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Stefan Grimm *Editor*

Anticancer Genes

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Dedicated – as always – to my parents
and my sister Susanne

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Chapter 1
Introduction

Stefan Grimm

Contents

1.1 The Quest for Novel Agents in Cancer Treatment

Cancer is a devastating disease with an estimated 14 million new cases occurring worldwide in 2012. This is a substantial increase from the 2008 number of 12.7 million. If these trends are projected into the future, globally, the burden of cancer will increase to 19 million new diagnoses each year by 2025. Cancer is also a leading cause of death with 8.2 million casualties (around 15 % of all deaths) in 2012, which has equally increased since 2008 [4, 5]. So evidently, despite huge efforts to tackle the disease, current treatments are largely ineffective and there is an urgent need for innovative ideas that deviate from conventional approaches.

The search for novel chemical agents against cancer has long been the mainstay of cancer research. The dye industry in the nineteenth century created an abundance of new chemicals some of which were found to specifically stain and kill pathogens such as the malaria parasite Plasmodium leading to the chemotherapy concept by Paul Ehrlich [10]. The fortuitous discovery of nitrogen mustard-based chemicals in the 1940s extended Ehrlich's idea to tumor cells and proved to be a successful, even though short-lived, treatment against lymphomas and lung cancer [1]. In the following decades the search for additional chemical anticancer agents intensified yielding

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many low molecular weight compounds, which are classically assigned to five general categories that are currently used in the clinic: alkylating agents (such as cisplatin), antimetabolites (such as methotrexat), topoisomerase inhibitors (e.g. etoposide), cytotoxic antibiotics (e.g. doxorubicin), and alkaloids (e.g. taxol). Each new chemical harnessed a different mode of action in the fight against cancer and hence constituted progress. Common to many of these anti-cancer compounds is that they activate apoptosis, the genetically regulated cellular suicide program. Based on the historical evidence, one can assume that any additional chemical entities, if found suitable for cancer treatment, will further extend the armamentarium against the disease and reveal additional apoptosis sensors in malignant cells. Consequently, uncovering novel chemicals for the treatment of cancer is an ongoing challenge, and extensive efforts are being made both in industry and academia to discover additional drugs that specifically target malignant cells. Despite this hard work, the development pipelines of pharmaceutical companies that specialize in cancer treatment have been drying up in recent years, possibly because most of the targets in cancer cells that can be reached with chemical compounds have already been discovered [2]. Moreover, all the known anticancer chemicals are of low molecular weight and consequently are limited in their specificity. Hence, they elicit toxic side effects in patients that preclude using higher and more efficacious doses and hence cannot unfold their full potential. Most importantly, many tumors are *ab initio* refractory or eventually become resistant to these chemicals through the accumulation of mutations. These observations reveal considerable shortcomings of conventional low molecular weight drugs.

1.2 Anticancer Genes

In this book we have compiled the evidence for a fundamentally novel class of agents that can target cancer: Genes and their encoded proteins. We call them Anticancer Genes [7].

Over the last couple of years research in the laboratories of the authors in this book has led to the discovery of a handful of genes with specific anti-cancer activities. Upon ectopic expression these genes cause cell death by apoptosis (or alternative forms of cell death) in tumor cells but spare normal cells. What could be the biological reason that such genes exist? To answer this question it is helpful to recall that, just like with oncogenes, the first Anticancer Gene (Apoptin, Chaps. 2 and 3) was discovered in the genome of a virus (chicken anemia virus, CAV) [3]. So, one could speculate that the specificity of Apoptin and other viral Anticancer Genes for tumor cells lies in the fact that the induction of apoptosis forms an obligatory phase of virus replication and as CAV can only replicate in certain lymphoid and haematopoietic cells, namely those that are actively proliferating, it is especially active in cells with limitless replication, one of the fundamental features of tumor cells. Apoptosis may then be a cellular defense mechanism of the host cells to prevent the virus from spreading and removes the infected cell without inducing an inflammatory reaction. Consequently, in this interpretation the

cell death observed with viral Anticancer Genes would not be a fate imposed by them but rather a defense mechanism of infected, actively proliferating target cells [12]. Apart from viral Anticancer Genes, research over the past couple of years revealed that the genome of humans and other mammals also contain Anticancer Genes and hence the above arguments cannot apply to those cellular Anticancer Genes. But why would Nature "invent" genes that specifically target transformed tumor cells? The simplest answer could be that cellular Anticancer Genes function as tumor suppressor genes, which, when transfected as wild type allele into cells, reconstitute an apoptosis pathway that is compromised in tumor cells: and indeed, in some cases, such as par-4 (Chap. 7), they encode tumor suppressor proteins. However, Anticancer Genes can also exert a neomorphic activity that goes beyond their normal function at physiological expression levels and that is only understood as a suicide signal by the transformed cancer cell based on the concept of synthetic lethality (see below). An example for such a gene would be ORCTL3, which normally functions as a transporter but upon overexpression inhibits an enzyme in fatty acid metabolism (Chap. 11). On the other hand, the majority of the cellular Anticancer Genes might exert the same effect as they do in normal cells, e.g. activate certain signalling pathways. Only that these signalling pathways create a conflict in tumor but not in normal cells thereby leading to apoptosis and seemingly conferring a novel function to the Anticancer Gene. For instance, it is conceivable that genes that halt the cell cycle are in conflict with the cell cycle machinery in cancer cells that is geared towards excessive proliferation. In fact, the substantial body of work on MDA-7/IL-24 for example supports targets that are part of the cell cycle machinery (Chap. 6).

1.3 Expectations of Anticancer Genes

Anticancer Genes are investigated with two main aims, which will be elaborated in the various chapters of this book:

I. Anticancer Genes and their proteins constitute novel agents for cancer treatment
II. Anticancer Genes allow determine the specific cellular signal they exert and hence reveal a unique vulnerability of cancer cells, which will uncover novel treatment options

The first aim is based on the realization that genes and their encoded proteins possess a number of distinct advantages over conventional low molecular weight compounds currently used in cancer treatment that make them promising therapeutic agents. One, in particular, is based on their superior structural complexity, which leads to more specific interactions of the proteins encoded by those genes. This makes them less limited by toxic side effects when used at high concentrations.

One key insight emerging from the discovery of cellular Anticancer Genes is that artificial, exogenous chemicals are not the only or even the best source of agents to control cancer cells. Rather it is endogenous genes that constitute potential

therapeutic reagents. But can we find ways to activate those genes? Overexpression is what makes Anticancer Genes exert their effect, so the most direct way to exploit this would be to cause an accumulation of their gene products in the tumor cells. The second possibility to make use of this insight will be discussed when we turn to the second aim connected with Anticancer Genes below.

The notion of Anticancer Genes as therapeutics leads to the concept of "genetic medicine" in gene therapy, in which genes are used against tumors, not to replace or repair a defect, but to accomplish a specific therapeutic effect by the transient overexpression of the gene. This assumes that in the tug of war with the tumor, much like in any game, one has to comply with the rules and use the appropriate tools, in this case genes and their encoded proteins, to best keep the opponent in check. With the accelerating progress being made in gene therapy to introduce genetic material into cells, the prospect of developing Anticancer Genes into therapeutics looks increasingly realistic. Transferring genes efficiently into tumor cells is still mostly achieved by viral gene delivery (Chap. 13). Nanoparticles as well are making significant advances as gene delivery vehicles in nanomedicine, one of the most dynamic fields of nanotechnology [9]. Alternatively, the proteins of Anticancer Genes can be developed into therapeutics through fusion with cell-penetrating peptides (CPP)/peptide transduction domains (PTDs) such as TAT and its functional derivatives including optimized versions exhibiting proton sponges to aid with specific endosomal escape. This "protein therapy" approach has become a viable alternative as highlighted by over 20 Phase I and Phase II clinical trials [13]. Also, additional innovative methods are under investigation such as sonoporation in which ultrasound in combination with micro-bubbles is used to transport genes into cells (Chap. 12) [8]. In fact the therapeutic potential of Anticancer Genes is investigated in several clinical trails [7] some of which are described in this compendium (Chaps. 3, 5, 6, and 8).

The second main expectation of Anticancer Genes is that their higher specificity can be exploited to determine how they target tumor cells by uncovering, using established molecular biology methods for e.g. protein-protein interactions, and the signalling pathways they engage. The complex signalling circuits governing the cell death response in combination with the principle of synthetic lethality (see below) provide the conceptual framework for these efforts.

In most cases the cell death mode caused by Anticancer Genes is the cellular suicide program of apoptosis but also other, equally genetically regulated, cell death responses such as excessive autophagy have been described [7]. All of them are governed by elaborate networks of signalling pathways that integrate various signals the cell is receiving at any given time. If correct what has been implied above, that Anticancer Genes activate pre-existing signalling pathways that create conflicts in the cells, it could be accomplished along the whole length of a signalling cascade. The modules of these pathways have been found to mostly consist of protein complexes that respond to inputs in the form of differentially activated or recruited pro- and anti-apoptotic signalling factors. If those protein complexes, the sensors of apoptosis, are changed in cancer, it would allow for specific activation in malignant but not in normal cells. This is the reason why the

concept of synthetic lethality as described below is so important for understanding Anticancer Genes. Consequently, these signalling circuits are not only key in explaining the activity and specificity of Anticancer Genes, but can also lead to additional interference options by activating them at more upstream positions, possibly even at the plasma membrane. This would circumvent the problem of having to introduce the genes into cells.

In genome databases an abundance of information on virtually any gene exists nowadays, which, in contrast to proprietary compounds, makes it much easier to integrate Anticancer Genes into a cellular context and assess how they exert their effect. While compounds mostly only inhibit proteins and their signalling circuits in the cell, Anticancer Genes can also activate signalling pathways and thereby generate novel signals for the specific destruction of malignant cells. The consequences can be pleiotropic, i.e. Anticancer Genes can impact on multiple signalling events in the cell. Hence, individual changes in cancer cells are not necessarily the only target for Anticancer Genes, it can also be the combination of tumor-specific changes that create specificity. This is exemplified in various chapters of this book with Anticancer Gene exerting a number of different activities. Thus, with genes not only more refined but also additional interference options are accessible and could overcome the treatment resistance of tumors. Moreover, the knowledge about their mode of action can lead to optimization through specific mutations and selection of the most potent variant as nicely exemplified by par-4 whose SAC domain is more powerful than the entire gene (Chap. 7).

1.4 Synthetic Lethality: The Genetic Basis of Anticancer Genes

The activity of Anticancer Genes is based on the principle of synthetic lethality. This phenomenon was first described in the early twentieth century in experiments on crossing fruit flies (Drosophila melanogaster). Certain non-allelic genes were found to be lethal only when combined, even though the homozygous parents that harbored either genetic defect were perfectly viable. The term “synthetic lethality” was only coined later when the same phenomenon was observed in another Drosophila species (pseudoobscura) [11]. “Synthetic” is used here for its ancient Greek meaning: the combination of two entities to form something new. Consequently, synthetic lethality is defined as a type of genetic interaction where the co-occurrence of two genetic alterations results in organismal or cellular death. In other words: a mutation is synthetic lethal with another genetic change when only in the presence of the latter the former can cause cell death. In the case of Anticancer Genes the tumorigenic mutations such as the deletion of tumor suppressor genes and the activation of oncogenes are responsible for the genetic background in which the second mutation, the overexpression of the Anticancer Gene, is lethal to the cell. Hence, completely novel functional interactions can be found and exploited by

Anticancer Genes for the specific removal of cancer cells as synthetic lethality can not only target the tumorigenic mutations themselves but also their downstream signals. This experimental setting has the advantage that it may help overcome the challenge associated with tumor suppressors, which are dysfunctional or even absent in transformed cells.

Tumors are not a uniform cell population. The sequencing of the cancer genome, sub-sections of tumors, and even individual tumor cells emphasize that they are composed of a very heterogeneous set of cells with respect to their genetic background [6]. For the synthetic lethality of Anticancer Genes this means that they have to function in various genetic backgrounds. In fact, most, if not all, so far known Anticancer Genes target a wide variety of transformed cells. This is probably made possible by the fact that Anticancer Genes have the ability to exert a range of signals in cells. Indeed, as the chapter on the Anticancer Gene Apoptin will reveal (Chap. 2), there are a number of targets in the cells to accomplish its effect. It is currently unknown to what extent the known Anticancer Genes overlap in terms of their targets and activities. Additional and so far unknown Anticancer Genes could be very specific in their requirement for a certain genetic background and hence are active only in certain, very defined tumor scenarios – much like chemical compounds that are known to be active only in certain tumor cells. Cisplatin, for example, is very successfully used in the treatment of testicular cancer and its derivative carboplatin is the main treatment option for ovarian cancer. Much less data on such efficacies exist for Anticancer Genes.

As promising as Anticancer Genes are, the field faces a number of challenges. In particular its application in clinical trials is the "acid test" for Anticancer Genes. The outcomes of the various clinical trials described in this book are promising and additional ones are currently being conducted. If Anticancer Genes can indeed be established as an alternative treatment option for established protocols, those trials are critical. Moreover, whereas compounds have various problems as indicated above, Anticancer Genes have one main issue: How to efficiently introduce the gene or its product, the protein, into cells. The last two chapters of this book present exciting novel and innovative techniques but they still have to be established for routine use with Anticancer Genes.

Cancer cells are known to develop resistance to treatments. As mentioned above, the diverse signalling effects of Anticancer Genes could make this more difficult but no data are available on how fast resistance to Anticancer Genes can develop. Moreover, the genetic alterations targeted by Anticancer Genes based on the principle of synthetic lethality might not underlie tumorigenesis – i.e. they are "passengers" rather than "drivers" in this process and hence when reversed would not compromise the tumor's growth as they are non-essential for malignant cells.

The conventional approach in cancer research is to determine the genetic changes in tumor cells in comparison with normal cells and then target those changes. The specificity of targeted therapy, i.e. drugs that are specific for certain mutations connected with cancer, is based on this. In contrast, with Anticancer Genes, as they were found based on their mere effect on cancer cells, their mode of action is a *priori* unknown. So, it is evident that much work remains to be done.

This book tries to give an overview of the known Anticancer Genes. We introduce viral Anticancer Genes, beginning with the first discovered Anticancer Gene, Apoptin, whose signalling for cell death as well as the results on its clinical trials are presented in Chaps. 2 and 3, respectively. This is followed by the sections on E4orf4 (Chap. 4), and, likewise of viral origin, NS1 (Chap. 5). The chapters on cellular Anticancer Genes then introduce mda-7/IL-24 (Chap. 6), par-4 (Chap. 7), TRAIL (Chap. 8), and Sirt6 (Chap. 9). The chapters on Bevinin (Chap. 10) and ORCTL3 (Chap. 11), which were isolated from amphibians and mice, respectively, reveal that also species other than humans harbor Anticancer Genes. The last two chapters focus on methods of introducing the genetic material of Anticancer Genes into cells. Viral vectors are presented for this purpose (Chap. 13) as well as an emerging technology that uses physical means such as sound waves and electrical fields to load cells with Anticancer Genes (Chap. 12).

