

Mario Mandalà
Emanuela Romano *Editors*

Mechanisms of Drug Resistance in Cancer Therapy



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Mechanisms of Drug Resistance in Cancer Therapy

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Preface

The remarkable advances in precision medicine and the development of targeted cancer treatments have generated significant optimism based on the assumption that a better knowledge of genetic or molecular features sustaining cancer growth could determine long-term control of many types of cancer and potentially cure the majority of patients. After several years of basic and translational research, the oncology community has tempered this optimism by the recognition that in only a minority of patients cure can be achieved by innovative strategies such as targeted therapies, immunotherapy and cytotoxic agents. In the vast majority of cancer patients, cancer wins the battle, and the reason for this failure is drug resistance. Ultimately, advanced cancer patients die because some or all of their tumour cells exhibit or develop resistance to available therapeutic strategies. The challenge of drug resistance therefore represents an important barrier that hinders the ultimate goal of cure or at least long-term control of cancer.

As a corollary of this, response rates in the setting of tumour progression/relapse are dismal. Despite the considerable importance of drug resistance to cancer therapies, our understanding of their biological mechanisms – and plausible therapeutic avenues to intercept them – remains highly incomplete.

Knowledge of specific resistance mechanisms can inform novel therapeutic approaches to counter this phenomenon, and improvement of our understanding of key driver genes could guide new therapeutics approaches capable of eliciting meaningful tumour responses in patients with advanced malignancies.

As previously reported with chemotherapy, it is reasonable to suppose that a combination of multiple targeted therapies and immunotherapy strategies as well as planned sequence of treatments will be necessary to effectively prevent and/or treat drug-resistant cancers. The potential number of therapeutic combinations is immense; thus, new preclinical paradigms are needed to prioritize high-yield combinations and define the genetic or molecular contexts in which they would most likely be efficacious. To improve outcome of patients a strict collaboration between academia and industry will be needed so that the appropriate resources and innovation may be brought to bear on this challenge. The goal to achieve durable control of many cancer subtypes will likely require dedicated, multidisciplinary teams of preclinical and clinical experts that work together guided by rigorous translational and analytical science.

With the increasing armamentarium of anticancer agents and the advent of powerful high-throughput screening techniques, there are now unprecedented opportunities to understand and overcome drug resistance through the clinical assessment of rational therapeutic drug combinations and the use of predictive and prognostic biomarkers to enable patient stratification and tailor treatments.

The main aim of this book is to offer to the readers an updated overview on the possible reasons of failure of new and promising therapeutic opportunities.

The first part of the book covers the basic mechanisms of such hot topics. The other chapters cover specific pathways of resistance that from the knowledge of basic mechanisms translate this information in clinical routine or in translational clinical research.

Readers will discover diverse perspectives of the contributing authors (such as basic scientists, clinical pharmacologists and clinicians) and extensive discussions of issues including pharmacodynamic and pharmacokinetic mechanisms of resistance, alterations in the drug target, activation of prosurvival pathways, ineffective induction of cell death and plasticity of microenvironment.

This book will be of interest to scholars and researchers, biologists, pharmacologists, medical oncologists, haematologists and immunologists.

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Contents

Molecular and Pharmacological Mechanisms of Drug Resistance: An Evolving Paradigm	1
Benedetta Colmegna, Lavinia Morosi, and Maurizio D’Incalci	
Major Physiological Signaling Pathways in the Regulation of Cell Proliferation and Survival	13
Huifang Tang and Gongda Xue	
Immune-Mediated and Hypoxia-Regulated Programs: Accomplices in Resistance to Anti-angiogenic Therapies	31
Diego O. Croci, Santiago P. Mendez-Huergo, Juan P. Cerliani, and Gabriel A. Rabinovich	
Mechanisms of Resistance to Target Therapies in Non-small Cell Lung Cancer	63
Francesco Facchinetti, Claudia Proto, Roberta Minari, Marina Garassino, and Marcello Tiseo	
Mechanisms of Drug Resistance in Melanoma	91
Matthew Winder and Amaya Virós	
Mechanisms of Resistance to Immune Checkpoint Antibodies	109
Rodrigo N. Ramos, Eliane Piaggio, and Emanuela Romano	
Immunotolerance as a Mechanism of Resistance to Targeted Therapies in Melanoma	129
Mario Mandalà and Daniela Massi	
Plasticity of Resistance and Sensitivity to Anti-Epidermal Growth Factor Receptor Inhibitors in Metastatic Colorectal Cancer	145
Andrea Sartore-Bianchi and Salvatore Siena	
Targeting DNA Repair	161
Giuseppe Curigliano	
Resistance to Hormonal Therapy in Prostate Cancer	181
Alfredo Berruti and Alberto Dalla Volta	

Mechanism of Resistance in Gastrointestinal Stromal Tumors	195
E. Tamborini	
Mechanisms of Resistance to Targeted Therapies in Chronic Lymphocytic Leukemia	203
Francesca Arruga and Silvia Deaglio	
Mechanisms of Resistance to Targeted Therapies in Chronic Myeloid Leukemia	231
Federico Lussana, Tamara Intermesoli, Paola Stefanoni, and Alessandro Rambaldi	
Mechanisms of Resistance in Multiple Myeloma	251
Athanasios Papadas and Fotis Asimakopoulos	
Cancer Evolution as the New Frontier of Precision Medicine	289
Giuseppe Curigliano	



Molecular and Pharmacological Mechanisms of Drug Resistance: An Evolving Paradigm

Benedetta Colmegna, Lavinia Morosi, and Maurizio D’Incalci

Contents

1	Introduction	2
2	Cellular Mechanism of Multidrug Resistance	4
2.1	Drug Activation and Inactivation	4
2.2	Expression of Drug Efflux Pumps	5
2.3	DNA Damage Repair	7
2.4	Deregulation of Apoptosis	8
2.5	Alteration of Drug Target	9
3	Conclusion	10
	References	11

Abstract

The high heterogeneity and genomic instability of malignant tumors explains why even responsive tumors contain cell clones that are resistant for many possible mechanisms involving intracellular drug inactivation, low uptake or high efflux of anticancer drugs from cancer cells, qualitative or quantitative changes in the drug target. Many tumors, however, are resistant because of insufficient exposure to anticancer drugs, due to pharmacokinetic reasons and inefficient and heterogeneous tumor drug distribution, related to a deficient vascularization and high interstitial pressure. Finally, resistance can be related to the activation of anti-apoptotic and cell survival pathways by cancer cells and often enhanced by tumor microenvironment.

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1 Introduction

Although significant improvement of the therapy of several human malignancies have been achieved in the last two decades with the use of small molecules or antibodies designed to hit cancer-specific molecular targets, for the majority of solid human tumors at advanced stage pharmacological treatments have only a transient antitumor effect with small or no impact on patients' survival. The major reason for this disappointing result is the phenomenon of drug resistance.

Although most recent research has been focused on molecular and cellular mechanism of resistance, it seems likely that in many cases the lack of antitumor activity is related to pharmacokinetic reasons, essentially associated with an insufficient drug concentration in the cancer cells.

