types rapamycin only partially inhibits mTORC2 assembly. No absolute correlation exists between the tissue of origin of a cell line and the sensitivity of mTORC2 formation to rapamycin, although many cell lines with this property are derived from the hematological system. Recent work provides the first evidence that mTORC2 function can be rapamycin-sensitive in patients. In more than 50% of patients with AML, rapamycin and its analogs inhibited AKT phosphorylation in primary leukemic cells and the inhibition correlated with the loss of intact mTORC2 [167]. So, rapamycin and its analogs are universal inhibitors of mTORC1 and S6K1, and cell-type specific inhibitors of mTORC2 and AKT. As the inhibition of mTORC2 by rapamycin is time and dose dependent, AKT activity in tumors will vary with the length of rapamycin treatment and the dosing regimen. It is important to keep in mind that, because inhibition of mTORC1 and mTORC2 will not always occur at the same time, markers of mTORC1 inhibition, such as loss of phosphorylated S6, will not necessarily reflect mTORC2 activity. The capacity to sometimes inhibit mTORC2 might help explain why the cellular effects of rapamycin vary among cancer cell lines. Moreover, in a tumor this inhibition might have the beneficial effect of preventing the activation of AKT, through inhibition of S6K1, that rapamycin would otherwise cause.

The kinase activity of PI3K was first reported to be associated with viral oncoproteins [168]. Subsequent studies employing mouse knockouts of both the regulatory and catalytic subunits of PIK3 resulted in a number of deficits including embryonic lethality, B cell defects, liver necrosis, and colorectal cancer [141]. Other investigations showed that the amplification of the *PI3K* locus as well as deletions of short nucleotide sequences resulted in elevated lipid kinase activity of the p110a catalytic subunit of PI3K (PIK3CA) in various cancer types with the implication that PI3K was functioning as an oncogene (reviewed in [169]). *PIK3CA* is a 34 kb gene located on chromosome 3q26.3 that consists of 20 exons coding for 1068 amino acids yielding a 124 kDa size protein. Gene amplifications, deletions, and more recently somatic missense mutations in the *PIK3CA* gene have been reported in many human cancer types including cancers of the colon, breast, brain, liver, stomach, and lung. These somatic missense mutations were proposed to increase the
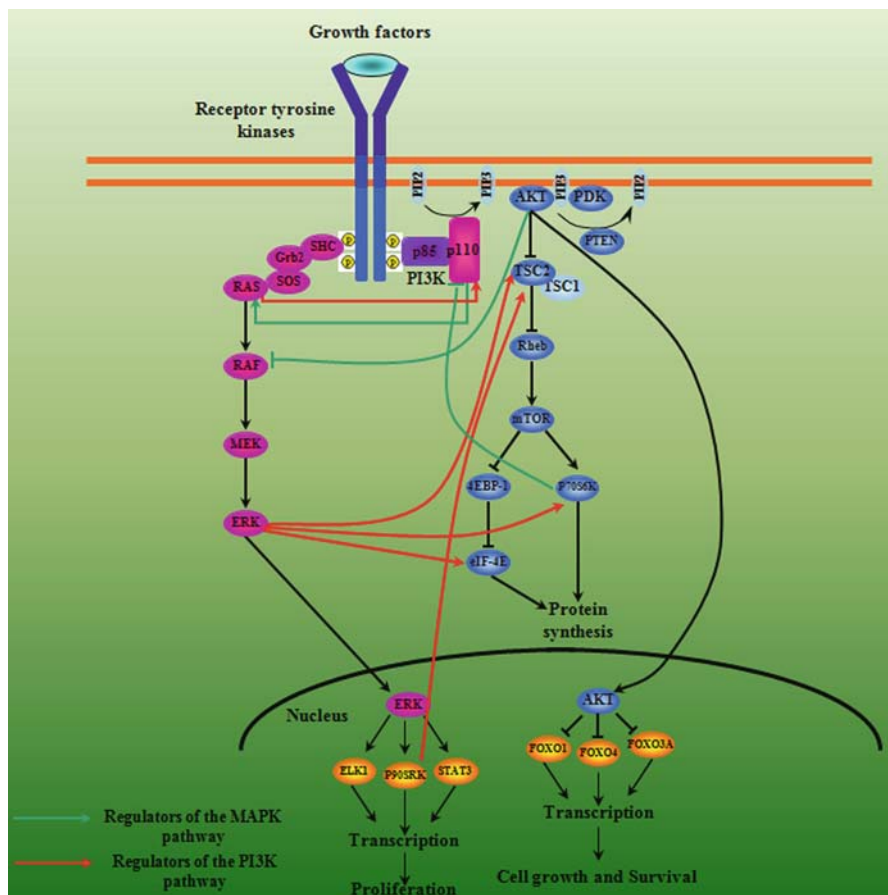
kinase activity of PIK3CA contributing to cellular transformation. The first of these mutational reports was published by Samuels et al. [170]. In this seminal paper, the authors initially analyzed the sequence of eight *PI3K* and eight *PI3K*-like genes in a relatively small number of primary colorectal tumors and discovered that *PIK3CA* was the only gene harboring somatic mutations. They subsequently expanded their sample size, which included tissues from primary tumors of the colon, brain, breast, stomach, and lung. Their results verified their initial observations and demonstrated that somatic mutations were found in all of these tissues at varying frequencies. Notably, colorectal, brain, and gastric cancers were found to have a high rate of *PIK3CA* gene mutation with frequencies of 32, 27, and 25%, respectively. Somatic missense mutations were scattered across most of the exons, but were predominantly found in the kinase and helical domains of the *PIK3CA* subunit [169]. Of note, "hotspot" or frequently recurring mutations were found in exon 9 (G1624A:E542K) and exon 20 (A3140G:H1047R) in this analysis. Based on all sequencing data, there now appear to be three hotspots mutations within *PIK3CA*: H1047R, E542K, and E545K. Bachman et al. subsequently demonstrated that, on average, 25% of breast cancers harbor missense mutations in the kinase, helical, or p85-binding domains [171]. Many other studies followed, examining *PIK3CA* mutations in various cancer types. Campbell et al. sequenced all of the 20 coding exons of *PIK3CA* from primary tumor samples of breast, ovarian, and colorectal cancers and reported new mutations found in exons 6, 7, and 9, as well as mutations previously reported by others [172]. They reported a *PIK3CA* mutation frequency of 18.8% in colorectal cancers and 40% in breast cancer samples. The frequency of ovarian cancers was reported as 6%, but of note, mutations clustered according to the histologic subtype with endometrioid and clear cell variants having a much higher rate than serous and mucinous ovarian cancers. In a more recent analysis by Saal et al. that examined a total of 292 primary breast cancers an overall *PIK3CA* mutation rate of 26% was found, with a statistically significant correlation between the presence of mutations and the presence of nodal metastases, estrogen/progesterone receptor positivity, and Her2/neu receptor overexpression/amplification [173]. They also demonstrated a statistically significant correlation between the presence of *PIK3CA* mutations and the presence of PTEN expression, an intriguing finding given the known roles of these two pathways and similar findings in brain cancers [174], where a mutational rate of 5% was found. Another recent study demonstrated a very high rate (36%) of *PIK3CA* somatic mutations in liver cancer [175]. Interestingly, the authors also found one *PIK3CA* mutation out of 88 acute leukemias (mutation rate 1.1%) that were analyzed in this study, suggesting that *PIK3CA* mutations are not limited to solid tumors of epithelial origin. An analysis of *PIK3CA* somatic mutations and amplifications in thyroid cancers did not reveal any *PIK3CA* mutations; however, this group did find *PIK3CA* gene amplification in 12% of thyroid adenomas, 5% of papillary thyroid cancers, 24% of follicular thyroid cancers, and 71% of thyroid cancer cell lines [176]. More recently, somatic mutations in genes downstream of the PI3K signaling pathway (i.e., *PDK1*, *AKT2*, and *PAK4*) have also been reported [177].