Who is this book for? It is indented for scientists, clinicians, students, journalists, editors, and laypersons. We provide an overview of the nascent field of Anticancer Genes, its genesis, current state, and prospect – to familiarize the reader with a novel concept in cancer research. The book tries to point out the therapeutic promises and challenges of those genes – to inform and inspire clinicians. Most of all, it describes the so far known Anticancer Genes in many cases by their discoverers – to educate students, researchers both in academia and industry, and laymen about a rapidly developing area of cancer research.

We, those scientists working on Anticancer Genes, strive to further establish a promising field of cancer research: Introducing the biologically most specific tools, genes and their encoded proteins, as a novel class of agents in the fight against cancer. We invite the reader to join us in this endeavor. We believe the journey has just begun.

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Part I
Viral Anticancer Genes

Chapter 2
Signalling of Apoptin

Jessica Bullenkamp and Mahvash Tavassoli

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Abstract The virus-derived protein Apoptin has the ability to induce p53-independent apoptosis in a variety of human cancer cells while leaving normal cells unharmed. It thus represents a potential anti-cancer therapeutic agent of the future but a proper understanding of Apoptin-induced signalling events is necessary prior to clinical application. The tumor-specific nuclear translocation and phosphorylation of Apoptin by a cellular kinase such as protein kinase C seem to be required for its function but otherwise the mode of tumor selectivity remains unknown. Apoptin has been shown to interact with several cellular proteins including Akt and the anaphase-promoting complex that regulate its activity and promote caspase-dependent apoptosis. This chapter summarizes the available data on tumor-specific pathways sensed by Apoptin and the mechanism of Apoptin-induced cell death.

Keywords Cancer • Tumor selectivity • Molecular therapy • Apoptosis • p53 family • Protein kinase C (PKC) • Targeted therapy • DNA damage

2.1 Introduction to Apoptin

Apoptin is among the first tumor selective anti-cancer genes that have been isolated. This small viral protein induces p53-independent tumor cell-specific cell death. In this chapter we will discuss cellular pathways that are important for cell death initiation by Apoptin and possible mechanisms which regulate its tumor selectivity.

Apoptin was originally identified as the apoptosis-inducing protein derived from Chicken Anemia Virus (CAV), a single-stranded DNA virus of the Gyrovirus genus [1]. Infection with the virus gives rise to a severe disease in young chicken characterized by lymphoid atrophy, anemia, thrombocytopenia and intramuscular hemorrhages with mostly fatal consequences [2, 3]. The major targets of the virus include myeloid progenitor cells in the bone marrow and T lymphocyte precursor cells in the thymus. When infected these cells undergo apoptosis causing depletion of mature erythroid, myeloid and lymphoid cells resulting in the characteristic disease symptoms [4]. The CAV genome contains three partially overlapping open reading frames encoding viral proteins from a single polycistronic mRNA: VP1 (capsid protein), VP2 (protein phosphatase, scaffold protein) and the death-inducing protein VP3 [3]. Expression of VP3 alone was shown to be sufficient to trigger cell death in chicken lymphoblastoid T-cells and myeloid cells but not in chicken fibroblasts and it was therefore renamed Apoptin [5].

Recently, several viruses resembling the structure and overall organization of CAV have been isolated from human specimens and have now been included in the Gyrovirus genus [6–9]. Like CAV the first identified human Gyrovirus (HGyV) contains a VP3 protein homologous to Apoptin which exhibits similar features as

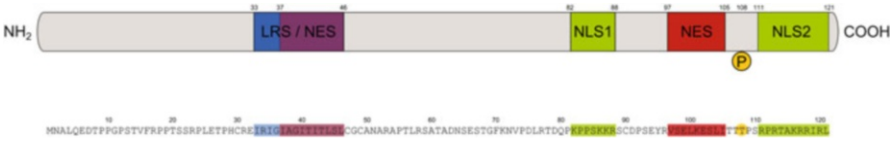

Fig. 2.1 Structure and sequence of Apoptin. The positions of key functional domains of the protein such as the nuclear localization sequence (*NLS*), nuclear export signal (*NES*) or the Thr-108 phosphorylation site are indicated. The *lower panel* shows the sequence of Apoptin; the corresponding amino acids of indicated domains are *highlighted*

CAV-Apoptin that are described below [10]. However, the importance of these viruses in human disease and the therapeutic potential of HGyV-Apoptin remain to be determined.

Since its discovery in the early 1990s Apoptin has become of interest in cancer research due to its ability to induce apoptosis in a variety of human tumor cells while leaving normal cells relatively unharmed [11–14]. Overall more than 70 cell lines from different tumor types, including melanoma, lymphoma, colon carcinoma and lung cancer, have displayed sensitivity to Apoptin-induced cell death. In addition SV40-transformed cells or UV-irradiated cells from patients with hereditary cancer-prone disorders are susceptible to Apoptin expression [11, 15, 16]. Thus, Apoptin seems to sense an early event of oncogenic transformation and induces cancer-specific apoptosis regardless of tumor type and presents a potential future anti-cancer therapeutic agent.

Several studies have been investigating strategies for the efficient delivery of Apoptin to the tumor in vivo, some of which will be discussed elsewhere in this book. In order to develop Apoptin into an effective anti-cancer drug it is important to understand Apoptin-induced signalling events. In addition, the identification of tumor-specific pathways activating Apoptin will help to develop novel and specific anti-cancer treatments. Hence this chapter focuses on the cellular pathways initiated by Apoptin and the mechanisms which determine Apoptin tumor cell selectivity.

2.2 Structure of Apoptin

Apoptin is a small protein of 14 kDa rich in proline, serine, threonine and basic amino acids and does not share any sequence homology with known cellular proteins described so far (Fig. 2.1). It comprises a C-terminal bipartite nuclear localization sequence (NLS) spanning amino acids 82-88 (NLS1) and 111-121 (NLS2) as well as a nuclear export signal (NES) which has been mapped to either the N-terminus or C-terminus of the protein. Both of these domains are required to facilitate nuclear shuttling of the protein [17–19]. Moreover, Apoptin contains several potential threonine and serine phosphorylation sites, including threonine 108 (Thr-108) within its C-terminus. These allow for modification by cellular kinases to regulate Apoptin activity and the interaction of Apoptin with other

proteins [20]. A short leucine-rich stretch (LRS) in the N-terminal domain (amino acids 33–46) is important for self-association of Apoptin and its binding to other cellular proteins such as promyelocytic leukemia protein (PML) [21].

Recombinant Apoptin fused to maltose binding protein (MBP-Apoptin) aggregates into stable globular complexes comprising 30–40 non-covalently attached Apoptin monomers in vivo. These multimers retain the ability to induce apoptosis and are stable in tumor cells but seem to be unstable and eventually diminish in normal cells [22, 23]. The formation of these complexes is facilitated through interactions of the hydrophobic regions located in the N-termini of Apoptin monomers (amino acids 1-69) and there seems to be little exchange of Apoptin subunits between multimers. Spectroscopic analyses did not reveal any ordered α-helical or β-sheet secondary structure of Apoptin monomers; however they adopt a stable conformation within the complexes. The three dimensional structure of Apoptin multimers is characterized by hydrophobic patches on the surface as well as the exposure of the C-terminal domains of Apoptin monomers, both of which may affect its binding to other cellular factors [24]. Importantly, Apoptin multimers have been shown to form superstructures with DNA with a preference for DNA strand ends in vitro and localize to DNA-dense heterochromatin and nucleoli in vivo [25]. However, the functional consequences of DNA binding for the cell and for the virus will have to be revealed by further studies.

2.3 Cell Type-Specific Localization of Apoptin

The mode of Apoptin's tumor cell-specific cytotoxicity so far remains unknown although several mechanisms have been proposed. One of the major differences observed is the subcellular distribution of Apoptin that correlates with its ability to induce apoptosis. In tumor cells or transformed cells Apoptin is expressed predominantly in the nucleus whereas in normal cells it is mainly localized in the cytoplasm (Fig. 2.2).

Importantly, nuclear translocation is at least partially required for the ability of Apoptin to induce cell death. Several deletion mutants of Apoptin defective in nuclear translocation thus induce apoptosis less efficiently than wild type Apoptin [14, 17, 26]. Moreover, targeting of Apoptin to the endoplasmic reticulum (ER) or the mitochondria instead of the nucleus reduced its ability to trigger cell death [12]. However, forced expression of Apoptin into the nucleus of normal cells on its own did not result in apoptosis, showing that additional factors are important for the activation of Apoptin. In addition, both the C-terminal and N-terminal domains of Apoptin are able to induce apoptosis on their own, albeit less potently than full-length Apoptin, even though only the C-terminus contains a NLS [17]. Thus nuclear localization is partially required but not sufficient for Apoptin-induced cell death. In a study by Wadia et al. nuclear localization of Apoptin in cancer cells was suggested to depend on the intracellular protein levels of Apoptin instead of the tumorigenic status of the cell [27]. However, nuclear import remained significantly

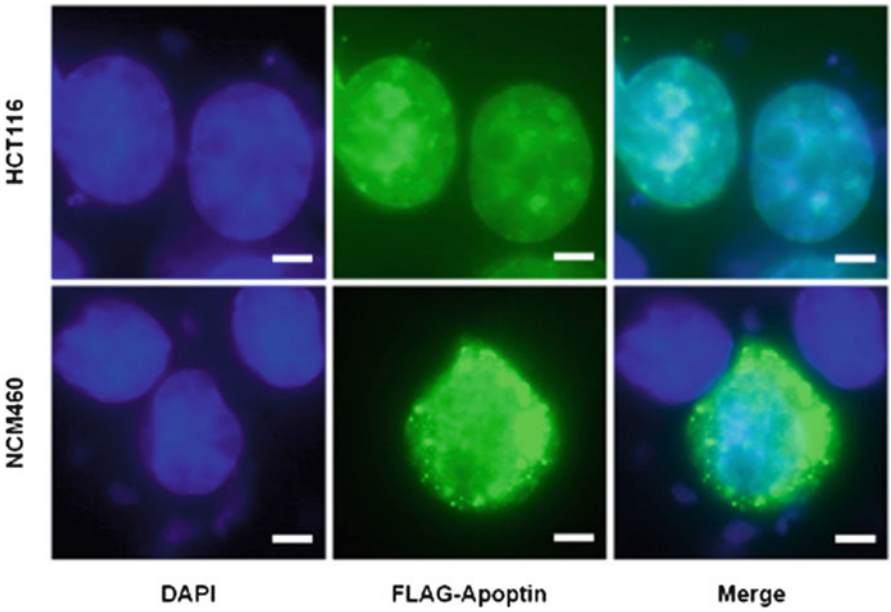

Fig. 2.2 Subcellular distribution of Apoptin in normal and tumor cells. HCT116 colorectal cancer cells and NCM460 normal colon mucosa cells were transfected with FLAG-Apoptin. At 48 h post-transfection cells were fixed and stained with a primary mouse anti-FLAG and secondary FITC anti-mouse antibody for detection of FLAG-Apoptin. In addition, nuclei were detected by counterstaining with DAPI. Scale bars indicate 20 μm

higher in transformed cells compared to untransformed fibroblasts under all transfection conditions tested. Furthermore, their study employed a C-terminal fragment of Apoptin lacking a putative nuclear export signal as well as the Apoptin multimerization domain which most likely affects nuclear shuttling compared to the full length protein. Moreover, the use of Apoptin tagged with green fluorescent protein (GFP-Apoptin) might favor Apoptin translocation to the nucleus even in normal cells. Despite this, GFP-Apoptin was still shown to accumulate in the nucleus to a greater extent in transformed cells compared to their non-transformed counterparts [28, 29].

The nuclear shuttling of Apoptin is regulated by nuclear localization and nuclear export signals that have been identified in mutagenesis studies. Mutation of either of the predicted nuclear localization sequences in the C-terminus resulted in reduced translocation to the nucleus, showing that both sequences form a bipartite NLS are required for efficient nuclear localization of Apoptin [17, 19, 30]. This NLS seems to be active in both normal and tumor cells as inhibition of Crm1-mediated export from the nucleus caused nuclear accumulation of Apoptin in normal cells, suggesting that the cell type-specific localization of Apoptin may be regulated by nuclear export rather than import [18]. While agreeing on the importance of Apoptin's C-terminus to trigger nuclear translocation, two separate

studies have proposed different sequences as potential nuclear export signals. Poon et al. described a NES within the C-terminus of Apoptin (amino acids 97-105) mediating Crm1-recognized nuclear export in normal cells. In their study mutations within the N-terminal LRS (amino acids 33-46) resulted in reduced nuclear accumulation of Apoptin in Saos-2 osteosarcoma cells, suggesting it as a nuclear retention sequence [19]. In contrast, a study by Heilman et al. reported that mutation of the putative N-terminal NES (amino acids 37-46) caused localization of Apoptin to the nucleus in normal cells. Expression of deletion mutants containing only the N-terminal NES resulted in Crm1-dependent nuclear export of Apoptin into the cytoplasm of H1299 non-small cell lung carcinoma cells [18].

In summary, the cell-type specific localization and nuclear shuttling of Apoptin is believed to be controlled by a C-terminal bipartite NLS as well as a NES located in either the C-terminal or the N-terminal half of the protein. Furthermore, the localization of Apoptin can also be affected by the interaction with cellular proteins resulting in its nuclear or cytoplasmic retention.

Although it is clear that nuclear translocation is important for Apoptin-induced killing of tumor cells, the link between subcellular distribution and induction of cell death is still missing. Despite its ability to bind DNA Apoptin does not act as a direct transcriptional repressor or activator but might still indirectly affect the activity of other cellular transcriptional regulators [17].

Interestingly, the induction of DNA damage response (DDR) signalling was shown to trigger nuclear localization of Apoptin and apoptosis induction even in non-transformed primary cells [31]. Conversely, inhibition of DDR signalling, e.g. through knockdown of ataxia telangiectasia mutated kinase (ATM) or DNA-dependent protein kinase (DNA-PK), resulted in cytoplasmic localization of Apoptin in tumor cells. Since many cancer cells are characterized by constitutive DNA damage signalling these data suggest a possible link between DDR, nuclear localization and induction of cell death by Apoptin in tumor cells.

Furthermore, in normal cells Apoptin localizes to the cytoplasm, forms aggregates and becomes epitope shielded, eventually leading to its elimination [23]. Early after injection into VH10 fibroblasts MBP-Apoptin could be detected as larger aggregates in the cytoplasm but later became undetectable and neutralized due to epitope shielding even though the protein was still present. However, eventually MBP-Apoptin was completely degraded in normal cells. In line with that, treatment with proteasomal inhibitors such as Bortezomib has been shown to stabilize Apoptin in normal but not in tumor cells [32]. However, the relevance of this differential sensitivity to proteasomal degradation depending on the cell type still needs further evidence.

In addition, nuclear translocation might be required for interaction with nuclear binding partners in the nucleus such as the anaphase promoting complex or PML bodies that are discussed below.

In summary, even though the role of nuclear translocation for Apoptin-induced cell death has been extensively investigated the precise link to apoptotic pathways remains unclear.