The pharmacokinetic factors that can be involved in the failure of treatment can be dependent on the route of administration. For example, in the case of orally administered drugs the variable absorption as well as the first pass effect can be responsible for an insufficient bioavailability. For many anticancer drugs the variable metabolism can be a clinically relevant problem, also in consideration of potential interactions with other drugs interfering with the activity of cytochromes involved in drug biotransformation. These pharmacokinetic factors can be investigated by monitoring plasma drug levels, thus allowing, at least in theory, a dose adjustment or a change in the route of administration.

However, the drug plasma levels that for other classes of drugs predict the drug concentration at the pharmacological target, for anticancer drug are often meaningless. The circulating drug concentrations in fact do not necessarily mirror those present in the neoplastic tissue and its metastases because of tumor architecture and microenvironment (Fuso Nerini et al. 2014).

It is known that the vascularization of solid tumors is often inefficient, as the angiogenic process occurring in growing tumors leads to unorganized capillary network with fenestrated vessels and significant leakage of proteins responsible for an increase in the interstitial pressure. Probably further factors contributing to the high interstitial pressure are the inefficient lymphatic drainage as well as a significant infiltration of inflammatory and mesenchymal cells that represent common features of many solid tumors. This peculiar abnormal vascularization and tissue architecture is responsible for a low and heterogeneous tumor drug distribution (Minchinton and Tannock 2006). The evidence of this heterogeneous distribution of anticancer drugs was already available several years ago by examining different parts of tumors. It was demonstrated that in necrotic or hypovascularized and hypoxic parts of the tumor the drug concentrations were lower than those that were normally perfused by blood.

Recently new powerful technologies have been developed to investigate drug distribution in different parts of the tumor by simultaneous visualization and quantification of the drug levels. Among these new approaches, mass spectrometry imaging (MSI) is an interesting technique to obtain specific information on the spatial distribution of a drug linking qualitative molecular information of compounds to their spatial coordinates and distribution within the investigated tissue (Morosi et al. 2013).

MSI can be applied to visualize potentially every tissue component localizing the molecule of interest on the base of its molecular mass and fragmentation pattern, without the need to label the analyte, a great advantage over other imaging techniques such as fluorescence microscopy, positron emission tomography, magnetic resonance spectroscopy, and autoradiography (Sugiura and Setou 2010).

MSI was applied to localize drug distribution and penetration inside solid tumors, thanks to its superior spatial resolution (20–100 μm) and specificity allowing the detection of parent compound and metabolites simultaneously (Buck et al. 2015; Connell et al. 2015).

MSI can also provide the quantitative amounts of target compounds in well-defined region of interest of the examined tissue, ideally in a single pixel. This goal is extremely challenging depending on the technical possibilities and limitations of the MSI instrument hardware, but equally on the chosen calibration/standardization strategy (Giordano et al. 2016a; Rzagalinski and Volmer 2016).

MSI has also some limitation: it is only applicable to molecules that are ionizable, the selection of optimal matrix is arduous and driven by empirical results, moreover the interpretation of quantitative data is often complex especially because of ion suppression effect and the chemical noise from the matrix ions covering the drug ion signal and finally, the sensitivity is quite limited (Prideaux and Stoeckli 2012).

By applying this methodology it has become clear that drug distribution in tumors is very heterogeneous comparing different tumor models having different histopathological characteristics (Giordano et al. 2016b). Figure 1 shows the intratumor distribution of the anticancer drug paclitaxel by MSI. The drug distribution is homogeneous only in the ovarian cancer model while it is highly irregular in the other examples of tumor types. As you can see in other models such as sarcoma or breast cancer, areas of the tumor where the drug is highly concentrated and areas where it is almost absent can be observed at the same time. Broad parts of the tumor where the drug is not present at all are present especially in mesothelioma xenograft. This aspect surely contributes to explain tumor resistance. Torok et al. recently correlate the antitumor activity of several antiangiogenic drugs with their intratumoral distribution data obtained by MSI (Torok et al. 2017). Moreover, Cesca et al. observed an enhancement in paclitaxel activity combined with bevacizumab associated with the improvement of its intratumor distribution (Cesca et al. 2016).

The possibility to superimpose MSI molecular images with histological and immune-histological evaluation could help understanding the factors that impair drug penetration. Finally, this technology could be used in future to evaluate the

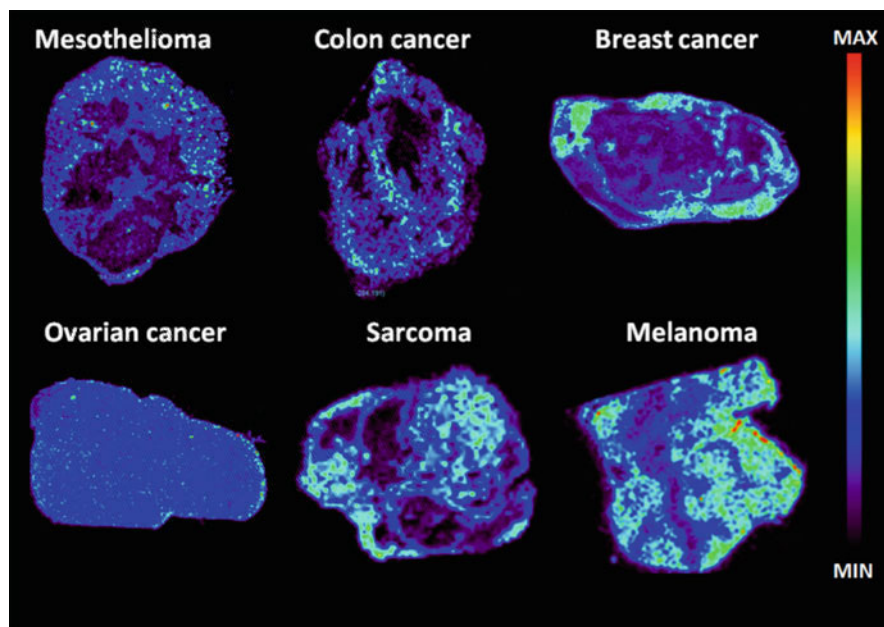


Fig. 1 Paclitaxel distribution in different cancer models analyzed by MALDI mass spectrometry imaging. *Light blue* and *green* mark the drug presence on the basis of the color scale on the right

effect of therapeutic strategies aimed at modifying tumor microenvironment or altering drug properties to facilitate drug uptake and intratumor distribution.

2 Cellular Mechanism of Multidrug Resistance

The hallmarks of cellular anticancer drug resistance fall into distinct categories:

2.1 Drug Activation and Inactivation

Altered local drug metabolism and detoxification are key resistance mechanisms highly specific for each class of drugs. Lack of prodrug activation or epigenetic silencing – by promoter methylation – of genes involved in drug processing are examples of potential cause of resistance and are frequently tumor-specific. Classical examples could be the inactivation of platinum drugs by the thiol glutathione (Meijer et al. 1992) or metallothioneins (Amable 2016), or by epigenetic mechanisms, e.g., silencing – by methylation – of gene encoding for thymidine phosphorylase, an enzyme involved in the conversion of capecitabine (prodrug) to 5-fluorouracil (active drug) (Longley et al. 2003). For many DNA interacting agents, e.g., alkylating agents, anthracyclines, and platinum drugs, an important

mechanism of inactivation is related to the overexpression of Glutathione-S-Transferases (GSTs) that metabolizes the drugs into inactive molecules (Kauvar et al. 1998).