Although the frequency of mutations and the discovery of hotspot heterozygous mutations strongly argue for the importance of *PIK3CA* in the carcinogenic process, functional analysis of these mutations has also been performed to confirm this supposition. Overexpression of common hotspot *PIK3CA* mutations, as well as gene deletion experiments using somatic cell knockouts, has demonstrated that these mutations are in fact oncogenic (reviewed in [169]). Kang et al. [178] overexpressed cDNAs containing the common PIK3CA mutations, E542K, E545K, and H1047R, in chicken embryo fibroblasts. Their study demonstrated that overexpression of these mutant PIK3CA proteins led to cellular transformation with concomitant phosphorylation of proteins in the AKT pathway. Through the use of somatic cell knockouts, Samuels et al. [179] reported that mutation of the *PIK3CA* kinase domain in the HCT116 colon cancer cell line and mutation of the helical domain in the DLD1 colon cancer cell line resulted in increased activity of the PIK3CA enzyme as manifested by increased cell signaling, cell growth, and invasion. Another functional study examining the E542K, E545K, and H1047R hotspots found that an increase in PIK3CA kinase activity and cellular transformation occurred when the above-mentioned mutant *PIK3CA* sequences were introduced into mouse NIH 3T3 cells [180]. On average, *PIK3CA* gene is mutated in approximately 15% of human cancers, although there is obviously great variability in the tissue type. In most tissue types, mutations predominantly cluster within the three aforementioned hotspots: E542K, E545K, and H1047R. It is now evident that cancers of the liver, colon, and breast harbor the most *PIK3CA* mutations with average mutational frequencies (across the reported studies) of 36, 26, and 25%, respectively. Despite a certain degree of discrepancy in the reported *PIK3CA* mutation rates, their high frequency and the discovery of hotspot mutations have important clinical implications for diagnosis, prognosis, and therapy.

2.3.3 Signaling Cross talk

Emerging evidence indicates that, although separate, the RAF/MEK/ERK and the PI3K/PTEN/AKT pathways are intimately linked (Fig. 2.1). Both signaling cascades are frequently deregulated in cancer and there is accumulating evidence that they may cooperate to promote the survival of transformed cells [80]. In fact, RAS activation regulates activation of both pathways [181]; moreover, both pathways may result in the phosphorylation of many downstream targets and impose a role in the regulation of cell survival and proliferation.

The PI3K pathway may impact on MAPK signaling at multiple levels. In some cell types, the PI3K pathway can directly modulate RAF kinase bypassing the GTPase RAS. RAF activity is negatively regulated by AKT indicating a cross talk between the two pathways. AKT phosphorylates c-RAF and B-RAF on Ser259, thereby inhibiting RAF activity and downstream MAPK signaling [182, 183]. In addition, the GTPase Rheb has also been shown to negatively regulate RAF [184, 185]. A novel mTOR-MAPK/ERK feedback loop has recently

Fig. 2.1 PI3K/PTEN/AKT/mTOR and RAF/MEK/ERK pathways and their potential interactions in transformed cells. The RAS/RAF/MEK/ERK and PI3K/PTEN/AKT/mTOR signaling cascades transduce many signals from growth factor receptors to regulate gene expression. These pathways interact with each other to regulate growth and in some cases tumorigenesis. The RAS signaling pathway can be triggered by a set of RTK that are activated by growth factors. RAS can then activate PI3K or RAF, as described. Several members of the PI3K (PI3K, AKT, p70S6K) control the activation status of the RAS-MAPK pathway (*green arrows*). On the other hand, the PI3K signaling pathway is also regulated by other pathways, such as signaling through the MEK/ERK module. The RAS-MAPK pathway modulates the PI3K pathway at multiple levels (*red arrows*): RAS can regulate the activity of PI3K; ERK can regulate the activity of TSC2, p70S6K, and eIF-4E; and p90SRK can regulate TSC2 activity

been demonstrated [186]. In this study, the authors reported the involvement of S6 kinase in the negative regulation of ERK activation, while treatment with mTOR inhibitors resulted in a hyperactive PI3K pathway, increasing the signal toward the RAS/RAF/MEK/ERK pathway [186].