2.4 Tumor-Specific Phosphorylation of Apoptin

Further studies on Apoptin also revealed another interesting property of the protein that provides a potential link to tumor-specific signalling pathways. In cancer cells but not in normal cells Apoptin becomes phosphorylated on threonine 108 (Thr-108) by a tumor-specific but as yet unknown cellular kinase [20]. The phosphorylation on Thr-108 could be detected in vitro and in vivo both in tumor cell lines and human tumor tissue lysates but not from healthy individuals. In addition, transient expression of the SV40 large T antigen (SV40 LT) was shown to trigger Apoptin phosphorylation in normal cell lines [16] suggesting that the Apoptin kinase can be activated by a transforming signal.

Apoptin phosphorylation was suggested to drive nuclear accumulation of Apoptin through inactivation of the adjacent putative NES in tumor cells. A gain-of-function point mutation of Thr-108 to a phospho-mimicking glutamic acid (T108E) led to increased nuclear translocation of Apoptin and cell death induction in VH10 normal fibroblasts [19, 20, 30]. In contrast, another study claims that phosphorylation of Thr-108 is dispensable for Apoptin's tumor-specific nuclear localization and partially also its apoptotic activity [33]. An unphosphorylatable Apoptin mutant or a C-terminally truncated protein lacking the Thr-108 phosphorylation site still retained a partial ability to accumulate in the nucleus and induce apoptosis. This might be explained by the fact that Apoptin contains two distinct pro-apoptotic domains and that Thr-108 phosphorylation is essential only for regulation and activation of the C-terminal death domain. Moreover, Apoptin might be phosphorylated alternatively on the adjacent threonine residues Thr-106 and Thr-107. In support of that, another study showed that both Thr-108 and Thr-107 were required to be mutated to completely abolish Apoptin-induced cell death [29]. The potential mechanism of Apoptin activation through phosphorylation and subsequent nuclear translocation is summarized in Fig. 2.3.

The tumor cell-specific activation of Apoptin through phosphorylation might be due to the presence of a cancer-associated pathway resulting in increased activity of a cellular kinase. Thus, several studies have attempted to identify the kinase or kinases responsible for mediating the phosphorylation of Apoptin in tumor cells. Identification of such kinases has immense importance not only for unravelling the function of Apoptin but may also provide novel targets for the development of selective anti-cancer drugs.

Maddika et al. have reported a sustained activation of the pro-survival PI3K/Akt pathway through direct interaction of Apoptin with the p85 subunit of phosphoinositide 3 kinase (PI3K) [34, 35]. Akt (or protein kinase B) is recruited and activated downstream of the PI3K-induced generation of lipid messengers and regulates processes including cell survival, cell cycle and metabolism [36–38]. In their study, expression of Apoptin triggered sustained activation and nuclear translocation of Akt which was required for Apoptin-induced cell death. However, neither PI3K nor Akt were able to phosphorylate Apoptin directly. Instead, interaction with Apoptin was shown to result in Akt-dependent activation of

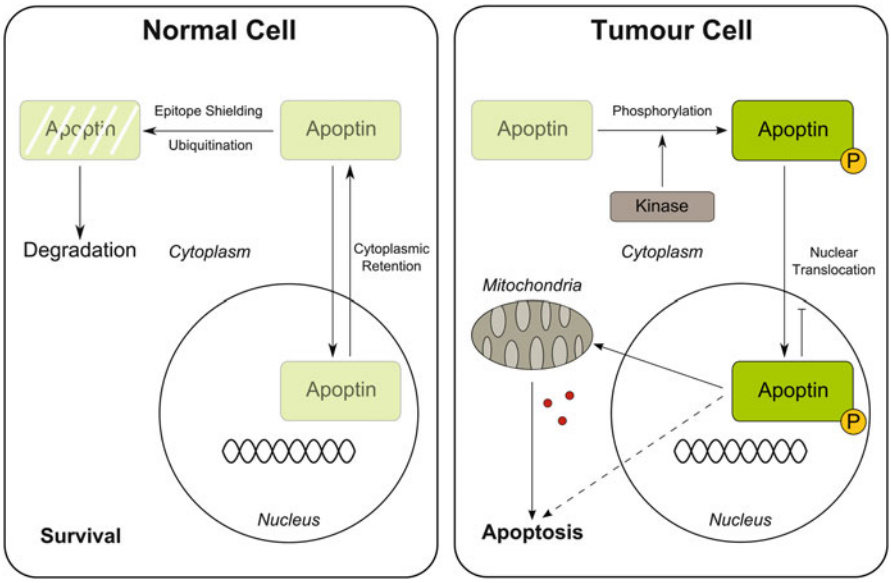

Fig. 2.3 Model of Apoptin activation in tumor cells versus normal cells. In tumor cells Apoptin is phosphorylated by a cellular kinase and translocates to the nucleus where it accumulates. Subsequently Apoptin activates the apoptotic machinery either through the mitochondria or other pro-apoptotic signalling pathways resulting in cell death. In contrast, in normal cells Apoptin shuttles back to the cytoplasm, becomes epitope shielded and eventually degraded in a proteasome-dependent manner

cyclin-dependent kinase 2 (CDK2) which in turn mediated the phosphorylation of Apoptin on Thr-108 in PC3 prostate cancer cells. Pharmacological inhibition or siRNA knockdown of either PI3K/Akt signalling or CDK2 protected cells from Apoptin-induced cell death [39].

In 2010 Jiang et al. proposed the β isozyme of protein kinase C (PKCβ) as a potential Apoptin kinase in a model of human multiple myeloma (MM) cell lines [40]. Dexamethasone-resistant MM1.R cells displayed higher susceptibility to Apoptin-induced cell death than the parentally matched MM1.S cells, correlating with increased Apoptin phosphorylation in MM1.R cells. Microarray expression analysis and RT-PCR revealed a differential expression pattern of several kinases including PKCβ which showed a ninefold higher mRNA level in MM1.R cells compared to MM1.S. Since recombinant PKCβ was able to phosphorylate MBP-Apoptin in an in vitro kinase assay, further experiments were conducted to confirm the link between PKCβ and Apoptin in vivo. Pharmacological inhibition of PKCβ but not of Akt as well as shRNA-mediated knockdown of PKCβ significantly reduced Apoptin phosphorylation in MM1.R cells. Further immunoprecipitation studies also identified a physical interaction between Apoptin and PKCβ in vitro and in vivo, supporting an important role for PKCβ in regulating Apoptin activity. This study also showed that Apoptin expression results in PKCβ upregulation and

activation as well as its translocation to the nucleus of HCT116 colon carcinoma cells together with Apoptin. Besides, expression of Apoptin also induced cleavage of PKCδ, a known pro-apoptotic PKC isoform that is able to activate caspases and in turn gets activated through proteolytic processing by effector caspases. However, whether PKCδ cleavage and activation is a causal factor or merely a consequence of Apoptin-induced cell death requires further investigation.

PKC is a group of serine-threonine kinases that comprises at least 12 known isoforms sharing a common structure and mechanism of activation [41]. They were originally identified as the receptors for tumor-promoting phorbol esters in the early 1980s [42] and many PKC isoforms can contribute to tumorigenesis and invasive or metastatic phenotypes of cancers. However, each isoform exerts different cell type-dependent effects on cellular pathways controlling apoptosis or cell survival. For example, upregulation of PKCβ2 seems to promote early colon carcinogenesis through increased proliferation and invasion of epithelial cells as well as proliferation in general [43–45] while PKCδ is mediating cell death acting as a tumor suppressor in many cell types [46, 47]. In multiple myeloma upregulated PKC signalling, in particular of PKCβ, has been implicated in pathogenesis and the PKC inhibitor Enzastaurin was shown to inhibit the survival and proliferation of MM cell lines [48]. Thus, in the study by Jiang et al. Apoptin senses a tumor-specific survival pathway and potentially redirects it towards induction of apoptosis by a yet unknown mechanism.

Recently, inactivation of the protein phosphatase PP2A through expression of the transforming SV40 small T antigen (SV40-ST) or RNA interference was shown to be sufficient to activate Apoptin in normal VH10 fibroblasts [49]. Thus, this study proposes the differential activation of phosphatases rather than kinases in regulating Apoptin's tumor selective phosphorylation and function. Inactivation or downregulation of PP2A has been linked to oncogenic transformation and it might represent a central regulator for Apoptin activation. However, further studies will be necessary to establish the role of PP2A, or dephosphorylation in general, for the regulation of Apoptin activity.

In summary, while it is clear that Apoptin phosphorylation on Thr-108 is important for its death-inducing ability, a single tumor-specific Apoptin kinase could not be identified so far. Most likely several cellular kinases are involved in phosphorylating Apoptin in cancer cells, depending on the tumor type. A common denominator seems to be the fact that Apoptin is able to turn established pro-survival pathways such as PI3K/Akt or PKC signalling, which are often upregulated in cancers, into a death-inducing signal. In addition, recent evidence also points to a potential role of protein phosphatases in regulating Apoptin phosphorylation and function.

2.5 Interacting Proteins

To exert its tumor-specific cytotoxicity Apoptin needs to become activated to be able to trigger cellular signalling pathways. Several cellular proteins have been identified to interact or co-localize with Apoptin and affect its cytotoxic function or

Table 2.1 Apoptin interaction partners

Protein	Biological function	References
Protein Kinase C β (PKCβ)	Binding of Apoptin to PKCβ results in PKCβ-dependent phosphorylation and activation of Apoptin and nuclear translocation of PKCβ	[40]
Fas-associated protein with death domain (FADD)	Overexpression causes co-localization of FADD and Apoptin in cytoplasmic death effector filaments, potentially interfering with death receptor signalling	[12]
Bcl-10	Apoptin and the NF-κB regulator Bcl-10 co-localize in cytoplasmic filaments with a yet unknown consequence	[12]
Protein Kinase G (PKG-I)	High levels of PKG-I in normal cells correlate with lower Apoptin activation, however the precise role for PKG-I during Apoptin-induced cell death remains unclear	(unpublished data)
PI3-Kinase (PI3K) and Akt	Apoptin binds to the p85 subunit of PI3K, activating PI3K which results in sustained activation and nuclear translocation of Akt. This triggers activation of CDK2 which in turn phosphorylates Apoptin	[34, 35, 39]
Heat shock cognate protein 70 (Hsc70)	Binding of Apoptin triggers nuclear translocation of Hsc70 which might be required for Apoptin-induced Akt activation and downregulation of p65	[50, 51]
Promyelocytic leukemia protein (PML)	Apoptin is sumoylated and targeted to nuclear PML bodies. However, the interaction with PML is not required for the cytotoxic function of Apoptin	[21]
Anaphase promoting complex 1 (APC1)	Apoptin binds to APC1, disrupting the APC/C and resulting in G2/M arrest and apoptosis	[18, 52]
Hip-1 protein interactor (Hippi)	In normal cells but not in tumor cells Apoptin and Hippi interact in the cytoplasm	[53]
Death effector domain-associated factor (DEDAF)	Co-expression of Apoptin and DEDAF results in enhanced apoptosis compared to expression of either protein alone	[54]
Peptidyl-prolyl isomerase-like 3 (Ppil3)	Overexpression of Ppil3 can promote cytoplasmic localization of Apoptin	[55]
N-myc interacting protein (Nmi)	Nmi was identified as an Apoptin binding partner but no functional studies are available yet	[56]
Breast cancer associated gene 3 (Bca3)	Bca3 enhances Apoptin phosphorylation and cytotoxic function by a still unknown mechanism	[49]

cellular localization. Many of these interacting partners have been shown to either directly activate Apoptin or to be involved in initiating signalling pathways which lead to induction of apoptosis (Table 2.1). These include PKC, p73, anaphase promoting complex 1 (APC1) or PI3K and Akt. For others the importance of Apoptin binding still needs to be further investigated such as heat shock cognate protein 70 (Hsc70), Bcl-10 or N-Myc-interacting protein (Nmi).

2.5.1 Protein Kinase C (PKC)

Apoptin was shown to interact with the PKCβ isozymes in vitro and in vivo [40]. PKCβ1 and PKCβ2 could be detected in complexes immunoprecipitated from MM1.R multiple myeloma cells expressing GFP-Apoptin using an anti-GFP antibody. Other cellular kinases including MERTK, Akt, PI3K/p85, PKCα and PKCδ were not found to interact with Apoptin in this model. The interaction of PKCβ with Apoptin resulted in PKCβ-dependent phosphorylation and activation of Apoptin as well as translocation of PKCβ together with GFP-Apoptin to the nucleus. This suggests a novel mechanism in which Apoptin expression induces PKC activation consequently leading to Apoptin phosphorylation and induction of apoptosis, either through Apoptin or the activation of other apoptotic signalling pathways.

2.5.2 Bcl-10 and Fas-Associated Protein with Death Domain (FADD)

At early stages of expression in Saos-2 cells Apoptin localizes to cytoplasmic filaments resembling so-called death effector filaments (DEF). These DEFs are usually generated upon overexpression of proteins from the death domain superfamily that are involved in the extrinsic pathway of apoptosis. In Saos-2 cells overexpression of GFP-tagged Fas-associated protein with death domain (FADD) or Bcl-10 resulted in their co-localization with Apoptin in cytoplasmic filaments [12].

FADD functions as an adaptor molecule required for the formation of the death inducing signalling complex (DISC) in response to apoptotic signals activating death receptors on the cell surface such as Fas or tumor necrosis factor receptor 1 (TNFR1) [57]. The interaction of FADD with the receptor through its C-terminal death domain (DD) results in exposure of the N-terminal death effector domain (DED) and subsequently recruitment of pro-caspase-8 to the DISC to initiate the proteolytic caspase cascade [58]. Thus Apoptin might potentially be involved in modulating components of the extrinsic apoptotic pathway but the significance of this association with FADD remains unclear.

Bcl-10 contains an N-terminal caspase recruitment domain (CARD) and has been shown to trigger apoptosis as well as the activation of nuclear factor kappa b (NF-κB). Together with other proteins it acts as an adapter protein during signalling from antigen receptors to the IKK complex and NF-κB stimulation, resulting in lymphocyte activation [59]. While NF-κB has been well established to promote cell survival and proliferation, recent studies also suggest a pro-apoptotic and tumor suppressor role for NF-κB. Amongst others NF-κB was shown to be involved in p53- and Fas-mediated apoptosis as well as cell death in response to certain viral infections [60–62]. Interestingly, Apoptin induces luciferase expression from an NF-κB-responsive element in a reporter assay that could be blocked by overexpression of inhibitor of NF-κB (IκBα) [63]. This finding suggests the activation of a pro-apoptotic NF-κB pathway in response to Apoptin expression.

Co-expression of Apoptin with Bcl-10 reduced the level of NF-κB activation induced by Bcl-10 alone which might indicate differential activation of pro- or anti-apoptotic NF-κB responses. However, the significance of the co-localization of Apoptin with Bcl-10 as well as the role of NF-κB still requires further investigation.