2.2 Expression of Drug Efflux Pumps

Plasma membrane transport proteins are key players in the uptake of nutrients, such as sugars, amino acids, nucleosides, and inorganic ions and the efflux of xenobiotic toxins, including many anticancer drugs. Transport proteins can be classified into two major families, the solute carrier (SLC) and the ATP-binding cassette (ABC) transporters and can affect drug absorption, distribution, and excretion conferring sensitivity or resistance to anticancer drugs.

The importance of ABC transporters in cancer therapy has been well documented while the impact of the solute carriers (SLC) on cancer therapy has not been extensively characterized (Table 1).

So far, the most characterized transporters belong to ABC family and include ABCB1 (also known as MDR1 or P-glycoprotein), ABCC1 (MRP1), and ABCG2 (BCRP or MXR).

Table 1 Reports the transporters whose activity has been associated with drug resistance

Transporters	Chemotherapeutic drugs effluxed
P-gp/MDR1	Anthracyclines, taxanes, vinca alkaloids, imatinib, etoposide
MRP1	Anthracyclines, methotrexate, camptothecins, etoposide, glutathione conjugates
MRP2	Doxorubicin, methotrexate, cisplatin, vincristine
MRP3	Methotrexate, etoposide
MRP4	Methotrexate, camptothecins, thiopurines
MRP5	Methotrexate, thiopurines
MRP6	Etoposide
MRP8	5-Fluorouracil
BCRP/MXR	Doxorubicin, mitoxantrone, topotecan, flavopiridol, SN-38
LRP	Doxorubicin, cisplatin, vincristine, etoposide, paclitaxel, melphalan
Human nucleoside transporters (hENT, hCNT)	Cytarabine, gemcitabine, fludarabine, clofarabine
Copper transporters (Ctr1, Ctr2, ATP7A, ATP7B)	Cisplatin
Multidrug extrusion transporter1 (MATE1)	Cisplatin
Organic cation transporter (OCT1, -2, -3)	Platinum drugs, SN-38, mitoxantrone, melphalan
Organic anion transporter (OAT1, -2, -3)	Methotrexate, 5-fluorouracil, paclitaxel

The ATP-binding cassette (ABC) transporters superfamily is a large group of membrane proteins that are responsible for the translocation of various cytotoxic molecules out of the cell reducing their intracellular concentration and thus their cytotoxicity. ABC transporters are codified by 48 genes (divided into 7 subfamilies) and characterized by a conserved quaternary structure containing 2 transmembrane domains (TMDs) and 2 nucleotide (ATP)-binding domains (NBDs). Their ATP-dependent activity is involved in the movement of a wide variety of xenobiotics – including drugs – lipids, peptides, and metabolic products across the plasma and intracellular membranes. The ABC transporters tissue distribution reflects the complex physiological network of these proteins and reveals their important role in absorption, excretion, and distribution of drugs.

P-glycoprotein (P-gp) is a 170 kDa glycoprotein coded by ABCB1 gene mapped to chromosome 7q21.1. Its presence in normal tissue (as liver, kidney, brain, intestine, etc.) reveals the ability of modulating bioavailability and transport of wide range of substrates, especially organic molecules containing aromatic groups, and suggests its role in determining multidrug resistance (MDR) phenotype. P-gp is overexpressed in many tumors (thus causing intrinsic drug resistance) and the expression of P-gp can also be induced by chemotherapy (thus resulting in the acquired development of MDR) (Thomas and Coley 2003). Expression of P-gp fluctuates with elevated expression level in untreated cancer into higher level upon relapse after chemotherapy and undetectable or low level in the expression in drug sensitive tumors. P-gp expression has been reported in 40% of breast cancer (Kao et al. 2001), 20% of ovarian cancer (Baekelandt et al. 2000), and in more than 50% of patients with acute myelogenous leukemia experiencing relapse (Leith et al. 1999) and is now associated with treatment failure in kidney, liver, and colon cancer (Ambudkar et al. 2003). It confers the strongest resistance to the widest variety of compounds including vinca alkaloids, anthracyclines, and taxanes contributing to the failure of chemotherapy (Szakács et al. 2006). Recent reports have suggested that molecularly targeted therapies, such as some kinases inhibitors, are also substrates for drug efflux proteins (Shervington and Lu 2008).

MRP1 is a transporter involved in glutathione-linked organic compound transport and is normally expressed in the basolateral membrane of cells from testis, kidney, oropharyngeal mucosa, etc. Recently the overexpression of MRP1 has been correlated with chemoresistance in prostate, lung, and breast cancer (Holohan et al. 2013) and confers resistance to several hydrophobic compounds that are also P-gp substrates.

Screens carried out with the NCI60 cell panel indicate that there is a strong correlation between the expression of several transporter (including ABC superfamily) and decrease in chemosensitivity.

The goal of reversing MDR in the clinic through the pharmacological inhibition of ABC transporter has been pursued for years and different generation of inhibitors have been developed. Only P-gp inhibitors have been evaluated in clinical studies. However, the results of clinical trials using modulators of P-gp have been disappointing mainly because of the required marked reduction of the anticancer drug doses. In addition it is now very clear that resistance is not only due to the

overexpression of P-gp, but also to many other mechanisms, thus making unrealistic to reverse tumor resistance by pump inhibition.

The resistance of tumors originating from tissues expressing high levels of P-gp (such as colon, kidney, or the adrenocortex) often extends to drugs that are not subject to P-gp-mediated transport, suggesting that “intrinsically resistant” cancer is also protected by non-Pgp-mediated mechanisms (Szakács et al. 2006).

Evidence linking P-gp expression with poor clinical outcome is more conclusive for breast cancer, sarcoma, and certain types of leukemia. P-gp expression in patients with AML has consistently been associated with reduced chemotherapy response rates and poor survival (Pallis and Russell 2004), in contrast with MRP1 whose expression is not a significant factor in drug resistance in AML (Leith et al. 1999).

The expression of these transporters as a mechanism of acquired resistance after a specific treatment is also responsible for the phenomenon of cross-resistance that must be taken into account especially for a clinical perspective.

2.3 DNA Damage Repair

The cellular response to DNA damage involves several mechanisms aimed at repairing the damage, thus restoring DNA integrity or alternatively activating cell death pathways.