The PI3K pathway also receives regulatory signals from the MAPK pathway. PTEN transcription is regulated by RAS in cancer cells leading to tumor progression [187, 188]. The TSC complex is also regulated by MAPK at two levels: p90 ^{RSK1} phosphorylates TSC2 on Ser1798, thereby inhibiting the tumor suppressor function of the tuberin/hamartin complex and resulting in increased mTOR signaling to S6K1 [189]; and ERK can phosphorylate TSC2 on Ser664 leading to TSC1–TSC2 inhibiting mTOR activity [190]. In addition, a novel link between the RAS/MAPK pathway and the mTOR signaling was recently described. In this study, the authors demonstrate that raptor is phosphorylated by p90 ^{RSK1} and p90 ^{RSK2} protein kinases in vitro and in vivo and that RSK-mediated phosphorylation of raptor positively regulates mTOR kinase activity [191].

Treatment of human disease with drug combinations might be exploited therapeutically. It has recently been demonstrated that even in aggressive melanoma cell lines that are resistant to single-pathway MEK or PI3K inhibition, the combination of MEK with PI3K inhibitors suppresses the growth and invasion of metastatic melanoma cells [192, 193]. These data support the hypothesis that in the treatment of melanoma, and perhaps many other cancers, it is not sufficient to inhibit only a single constitutively activated signaling pathway and that an effective treatment strategy must take into account more than one deregulated signaling pathway. In a subsequent study, the authors reported the effects of simultaneous treatment with an inhibitor of MEK-1/2 (PD0325901) and mTOR (rapamycin) using PTEN deficient transgenic prostate cancer. They reported that these agents effectively inhibited their targets and, when combined, interacted synergistically to prevent prostate cancer cell growth both in vitro and in vivo. In patient specimens, activation of ERK and the PI3K/PTEN/AKT/mTOR pathway was associated with prostate cancer progression; moreover, the authors found that combined MEK/ERK and mTOR inhibition was effective in the adjuvant setting. The authors concluded that a strategy combining MEK/ERK and mTOR inhibition may be effective in the treatment of advanced cancer [194, 195].

2.4 Oncogenic Addiction

As mentioned above, carcinomas of the lung, colon, breast, and other organ sites often display mutations in multiple oncogenes and tumor suppressor genes, harbor epigenetic abnormalities that result in increased or decreased expression of hundreds of genes, and contain chromosomal abnormalities that include aneuploidy and loss of heterozygosity at numerous loci. It is therefore surprising that despite this extensive disruption in the genomes of cancer cells, there are several examples in both experimental systems and cancer patients whereby the reversal of only one or a few of these abnormalities can profoundly inhibit the growth of cancer cells and, in some cases, lead to improved survival rates. A few years ago this phenomenon was termed as "oncogene addiction," to emphasize the apparent dependency of some cancers on one or a few genes for both maintenance of the malignant phenotype and cell survival.

Evidence to support the concept of oncogene addiction has been obtained in three diverse systems: genetically engineered mouse models of human cancer; mechanistic studies in human cancer cell lines; and clinical trials involving specific molecular targeted agents [196, 197]. Several investigators have generated transgenic mice that overexpress an oncogene in a specific target tissue under conditions in which the oncogene can be switched on or off: Felsher and Bishop used this model system and found that switching on the c-myc oncogene in the hematopoietic cells of mice led to the development of T cell and myeloid leukemias; however, when this gene was subsequently switched off the leukemia cells stopped dividing and displayed differentiation and apoptosis [198]. Dependence on continued expression of a single oncogene for maintenance of the neoplastic state has also been seen in similar murine models of other tissues, including: myelocytic leukemia induced by the Bcr-Abl oncogene; melanoma induced by the H-ras oncogene; lung tumors induced by the KRAS oncogene; pancreatic β-cell tumors and osteogenic sarcoma induced by the c-myc oncogene; breast (mammary) tumors induced by the Her-2/neu oncogene; breast tumors induced by the c-myc oncogene; and breast tumors induced by the Wnt oncogene (reviewed in [196]). In the c-myc breast cancer model, when the c-myc oncogene was switched off 50% of the breast tumors regressed, but the remaining 50% showed only partial regression. Furthermore, breast tumors that recurred were found to be c-myc independent and some of these displayed an activated KRAS oncogene [199]. Similarly, in the Her-2/neu breast tumor model, tumors that recurred were found to be Her-2/neu independent, possibly in relation to increased expression of the transcription factor Snail [200, 201]. In the Wnt-1 murine model, even though downregulation of Wnt-1 resulted in rapid and extensive regression of aneuploid and invasive breast tumors and pulmonary metastases, a number of breast tumors recurred that were Wnt independent. Apparently, recurrence was caused by acquisition of mutations in the p53 tumor suppressor gene [202]. Despite the aforementioned examples of "escape from oncogene addiction," a variety of studies using human cancer cell lines also indicate that although these cells are aneuploid and carry numerous genetic and epigenetic abnormalities, they can also be highly dependent on the activity of a single oncogene for maintaining the malignant phenotype. Blocking the expression of HER2, cyclin D1, KRAS, β-catenin, cyclin E, B-Raf, or microphthalmia transcription factor (MITF) using either antisense DNA or RNA interference (RNAi) strategies can markedly inhibit the in vitro growth of various types of human cancer cells (reviewed in ref [196]). In some cases, blocking oncogene expression also increases the sensitivity of these cells to specific chemotherapy agents and inhibits their tumorigenicity in mice [203]. As a result of the efficacy of the RNAi method for inhibiting the expression of specific genes, the list of such examples of oncogene addiction is now rapidly expanding. The most convincing and clinically relevant evidence for the concept of oncogene addiction comes from the increasing number of examples (i.e., prospective randomized trials) of the therapeutic efficacy of antibodies or drugs that target specific oncogenes in human cancers. One of the earliest examples is the use of the antibody trastuzumab, which targets the receptor tyrosine kinase HER2 (see also Chapter 13). This membrane-associated receptor is overexpressed in 20–30% of