2.5.3 Protein Kinase G (PKG-I)

Recent data from our laboratory suggest a protective effect of protein kinase G-I (PKG-I) expression in normal cells against Apoptin-induced cell death (Daryl Cole, PhD Thesis). Using a pair of colon cell lines it was demonstrated that the resistance of normal colon mucosa cells (NCM460) to Apoptin correlated with high levels of PKG-I as well as low expression of PKCβ1. In addition, in normal human 1BR3 fibroblasts the siRNA-mediated knockdown of PKG-I triggered nuclear translocation and activation of Apoptin (Fig. 2.4). Several studies have shown a loss of PKG-I expression during the process of tumorigenesis in tumor tissues compared to normal cells [64, 65]. Thus, reduced PKG-I levels in cancers might represent a tumor-specific alteration that affects the function of Apoptin. However, the precise effect of PKG-I on Apoptin-mediated signalling remains to be determined.

2.5.4 PI3-Kinase (PI3K) and Akt

Apoptin associated with the p85 subunit of PI3 Kinase (PI3K) in several tumor cell lines by binding through its proline-rich region to the SH3 domain of p85. This interaction resulted in the activation of PI3K which seems to be important for Apoptin's pro-apoptotic function [35]. Apoptin mutants lacking the proline-rich region failed to bind and activate p85 and displayed a reduced ability to induce apoptosis. In addition, siRNA-mediated downregulation of p85 caused an impaired nuclear accumulation and cytotoxic function of Apoptin. Apoptin was also shown to interact with the downstream PI3K target kinase Akt in PC3 prostate cancer cells. Binding of Apoptin to Akt (and PI3K) triggered sustained activation and nuclear translocation of Akt which was suggested to promote cell death instead of survival [34]. Neither Akt nor PI3K were able to directly phosphorylate and activate Apoptin. However, activated Akt has been described to phosphorylate several nuclear target proteins including cyclin-dependent kinase 2 (CDK2) which was shown to phosphorylate Apoptin and regulate its activity [39]. These results suggest an important role for sustained activation of the PI3K/Akt pathway through direct binding by Apoptin, which in turn promotes Apoptin-induced apoptosis in a CDK2-dependent manner.

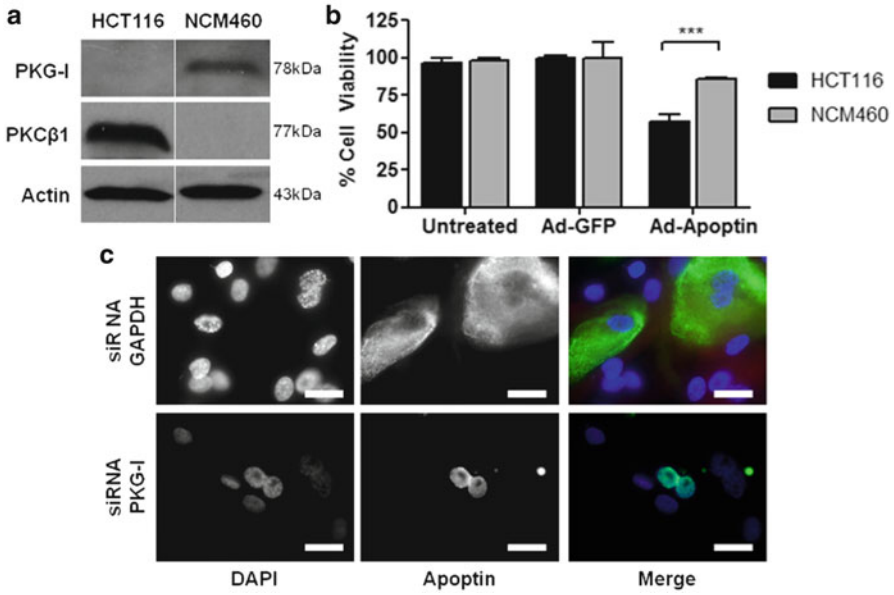

Fig. 2.4 Role of PKG-I in Apoptin-mediated cell death. (**a**) The expression levels of PKG-I and PKCβ1 respectively in HCT116 colorectal cancer cells and NCM460 normal colon mucosa cells were determined by Western blot analysis (blots were cut and combined at the indicated line). (**b**) NCM460 and HCT116 cells were left untreated or infected with Ad-Apoptin or Ad-GFP at an MOI of 10. After 48 h cell survival was analyzed by MTT assay. The results are shown as percentage of viable cells normalized to untreated control cells. Error bars indicate standard deviation from experiments performed in triplicate (*** $p < 0.0005$). (**c**) 1BR3 normal human fibroblasts were transfected with siRNA to knock down PKG-I or control siRNA, infected with Ad-Apoptin and fixed for immunofluorescence. Apoptin was visualized with FITC-conjugated antibodies and nuclei were counterstained with DAPI

2.5.5 Heat Shock Cognate Protein 70 (Hsc70)

Recently, Chen et al. proposed a role of heat shock cognate protein 70 (Hsc70) for Apoptin-induced cell death. Hsc70 is a constitutively expressed member of the heat shock protein 70 protein family and is involved in the regulation of multiple cellular processes. Chen et al. were able to show an interaction of both exogenous and endogenous Hsc70 protein with Apoptin in a cell line of spontaneously transformed chicken fibroblasts [51]. In the same cells Apoptin expression triggered translocation of Hsc70 from the cytoplasm to the nucleus and Hsc70 was required for Apoptin-induced downregulation of p65, a member of the NF-κB transcription factor family. However, whether this holds true for human cells and is a general molecular mechanism for Apoptin function remains to be established. In a concurrently published study they confirmed the interaction of Apoptin and Hsc70 as well as Hsc70 nuclear translocation in transformed human embryonic kidney (HEK293T) cells [50]. Moreover, downregulation of Hsc70 was shown to inhibit

Apoptin-induced phosphorylation and activation of Akt. These data suggest a potential role of Hsc70 in the activation of PI3K/Akt by Apoptin described above but the importance of such interactions requires further investigation.

2.5.6 Promyelocytic Leukemia Protein (PML)

In tumor cells Apoptin displays a distinct expression pattern forming subnuclear aggregates. These resemble so-called PML bodies, nuclear matrix domains organized by the promyelocytic leukemia protein (PML). Poon et al. demonstrated that Apoptin co-localization with PML bodies in Saos-2 cells is dependent on its N-terminal LRS (amino acids 33-46) with a yet unknown functional consequence [19]. PML bodies have been implicated in many cellular processes such as cell cycle regulation, apoptosis and anti-viral responses [66, 67]. In addition to PML, several other proteins including regulators of apoptosis are physiologically recruited to PML bodies. In many cases targeting to PML bodies is achieved by covalent attachment of small ubiquitin-like modifier (SUMO) to lysine residues known as sumoylation [68]. Indeed, Janssen et al. showed that Apoptin was sumoylated at several leucine or isoleucine residues within the LRS, interacted with PML protein and associated with PML bodies in U2OS osteosarcoma cells [21]. However, the pro-apoptotic activity of Apoptin was not affected by knockout of PML or mutation of the LRS which eliminates Apoptin sumoylation. While sumoylation and association with PML bodies do not seem to be required for Apoptin-induced apoptosis it was speculated that these modifications might be relevant for CAV replication.

2.5.7 Anaphase Promoting Complex 1 (APC1)

Teodoro et al. have identified the APC1 subunit of the anaphase-promoting complex/cyclosome (APC/C) as an Apoptin binding protein using mass spectrometry [52]. The APC/C functions as an E2 ubiquitin ligase and targets cell cycle proteins for proteasomal degradation to promote the transition from metaphase to anaphase [69]. In human cancer cells lacking functional p53 but not in normal cells Apoptin associated with APC1, disrupting the APC/C and inhibiting its function. Overexpression of Apoptin and its association with APC1 was shown to result in G2/M arrest and apoptosis similar to siRNA-mediated downregulation of APC1 in p53-deficient cells. The interaction of Apoptin with APC1 was mediated by a domain located within the C-terminus of Apoptin that partially overlaps with the bipartite NLS (amino acids 80-121). Moreover, Apoptin was shown to trigger the formation of PML bodies in H1299 non-small cell lung carcinoma cells and lead to the sequestration of APC/C components in these PML bodies [18]. In a recent study Apoptin was found to co-localize with MDC1, an APC-associated

mediator of DNA damage signalling, in PML bodies resulting in proteasome-dependent degradation of MDC1 [31]. Taken together these results provide evidence that APC/C could be a potential target for anti-cancer drug development in addition to suggesting a novel mechanism for Apoptin-induced cell cycle arrest and apoptosis.

2.5.8 Hip-1 Protein Interactor (Hippi)

Cheng et al. identified Hippi (Hip-1 protein interactor and apoptosis co-mediator) as an Apoptin binding partner in a yeast two-hybrid screen [53]. The complex of Hippi and huntingtin interacting protein 1 (Hip-1) can recruit and activate pro-caspase-8 and thereby induce apoptosis via the extrinsic pathway albeit independently of death receptors [70]. This might contribute to neuronal cell death in Huntington disease as expansion of the polyglutamine repeat on huntingtin was shown to reduce Hip-1 binding to huntingtin which liberates it for binding to Hippi and subsequent caspase-8 activation [71]. In confirmation of the initial screen, Apoptin and Hippi were shown to interact both in vitro and in vivo. The respective binding regions were mapped to the self-multimerization domain of Apoptin and the C-terminal half of Hippi which contains a pseudo-DED motif. In normal cells Apoptin and Hippi were shown to interact and co-localize in the cytoplasm whereas in cancer cells Apoptin translocated to the nucleus while Hippi remained in the cytoplasm. The binding of Apoptin to Hippi in normal cells might reduce the sensitivity of cells to caspase-8-mediated apoptosis. However, Apoptin-induced cell death seems to be independent of caspase-8 activation as cells deficient in caspase-8 remain sensitive to Apoptin [72]. Otherwise, binding of Hippi to Apoptin might sequester Apoptin in the cytoplasm and prevent its nuclear accumulation in normal cells, but overexpression of Hippi in cancer cells is not able to retain Apoptin in the cytoplasm. Thus, the significance of this interaction remains to be discovered.

2.5.9 Death Effector Domain-Associated Factor (DEDAF)

Death effector domain-associated factor (DEDAF) interacts with several DED-containing proteins such as FADD, pro-caspase-8 and pro-caspase-10 to promote apoptosis [54]. Like Apoptin, transient overexpression of DEDAF resulted in the induction of apoptosis in several tumor cell lines but not in normal fibroblasts [73]. Apoptin itself was shown to interact with DEDAF in tumor cells in the nucleus and overexpression of DEDAF increased the rate of cell death induced by Apoptin. Whether this is due to DEDAF-mediated transcriptional repression and whether DEDAF is required for Apoptin-induced apoptosis is still unclear.

2.5.10 Peptidyl-Prolyl Isomerase-Like 3 (Ppil3)

The binding of Apoptin to peptidyl-prolyl isomerase-like 3 (Ppil3) was shown to regulate Apoptin localization in normal and tumor cells [55]. Ppil3 belongs to the cyclophilin family which catalyzes the cis-trans isomerization of peptide bonds at proline residues and can regulate protein stability. Members of this family are involved in mitochondrial maintenance, apoptosis and cell cycle progression [74]. Ppil3 was initially identified as an Apoptin binding partner in a yeast two-hybrid screen and confirmed using an in vitro GST pull-down assay. Overexpression of Ppil3 resulted in increased retention of Apoptin in the cytoplasm. This was dependent on the proline-109 residue of Apoptin and binding of Ppil3 in this region was suggested modify the activity of the nearby NES and/or NLS to regulate Apoptin localization. However, the importance of Ppil3 for Apoptin pro-apoptotic function is currently unclear.

2.5.11 N-Myc Interacting Protein (Nmi)

Using a yeast two-hybrid system, N-Myc-interacting protein (Nmi) was identified as an Apoptin binding partner [56]. Nmi is an interferon-inducible protein and can enhance the activity of Myc proteins [75]. Transcription factors of the Myc protein family are key regulators of cell proliferation and differentiation and their upregulation can contribute to tumorigenesis [76]. This interaction might account for Apoptin's tumor-selective activity as Nmi expression levels in normal tissues are low while it is upregulated in transformed cells. However, so far no further functional studies on Nmi involvement during Apoptin-triggered cell death are available.

2.5.12 Breast Cancer Associated Gene 3 (Bca3)

In a recent study the breast cancer associated gene 3 protein (Bca3) was identified as an Apoptin binding partner in normal human fibroblasts [49]. Overexpression of Bca3 was shown to enhance Apoptin phosphorylation and increase apoptosis induction in Saos-2 cells. However, the significance of Apoptin interaction with Bca3 for Apoptin-induced cell death remains to be discovered. Bca3 (or AKIP1) interacts with several other cellular proteins including protein kinase A (PKA) and TAp73 to modify cellular signalling pathways [77–79]. The authors of the present study argue that Bca3 could negatively affect PKA-mediated activation of the phosphatase PP2A resulting in decreased Apoptin phosphorylation. Furthermore, Bca3 might also promote NF-κB activation in a PKA-dependent or PKA-independent manner and enhance the transcriptional activity of TAp73 in response to Apoptin expression.

2.6 Mechanism of Apoptin-Induced Cell Death

2.6.1 Apoptotic Pathways

Apoptosis, or programmed cell death, is a physiological process that is essential during the development and the life of multicellular organisms. Inhibition of apoptosis allows for cell proliferation in an uncontrolled fashion, resulting in the persistence of potentially dangerous and unwanted cells. When undergoing apoptosis, cells display a characteristic morphology clearly distinct from that of healthy or necrotic cells [80]. To execute the apoptotic program, cells need to carry out a series of tightly regulated intracellular events. All apoptotic stimuli result in the activation of caspases, specific cysteine proteases that reside as inactive zymogens and require activation by proteolytic processing [81]. The initiator phase is marked by the recognition of an apoptotic stimulus and activation of initiator caspases which in turn activate effector caspases that specifically cleave cellular substrates leading to cell disintegration. The extrinsic pathway of apoptosis is triggered by the engagement of death receptors of the tumor necrosis family such as TNF-R1 or CD95/Fas by their corresponding ligands [82]. Ligand binding induces receptor trimerization and a conformational change exposing the receptor's death domain. This results in recruitment of adapter proteins such as FADD containing both a death domain and a death effector domain (DED). Pro-caspase-8 can associate with this DED to form the DISC which triggers its proteolytic activation [83]. Activated caspase-8 can then directly cleave effector caspases (type I pathway) or trigger the intrinsic apoptotic pathway through cleavage of the BH3-only protein Bid (type II pathway) [84].

The intrinsic or mitochondrial pathway is activated by various stress stimuli including DNA damage, growth factor deprivation or ER stress. A hallmark of the intrinsic pathway is the permeabilization of the mitochondrial outer membrane (MOMP) which leads to the release of numerous pro-apoptotic factors from the mitochondrial intermembrane space into the cytoplasm [85]. These include cytochrome c, which associates with apoptotic protease-activating factor (Apaf-1) and pro-caspase-9 to trigger formation of the apoptosome. In this complex caspase-9 is activated and can in turn cleave caspase-3 and other effector caspases [86]. The main regulators of the intrinsic pathway controlling MOMP belong to the Bcl-2 protein family [87]. Stress signals are sensed by BH3-only proteins, which accumulate to overcome the anti-apoptotic effect of pro-survival Bcl-2-like proteins. Consequently, the pro-apoptotic proteins Bax and Bak can be activated to oligomerize on the outer mitochondrial membrane leading to the formation of pores and MOMP.