Many chemotherapeutic drugs induce DNA damage either directly (for example, platinum-based drugs) or indirectly (for example, topoisomerase inhibitors). Without repair, this damage can result in genetic instability, acquisition of further mutations, and cytotoxic effects (Holohan et al. 2013). The different sensitivity to DNA damaging agents can be due to a different ability to repair drug-induced damage. For example, cancer cells deficient in Homologous Recombination (HR), e.g., because of mutations of BRCA1 or BRCA2 genes, are very sensitive to DNA damage caused by platinum drugs. HR deficient cancers are very sensitive to PARP inhibitors that cause an accumulation of DNA single strand breaks. In HR proficient cells, DNA single strand breaks generate DNA double strand breaks during DNA replication that are repaired before mitosis. Instead in HR deficient cells the inhibition of base excision repair by PARP inhibitors leads to unrepairable DNA double strand breaks with consequent cell death. This is the concept of synthetic lethality whose application has shown promising results in patients with breast and ovarian tumors (Farmer et al. 2005). Even in this case resistance can occur because of the acquisition of secondary mutations restoring HR normal function (Ashworth 2008).

Other pathways involved in DNA repair that have been reported to be relevant for drug resistance are nucleotide-excision repair (NER) and mismatch repair (MMR). The first one is required for the repair of DNA damage caused by many DNA-damaging drugs, such as platinum-based drugs. High expression of a key component, excision repair cross-complementing 1 (ERCC1), has been linked with poor responses to chemotherapy in non-small-cell lung carcinoma (NSCLC),

gastric, and ovarian cancer (Kirschner and Melton 2010). Notably, testicular cancers, which are very sensitive to cisplatin treatment, have very low levels of ERCC1 (Usanova et al. 2010).

The mismatch repair (MMR) system, whose key players are MLH1 and MSH2 genes, is crucial for maintaining genomic integrity, and its deficiency has been linked to high mutation rate of a large number of genes including those involved in immunogenicity and drug sensitivity. This explains why hypermethylation of MLH1 causes resistance to cisplatin and carboplatin (Fink et al. 1998). More recently, a synthetic lethal interaction between MSH2-targeted short interfering RNA (siRNA) and methotrexate was identified in MMR-deficient cancer cells; methotrexate caused the accumulation of oxidative lesions such as 8-oxoguanine (8-oxoG) in MSH2-deficient cells, which resulted in a loss of viability through apoptosis. This has led to an on-going Phase II clinical trial with methotrexate in patients with MSH2-deficient metastatic colorectal cancer using measurement of 8-oxoG lesions as a biomarker (Holohan et al. 2013).

Another DNA protein involved in resistance is *O*-6-methylguanine-DNA-methyl transferase (MGMT), whose overexpression is responsible for the resistance to methylating agents such as temozolomide. The hypomethylation of the promoter of the MGMT gene has been associated with resistance of human glioblastoma to temozolomide (Thomas et al. 2017).

2.4 Deregulation of Apoptosis

The resistance to anticancer drugs can be due to the fact that in cancer cells, apoptotic pathways are frequently dysfunctional. Numerous intrinsic adaptive responses can be triggered and promote survival of cancer cells exposed to DNA-damaging agents. Anti-apoptotic BCL-2 family members, inhibitor of apoptosis proteins (IAPs), and the caspases are key proteins of this mechanism. Mutations, amplifications, chromosomal translocations, and overexpression of the genes encoding for these proteins have been associated with various malignancies and linked to resistance to chemotherapy and targeted therapies.

BCL-2 family proteins have a pivotal role in dictating cell fate following chemotherapy treatment. The balance between the anti-apoptotic BCL-2 family members (such as BCL-XL and MCL1) and pro-apoptotic family members (such as BAX, BAD, and BAK, as well as various BH3-only proteins) is critical in determining the activation or not of a common pathway generally stimulated from all cytotoxic drugs that causes the mitochondrial outer membrane permeabilization (MOMP) and finally cell death. Overexpression of one or more anti-apoptotic proteins or underexpression of one or more pro-apoptotic proteins or a combination of both dysregulate apoptosis. For example, the overexpression of Bcl-2 protected prostate cancer cells from apoptosis while led to inhibition of TRAIL-induced apoptosis in neuroblastoma, glioblastoma, and breast carcinoma cells. In colorectal cancers bax(G)8 frameshift mutations could contribute to resistance of cells to anticancer treatments. In the case of chronic lymphocytic leukemia (CLL), the

malignant cells have an anti-apoptotic phenotype with high levels of anti-apoptotic Bcl-2 and low levels of pro-apoptotic proteins such as Bax (Wong 2011). The IAPs are a group of structurally and functionally similar proteins that regulate apoptosis by inhibiting caspase activity and promoting degradation of active caspases. Dysregulated IAP expression has been reported in many cancers. For example, drug resistance correlated with the expression of cIAP-2 in pancreatic cells while Apollon was found to be upregulated in gliomas and was responsible for cisplatin and camptothecin resistance (Chen et al. 1999).

The caspases are a large group of proteins that play a central role in apoptosis. As they work at two levels, *initiator caspases* (e.g., caspase-2, -8, -9, and -10) responsible for the initiation of the apoptotic pathway and *effector caspases* (caspase-3, -6, and -7) responsible in the actual cleavage of cellular components during apoptosis. Therefore low levels of these proteins or impairment function can decrease apoptosis thus contributing to resistance.

The p53 protein, also called tumor protein 53 (or TP 53), is one of the best known tumor suppressor proteins whose mutation acquired oncogenic property. Defects in the p53 tumor suppressor gene have been linked to more than 50% of human cancers (Bai and Zhu 2006) because it is not only involved in the induction of apoptosis but it is also a key player in cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation, and cellular senescence. A recent study reported that some target genes of p53 involved in apoptosis and cell cycle regulation are aberrantly expressed in melanoma cells and possibly related to resistance (Avery-Kiejda et al. 2011).

Several abnormalities in the death signaling pathways that can lead to evasion of the extrinsic pathway of apoptosis have been identified. Death receptors such as Fas, DR3, Trail-1, Trail-2, and ligands of the death receptors are key players in the regulation of extrinsic pathway of apoptosis. Downregulation or impairment of receptor function, abnormal expression of decoy receptors, as well as a reduced level in the death signals can contribute to impaired signaling and hence a reduction of apoptosis and acquisition of drug resistance.

2.5 Alteration of Drug Target

One of the most common drug resistance mechanisms involves genetic alterations of drug target such as mutations or changes in expression levels. These modifications could exist at low levels before drug treatment and undergo positive selection during exposure to chemotherapy. In oncology, genomic characterization of cancer has highlighted the importance of driver somatic mutations that give rise to an unusual reliance of cancer cells on a particular molecular pathways and their specific oncogenic kinases. These proteins are targets for many drugs and can be altered by different mechanisms.

Main categories of this type of resistance could be:

- Downregulation of gene expression (e.g., effect of doxorubicin on topoisomerase II α) (Di Nicolantonio et al. 2005)
- Gene amplification (e.g., BCR-ABL amplification detected both in vitro and in imatinib-resistant CML specimens) (Gorre et al. 2001)
- Mutations in *gatekeeper residue* (e.g., EGFR-T790M in NSCLC after treatment with tyrosine kinase inhibitor (TKI) gefitinib and erlotinib (Bell et al. 2005); BCR-ABL T315 in CML patient treated with imatinib) (Gorre et al. 2001)
- Mutations that alter the conformation (e.g., mutations in ALK – F1174L, C1156Y, and L1152R – confer an increase in ATP affinity and clinical resistance to the ALK inhibitor crizotinib) (Choi et al. 2010)
- Alternative spliced form (e.g., p61BRAF^{V600E} detected in both vemurafenib in vitro-resistant cells and from resistant patient tumor biopsies produces enhanced dimerization with other RAF family members and resistance to vemurafenib but not MEK inhibitors) (Poulikakos et al. 2011).