breast cancers and it is now established that use of trastuzumab in these patients can markedly inhibit tumor growth and prolong patient survival in both the adjuvant and metastatic settings [204–207]. Within the past few years several low molecular weight drugs have been developed that target and inhibit the activity of other specific protein kinases that have key roles in the growth and survival of human leukemia and carcinoma cells [80, 108, 208, 110, 209, 210, 111]. The remarkable therapeutic efficacy of some of these compounds provides direct evidence for the concept of oncogene addiction: examples include imatinib, which targets the oncogenic BCR/ABL protein in CML and the mutant oncogene *c-kit* in GIST [211, 212] and the EGFR-targeted drugs NSCLC (see above), colorectal, head, and neck, and pancreatic cancer, as well as glioblastoma [213]. Such clinical studies also provide mechanistic insights into the phenomenon of oncogene addiction. For example, in a subset of patients with CML who initially responded to imatinib but later suffered a relapse, examination of the leukemic cells showed a de novo mutation in the kinase domain of the BCR/ABL protein, which blocked the inhibitory activity of imatinib [214]. A similar "resistance" mechanism has also been described for NSCLC with a mutated *EGFR*, who relapse after an initial dramatic response to gefitinib ([57–60] and reviewed in [39]). The strong selective pressure for emergence of cells that carry de novo mutations in the respective oncogenes indicates the remarkable dependence of these neoplastic cells on these oncogenes and provides further evidence for the concept of oncogene addiction. At the same time these findings reveal the emergence of resistance mechanisms to molecular targeting agents. Studies in progress indicate that, in the case of the *Bcr*/Abl oncogene, there are other drugs that can inhibit the kinase activity of the mutant BCR/ABL protein [215] and it could be possible to develop similar drugs that act on resistant mutants of the EGFR and resistant forms of other protein kinases [216]. Furthermore, it might be possible to suppress the emergence of these types of resistant cells by combining a specific protein kinase inhibitor with an agent that inhibits cell proliferation via a different mechanism; this approach would limit the likelihood of the emergence of mutant clones.

It has been proposed that the phenomenon of oncogene addiction is a consequence of the fact that the multistage process of carcinogenesis is not simply a summation of the individual effects of activation of multiple oncogenes and inactivation of multiple tumor suppressor genes [217, 218]. This proposal is consistent with the fact that the proteins encoded by these genes often have multiple roles in complex and interacting networks, which display both positive and negative feedback control. The function of these proteins is also influenced by their levels of activity and the context in which they are expressed. Thus, a given oncogene can enhance cell proliferation but it can also enhance apoptosis. Furthermore, throughout the multistage carcinogenic process, the evolving cancer cell must maintain a state of homeostasis between positive-acting and negative-acting factors in order to maintain structural integrity, viability, and the capacity to replicate. For these reasons, the intracellular circuitry or "wiring diagram" that regulates signal transduction and gene expression in cancer cells is very different, i.e., "bizarre," when compared to that of normal cells. In cancer cells a given oncogene may play a more essential and

qualitatively different role in a given pathway or "module" compared with its role in normal cells. Thus, cancer cells may be much more dependent on the activity of a specific oncogene than normal cells. Within the context of disordered cell circuitry, specific mechanisms have been proposed to explain why inactivation of an oncogene might lead to selective growth inhibition, differentiation, and/or apoptosis in cancer cells but not in normal cells that express the same oncogene. One explanation is that, in order to maintain homeostasis, the proliferation-enhancing effects of a specific oncogene in cancer cells might be partially buffered through negative feedback mechanisms, through increased expression of proliferation-inhibitory factors. If this oncogene is then inactivated the cancer cells might suffer a relative excess of the latter inhibitory factors and thus undergo apoptosis, before a new level of homeostasis can be achieved. The apparent propensity of some cancer cells to undergo apoptosis when stressed could enhance this process. A second mechanism is based on the concept of "synthetic lethality" originally derived from studies in lower organisms [219]. According to this concept, two genes are said to be synthetic lethal if mutation of one of the two genes is compatible with survival but mutation of both genes causes cell death. For example, certain cancer cells might be highly dependent on a given oncogene because during their development they lost the function of another gene that normally performs a similar function. A drug that inhibits the activity of the oncogene would, therefore, selectively target the cancer cells and spare the normal cells. Furthermore, because of the bizarre circuitry of cancer cells, pairs of genes in cancer cells that have a synthetic lethal relationship may differ from those in normal cells, thus increasing the dependence of tumor cells on a specific oncogene. A related explanation for oncogene addiction is that, during the multistage carcinogenesis process, cancer cells become highly dependent on specific oncogenes and their related pathways because of the large numbers of mutated and inactivated genes that normally function in other pathways. This dependence could render cancer cells less adaptable than normal cells [220]. As highlighted above, only a subset of patients with NSCLC (about 10–20%) display favorable and often impressive clinical responses to the EGFR inhibitor gefitinib, and this response is often associated with tumors that have specific activating mutations in the kinase domain of *EGFR*. For reasons that are not understood, patients with these activating mutations are also more likely to have adenocarcinomas, be female, non-smokers, and of Japanese origin [52–56, 39]. Thus, addiction to a specific oncogene might occur only in a subset of specific types of cancers with a distinct etiology, and only when that oncogene is mutated and not simply activated. Normal EGFR activation results in induction of multiple downstream signaling pathways, some of which enhance cell proliferation while others enhance cell survival (i.e., inhibit apoptosis). An experimental study indicated that mutations in the EGFR can preferentially enhance activation of the survival, AKT-associated pathway [65]. This could explain why NSCLC cells that harbor this mutation in *EGFR* are highly dependent on this activated oncogene for survival. Similarly, the presence of specific deletion mutations in the *EGFR* gene in glioblastoma was recently shown to correlate with clinical responses to an EGFR inhibitor [221].