2.6.2 Induction of Apoptotic Pathways by Apoptin

The precise mechanism of Apoptin-induced cell death is not clearly understood although several key events and molecular players have been identified and discussed. Expression of Apoptin ultimately results in the activation of effector caspases, in particular caspase-3, to execute the apoptotic program. Treatment with the pan caspase inhibitor zVAD-fmk or co-expression of the viral caspase inhibitor p35 reduced the sensitivity of cancer cells to Apoptin in two separate studies, indicating that caspase activation is required for Apoptin-induced cell death [88, 89]. However, Danen-van Oorschot et al. argue that activation of caspase-3 occurs only in Apoptin-expressing cells at late apoptotic stages and might thus represent a consequence of apoptosis induction rather than a cause [89]. Furthermore, other effector caspases such as caspase-6 or caspase-7 are able to compensate for caspase-3 deficiency in MCF-7 cells during Apoptin-induced apoptosis.

To investigate whether Apoptin triggers apoptosis through the extrinsic pathway Maddika et al. tested the sensitivity of Jurkat cells deficient in key regulators of the death receptor pathway including caspase-8 and FADD to treatment with Apoptin fused to the HIV-TAT protein transduction domain (TAT-Apoptin) [72]. A previous study had shown that Apoptin co-localizes with Bcl-10 and FADD in cytoplasmic filaments in Saos-2 cells suggesting a role for the extrinsic pathway in Apoptin-induced cell death [12]. However, Jurkat cells lacking functional caspase-8 or FADD remained sensitive to Apoptin and they could not detect activation of caspase-8, indicating that Apoptin probably mediates cell death independently of death receptor signalling. Nevertheless, since several Apoptin binding partners are known to be involved in death receptor signalling the possibility that in other cellular systems Apoptin induces activation of the extrinsic pathway should not be excluded.

In the same study by Maddika et al. treatment with TAT-Apoptin caused a loss of the mitochondrial membrane potential and release of cytochrome c, both representing key events in the intrinsic pathway [72]. Knockdown of the adapter protein Apaf-1 blocked Apoptin-induced cell death in transformed fibroblasts [88]. Taken together these data indicate that Apoptin-triggered apoptosis is executed via the classical mitochondrial pathway and might thus be regulated by members of the Bcl-2 protein family. However, the role of the anti-apoptotic protein Bcl-2 in Apoptin-induced apoptosis remains controversial. Several studies suggest that Apoptin triggers cell death independently of the Bcl-2 status and that overexpression of Bcl-2 rather sensitizes cells to induction of cell death by Apoptin [90, 91]. Another study demonstrated that overexpression of the pro-survival protein Bcl-x_L did not impair Apoptin-induced cell apoptosis in a head and neck squamous cell carcinoma cell line [92]. By contrast, Burek et al. showed that co-expression of either Bcl-2 or Bcl-x_L with Apoptin protected MCF-7 cells from cell death while the pro-apoptotic family member Bax promoted Apoptin-induced apoptosis [88]. These opposing effects of Bcl-2 on cell death triggered by Apoptin seem to be cell type-specific and might be due to the differential activity of other cellular signalling factors that control Bcl-2 function. One of these potential factors

is Nur77, an orphan receptor that has a regulatory role in both cell growth and apoptosis. Upon various apoptotic stimuli phosphorylated Nur77 translocates from the nucleus to the mitochondria where it activates the intrinsic apoptotic pathway either directly or indirectly, e.g. by changing the conformation of Bcl-2 to promote apoptosis instead of suppressing it [93–95]. Indeed, treatment of MCF-7 cells with TAT-Apoptin triggered Nur77 shuttling to the mitochondria [72]. In addition, inhibition of Nur77 expression protected cells against Apoptin-induced killing. While this indicates a potential role for Nur77 in the cell death pathway induced by Apoptin, the actual mechanism of Nur77 in this apoptotic program so far remains unclear.

2.6.3 Role of the Tumor Suppressors p53 and p73

Another protein that is known to transmit apoptotic signals from the nucleus to the mitochondria is the tumor suppressor p53. Apart from inducing the transcription of pro-apoptotic target genes like p53-upregulated modulator of apoptosis (PUMA) or Noxa, p53 can also translocate to the mitochondria upon stress stimuli and exert a direct pro-apoptotic function there by interacting with members of the Bcl-2 protein family [96]. However, Apoptin is able to induce apoptosis even in cells that lack functional p53 such as Saos-2 cells [14]. This p53-independent function of Apoptin is an important feature for its potential as a future anti-cancer therapeutic agent because many tumors contain mutated, non-functional p53.

Another p53 family member, p73, shares significant homology with p53, particularly in the DNA-binding domain. It is expressed as multiple isoforms that result from alternative splicing in its C-terminus as well as the use of two distinct p73 promoters which generates either transactivation-competent (TA) or N-terminally truncated (ΔN) forms. While TAp73 isoforms are able to induce the transcription of genes involved in apoptosis and cell cycle arrest, ΔNp73 isoforms function as dominant negative inhibitors of TAp73 or p53 activity through a negative feedback loop [97, 98]. In response to genotoxic stress TAp73 isoforms become stabilized and activated while ΔNp73 levels decrease, changing the equilibrium between pro-apoptotic and anti-apoptotic isoforms to induce apoptosis. This process is controlled by a network of post-translational modifications of p73 such as phosphorylation, acetylation and ubiquitination [99].

Two recent studies provide evidence for an important role of specific p73 isoforms during the regulation of p53-independent Apoptin-induced cell death. Apoptin expression in p53-deficient H357 head and neck squamous cell carcinoma cells resulted in increased protein levels of TAp73α and reduction in the expression of ΔNp73α, probably through selective protein stabilization [100]. Furthermore, exogenous expression of TAp73, in particular TAp73β, but not ΔNp73 further enhanced the sensitivity of Saos-2 cells to Apoptin-induced cell death (Fig. 2.5).

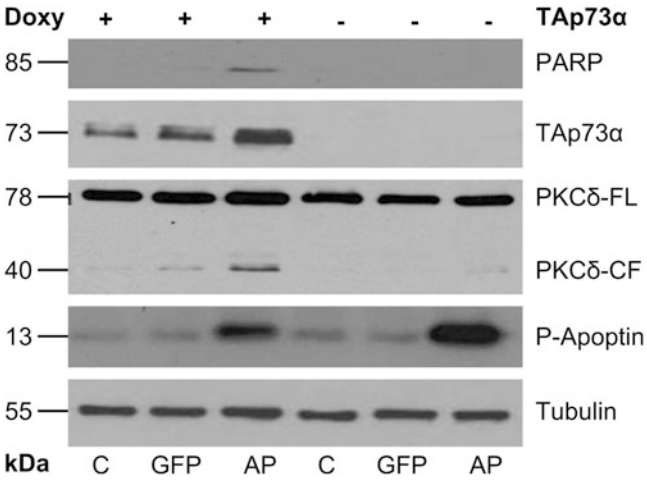

Fig. 2.5 Importance of p73 isoforms for Apoptin-induced cell death. TAp73α Saos-2 inducible cells were infected with either Ad-GFP (GFP) or Ad-Apoptin (AP) at an MOI of 10 or left untreated in the presence or absence of doxycycline to induce expression of TAp73α. Cells were collected after 24 h post-infection and subjected to Western blot analysis with the indicated antibodies

Apoptin-mediated activation of TAp73 isoforms also led to increased expression of the pro-apoptotic BH3-only protein PUMA resulting in the activation of the intrinsic pathway of apoptosis in response to Apoptin.

A recent study in our laboratory further highlighted the importance of p73 and PUMA for Apoptin-induced cell death. While knockout of p53 did not affect the sensitivity of HCT116 cells to Apoptin, siRNA-mediated downregulation of p73 or inhibition of PUMA significantly decreased the levels of apoptosis induction [101]. Further investigations revealed that the initial Apoptin-induced stabilization of TAp73α resulted in p73-mediated induction of p73-induced RING2 protein2 (PIR2). PIR2 had previously been identified as a transcriptional target of p73 and functions as an ubiquitin ligase to mediate the preferential polyubiquitination and degradation of ΔNp73 isoforms [102]. Expression of Apoptin led to the PIR2-mediated proteasomal degradation of ΔNp73 isoforms, relieving the inhibitory effect of ΔNp73 on TAp73 and thus facilitating the induction of apoptosis. These data suggest a novel mechanism of Apoptin-induced changes in the equilibrium between pro-apoptotic and anti-apoptotic p73 isoforms to induce cell death in cancer cells. However, the signalling pathway linking Apoptin expression to the initial activation of TAp73 has not yet been identified. Stabilization of TAp73 might involve phosphorylation by kinases such as PKCδ and PKCβ or components of the DDR, which have already been implicated in Apoptin signalling (Fig. 2.6).

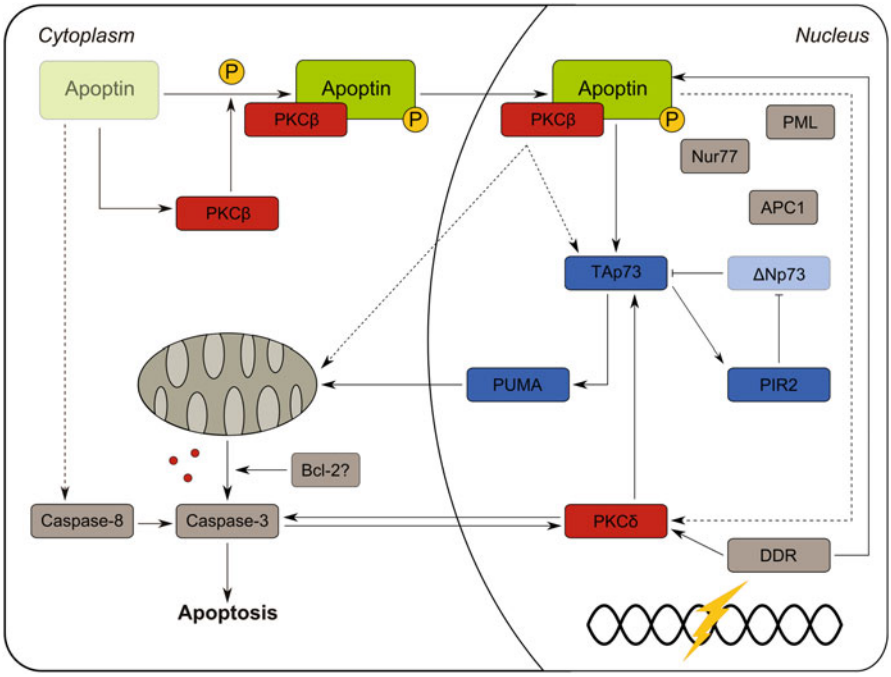

Fig. 2.6 Proposed model of Apoptin action in tumor cells. In tumor cells Apoptin expression induces activation of protein kinase C β (PKCβ) which in turn binds to and phosphorylates Apoptin. Both proteins translocate to the nucleus where Apoptin triggers activation of pro-apoptotic TAp73 isoforms. This results in transactivation of the ubiquitin ligase PIR2 which selectively promotes the degradation of ΔNp73 isoforms to relieve their inhibitory effect on TAp73. Stabilized TAp73 can then induce the expression of pro-apoptotic proteins such as PUMA that transmits the apoptotic stimulus to the mitochondria to trigger cytochrome c release and subsequent caspase activation and cell death. A constitutively activated DNA damage response (DDR) in cancer cells supports this model by activation of PKCδ, which might also be activated by Apoptin itself, and promoting nuclear translocation of Apoptin. Whether Apoptin also induces apoptosis through a caspase-8-dependent pathway and whether PKCβ can directly activate TAp73 or other pro-apoptotic pathways is currently under investigation. In addition, several other proteins including Nur77, PML and APC1 have been implicated in Apoptin-induced cell death

2.6.4 Induction of G2/M Arrest

Cell cycle arrest at the G2/M checkpoint allows for the repair of damaged DNA prior to the initiation of mitosis and may eventually lead to apoptosis of arrested cells. Teodoro et al. reported that Apoptin expression in p53-deficient cells resulted in G2/M arrest and apoptosis through association with the APC/C and inhibition of its function [52]. In addition, Apoptin was shown to induce accumulation of Saos-2 cells in G2/M phase, linking it to the activation of pro-apoptotic p73 isoforms [101]. These data provide a novel mechanism of Apoptin-induced cell

death due to G2/M arrest that might be induced by interference of Apoptin with DDR signalling, the APC/C or other signalling components still to be identified.

2.6.5 Mitotic Catastrophe

Mitotic catastrophe is considered to be a protective mechanism against genetic or chromosomal instability and thus suppresses tumor progression [103]. A recent study by Lanz et al. suggests that Apoptin triggers mitotic catastrophe and subsequently cell death by sensing inaccuracies in the spindle assembly checkpoint [104]. A majority of Saos-2 cells expressing Apoptin displayed abnormal spindle formation and slower progression through mitotic phases compared to control cells. This eventually resulted in cell death either during mitosis or the following interphase that showed characteristics of apoptosis such as membrane blebbing and DNA fragmentation. The fact that Apoptin interacts with and inactivates the APC/C as discussed above might provide a link from Apoptin to the final induction of cell death through mitotic catastrophe. However, in this study the apoptosis-inducing activity of Apoptin was not confined to cycling cells and mitotic catastrophe might thus represent only one aspect of Apoptin-induced cell death.

2.6.6 Ceramide Signalling

As a further potential mechanism for Apoptin-mediated apoptosis Liu et al. have proposed that Apoptin signalling involves modulation of the sphingolipid-ceramide metabolism [105, 106]. Expression of GFP-Apoptin in a prostate cancer cell line resulted in elevated ceramide levels with a concurrent decrease in sphingomyelin levels. Apoptin-treated cells displayed increased acid sphingomyelinase (ASMase) and decreased acid ceramidase (ACDase) activities respectively compared to control cells, both of which result in accumulation of ceramide. Downregulation of ceramide by inhibition of ASMase attenuated Apoptin-induced apoptosis while co-treatment with a ceramide analogue increased the induction of cell death. Ceramide is upregulated in response to several cellular stress signals such as tumor necrosis factor α (TNFα) stimulation or irradiation and functions as a second messenger activating pro-apoptotic pathways. To promote their survival cancer cells often upregulate enzymes that metabolize ceramide such as ACDase. A study on primary prostate cancer tissues revealed increased ACDase levels in a majority of samples compared to normal tissues, making it a potential therapeutic target. Indeed, combination treatment of tumor cells with Apoptin and an ACDase inhibitor induced enhanced cell death both in vitro and in vivo.

2.6.7 Summary

In summary, a number of known pro-apoptotic pathways and proteins have been implicated in Apoptin-induced cell death. The specific mechanism of cell killing by Apoptin might depend on the tumor type, the cellular background regarding oncogenic genetic alterations and the mode of Apoptin delivery to the cell. While it is established that Apoptin triggers caspase-dependent apoptosis of tumor cells, the precise mechanism resulting in caspase activation remains unclear and the possibility of caspase-independent cell death induction cannot be excluded.