A possible consequence of the inhibition of specific target is also the activation of a so-called bypass signaling mechanism. This results in the activation of a critical downstream signaling effector – normally activated by the kinase and extinguished by a kinase inhibitor – through a parallel mechanism that is indifferent to the kinase-directed therapy. An illustrative example of bypass-mediated resistance has been described in EGFR-mutant NSCLC. The bypass resistance mechanisms may also involve the modulation of positive or negative feedback loops and an example could be the augmentation of AKT signaling by MEK inhibitors (Garraway and Jänne 2012).

3 Conclusion

This review article shortly summarizes the main mechanisms of resistance to anticancer drugs. In order to define possible strategies to counteract resistance mechanisms, it can be useful to divide them in *pre-target*, *target related*, and *post-target* mechanisms.

As far as *pre-target* mechanisms – that can also be classified as pharmacokinetics mechanisms – we envisage the possibility to circumvent them by increasing drug doses and improving drug delivery testing combinations with modulators of drug transport and of tumor microenvironment.

On the other side, a deeper and deeper knowledge of *target related* mechanisms, e.g., target mutations, downregulation, amplification, is instrumental to identify alternative drugs, equally effective against cancer cells, to circumvent these types of resistance.

Finally as regards *post-target* mechanisms, the modulation of apoptosis appears to be a potentially feasible approach as recently demonstrated by Croce and Reed (2016).

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Major Physiological Signaling Pathways in the Regulation of Cell Proliferation and Survival

Huifang Tang and Gongda Xue

Contents

1	Introduction	14
2	Signaling Pathways Involved in the Regulation of Cell Proliferation	14
2.1	Biological Regulation of Proliferation by mTOR/PI3K/Akt Signaling	15
2.2	Ras/MAPK Signaling-Regulated Cell Proliferation, Differentiation, and Survival ...	17
2.3	JAK/STAT Signaling in the Regulation of Cell Proliferation and Survival	20
3	Signaling Interaction Network Between PI3K/Akt, Ras/MAPK, and JAK/STAT Axes ...	20
4	Output Signals of Integrated Signaling Pathways	22
4.1	Redundant Targeting of Shared Substrates in Cell Growth and Metabolism	22
4.2	Differential Phospho-Regulation of Shared Substrates in Cell Proliferation and Survival	23
5	Impact of Signaling Interaction Network in Clinical Cancer Therapies	23
6	Final Remarks	24
	References	25

Abstract

Multiple signaling pathways regulate cell proliferation and survival and are therefore important for maintaining homeostasis of development. The balance between cell growth and death is achieved through orchestrated signal transduction pathways mediated by complex functional interactions between signaling axes, among which, PI3K/Akt and Ras/MAPK as well as JAK/STAT play a dominant role in promoting cell proliferation, differentiation, and survival. In clinical cancer therapies, drug resistance is the major challenge that occurs in almost all targeted therapeutic strategies. Recent advances in research have suggested that the intrinsic

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pro-survival signaling crosstalk is the driving force in acquired resistance to a targeted therapy, which may be abolished by interfering with the cross-reacting network.

Keywords

Apoptosis • Cell cycle • Drug resistance • JAK/STAT • Lung cancer • Melanoma • mTOR/PI3K/Akt • Proliferation • Ras/MAPK • Signaling crosstalk

1 Introduction

The development of a multicellular eukaryote starts from the division of a zygote that eventually produces many types of functionally specialized cells. Proper tissue formation and growth requires a homeostatic regulation of cell cycle events that control cell proliferation and the cellular metabolism that often associates with apoptosis, a process of programmed cell death that surveils a balanced cell proliferation rate and cell number. Such regulation is usually governed by defined activation and inactivation of individual genes at different developmental stages. The selective pattern of gene expression functionally directs cell proliferation, cell differentiation, cell migration, and cell–cell communication to maintain a developmental integrity of the complex formation of tissues and organs. This phenotypic complexity is precisely mirrored by the genetic control system, an orchestrated signaling network coordinated among a few key evolutionarily conserved pathways. In this chapter, we intend to discuss recent advances of two important pathways, mTOR/PI3K/Akt and mitogen-activated protein kinase (MAPK), whose activation and interaction are critical for development and are the key regulatory elements in pathological circumstances such as malignant transformation of neoplasia and drug resistance in clinical cancer therapy.

2 Signaling Pathways Involved in the Regulation of Cell Proliferation

Cell proliferation is integrated with the overall needs of the organismal development in coordination with cell growth. It is principally mediated by cell cycle, a fundamental process that occurs when a parental cell produces two daughter cells via a series of genetic events (Hartwell and Kastan 1994; Nakayama and Nakayama 2006). Each of the daughter cells theoretically obtains the same copy of the genetic material inherited from the parental cell and are therefore genetically identical and morphologically undistinguishable. Similar to their parent, the mature daughter cells may initiate and complete the cell cycle when triggered by distinct signals in favor of the needs for growth and metabolism. Under physiological conditions, the proliferative cells are generally restricted to cells that supplement the tissue. Most tissues contain a group of multipotent cells, usually called stem cells, which can not only renew themselves but also divide asymmetrically to generate a new stem cell and a progenitor cell. In a context

dependent manner, progenitor cells may undergo further divisions, or enter terminal differentiation with endowed specialized functions. Thus, in order to maintain proper tissue development, cell proliferation is programmed to be tightly controlled and its deregulation is a hallmark of malignant transformation found in the clinic (Hanahan and Weinberg 2000, 2011).

Maintenance of an appropriate cell number is also contributed by programmed cell death, apoptosis, which actively eliminates unwanted cells including aged and damaged cells (Elmore 2007; Taylor et al. 2008). Importantly, these two cellular functions are under a variety of stringent controls to ensure tissue homeostasis. Aberrant alterations of each functional node, either hyperactivation of proliferation or deactivation of apoptosis, will ultimately result in uncontrolled cell proliferation and growth that leads to tumor formation.

Mechanistic studies have revealed a number of biological signaling axes important for controlling cell proliferation and apoptosis. These signaling pathways commonly form an integral signaling network through diverse functional interactions, which are mediated by specific feedback signaling loops. Recent advances from clinical studies have shown that therapeutic disruption of the indispensable signaling loops during tumorigenic development is one of the leading causes of drug resistance and is attributed to over-activation of parallel proliferative signaling pathways (Holohan et al. 2013; Wicki et al. 2016).