2.4.1 Oncogenic Shock

Closely related to the concept of oncogene addiction are the concepts of "oncogenic shock" and "oncogene amnesia." As commonly understood alterations of the signal transduction pathways in cancer cells are thought to underlie drug hypersensitivity, as highlighted in the previous paragraph. Based on modeling studies in vitro, Sharma et al. have recently proposed that unbalanced pro-apoptotic and pro-survival signals may lead to a phenomenon referred to as "oncogenic shock," which might account for the observed apoptotic outcome following the acute inactivation of a crucial oncogene in an addicted cancer cell [222, 39]. According to this model, an addicting oncogene gives rise to both pro-apoptotic and pro-survival signal outputs. While the oncogene is active, the pro-survival signals predominate and keep the pro-apoptotic signals in check, enabling the survival and proliferation of the cancer cell. After acute oncogene inactivation, the relatively short-lived pro-survival signals decay first, whereas the longer lasting pro-apoptotic outputs are maintained during a crucial window of time. Therefore, differential signal decay leading to a signal imbalance and a temporary predominance in pro-apoptotic outputs sets in motion the apoptotic cascade and commits the cell irrevocably to apoptosis, even if the signaling imbalance is subsequently redressed. In support of the oncogenic shock model, the apoptotic response to oncogene inactivation in oncogene-addicted cells is abrogated if the disruption of oncogene-derived signals is extended over a period of time, rather than being acute, or if pro-survival signals are transiently applied during the crucial window of time following acute withdrawal [222, 39]. Therefore, the cell is not hard-wired to depend on a given oncogene, but rather it requires time to adapt to the loss of such a signal and is highly susceptible to apoptosis during that window of time. The implications of this model for clinical practice, if confirmed, are considerable, as it would argue against the co-administration of TKIs with chemotherapy drugs that, by virtue of their own effects on DNA-damage checkpoints, might attenuate the acute effect of growth factor signal withdrawal. For RTK-like EGFR, it is also possible that the acute effect of EGFR-TKI in abrogating kinase activity might be qualitatively different from that of anti-receptor antibodies, which might enable a more gradual signal attenuation, therefore explaining the differential effect of these two classes of agents on EGFR-mutant NSCLC [223]. Implicit in the oncogenic shock model is the paradoxical requirement that activated oncogenes generate pro-survival and pro-apoptotic signals simultaneously [224]. Such a coupling of antagonistic signals is well documented for *Ras*, *Src*, *BCR-ABL*, *EGFR*, *MYC*, and even viral oncogenes such as adenoviral *E1A* (reviewed in [39]). Taken together in the context of NSCLC, mutated *EGFR* might represent the genetic lesion to which the tumor is addicted, and the acute withdrawal of these signals by EGFR-TKI might trigger oncogenic shock and tumor cell apoptosis.

2.4.2 Oncogene Amnesia

An alternative model that might help explaining how oncogenes initiate and are restrained from causing tumorigenesis, and why oncogene inactivation induces

tumor regression, is that of "oncogene amnesia" recently proposed by Felsher and coll. [225]. This model postulates that tumor-cell dependence on aberrant signaling through a specific oncogene is a direct consequence of the fact that specific oncogenes play a direct role in the regulation of physiologic safety switches that regulate mortality/self-renewal, differentiation, and/or DNA repair. Such model has been developed to also accommodate the notion that cell autonomous host mechanisms play a role in the mechanisms by which oncogenes initiate and sustain tumorigenesis. It is axiomatic that many oncogenes contribute to tumorigenesis by inducing unrestrained cellular proliferation and growth, and by overcoming physiologic controls or safety switches. Analogously, oncogene activation has been shown to be restrained from causing tumorigenesis because this results in genotoxic stress – actual genomic damage – and that this stress activates cellular mechanisms that restrain any individual oncogene from causing tumorigenesis by activating cellular programs that induce proliferative arrest, cellular senescence, and apoptosis [226, 227]. Hence, cancer is postulated to arise only after these physiologic barriers have been overcome. Indeed, one of the most characteristic features of cancer is that they not only exhibit autonomous proliferation and growth but exhibit genomic instability, suggesting that they have lost control of regulatory mechanisms that maintain genomic integrity. Indeed, oncogenes have been shown to contribute to genomic damage precisely because they override physiologic checkpoints that regulate DNA replication and repair. Yet despite these pervasive genomic disruptions that in normal cells would prompt an aggressive response inducing proliferative arrest, senescence, and/or apoptosis, tumors seem to be oblivious or amnesic to their genomic disruption. For a tumor to arise, these physiologic safety switches must be shut off, and no single oncogenic lesion is sufficient to do this. Thus, when an individual oncogene is activated, this does block some of the safety switches and this indeed can cause genotoxic stress, actually DNA damage, and this activates the other safety switches and the cells arrest, die, or undergo senescence. Thus, for cancer to arise, other "genetic events" must occur to block enough of the other safety switches to correspondingly block the arrest/senescence/apoptosis response. Then, it may be presumed that by inactivating one of the oncogenes, you would necessarily restore at least some of these safety switches that had been "epigenetically" blocked by the "genetic" oncogenic event, awakening from their slumber the relevant physiologic programs. Indeed, upon oncogene inactivation, tumors exhibit a restoration of physiologic programs that is analogous to a physiologic response to DNA damage: proliferative arrest, differentiation, apoptosis, and/or senescence. At first glance, this seemed a paradox, for if cell cycle arrest, apoptosis, and cellular senescence are the barriers to oncogene initiation of tumorigenesis, then should not these pathways be abrogated in an established tumor, and hence, oncogene inactivation should not result in arrest, apoptosis, or senescence? However, the explanation could be that oncogene inactivation may induce tumorigenesis precisely because these gene products often play a direct role in the regulation of physiologic programs that govern not only cell cycle checkpoint mechanisms but also self-renewal/mortality and senescence programs. Hence, oncogene inactivation would necessarily uncover precisely the specific physiologic programs that the oncogene antagonized to promote