2.7 Conclusive Remarks

A major goal of cancer treatment is the eradication of malignant cells while minimizing damage to normal cells. A number of proteins such as TRAIL and HAMLET have been reported to selectively kill tumor cells by sensing essential molecular and cellular changes during carcinogenic transformation and redirecting these pathways towards cell death. Since its identification in the early 1990s as the apoptosis-inducing component of CAV, Apoptin has become of interest due to its ability to induce p53-independent cell death specifically in transformed cells. Apoptin is activated by a cancer-associated pathway and has been linked with several cellular signalling pathways leading to apoptosis although the precise mechanism of Apoptin-induced cell death remains unclear. Further investigation of the pathways contributing to the cytotoxic function and tumor-selective mode of Apoptin will help to identify novel targets for the development of anti-cancer therapies.

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Chapter 3
Apoptin Towards Safe and Efficient Anticancer Therapies

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Contents

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Abstract The chicken anemia virus derived protein apoptin harbors cancer-selective cell killing characteristics, essentially based on phosphorylation-mediated nuclear transfer in cancer cells and efficient cytoplasmic degradation in normal cells. Here, we describe a growing set of preclinical experiments underlying the promises of the anti-cancer potential of apoptin. Various non-replicative oncolytic viral vector systems have revealed the safety and efficacy of apoptin. In addition, apoptin enhanced the oncolytic potential of adenovirus, parvovirus and Newcastle disease virus vectors. Intratumoral injection of attenuated Salmonella typhimurium bacterial strains and plasmid-based systems expressing apoptin resulted in significant tumor regression. *In-vitro* and *in-vivo* experiments showed that recombinant membrane-transferring PTD4- or TAT-apoptin proteins have potential as a future anticancer therapeutics. In xenografted hepatoma and melanoma mouse models PTD4-apoptin protein entered both cancer and normal cells, but only killed cancer cells. Combinatorial treatment of PTD4-apoptin with various (chemo)therapeutic compounds revealed an additive or even synergistic effect, reducing the side effects of the single (chemo)therapeutic treatment. Degradable polymeric nanocapsules harboring MBP-apoptin fusion-protein induced tumor-selective cell killing *in-vitro* and *in-vivo* and revealed the potential of polymer-apoptin protein vehicles as an anticancer agent.

Besides its direct use as an anticancer therapeutic, apoptin research has also generated novel possibilities for drug design. The nuclear location domains of apoptin are attractive tools for targeting therapeutic compounds into the nucleus of cancer cells. Identification of cancer-related processes targeted by apoptin can potentially generate novel drug targets. Recent breakthroughs important for clinical applications are reported inferring apoptin-based clinical trials as a feasible reality.

Keywords Adenovirus • Anticancer therapy • Apoptin • Apoptin interacting proteins • Bacterial and viral vector systems • Bcl2 • Bcr-Abl • Cancer-selective apoptosis • Cancer stem cells • Ceramide • Combinatorial therapy • Gene therapy • Mitotic catastrophe • Nanocapsules • Nuclear targeting • Oncolytic viruses • p53 • Photodynamic therapy • Plasmid vectors • Proteasomes • Protein-based therapeutics • Proteins killing tumor cells (PKTC) • Protein phosphatase 2A (PP2A) • Radiotherapy • Sensing drug targets

3.1 Cancer

World-wide, cancer is the most occurring cause of death in developed countries and the second cause in developing countries. In 2008, approximately 12.7 million tumor cases were diagnosed and about 7.6 million cancer deaths registered [1].

In cancer cells both genetic and epigenetic instability are the underlying driving forces resulting in sustained proliferation, evasion of growth inhibitory signaling, replicative immortality, prevention of cell death, stimulation of angiogenesis and

invasion and metastasis. Hanahan and Weinberg ([2], [93]) named these six derailed processes, together with reprogramming of energy metabolism and circumventing immune destruction, "Hallmarks of Cancer". Their landmark articles represent an informative source of the characteristics of all cancer cells. Cancer can be described as a dynamic and heterogenous cellular situation with the need for multi-targeted therapies. Mutations in tumor suppressors such as p53 or overexpression of anti-apoptosis proteins as Bcl-2 represent major derailments in cancer cells [3].

Approximately 15 % of all human cancers are induced by viral infections [1]. For instance, human papilloma virus (HPV) infections causes cervix carcinoma by negatively interfering with guardian factors such as tumor suppressors p53 and retinoblastoma [4]. The DNA tumor virus SV40 (simian virus 40) has, as model system, led to the identification of many cancer-related processes [5]. Both the large T (LT) and small T (ST) antigens generate derailments resulting in cell transformation by interfering with cellular control factors such as tumor suppressor p53 or protein phosphatase 2A [6].

3.2 Cancer Therapies

Apoptosis removes damaged cells in a normal cellular microenvironment preventing cancer formation. Resistance to conventional anticancer therapeutics is often due to derailments in the apoptotic pathways [7]. Molecular mechanisms resulting in diminishing the anticancer drug activity or additional genetic alterations in the DNA damage repair pathways cause cancer resistance [8]. Besides apoptosis, other types of cell death such as autophagy and necrosis are crucial for cancer development and therapies. Evidence is gathered that a cellular network connects apoptotic and non-apoptotic pathways. Molecular identification and pharmacological knowledge of these elementary processes will provide novel therapeutics against cancer [9].

One of the major problems of cancer therapies is their huge level of toxicity of non-cancerous organs and tissues. These side effects cause tremendous discomfort, stress and traumatic experiences to the cancer patients. As important, the side effects limit severely treatment tolerability and survival rates of the treated cancer patients [10]. For instance, cancer treatments can negatively affect bone tissues. Examples are treatments of female patients suffering breast cancer or males with prostate cancer. Improvement in detoxifying anticancer therapies is ongoing, but much effort still has to be performed [11].

Besides concepts targeting (epi)genetic tumor features, aberrant kinase activities, an extending group of genes has been identified encoding proteins inducing cancer-selective cell death [12]. These proteins are named PKTC (proteins killing tumors cells) [13]. Apoptin, E1A, E4ORF4 and NS1 are of viral origin, whereas TRAIL and mda-7 are cellular proteins and HAMLET can be derived from human mother milk [14]. In this review, we will discuss the anticancer gene apoptin, which

Table 3.1 Apoptin based therapeutic approaches

Gene	Protein
Plasmid-based	Protein transduction tag
Apoptin	TAT-apoptin
Flag-apoptin	PTD4-apoptin
Asor-apoptin	
Virus-based	Fusion proteins
Adenovirus/lentivirus	MBP-apoptin
Oncolytic viruses	hC-SOD3-apoptin
Fowlpox virus	
Pseudotype baculovirus	
Bacterial delivery	Nano-capsules
Attenuated S. typhimurium	MBP-apoptin
Protein/polymer-coupled	
H4TL-histone based	
PAM-RG4 based	
Combinatorial therapy (see Fig. 3.1)	

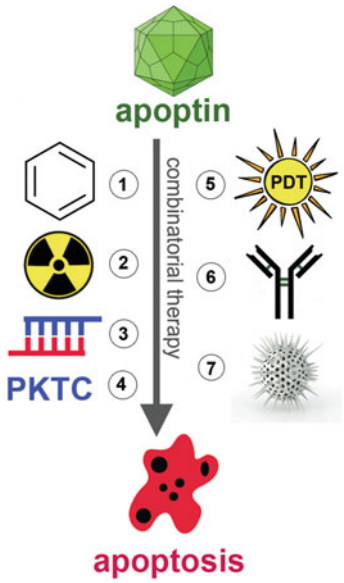

Fig. 3.1 Schematic representation of the various therapies used sofar in combination with apoptin in combinatorial regimes: chemotherapy (*1*), radiotherapy (*2*), RNA interference (*3*), combination with other proteins killing tumor cells such as TRAIL (*4*), photodynamic therapy (*5*), immunotherapy (*6*) and oncolytic viral therapy (*7*)

was the first protein with tumor-selective apoptosis characteristics [91]. Various preclinical studies developing therapeutic approaches (Table 3.1), ranging from gene-delivery systems, protein therapy and combinatorial strategies (Fig. 3.1), are highlighted. Also, the tumor-selective sensor capabilities of apoptin, leading to the identification of potential processes for drug interference, are described (Fig. 3.2).

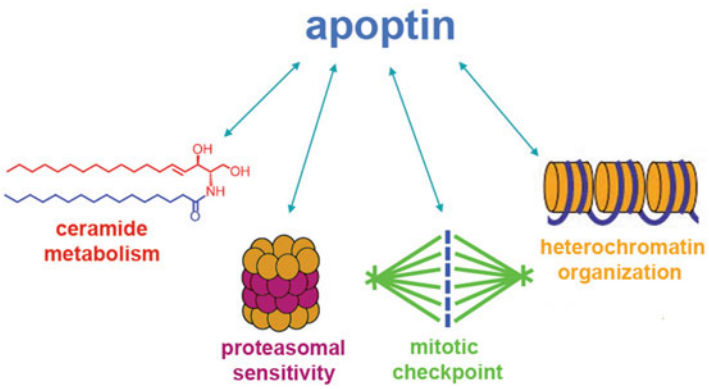

Fig. 3.2 Schematic representation of the various cellular processes sensed and/or affected by apoptin. Derailment in some or all of these processes may be responsible for the tumor selectivity of apoptin

3.3 Virus-Derived Vectors

3.3.1 Adenovirus Vectors

One of the crucial prerequisites of cancer gene therapy is its specificity [15]. Pietersen et al. [16] showed for the first time that infection of cancer cells *in-vitro* with the non-replicative adenovirus vector AdMLPVP3 expressing apoptin induced significant cell death in cancer HepG2 cells, but not in normal rat hepatocytes. Local administration of AdMLPVP3 to xenografted hepatocarcinoma resulted in a significant reduction of tumor growth and in several cases complete tumor regression could be observed. No side effects were observed. Repeated intravenous AdMLPVP3 injections did not induce detectable side effects throughout the treatment of the animals [17].

Cholangiocarcinoma has a poor prognosis. Chemo- and radiation therapy are unable to remove the malignancy completely. Pietersen et al. [18] showed that *in-vitro* apoptin delivery by the adenoviral vector AdMLPVP3 induced significant levels of cell death in various cholangiocarcinoma cell lines, regardless of the presence of inactivating oncogenic mutations in p16 and/or p53. These observations make apoptin an attractive candidate for treating bilary tract cancer.

One of the limitations of the above adenoviral vectors is that they are non-replicative, whereas oncolytic virus therapy is an attractive strategy for cancer gene therapy. Li et al. [19] generated the conditional replication-competent adenovirus Ad-hTERT-E1a-Apoptin, where the E1a gene is regulated by the cancer-specific TERT promoter. *In-vivo*, intratumoral injection or systemic delivery of Ad-hTERT-E1a-Apoptin resulted in an effective suppression of primary and metastatic tumors. Liu et al. [20] reported similar *in-vivo* results with Ad-hTERT-E1a-apoptin in a gastric carcinoma mouse model. Zhang et al. [21] described that Ad-hTERT-apoptin in *in-vivo* prostate cancer models significantly inhibited

tumor growth and extended the lifespan of the treated animals in comparison to those treated with Ad-hTERT-E1A. Zhang et al. [22] inserted the apoptin gene in the double-regulated oncolytic adenovirus AD55 in which E1A is regulated by the alpha fetoprotein promoter and E1B 55kDA is deleted. AD55-apoptin treatment revealed an obvious tumor growth reduction on the xenografted Huh-7 liver carcinoma, whereas the normal liver cells remained unaffected.

3.3.2 *Apoptin Enhances Oncolytic Properties of Newcastle Disease Virus and Parvovirus-H1*

The oncolytic adenovirus vectors were made by means of genetic engineering. Newcastle disease virus (NDV) is a naturally occurring oncolytic virus with the potency to kill cancer cells in (pre)clinical studies. Wu et al. [23] developed a recombinant NDV-apoptin strain named rFMW/AP. The recombinant strain exhibited a higher antitumor activity in xenografted breast cancer mouse models in comparison to the ones treated with the parental strain FMW.

Rodent parvoviruses (PVs) are also well-known oncolytic viruses [24]. Although, in numerous experiments PVs was shown to irradicate tumors in various *in-vivo* tumor models, Nuesch et al. stated that the oncosuppressive activity of PVs has to become improved before they can be used in clinical trials [25]. A possible solution could be found in the application of recombinant PV-apoptin vehicles. Olijslagers et al. [96] reported that apoptin enhances the oncolytic activity of PV. Tumor cells that were resistant to the basal cytotoxicity of PV, underwent significantly apoptosis upon infection with recombinant PV-apoptin.

3.3.3 *Alternative Viral Systems*

Besides oncolytic adenovirus, parvovirus and Newcastle disease virus, successfully developed for apoptin-based anticancer therapies, other viral-derived systems generating tumor-selective apoptosis via apoptin expression were also described.

A recombinant fowlpox virus (vFV-apoptin) expressing apoptin was generated for *in-vitro* and *in-vivo* anti-tumor studies and compared to wild-type fowlpox (vFV) [26]. HepG2 cells were to a certain extent resistant to vFV-induced cytotoxicity, but vFV-apoptin infection resulted in a significant increase of apoptotic HepG2 cells. Intratumoral infection of aggressive H22 mouse hepatomas in C57BL/6 mice with vFV-apoptin vehicles showed a clear therapeutic antitumor effect by inducing apoptosis in the H22 tumor cells.

In-vitro and *in-vivo* treatment of H22 hepatoma cells with a pseudotype baculovirus vector expressing apoptin significantly resulted in induction of

apoptosis and tumor growth suppression [27]. Another approach used systemic delivery of secretable TAT-apoptin by means of a lentivirus vector [28]. Almost all hepatocellular carcinoma xenografts disappeared upon systemic delivery of the recombinant lentiviral vector expressing apoptin. On the contrary, treatment with a recombinant lentivirus vector expressing secretable TAT-tagged control protein GFP did not affect tumor growth.

3.4 Plasmid-Based Vectors

Besides, viral vector systems also plasmid containing vehicles have been described. Peng et al. [29] described a systemic delivery system that linked the plasmid containing the apoptin gene to asialoglycoprotein, which targets apoptin only to hepatocytes via interaction with the asialoglycoprotein receptor (Asor-apoptin). Systemic delivery of Asor-apoptin *in-situ* to hepatocarcinoma bearing nude mice resulted in a specific and efficient uptake of the apoptin gene in normal liver cells and hepatocarcinoma tissue. Five days after the Asor-apoptin treatment, the hepatocarcinoma tissues showed significant signs of regression, whereas the surrounding normal hepatocytes were not affected. Systemically delivered Asor-LacZ controls did not inhibit the hepatocarcinoma growth.

Another protein-based delivery system for the apoptin gene was described by Wang and Zhang [30]. Modified wheat histone H24 protein, H4TL, was used to deliver apoptin-bearing plasmid DNA into human ovarian carcinoma HO8910 cells. Apoptin diminished the growth rate of the treated cancer cells and induced loss of mitochondrial potential.