2.1 Biological Regulation of Proliferation by mTOR/PI3K/Akt Signaling

In response to the stimulation of a variety of extracellular signaling cues, a number of membrane receptors with kinase activities can be activated via oligomerization. Activated membranous kinases subsequently create binding motifs in their intracellular segments that recruit and further activate the essential signal-mediator kinases. One of these intracellular messengers is lipid kinase phosphoinositide 3 kinase (PI3K), an evolutionarily conserved intracellular kinase that can convert Phosphatidylinositol (4,5)-bisphosphate (PIP2) into the secondary messenger molecule Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on the plasmic membrane (Engelman et al. 2006). This process can be reversed by the phosphatase and tensin homolog (PTEN), which pairs with PI3K to constitute a functional switch of PIP3-dependent signaling transduction. Membrane-targeted PIP3 recruits certain proto-kinases to the inner surface of cytoplasmic membrane through binding to the pleckstrin homology (PH) domain with elevated affinity. Several important kinases are dependent on PIP3-mediated membrane targeting for activation. Akt and phosphoinositide-dependent kinase 1 (PDK1), two PH-domain-containing kinases, recognize and bind to newly generated and enriched PIP3, resulting in transient membrane anchoring and consequent activation.

Akt, also called protein kinase B (PKB), is a serine/threonine kinase that belongs to the AGC protein kinase family (Pearce et al. 2010). It comprises three isoforms in mammals with a high degree of sequence identity. Although the tissue distribution pattern is slightly different, Akt isoforms are virtually expressed in all cell types and

act as one of the most important signaling nodes to direct proper responses to exogenous or endogenous stimuli (Fayard et al. 2010).

Activation of Akt is a multistep process with characteristic phosphorylation of two key amino acids, threonine 308 (T308) in the “activation loop” and serine 473 (S473) in the “carboxy-terminal hydrophobic motif,” both of which are conserved in all three isoforms. T308 phosphorylation is contributed by PDK1 when both proteins are recruited to the plasma membrane by PIP3. Although not exclusively required, it is generally accepted that the full kinase activity of Akt is stimulated upon phosphorylation of S473 by the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) (Cybulski and Hall 2009). Recent studies have also uncovered another kinase, DNA-dependent protein kinase (DNA-PK), that is capable of phosphorylating Akt on the same amino acid in response to DNA double-strand break (Bozulic et al. 2008; Surucu et al. 2008). Therefore, under physiological or pathological conditions, Akt acts as a direct functional target downstream of PI3K under the stringent control of PDK1 and mTORC2 or DNA-PK, which consolidates the theoretical basis for understanding the current model (Fig. 1). Genetic knockout of each Akt isoform or pharmacological inhibition of its activity results in a universal defect in growth in mouse models, indicating a central role of the Akt family in growth control (Fayard et al. 2010). In consistence with its roles in development, Akt is widely observed to be hyperactivated in a broad panel of cancers (Fruman and Rommel 2014; Vivanco and Sawyers 2002). In this regard, the signaling axis mTOR/PI3K/Akt has become one of the most attractive targeting pathways to restrain cancer cell proliferation. In spite of its three major upstream regulators, other kinases such as serine–threonine kinases IKK (Xie

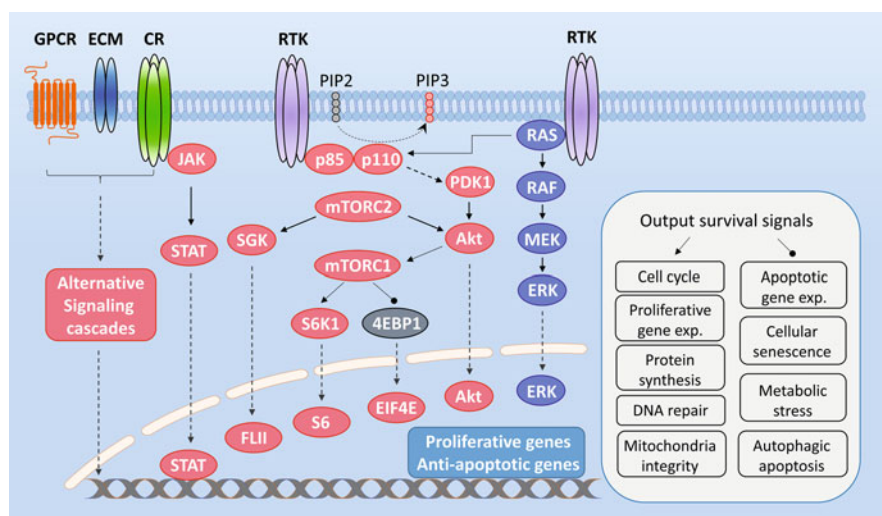


Fig. 1 Introduction to major proliferative signaling pathways. Three major signaling axes, mTOR/PI3K/Akt, Ras/MAPK, and JAK/STAT, are dissected to show their downstream targets that promote cell cycle progression. RTK receptor tyrosine kinase, CR chemokine receptor, ECM extracellular matrix, GPCR G-protein-coupled receptor

et al. 2011) and TBK1 (Ou et al. 2011; Xie et al. 2011), together with three tyrosine kinases Src (Chen et al. 2001; Haynes et al. 2003), Ack1/TNK2 (Mahajan et al. 2010; Mahajan and Mahajan 2015), and PTK6 (Zheng et al. 2010), are also reported to modulate Akt activity with distinct regulatory mechanisms. Therefore, Akt appears to be a universal intracellular mediator converged from a number of membrane signaling cascades. Indeed, not only phosphorylation but also many other types of post-translational modification of Akt have been discovered to collaboratively control Akt to maintain cellular homeostasis (Risso et al. 2015).

Akt activation triggers a number of functional events including cell proliferation, growth, survival, protein synthesis, and metabolism. These cellular functions are differentially promoted by multiple downstream targets of Akt, which can be grouped into five categories with functional specificities in regulations of cell cycle: gene synthesis and protein translation, cell survival and anti-apoptosis, developmental progress/stress and metabolism, and crosstalk with other signaling pathways. Akt-regulated cellular functions are preferentially controlled by its specific substrates with distinct roles (Manning and Cantley 2007) (Fig. 2a) as well as a few physical interacting partners that can also regulate its activity.

2.2 Ras/MAPK Signaling-Regulated Cell Proliferation, Differentiation, and Survival

In parallel to the PI3K/Akt axis, another intracellular signaling pathway can be found downstream of membrane-coupled signalosomes; this pathway is the small GTPase-mediated MAPK cascade. The MAPK family is highly conserved throughout mammalian evolution to regulate diverse developmental processes such as cell cycle regulation, differentiation, cell motility, physiological metabolism, stress response, and pro- or anti-apoptosis (Widmann et al. 1999). The mammalian MAPK family contains more than 20 protein kinases characteristically organized into three-tiered kinase cascades (Chang and Karin 2001). Activation of the last tier messenger MAPK proteins is mediated by directed phosphorylation on threonine (Thr) and tyrosine (Tyr) residues within a conserved Thr-X-Tyr motif (X represents glycine, glutamine, or proline) that is located in the activation loop of the kinase domain. Based on the extracellular stimuli and their distinct membrane-associated signaling receptors, the MAPK signaling pathway can be categorized into four major branches characterized by distinct MAPK family members including extracellular signal-regulated kinases (ERK), c-Jun NH₂-terminal kinases (JNK), p38, and ERK5 (Pearson et al. 2001; Qi and Elion 2005). These MAPK messenger kinases have been shown to implement different cellular functionalities (Fig. 2b).