immortality/self-renewal. In some cases, this may simply restore physiologic programs such as cellular differentiation. In many cases, oncogene inactivation would then permit tumors to recognize that they are genomically damaged and result in cellular senescence. Oncogene inactivation can restore the checkpoints that it had blocked. In some cases, this results in the permanent ability of tumor cells to attain a neoplastic phenotype. As proposed by Felsher [225], this circumstance is analogous to the classic story of Dr. Jekyll and Mr. Hyde. As Mr. Hyde, under the influence of the potion of oncogenic activation, tumors behave without moral restraint – autonomous and out of control with regard to the destruction of themselves or others. Then, as this consuming potion wears out, upon oncogene inactivation, there is a restoration of the ability of tumor cells to become aware of their genomic disruption and with self-consciousness. Aware of his misdeeds as Mr. Hyde, Dr. Jekyll in moral indignation feels compelled to permanently destroy himself through death. Of course, experimental observations suggest that Dr. Jekyll also has two other possible outcomes: to learn from his ways and mature into a well differentiated more restrained scientist; or under the distress of moral indignation, rapidly ages into a senescent and now permanently innocuous senior colleague. The important discriminating point of oncogenic amnesia and the oncogene addiction models is that tumor regression following oncogene inactivation in the former is a direct consequence of the restoration of physiologic pathways. Thus, tumorigenesis is "restrained" because oncogenes block only some but not all of the safety switches which results in DNA damage and a physiologic response. Cancer is reversed because oncogene inactivation restores the programs that were blocked by that particular oncogene. Importantly, this model recognizes that the complete inactivation of an oncogene is not required to induce tumor regression, but simply the restoration of the oncogene to physiologic levels so that physiologic programs are resumed. The consequences of oncogene inactivation would be predicted to be different depending on the particular oncogene and the particular genetic and epigenetic features of a tumor. Tumors that were defective for other reasons in apoptosis pathways would be more likely to differentiate or senesce. Tumors defective in genes that are involved in mediating many pathways would exhibit greatly impaired or transient tumor regression.

2.5 Open Issues in the Clinical Development of Signal Transduction-Targeted Anticancer Agents

As our knowledge about molecular targets in cancer initiation and progression grows at an unprecedented pace, our vision of an "ideal medicine" is shifting from a medicine for the "entire population" toward that of a medicine for "the individual." With a handful of notable exceptions, the translation of exciting preclinical findings into the clinical arena using traditional clinical development strategies has been so far disappointing. Indeed, four outcome patterns are commonly observed in randomized trials of molecularly targeted agents: (1) studies reporting a statistically

significant, albeit small, survival benefit for the targeted agent (e.g., erlotinib versus placebo in advanced pretreated NSCLC [228]); (2) studies reporting a statistically significant, while clinically negligible, survival benefit for the targeted agent (e.g., erlotinib plus gemcitabine versus gemcitabine alone in advanced, untreated, pancreatic adenocarcinoma [229]); (3) studies reporting no significant differences in survival (e.g., gefitinib versus placebo in advanced pretreated NSCLC [230]); and (4) studies reporting an unexpected significantly detrimental effect of the targeted agent (e.g., maintenance gefitinib versus placebo after chemotherapy for locally advanced NSCLC [231]). To stick to EGFR (but many other similar examples may be cited), the beautiful biology behind EGFR as a therapeutic target does not seem to have fulfilled its promise more effectively than any other seemingly "untargeted" chemotherapeutic agent we have developed in the past 30 years. Indeed, medical oncology represents the field of clinical medicine with the highest failure rate for late-stage clinical trials, as compared to other specialties, and with the most time- and resource-intensive drug development process, with more than 800 million US dollars to bring a new drug to market. So, what is wrong with the classical clinical development strategy we have used in oncology for chemotherapy agents and their combinations when targeted agents are used? This issue is of paramount importance in determining the future of the ever increasing number of novel promising anticancer agents in clinical development and of signal transduction inhibition as a general therapeutic strategy, especially in a time of limited financial and patient resources.

While there is little doubt that clinical trial design methodology needs to be updated, given the "confusion" generated by the discovery of new molecular targets, which identify (in many, if not all, cases) distinct patients' subgroups, the way forward remains hotly debated. Some key points to address are whether: (1) response rate is an adequate end-point for phase II trials with targeted agents; (2) the randomized phase II design represents a real step beyond; and (3) which kind of phase III are most appropriate for targeted agents. On the other hand, should we restrict the application of novel clinical trial designs to drugs with a known target population (and so apply a "targeted design") and should we maintain a more traditional approach to develop drugs for which a subpopulation of patients that clearly benefit cannot be identified (and so apply an "untargeted design")? The metastatic breast cancer scenario provides both examples. Trastuzumab entered the market based on the results of a relatively small trial (469 patients), performed in a molecularly selected patient population (HER-2 overexpressing), in which a relatively big survival difference (5 months) could be detected [207]; had a traditional, untargeted, design been adopted more than 23 thousands of patients would have been required, considering a 20–30% prevalence of the HER-2 positive population and an absolute treatment-related benefit of 10% [232]. Conversely, the untargeted approach allowed the registration of bevacizumab based on a small, albeit statistically significant absolute benefit in progression-free survival [233]; however, retrospective evidence is emerging, indicating that certain genetically determined subsets of patients would maximally benefit from the addition of bevacizumab to chemotherapy [234].

2.5.1 The Role of "Early Phases": Are Phase II Studies Still Necessary?

An "average" drug development process carried out by the best multicenter, cooperative, international group encompasses a 1-year phase I to find the safe dose of the new drug and its toxicities, a 1- to 2-year formal phase II to test activity and tolerability (on the basis of a hypothesis formulated on historical data), and a 2- to 5-year classical phase III to see how the new drug compares with standard treatment. In the described best case-scenario, it easy to understand that the role of early phases of development (preclinical, phase I and II) is crucial to obtain positive results in phase III. After a good (and independent, unbiased) preclinical development, in the first 1–3 years of clinical development it is easy to control drug effects, monitor biological and clinical activity, and identify the relevant drug target (if present). Moreover, this is the phase of development when it is possible to screen for putative surrogate molecular markers of efficacy. Once a drug enters phase III, it is difficult to obtain such information, given the presence of strict statistical borders; only built-in stopping rules within pre-planned interim analyses are allowed (with all their related concerns). Thus, phase I and II studies are crucial. What are the limitations of the phase II study design? A single-arm formal phase II is designed upon response limits weighed on the basis of historical data or clinical experience of standard treatment, which constitute the benchmark response rate. The choice of such border is influenced by several biases, according to a recent report by Vickers et al. [235]. When appropriate criteria for citation of prior data were fixed, studies that met them were significantly less likely to reject the null hypotheses than those that did not meet the criteria (33% versus 85%, respectively; $p < 0.006$) [235]. Therefore, the decision to proceed to phase III can be biased by the lack of accurate reporting of historical data; if this happens, the wrong hypothesis is tested and the chance of a positive, reliable result in the following phase III is reduced. It affords from the above that unbiased evidence with accurate hypothesis testing is needed to improve the success rate of a new drug in a randomized trial [236].