Treatment of brain tumors are still hampered due to lack of specificity. An et al. [31] delivered the apoptin gene by means of a polyamidoamine dendrimer with an arginine surface (PAM-RG4) into human brain tumor cells. Transfection of the brain tumor cells with PAM-RG4-apoptin resulted in increased caspase-3 activity and induction of cell kill. In independent experiments, the transfection rate was approximately 40 %. In vivo, PAM-RG4 was intratumorally injected into intradermally located U87MG brain tumors in nude mice. 35 % of the tumor was positive for apoptin expression. The apoptin-treated tumors showed a clear growth reduction and signs of apoptosis.

All these studies using either virus-derived or plasmid vectors clearly highlight the anticancer potential of apoptin.

3.5 Protein Therapy

Besides the development of various gene-delivery systems also a lot of effort has been put in the generation of apoptin-protein-based therapeutic approaches. Bacterially produced apoptin protein revealed its tumor-selective apoptosis activity [32].

For instance, micro-injected recombinant maltose-binding-protein (MBP)-apoptin in the cytoplasm of normal human cells showed no signs of cellular apoptosis nor nuclear entry. In contrast, microinjection of MBP-apoptin in cancer cells resulted in nuclear location of apoptin and subsequent induction of cell death.

Guellen et al. [33] developed an effective apoptin protein transduction vehicle by fusing the HIV-derived TAT peptide to the N-terminus of apoptin (TAT-apoptin). TAT-apoptin was produced in bacteria, purified and added to normal and cancer cells. Under *in-vitro* conditions, TAT-apoptin protein entered both normal and tumor cells, but induced apoptosis only in cancer cell lines. Li et al. [34] reported that TAT-apoptin treatment of human bladder cancer EJ cells resulted in a dose-dependent induction of cell death due to significant down-regulation of the anti-apoptotic proteins Bcl-2 and survivin and increased levels of the pro-apoptotic proteins Bax and caspase 3.

Flinterman et al. [35] developed an alternative method based on a TAT-apoptin derivative, allowing secretion of the recombinant apoptin protein by producing eukaryotic cells and uptake by targeted cancer cells. The presence of phosphorylated apoptin and induction of apoptosis in the targeted cancer cells showed that the secreted recombinant apoptin proteins are functional in the targeted cancer cells. Zhao et al. [36] developed another protein transduction strategy for apoptin protein delivery. The C-terminus of human extracellular superoxide dismutase (hC-SOD3) transports cargo proteins into cells. The hC-SOD3-apoptin protein was produced in a bacterial system, purified and analyzed in HeLa cells. The fusion protein efficiently entered the HeLa cells resulting in a significant level of cell kill.

Sun et al. [37] showed that apoptin protein therapy is efficient and safe in an *in-vivo* tumor model. They have used the protein transduction domain 4 (PTD4) to mediate transduction of bacterially produced recombinant PTD4-apoptin protein. *In-vitro* studies revealed that PTD4-apoptin harbored tumor-selective characteristics as described for ectopically expressed plasmid-derived apoptin. PTD4-apoptin enters the nucleus of transduced tumor cells and stayed in the cytoplasm of normal cells. Treatment of xenografted human hepatocarcinoma for 3 weeks resulted in a significant reduction of tumor growth in comparison to treatment with control PTD4-fused green fluorescence protein or saline. The tumors treated with PTD4-apoptin protein were positive in a TUNEL-assay and disruption of the tumor tissue indicated induction of cell killing. Besides the tumor tissue, the epidermal tissue covering the tumor as well as several organs such as liver and lungs contained PTD4-apoptin protein, but none showed signs of cell kill. The *in-vitro* and especially *in-vivo* data indicated that combining the tumor-selective apoptosis activity of apoptin with efficient protein transduction tags provides an efficient therapy for human cancers.

Recently, Zhao et al. [38] have reported that water soluble degradable polymeric nanocapsules containing MBP-apoptin protein are taken up efficiently by various cancer cell lines including HeLa, MCF-7, MDA-MB-231 resulting in nuclear localization of MBP-apoptin and massive cell kill. In contrast, in normal human foreskin fibroblasts (HFF) the nanocapsule delivery of MBP-apoptin resulted in a cytoplasmic location and no cell kill could be observed. *In-vivo* studies

demonstrated that intratumoral injection of the apoptin-containing nanocapsules strongly inhibited the growth of xenografted breast cancer MCF-7 cells in a nude mouse tumor model. The combination of a water-soluble intracellular degradable polymer with apoptin protein represents a very powerful and promising novel anticancer therapy.

3.6 Combinatorial Therapies

In general, combinatorial anti-cancer therapies harbor more potential in comparison to single treatments. Here, we discuss several preclinical studies examining the beneficial effect of combining apoptin with other anticancer therapeutics (Fig. 3.1).

Jin et al. [39] analyzed the efficiency and safety aspects of PTD4-apoptin combined with dacarbazine in a C57BL/6 mouse B16-F1 melanoma model. PTD4-apoptin protein and dacarbazine treatment reduced the tumor growth, whereas the PTD4-apoptin/dacarbazine combinatorial treatment significantly increased the anti-tumor effect. Remarkably, a combined treatment of PTD4-apoptin and 50 % dacarbazine resulted in a similar tumor growth reduction as observed with PTD4-apoptin and the full dose of dacarbazine. More important, no hematologic side effects were measured with the combinatorial treatment containing the reduced dacarbazine dose.

In vitro, human cancer cells were treated with the chemotherapeutic agents paclitaxel or etoposide in combination with the recombinant adenovirus vector AdAptVP3 encoding apoptin. Both paclitaxel and etoposide treatment combined with apoptin significantly induced an additive growth reduction in various p53-negative and p53-positive cancer cell lines at low doses of the chemotherapeutic agent [40]. Overall, these results indicate that apoptin can be combined with chemotherapy and due to its tumor-selectivity the doses of the chemotherapeutic agents can be lowered, minimizing as such traumatic side effects for patients.

Lian et al. [41] reported that the growth of established Lewis lung carcinoma in C7BL/6 mice was significantly inhibited upon IL-18 immunization and expression of apoptin by means of gene transfer. Treatments with IL-18 and an empty vector or with apoptin alone revealed a much lower tumor growth reduction. The authors provided immunological evidence of the observed combinatorial effect of apoptin and IL-18. Indeed, the IL-18 enhanced Th1-type dominant immune response contributing to the regression of tumor cells is potentiated by apoptin.

Tumor growth is influenced by a large variety of different derailed genes. Survivin is one of the prominent anti-apoptosis proteins and is overexpressed in most human tumors. In the next set of experiments apoptin expression in human cancer cells was combined with survivin downregulation. Silencing of survivin was achieved by means of RNA interference and expression of apoptin was enabled by using an RNAi vector producing apoptin protein. Apoptosis and proliferation assays revealed that the combinatorial treatment of survivin-knockdown combined with apoptin expression inhibited cancer cell growth significantly more efficiently than a single treatment [42, 43].

Photodynamic therapy (PDT) is clinically suitable for the treatment of cancer [44]. Fang et al. [45] studied the feasibility of combining apoptin and photodynamic therapy in the treatment of nasopharyngeal carcinoma (NPC). Xenografted NPC were transfected with a plasmid encoding apoptin. The combinatorial treatment of apoptin and photodynamic therapy generated the strongest antitumor growth and cell death effect in the xenografted NPC tumors. These results indicate that apoptin is a potential candidate for the improvement of photodynamic therapy.

One of the obstacles in treating cancers is their radioresistance [46]. New approaches are under investigation for sensitizing these radioresistant tumors by combining radiotherapy with other anticancer strategies. Schoop et al. [99] studied the effect of apoptin on irradiated radioresistant SQD9 human head and neck squamous cell carcinomas under in-vitro conditions. SQD9 cells only irradiated did not secrete cytochrome c and cleavage of caspase-3 could not be observed. Both features are hallmarks of apoptosis. In contrast, combined adenovirus vector-regulated apoptin expression and irradiation treatment of SQD9 cells resulted in a significant release of cytochrome c and cleavage of caspase-3. Han et al. [47] reported that hepatic carcinoma HepG2 cells that are also sensitized by apoptin to radiation-induced apoptosis. Combining lentivirus expressed apoptin and irradiation resulted in HepG2 cells in a significant cytochrome c release, cleavage of caspases-3, -7 and -9 and upregulation of the pro-apoptotic tumor suppressor p53. Both studies suggest that apoptin treatment represents an effective way for applying irradiation strategies to tumors that are resistant or poorly responding to radiotherapy.

3.7 Attenuated Salmonella Typhimurium

Bacteria such as Salmonella, Clostridium or Escherichia coli harbor tumor-colonizing features. Therefore, attenuated live bacterial systems are potential tools for the development of novel anticancer therapeutics [48]. In addition, bacterial systems induce systemic T-cell responses which are beneficial for the regression of the treated tumors. Different strategies such as bactofection, DNA vaccination and bacterially mediated RNA interference and cytotoxic protein delivery based on modified bacterial systems are developed as anticancer agents [49].

Cao et al. [50] developed a Salmonella typhimurium strain containing genes encoding Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) and apoptin. TRAIL and apoptin induce apoptosis in human cancer cells via two different apoptosis cascades, either extrinsic or intrinsic. TRAIL and apoptin could be successfully delivered in xenografted gastric cancer cells in mice by the attenuated Salmonella typhimurium. Both bacterially delivered anticancer genes TRAIL and apoptin induced a significant regression of the gastric cancer *in-vivo*. TRAIL and apoptin expression induced apoptotic features by increasing the levels of caspase-3 and caspase-9. The combination of the anti-tumor activity of Salmonella typhimurium, TRAIL and apoptin harbors a strong potential as novel anticancer therapy against gastric cancer.

3.8 Apoptin-Derivates Act as Targeting Tool

Apoptin harbors several tumor-selective characteristics. Thus far, we have mainly focused on its apoptotic activity. In 2003, [92] described that apoptin contains a bipartite nuclear location signal (NLS) that transfers apoptin selectively in the nucleus of tumor cells, which was confirmed by studies of Alvisi et al. [51]. They showed that other nuclear location signal containing proteins fail to generate differential location in human cancer cells in comparison to their normal counterparts. They drew the conclusion that the C-terminal region of apoptin represents a unique nuclear targeting vehicle: Anticancer drugs only active in the nucleus could be transferred by the apoptin NLS selectively into the nucleus of cancer cells [52]. For instance, the NLS of apoptin could deliver photosensitizing drugs or DNA molecules encoding cytotoxic compounds to the nucleus of cancer cells, thereby promoting tumor-selective cell death. However, thus far, these approaches are hypothetical and have not yet been tested in preclinical or clinical settings [53].

3.9 Sensor of Potential Drug Targets

The capability of apoptin to induce tumor-selective apoptosis has to be based on the sensing capacity of apoptin to recognize tumor-related targets. In cancer cells, apoptin transfers to the nucleus prior to induction of cell kill, whereas in normal cells apoptin remains in the cytoplasm [91]. Immuno-electron microscopy of apoptin-positive cancer cells revealed that apoptin is largely located in heterochromatin areas and nucleoli [54]. Bacterially produced MBP-apoptin consists of complexes of 30–40 MBP-apoptin monomers. *In-vitro*, apoptin was shown to bind directly to DNA resulting in superstructures of 20 multimeric apoptin complexes covering approximately 3 kb of DNA. The consequences for these apoptin features remain unknown [54].

In the past, we have described that apoptin becomes phosphorylated in a cancer-selective manner [55]. Thus far, no clear evidence for the identification of the relevant cancer-related apoptin kinase has been obtained. Lanz et al. [72] reported the biochemical characterization of the apoptin kinase activity in cancer cells. *In-vitro*, the kinase requires ATP in a broad salt range. The apoptin-kinase is constitutively present in the cytoplasm and nucleus of cancer cells. *In vitro*, apoptin is phosphorylated at positions T107 and T108. Cyclin-dependent kinase 2 and protein kinase C beta, kinases reported for other cellular systems [56, 57] could be excluded. Possibly, apoptin could become phosphorylated by different kinases depending on the cancer cell type. Reversibly, it might depend on the inactivation of protein phosphatases. In this perspective it is interesting to mention that in the

CAV genome apoptin (VP3) overlaps with another gene (VP2) coding for a potential phosphatase [58]. Below, we will discuss the role of the cancer-related inhibition of protein phosphatase 2A in the activation and phosphorylation of apoptin.

Apoptin interacting partners such as DEDAF, N-myc interaction (Nmi) protein, Nur77 or anaphase promoting complex/cyclosome (APC/C) subunit APC/C1 have been identified (reviewed in [59]). Recently, we have found that apoptin associates with BCA3/AKIP1 [60] and RSF-1, a chromatin remodeling factor [61]. While BCA3/AKIP1 links apoptin to PP2A regulation (see below), RSF-1 reflects the interaction of apoptin with heterochromatin and nucleoli [54].

Another striking feature of apoptin is that it induces apoptosis in cancer cells, where other chemotherapeutic agents fail [62]. The role of Bcr-Abl and ceramide, known to inhibit chemotherapeutic treatments, in apoptin-induced apoptosis will be described. Finally, we will extensively describe the role of derailed mitotic events in cancer cells [97] as a trigger for apoptin-induced tumor-selective apoptosis.

3.10 Bcr-Abl

The Bcr-Abl oncoprotein is expressed in chronic myeloid leukemia (CML). Due to chromosomal translocations the genes encoding Bcr and Abl are fused, which results in the expression of a constitutively phosphorylated active Abl kinase. The Bcr-Abl kinase activates various downstream pathways stimulating growth and survival [63]. The small chemical compound imatinib inhibits the Bcr-Abl kinase activity and represents a very effective anticancer strategy for treatment of early-phase CML. Despite the encouraging results, second generation imatinib compounds and alternatives are under development because a high mutation rate within the Abl kinase domain generates imatinib resistance [64].

Zhuang et al. [65] have reported that apoptin can induce apoptosis in leukemia and lymphoma cell lines containing Bcr-Abl protein and it is also effective in cancer cell lines overexpressing Bcl-2, known to inhibit chemo-therapeutic strategies. Apoptin could enter the nucleus of Bcr-Abl-positive or Bcl-2 overexpressing cancer cells in similar rates to other cancer cell lines and induce apoptosis. Recently, Panigrahi et al. [66] showed by means of immunoprecipitation experiments and computational predictions that apoptin interacts directly with Bcr-Abl. Apoptin induced apoptosis rates in Bcr-Abl-positive K562 cells to similar extents as imatinib treatment. Apoptin reduced the phosphorylation of Bcr-Abl and thereby its functional activity. The Bcr-Abl regulated downstream pathways stimulating growth or inhibiting apoptosis will collapse upon the presence of apoptin. Interestingly, cells that are imatinib-resistance are still sensitive for apoptin, which makes apoptin a promising therapeutic alternative for patients becoming resistant to imatinib treatment (Marek Los, personal communication).

3.11 Ceramide

The sphingolipid ceramide regulates the cellular fate by acting as a tumor suppressor and driving cells upon derailment into apoptosis. A defective ceramide pathway results in the development of cancer and is an important factor in resistance to chemotherapy [67].