In response to stimulation of growth factors, conversion of Ras^{GDP} to Ras^{GTP} activates Raf kinase, an MAPK kinase kinase (MAPKKK) that can phospho-activate its downstream target MEK, an MAPK kinase (MAPKK). MEK rapidly phosphorylates ERK1/2 and subsequently stimulates cell proliferation through activating a broad range of substrates of ERK. These substrates include intracellular kinases p90 ribosomal S6 kinases (RSK) (Carriere et al. 2008b), mitogen- and stress-activated kinases

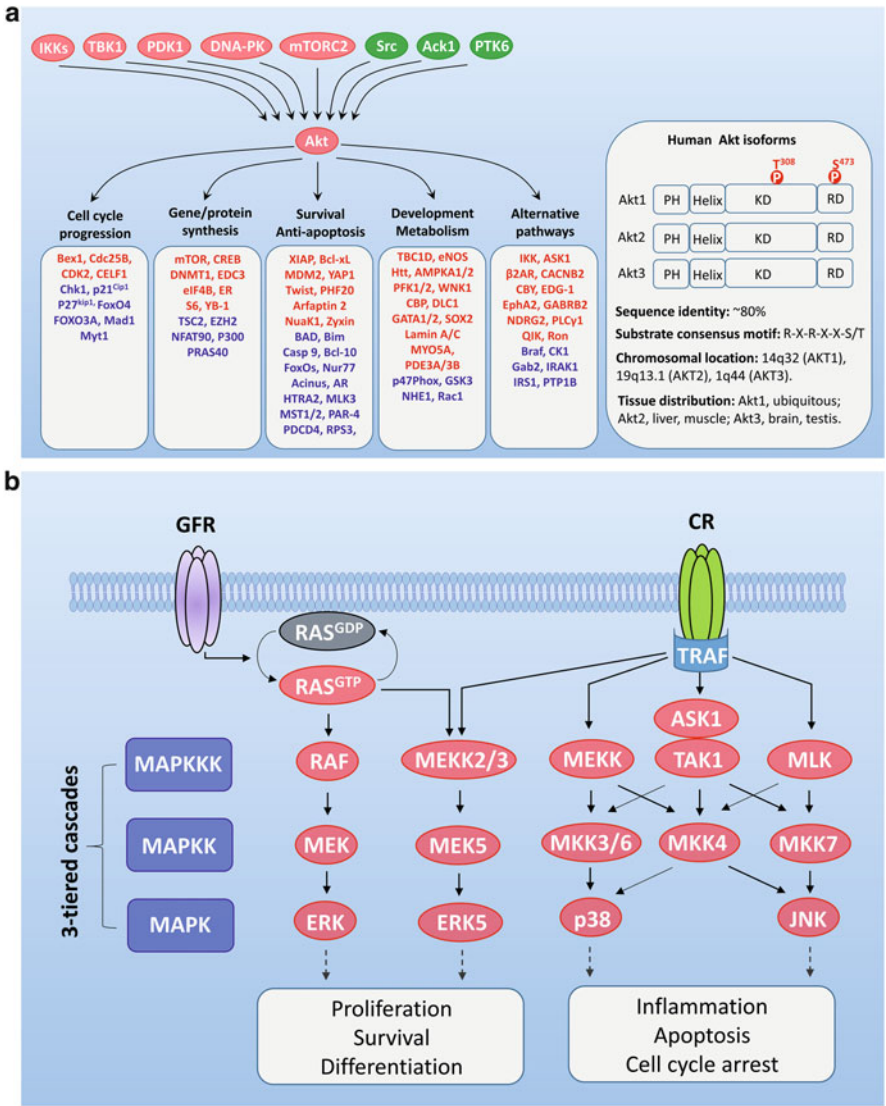


Fig. 2 Mechanistic regulation of cell proliferation by PI3K/Akt and MAPK pathways. **(a)** Up- and downstream targets of Akt that regulate differential gene expression in promoting cell proliferation and survival. Upstream kinases highlighted in *red* represent serine–threonine kinases, while kinases highlighted in *green* are phosphorylating tyrosine residues on Akt. Downstream of Akt are its substrates important in cell growth: the substrates highlighted in *red* are phospho-activated by Akt and subsequently promote cell proliferation and survival, while those highlighted in *blue* are pro-apoptotic and phospho-inactivated by Akt. **(b)** Schematic dissection of MAPK family members and their signaling interaction in regulating and determining cell fate. *GFR* growth factor receptor

(MSK) (Arthur 2008), MAPK-interacting kinases (MNKs) (Waskiewicz et al. 1997), and death-associated protein kinase (DAPK) (Chen et al. 2005). A number of transcriptional factors including NF-AT, STAT3, MEF2, c-Myc, ELK-1, and c-Fos (Cargnello and Roux 2011; Gaestel 2015) are also involved. Basal activation of the ERK1/2 cascade is important for efficient G1-S transition during cell cycle. Therefore, ERK1/2 conventionally acts as a positive regulator of cell cycle progression contributing to cell proliferation under physiological conditions. In fact, this proliferative role of activated ERK is directly reflected in many types of clinical disorders, such as cancer, in which ERK activation is dramatically deregulated to favor an uncontrolled proliferative phenotype of cancer cells.

In contrast to ERK activation in response mainly to growth factors (Ramos 2008), other classes of stimuli like cytokines potently induce JNK and p38 activation through three well-defined signaling routes: MEKKs-MKK3/6-p38, ASK1/TAK1-MKK4-JNK/p38, and MLK-MKK7-JNK. Similar to ERK, JNK and p38 also amplify the transduced signals in response to stimulation of pro-inflammatory factors and environmental stress through their specific substrates including c-Jun, p53, Bax, Tau, ATF-2, HuR, EST1, HSF-1, JunB, cPLA2, GADD153, and MK2/3 in spite of some shared substrates with ERK kinases such as ELK-1, NF-AT, STAT3, c-Myc MNK, MEF2, and MSK. Unsurprisingly, both proliferative and proapoptotic phenotypes can be triggered due to the complexity of these substrates that may induce both apoptosis and survival (Wada and Penninger 2004). Therefore, the cell fate regulated by MAPK signaling pathways is possibly determined by the equilibrium of the input signals of proliferation and apoptosis, where overexpression of either interferes with cellular homeostasis (Royuela et al. 2002). Another branch in the canonical MAPK signaling is mediated by ERK5, previously called BMK1 (big MAPK kinase 1). Knockout of ERK5 in mice induces early embryonic lethality due to impaired angiogenesis and cardiac development. Tissue-specific depletion of ERK5 leads to impaired endothelial cell development and vascular integrity (Hayashi and Lee 2004). Further studies dissect its role in the regulation of cell survival in response to the stimulation of both growth factor and pro-inflammatory pathways (Drew et al. 2012).