Do we have phase II-related predictors of success in subsequent phase III studies? A recent analysis of a series of phase II with targeted agents reports that the presence of positive results ($p = 0.027$), the sponsorship of a pharmaceutical company ($p = 0.014$), the short interval between the publication of phase II and III ($p < 0.001$) and the multi-institutional nature of the trials ($p = 0.016$) are all independent predictors of phase III success at multivariate analysis [237]. Another important finding (which is commonly reproduced in many phase II studies with targeted agents) is that if the rate of disease progression is chosen as measure of drug effect instead of the "classical" response rate, the chance of a positive following phase III is higher [237].

At least two "myths" are perceived to be specific features of targeted agents. The first one is that, as opposed to classical cytotoxics, targeted agents would selectively hit a specific molecule or enzyme and that their functional and clinical effects would be directly related to the level of target inhibition. By elegantly using kinase dendrograms, recent work from Karaman et al. visually shows that many commonly

used signal transduction inhibitors (e.g., sunitinib) actually hit several intracellular enzymes, while others really seem to restrict their action to one or two signaling molecules (e.g., lapatinib) [238]. It would be interesting to understand how much classical cytotoxics would differ from the so-called targeted agents in such kind of analysis. Indeed, recent reports strongly suggest a "targeted" effect of several conventional chemotherapeutic drugs [239].

The second "myth" to redefine is that targeted agents are "cytostatic" in nature, i.e., they slow down tumor growth, but seldom shrink pre-existing tumor masses. This seems to be the case for sorafenib in the setting of hepatocellular carcinoma, where hardly any objective response was observed in either the sorafenib or the placebo arm [240], although sorafenib treatment proved effective in a highly statistically significant fashion in both delaying radiological progression and prolonging overall survival [240]. Such example supports the notion that the activity of drugs interfering with cancer signaling pathways is best evaluated by survival/efficacy end-points, rather than classical objective response. However, another TKI, sunitinib, obtains a dramatic improvement in objective responses, as compared to interferon-α, in advanced renal cell carcinoma, an effect that strikingly correlates with both progression free and overall survival [241]. Another setting in which the "cytostatic" paradigm is strikingly dismantled is the use of EGFR TKIs in NSCLC patients harboring EGFR mutations (see above). In a recently reported phase II study performed by the Spanish Lung Cancer Group with erlotinib in molecularly selected, *EGFR*-mutated, NSCLC patients objective responses were seen in 82% of treated patients, an unprecedented finding in any setting for such disease [55].

2.5.2 Phase II Randomized Studies: A New Tale with Targeted Agents

An important bias of single-arm, uncontrolled, phase II studies is that the observed response rate could be related more to patient selection (even when the historical benchmark border is correctly chosen) than to a true drug effect. A possible solution is offered by randomized phase II studies, where, according to the selection design, multiple experimental drugs or regimens are concurrently tested together, and the winner is "picked" and proposed for the further phase III testing. The overall number of randomized phase II studies has significantly increased with the introduction of new drugs, as reported in a recent analysis of 89 phase II trials involving targeted agents performed by El-Maraghi et al. [242]: 30% of such studies were indeed designed in a randomized fashion.

Classically, randomized phase II trials have to (1) test experimental drugs or combination and pick the winner for further phase III; (2) be aimed to safety and activity (i.e., response rate); (3) not use survival end-points; and finally (4) never compare treatment arms. What is new with the introduction of targeted agents? The issue should be approached balancing risks and benefits of two different options. If we use randomization as a control tool, the question is in order to obtain more

accurate results from early studies with targeted agents, what is less dangerous? An uncontrolled single-arm phase II, with response as end-point, or a controlled multiple-arm randomized phase II, with survival (or similar efficacy parameter) as end-point? Taking into account the issues raised by Ratain et al. [236], uncontrolled designs (i.e., "classical" phase II), have high efficiency in identifying non-active drugs (high negative predictive value), but low efficiency in selecting the best challengers for phase III (low positive predictive value), while controlled designs (i.e., "comparative" phase II randomized) have increased positive predictive value, must be conducted with permissive statistical error criteria (higher alfa-error), and must be followed (if positive) by a classical phase III with traditional rules.

2.5.3 Targeted Agents: Moving into Phase III

Moving to phase III trials with new targeted agents it must be considered that the vast majority of cancer therapies do benefit only a subgroup among all treated patients. If we will be able to target treatment to the right patients we will maximize the patient benefit, optimize cost-effectiveness, and finally (but more relevant for clinical research) get more information for successful clinical trials. Unfortunately, information regarding the possible preferential effect of a targeted agent on a population of patients characterized by a specific molecular aberration (mutation, overexpression, amplification, etc.) is mostly provided by retrospective analyses of large randomized trials exploring the benefit of the tested drug in an unselected population. Thereafter, subgroup analyses (usually unplanned) are performed and, if the studied molecular parameter requires either fresh or paraffin-embedded tumor tissue, these are usually done on a small subset of the entire patient population, i.e., in those patients for whom tissue is available. With these premises, it seems obvious that such analyses should be considered exploratory and hypothesis-generating, rather than conclusive, and their strength should take into account the actual statistical power of the original analysis for which the trial was originally designed. Moreover, the subgroup analysis process itself is biased by many risks of data distortion. According to the brilliant paper published by Lagakos et al. if you test 10 subgroups, your chance to occur into more than 3, more than 2, and more than 1 false-positive results is around 2, 9, and 40% [243]. With all these considerations, the risk of misinterpretation of subgroup analyses, which is high by itself, does increase when molecular characteristics are included. With regard to the last point, prospectively specified analysis plans for randomized phase III studies are fundamental to achieve reliable results. Paradoxically, many of the currently ongoing trials for adjuvant treatment of resected NSCLC are designed in order to select patients on the basis of genetic features when "old-fashioned" chemotherapeutics are experimented (i.e., the Spanish Customized Adjuvant Treatment, SCAT, randomizing patients on the basis of BRCA overexpression, and the International Tailored Chemotherapy Adjuvant trial, ITACA, with a two-step randomization taking into account both levels of ERCC1 and TS tissue expression) and with a

non-selection strategy, when adopting "new and targeted" agents (i.e., erlotinib and bevacizumab in the RADIANT, and in the ECOG E1505 trial, respectively).