Although the canonical MAPK pathway is ubiquitously present, biochemical studies have revealed a set of unique kinases remotely linked to the conventional branches. These kinases fall into three subgroups based on individual messenger MAPKs. It is not clear whether these kinases can be interpreted by the characteristic three-tiered cascades as many of the components have not been identified (or do not exist at all). However, it is demonstrated that the unique messenger MAPKs, such as ERK3/4, ERK7/8, and NLK, do not harbor the same consensus phosphorylation motif Thr-X-Tyr (Coulombe and Meloche 2007). Because of many less-understood observations, it is still too early to review the functional relevance and importance of these atypical MAPKs in physiology, although recent studies have shed some light in this field (Aberg et al. 2009; Al-Mahdi et al. 2015; De la Mota-Peynado et al. 2011; Long et al. 2012; Sirois et al. 2015). Taken together, the complex signaling networks established by MAPKs are capable of generating highly specific signals to induce remarkable diversities of cellular responses to regulate the machinery of cell fate control.

2.3 JAK/STAT Signaling in the Regulation of Cell Proliferation and Survival

The third appreciated intracellular signaling axis is Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Notably, recent studies from clinical disorders reveal that JAK/STAT is one of the most deregulated signaling routes in secondary hyperactivation of parallel survival signaling in acquired therapeutic resistance to cancer therapies (Britschgi et al. 2012; Sun and Bernards 2014). Despite that the deregulated activation by RTKs frequently occurred during oncogenic progression (Wicki et al. 2016), JAK kinases are classical non-receptor tyrosine kinases that transduce cytokine-mediated signals. Compared to the MAPK signaling network, the JAK/STAT module seems to be simple; upon binding of individual cytokines to their corresponding receptors, four JAK family members (JAK1–3 and TYK2) can be differentially recruited to cytokine receptors and activated by trans- and/or autophosphorylation. Activated JAK kinases subsequently induce specific tyrosine phosphorylation on the intracellular tail of cytokine receptors that serve as a docking site for inactive cytosolic STAT proteins. To date, seven STAT family members have been identified in mammals. Direct phospho-activation by JAKs leads to oligomerization of STAT proteins, either homo- or heterodimerization, and rapidly translocates into the nucleus to activate distinct transcriptional programs (Levy and Darnell 2002). Several well-studied genes are directly regulated by STAT transcription factors. For example, STAT3 can upregulate MMP2 and MMP9 that increase cell motility, and VEGF, bFGF, and HIF-1 α that stimulate angiogenesis. Importantly, activated STATs can directly upregulate anti-apoptotic genes such as Bcl-X_L and cyclin D1, providing an enhanced anti-apoptotic potential to cells under stress conditions, implying a pro-survival role of JAK/STAT-mediated cytokine signaling (Rawlings et al. 2004).

3 Signaling Interaction Network Between PI3K/Akt, Ras/MAPK, and JAK/STAT Axes

Homeostatic development requires a balanced activity of each signaling cascade. Multiple physiological mechanisms have been shown to regulate cell growth and aging. In principle, these mechanisms can be characterized into two categories: autonomous signaling feedback loop and dependent signaling crosstalk loop, both of which can be integrated at transcriptional and translational levels. When this physiological functionality is taken by the cancer cells in clinical treatment, the pharmacologically stressed cancer cells may evolve to neglect the single-pathway-suppression-induced cytotoxicity. If such aptitude is (epi-)/genetically established, the acquired resistance of cancer cells to pathway inhibitors will inevitably be developed. Notwithstanding the well-understood negative self-feedback signaling mediated by the docking proteins insulin receptor substrate (IRS), GRB2-associated binder (GAB), and FoxO along the mTOR/PI3K/Akt axis (Chandarlapaty et al. 2011; Hsu et al. 2011; Laplante and Sabatini 2012; Yu et al. 2011), clinical observations evidently confirm that the interaction network

between PI3K/Akt, Ras/MAPK, and JAK/STAT has been implicated to play a vital survival role in promotion of cancer cells defending apoptotic induction, particularly in the context of therapeutic treatments (Fig. 3).

Signaling crosstalk can be either cross-activation or cross-inhibition. Activation of Akt may directly phospho-inhibit Raf (Moelling et al. 2002; Reusch et al. 2001) to maintain adequate MAPK activity, while preventing from stress-induced apoptosis by phosphorylating ASK1 (Kim et al. 2001; Zhang et al. 2005) and MLK (Figuroa et al. 2003). Meanwhile, Akt can promote its activation by transcriptional reprogramming through its downstream substrates, such as Twist (Cheng et al. 2007; Xue et al. 2012; Yang et al. 2015), that complies a feedforward signaling loop; in contrast, to avoid unwanted hyperactivation, it can suppress mTORC1-directed IRS1/GAB-mediated upstream RTK signalosomes. Moreover, mitogen-activated ERK was also reported to enhance mTORC1 activation through direct phosphorylation of Raptor, a key regulatory subunit of mTORC1 (Carriere et al. 2011), and indirectly through its downstream kinase RSK that may phosphorylate Raptor (Anjum and Blenis 2008; Carriere et al. 2008a). Such cross-activation-resulted enhancement of the mTOR pathway is also reflected by ERK-dependent inactivation of the intrinsic inhibitory Tuberous Sclerosis Complex 2 (TSC2) that behaves similar to the mode of Akt and suppresses mTOR activity (Huang and Manning 2009; Inoki et al. 2002; Ma et al. 2005). Reciprocal elevation of ERK activity can also be triggered by inactivation of GSK3 in an Akt-dependent manner (Ding et al. 2005). Interestingly, *in vitro* kinase assay revealed that PDK1 is able to promote MAPK activation by phosphorylating MEK1 and MEK2 (Lee et al. 2012).

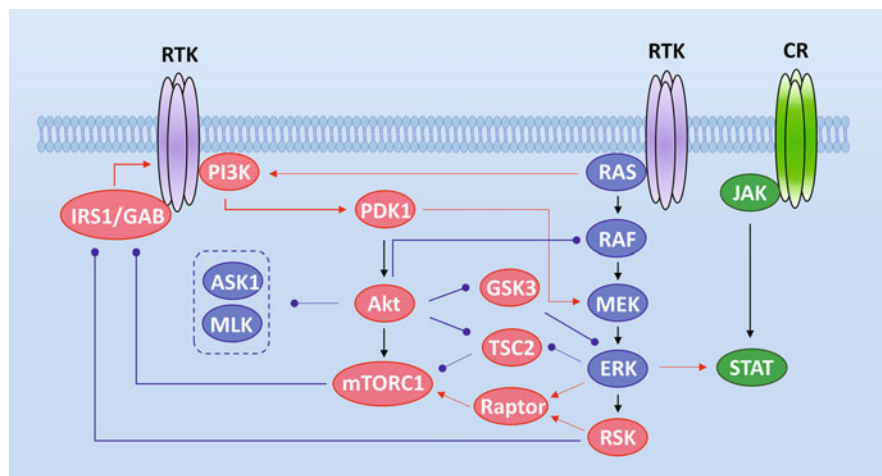


Fig. 3 Molecular interactions between three pathways of PI3K/Akt, Ras/MAPK, and JAK/STAT. The molecules highlighted in red are key components in the PI3K/Akt pathway, while in blue and in green belong to the MAPK and JAK/STAT pathways, respectively. The arrows in red represent “phospho-activating” and in blue represent “phospho-inactivating”