A recent trial exploring the effect of cetuximab over best supportive care (BSC) in advanced pretreated colorectal cancer patients according to the KRAS gene mutation gives the opportunity to speculate about both the prognostic role of such molecular feature and the issue regarding the interpretation of data coming from retrospective analyses [244]. The results are very impressive and consistent with those recently presented at the last ASCO meeting, which also restrict the benefit of cetuximab to KRAS wild-type patients. According to the overall survival data, KRAS status seems to not have any prognostic role in patients receiving BSC, while in the randomized trial recently published by Amado et al., testing the effect of panitumumab over BSC, a prognostic effect on OS of the KRAS status was observed also in the control arm [245]. This discrepancy raises the issue of the possible misinterpretation of data coming from retrospective analyses; indeed, this apparently inconsistent behavior of KRAS status in a very similar population of patients, all receiving BSC, could be due to selection, which allowed to recruit 68.9 and 92% of the original trial samples, respectively [245, 244]. Do we all still trust "retrospective" data interpretation for clinical practice?

Nevertheless, conducting a phase III trial in the traditional manner without strict eligibility criteria may result in a false-negative trial, unless a sufficiently large part of the treated patients have tumors in which the target is expressed. So, the more the target is underrepresented in the original sample, the more the chance to find right answer decreases. Greater emphasis should be probably given, when planning a clinical trial and when interpreting its results, to the great impact that the molecular heterogeneity of tumors, affecting sensitivity to the experimental treatment, may have on the results of a clinical trial. This concept has been never taken into account in the planning and the analysis of clinical trials with cytotoxic agents, but it should be necessarily considered in clinical trials with molecular targeted agents. In a simplified situation, in which the whole population of patients is divided in two distinct genotypes (A and B) – where genotype A is characterized by sensitivity to the experimental treatment producing in this group an outcome better than in the control group, and the genotype B is characterized by absence of difference in efficacy between experimental and standard treatment – the higher the proportion of patients with genotype B in the study sample, the lower the power of the clinical trial to show a positive result. The statistical power of the study is even lower if we postulate that the genotype B determines a detrimental effect of experimental treatment compared to control. Also, in the case that the targeted population is well represented and the trial gives positive results in favor of the new drug, this means that the effect is driven by the subset of "sensitive" patients, while the treatment is administered to many patients who do not really benefit.

In an ideal scenario, when complete information on predictive factors and proper selection of patients can be definitively obtained in the early phases of drug development, the conduction of subsequent phase III study could be optimized. Unfortunately, this ideal scenario rarely occurs, even with targeted agents. When planning a phase III trial comparing an experimental treatment with the standard,

we often have evidence supporting a predictive role of a marker (M) about the efficacy of the experimental treatment: according to that evidence, patients with expression of the marker (M+) are expected to potentially benefit from the experimental treatment, and patients with absence of expression of the marker (M–) are not. In such a scenario, different strategies based on prospective determination of marker status are theoretically possible: (a) *randomize-all* strategy, randomization between standard and experimental treatment without selection, but with stratification based on the status of the marker; (b) *targeted* design, randomization between standard and experimental treatment only in patients selected according to the status of the marker; (c) *customized* strategy (also called *marker-based strategy*), randomization between standard arm, in which the treatment is the same for all patients, and a personalized arm, in which treatment is chosen based on the marker status of each patient. The *randomize-all* strategy is useful if investigators are not sure of the complete lack of efficacy of experimental treatment in M– patients. Marker is prospectively assessed in all patients, allowing stratification, but all patients are randomized, regardless of the marker status. Interaction between marker status and treatment effect can be formally tested by an interaction test. On the contrary, the predictive role of the marker should not be addressed with separate comparison in M+ and M– patients, because this approach, as stated before, would be associated with a high risk of false results [243]. An alternative strategy (*targeted design*) is to test the status of the marker M, randomizing only M+ patients. This strategy is acceptable only in cases where investigators have already enough evidence to completely rule out the efficacy of the experimental treatment in M– patients. Due to the absence of M– patients, targeted design allows investigators to avoid potential dilution of the results. A third approach is the so-called *strategy design.* According to this design, the experimental arm will receive a personalized treatment based on the status of predictive marker, while all patients assigned to the control arm receive standard treatment. A great limit of strategy design is related to the proportion of M+ patients on the overall population. If M+ patients are a small minority, treatment received will be nearly the same in both arms and the study will provide little information on the efficacy of experimental treatment. On the contrary, the strategy design will be particularly effective when both M+ and M– patients represent a significant proportion of the patients.

   In conclusion, the success of a targeted drug development (and the patient benefit) strongly depends on extensive preclinical and early clinical modeling (good science). Early phases, and in particular phase II studies, remain crucial for development of targeted drug, because this is the moment in which it is possible to explore surrogate and potential selection biomarkers. With this perspective in mind, phase II trials should be hypothesis-generating and should signal either to progress to phase III or to go back to the lab. How should the clinical trial design with targeted agents be improved and fastened to realize the real "bench to bedside" medicine? Targeted agents should be studied in early phases with the newest adaptive design [246], with a more realistic basic hypotheses, and be "tailored" on a clearly specific molecular feature or signaling. This pivotal process will come up into more accurate early studies, providing few positive studies but with stronger and more reliable results.

Fewer drugs will enter phase III, thereby increasing the chance to win over the standard. The following phase III trials (which remain always mandatory) will be more frequently able to test superiority hypotheses, providing big differences, with less patients to be enrolled and shorter time for completing the studies.

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