Liu et al. [68] examined the induction of apoptosis by apoptin in prostate cancer-derived cell lines overexpressing anti-apoptosis proteins such as Bcl-2, survivin or XIAP. Under these conditions, apoptin expression resulted in an increased level of the tumor suppressor ceramide. Ten out of 15 analyzed primary prostate tumors were shown to overexpress acid ceramidase (AC). Ceramide deacylation by AC decreases the activity of ceramide leading to an anti-apoptotic environment. Apoptin inhibits AC activity and combined treatment of prostate cancer cells overexpressing AC with apoptin and the AC inhibitor LCL204 resulted in an augmented cell killing both under *in-vitro* and *in-vivo* conditions. Also xenografted DU145 cancer cells were significantly reduced in their tumor growth upon combinatorial treatment with AdGFP-apoptin and LCL204.

These results imply that apoptin is a promising anticancer approach for prostate cancer in combination with AC inhibitors.

3.12 Proteasomes

In contrast to the situation in various cancer cells, apoptin or MBP-apoptin were located in the cytoplasm of normal non-transformed human cells and did not induce apoptosis. Zhang et al. [69] reported that in normal cells the cytoplasmic recombinant MBP-apoptin was cleared from the cytoplasm. Early after microinjection of MBP-apoptin protein, it was present in the cytoplasm as small particles. These apoptin particles became aggregated and epitope-shielded. Eventually the apoptin protein became degraded. Zhang et al. [69] reported that apoptin protein aggregates did not localize within lysosomal structures.

The *in-vitro* observations of apoptin protein degradation in normal human cells were strengthened by *in-vivo* data. In apoptin transgenic mice [70], despite high apoptin RNA levels in thymus and spleen, extremely low levels of apoptin could be determined. After treatment of isolated splenocytes with proteasome inhibitors CBZ/LLL or lactacystin, high amounts of apoptin protein could be detected, indicating that apoptin protein is easily degradable in non-transformed normal cells.

The situation in normal cells can be radically changed by cell-transforming agents. Ectopic expression of apoptin in stably SV40 transformed fibroblasts or co-transfection of plasmids encoding apoptin and the transforming protein SV40 large T antigen in normal human fibroblasts induced nuclear localization of apoptin, no degradation of apoptin and induction of apoptosis [71].

Recently, Lanz et al. [72] studied the role of proteasomes regarding the fate of apoptin protein in normal versus cancer cells. In normal cells, inhibiting the

proteasomal activity with bortezomib and Ada-Ahx(3)L(3)VS resulted in the stabilization of apoptin protein. The observed enhanced concentration of apoptin protein upon increasing the proteasome inhibitor levels resembled the stabilization of the tumor suppressor protein p53. In cancer cells, the situation was completely different. Increasing amounts of bortezomib and Ada-Ahx(3)L(3)VS resulted in a gradual increase of the p53 protein level in a similar way as observed in normal cells. However, the amount of apoptin protein was not affected by either proteasome inhibitor. In the presence or absence of proteasome inhibitors the level of apoptin in cancer cells exceeds the amount in normal cells by approximately 15 times. Apoptin protein becomes degraded in normal cells but is insensitive to proteasomal degradation in cancer cells.

Lanz et al. [72] hypothesized that apoptin alike other viral proteins [73] might affect proteasomal processes. The anaphase promoting complex or cyclosome (APC/C) E3 ligase regulates proteasomal activity responsible for a correct and efficient cell cycle progress by degrading relevant mitotic proteins. Several APC/C subunits are mutated in cancer cells. Apoptin associates with APC/C in cancer cells resulting in destabilization of the anaphase promoting complex [74] and possibly its own stabilization.

The observed differential proteasomal degradation of apoptin protein reveals again its tumor-selective characteristics. As important, this feature of apoptin contributes to its potency of becoming a safe and efficient anticancer therapeutic agent. As proteasomal activity in cancers can become elevated [75] compounds such as bortezomib are clinically used for the treatment of non-Hodgkin's lymphoma. Currently, investigations are ongoing to improve the clinical effects of bortezomib by combining it with other therapeutics [95]. The fact that bortezomib seems not to affect apoptin protein in cancer cells makes apoptin a promising combinatorial candidate for bortezomib treatment.

3.13 Inactivated Phosphatase 2A Promotes Tumor Formation and Activates Apoptin

SV40 T antigens are involved in cancer development by derailing various cellular signaling pathways [98]. For a long period, the interaction of large T (LT) antigen domains with the tumor suppressors p53 and Rb were regarded to be involved in the transformation process of normal cells resulting in cancer cells [76]. Sablina et al. [77] reported that expression of the SV40 small T (ST) antigen contributes to the transformation of normal cells via negatively interfering with protein phosphatase 2A (PP2A). PP2A consists of several catalytic and scaffold subunits and a large range of regulatory B units [78]. Various cancer cell types are known to harbor reduced PP2A activities, which underlines the involvement of derailed PP2A in cancer development [79]. PP2A plays crucial roles in cell proliferation, signal transduction and apoptosis [80]. PP2A is also of importance in the mitotic

exit [81]. Altogether, derailed PP2A is one of the main factors underlying tumor formation.

Anticancer genes such as apoptin can act in a tumor-selective manner by sensing tumor-related derailments. We asked ourselves which minimal transforming steps are required for triggering apoptin-induced apoptosis in normal cells. Zimmerman et al. [60] have shown that expression of nuclear-targeted ST triggers the phosphorylation and apoptosis activity of apoptin in normal human fibroblasts. Expression of nuclear ST with mutations in its PP2A-binding domain, however, did not result in the activation of apoptin. RNA-interference studies revealed that knockdown of the ST-interacting PP2A-56γ subunit was sufficient to induce phosphorylation of apoptin. Downregulation of PP2A-56δ, which is targeted by protein kinase A (PKA) also led to phosphorylation of apoptin. Inhibition of PKA activity is related to tumor formation. Remarkably, apoptin interacts with the PKA-inhibiting protein BCA3/AKIP1 known to be upregulated in human breast and prostate tumor cell lines [82].

Our observations not only point to a relationship between tumorigenic PP2A and activation of apoptin, but also underline the concept that apoptin senses tumor-related processes.

3.14 Mitotic Catastrophe

Many cancer cells are aneuploid and contain aberrant amounts of chromosomes. Incorrect spindle assembly results in incorrect distribution of the chromosomes. The spindle assembly checkpoint (SAC) controls during mitosis the correct attachment of spindles to metaphase chromosomes. However, many cancer cells have a weakened SAC due to inactivating mutations or altered SAC protein levels [83]. Higher eukaryotes have developed counteracting measures for preventing survival of cells with an improper chromosomal distribution. One such process is mitotic catastrophe, driven by a barely understood signaling cascade [84]. Mitotic catastrophe can be regarded as a tumor suppressive mechanism preceding apoptosis and other related cell death processes [85]. Unfortunately, mitotic catastrophe is often impaired in cancer cells resulting in genetic instability and cancer progression. The induction of mitotic catastrophe in cancer cells represents a therapeutic endpoint [85].

The APC/C (see above) is one of the crucial targets of the SAC, which together constitute the major mitotic checkpoint. In human cancer, but not in normal cells, apoptin interacts with the (APC/C) subunit APC1 [86]. APC/C acts as a crucial element of the mitotic checkpoint apparatus. Expression of apoptin in transformed p53 null cells or knockdown of APC1 mRNA induces G2/M arrest resulting in apoptosis. Recently, Lanz et al. [87] reported that expression of apoptin in dividing cancer cells resulted in a disturbed transition from the meta- to the anaphase. Time-lapse microscopy illustrated that apoptin-positive cells contained aberrant mitotic structures and a heavily prolonged mitotic period ending in apoptosis during mitosis or in the following interphase. These findings strongly infer that apoptin

senses mitotic inaccuracies in cancer cells and has the ability to drive such cells into cell death. Possibly, a weakened SAC could be one of the targets sensed by apoptin.

The killing activity of apoptin is not restricted to dividing cells. Apoptin can also induce caspase-3 activity resulting in apoptosis in non-mitotic cell cycle-arrested cancer cells [87]. This feature implies that apoptin might also be able to kill poorly dividing cancer stem cells. During the last decade, various studies have provided convincing evidence that aberrant stem-cell maturation and/or differentiation is crucial for cancer development [88, 89]. Therefore, novel therapies should be developed targeting cancer stem cells [90].

3.15 Conclusions

Cancer therapies are hampered by lack of specificity. Fundamental and preclinical studies on the anticancer gene apoptin have shown that apoptin harbors tumor-selective apoptosis activity.

In-vitro and *in-vivo* studies with various delivery systems carried out by research groups world-wide revealed the anticancer efficacy of apoptin without any detectable toxic effects on normal healthy cells. The vehicles delivering the apoptin gene or apoptin protein range from viral vectors, attenuated bacterial strains, plasmids expressing the apoptin gene up to PTD4-apoptin protein and water degradable nanocapsules containing the recombinant MBP-apoptin protein (Table 3.1). Importantly, all used delivery systems have revealed the tumor-selective apoptosis activity of apoptin.

Combinatorial therapeutic strategies revealed that apoptin enhances the anticancer activity of chemotherapeutics, allowing the use of lower doses and reducing traumatic side effects.

Recent fundamental studies from our group and from others show the potential of apoptin to unravel potential drug targets by identifying derailed tumor-related processes sensed by apoptin.

The last decade, many research groups have proven that apoptin is a safe and efficient anticancer therapeutic. By now, apoptin seems to be ready to go towards clinical trials.

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Chapter 4
Induction of Cancer-Specific Cell Death by the Adenovirus E4orf4 Protein

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Contents

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Abstract The adenovirus E4orf4 protein is a multifunctional viral regulator that contributes to temporal regulation of the progression of viral infection. When expressed alone, outside the context of the virus, E4orf4 induces p53-independent cell-death in transformed cells. Oncogenic transformation of primary cells in tissue culture sensitizes them to cell killing by E4orf4, indicating that E4orf4 research may have implications for cancer therapy. It has also been reported that E4orf4 induces a caspase-independent, non-classical apoptotic pathway, which maintains crosstalk with classical caspase-dependent pathways. Furthermore, several E4orf4 activities in the nucleus and in the cytoplasm and various protein partners contribute to cell killing by this viral protein. In the following chapter I summarize the current knowledge of the unique mode of E4orf4-induced cell death and its underlying mechanisms. Although several explanations for the cancer-specificity of E4orf4-induced toxicity have been proposed, a better grasp of the mechanisms responsible for E4orf4-induced cell death is required to elucidate the differential sensitivity of normal and cancer cells to E4orf4.

Keywords Adenovirus • E4orf4 • PP2A • Src • ACF • APC/C • Ynd1/Golgi UDPase • Actin • Recycling endosomes (RE) • Chromatin remodeling • Cell death • Apoptosis • Cancer • *Drosophila* • Yeast • Acf1 • SNF2h • ISWI • Phosphatase 2a • B55 subunit • Cdc55 • ENTPDase • Saccharomyces cerevisiae • Cancer • Virus

4.1 E4orf4 Induces a Unique Form of Cancer-Specific Cell Death

It was shown over a decade ago that oncogene expression sensitized primary cells grown in tissue culture to cell killing by the adenovirus E4orf4 protein [1]. Thus E4orf4 reduced the number of foci obtained by expression of various oncogene combinations in baby rat kidney cells by 7- to 33-fold, and it induced high levels of cell death in the presence of oncogene combinations but not in their absence. A mutant E4orf4 protein that lost the ability to bind an active protein phosphatase 2A (PP2A) did not kill either control or oncogene-transformed cells [1]. It was later alleged in a review paper that E4orf4 induced cell death in 40 human cancer cell lines, but not in several primary human cell types derived from various tissues [2].

The mechanisms underlying the differential response of normal and cancer cells to E4orf4-induced cell death are not yet clear, however, several possible mechanisms have been suggested, which may all contribute to the cancer specificity of E4orf4. It has been suggested that activation of the oncogenic state leads to induction of latent apoptotic signals that are uncoupled from the basic apoptotic machinery, thus providing a lower threshold for activation of cell death by additional signals [3]. It was also reported that cancer cells become addicted to crucial oncogenic pathways [4] and it

may be possible that E4orf4 targets these pathways leading to cell death of the oncogene-addicted cells but not of normal cells. Alternatively, E4orf4 may exploit activated oncogenes in cancer cells, such as Src, for induction of cell death [5]. It might further be suggested that because cell cycle checkpoints in cancer cells are defective to some extent [6] these cells would be more susceptible to E4orf4, which further disrupts mitotic checkpoints. Induction of mitotic catastrophe by E4orf4 was indeed reported in one cell line [7], and mitotic catastrophe can lead to apoptosis or other forms of cell death [8]. Finally, we showed in the *Drosophila* model system that E4orf4 can inhibit classical apoptosis in normal fly tissues [9], and it can be hypothesized that this E4orf4 function is lost in cancer cells, leading to a more effective cell killing [10]. A better grasp of the mechanisms underlying E4orf4-induced cell death is required to facilitate understanding of the differences in susceptibility to E4orf4 toxicity in normal and cancer cells. Below I summarize the current knowledge of E4orf4 function, with an emphasis on its ability to induce a non-canonical form of programmed cell death.

4.2 E4orf4 in Virus Infection

4.2.1 The Adenovirus Early Region 4 Transcription Unit (E4) and E4 Proteins

The adenovirus (Ad) E4 transcription unit is one of five early Ad transcription units. The E4 promoter is activated very early after infection by the E1A transcriptional activator protein and transcription continues into the late phase of infection [11, 12]. At later times transcription declines due to repression by the E2A gene product [13, 14] and feedback inhibition of E1A-mediated transactivation by the E4orf4 protein [15]. Additionally, production of E4 proteins is temporally regulated at the post-transcriptional level by alternative splicing [16, 17]. The E4 unit of the most studied group C adenoviruses, including Ad2 and Ad5, contains seven predicted open reading frames (orfs) encoding at least six proteins known as E4orf1 to E4orf6/7. The E4 region in many other human Ad serotypes has a similar genomic organization.

The E4 proteins perform several functions that are required for efficient virus replication, including control of gene expression at various levels and protection of the virus against host defense mechanisms. Deletion of the entire E4 region severely impairs virus growth [18, 19] as it results in defects in many processes, including viral DNA replication, accumulation of late viral mRNAs and proteins, virus particle assembly, shut-off of host protein synthesis, and inhibition of anti-viral defense mechanisms (reviewed in [20, 21]). Genetic studies have revealed a functional redundancy between E4orf3 and E4orf6 [22, 23], and that the other E4 gene products are dispensable for lytic growth of the virus in cell culture, though they mildly affect its efficiency [18, 23]. There is no functional information about

Fig. 4.1 The predicted E4orf4 structure. The E4orf4 structure was predicted by ab-initio modeling, which generated ten different models [26]. (**a**) The three most divergent E4orf4 models are shown. They share the same α-helical structure with highly stabilizing H-bonds. These models are different in the orientation of the helices, as can be seen by the R81 residue, protruding out of the helix at different angels with stabilizing H-bonds or without them. (**b**) A superposition of the three most divergent E4orf4 models is shown, demonstrating the high similarity in the α-helical structure and the differences in inner angels and position of the N-terminal loop (This research was originally published in J Biol Chem by Horowitz et al. [26]. © The American Society for Biochemistry and Molecular Biology)

the gene products of E4orf2 and E4orf3/4, although the former has been detected as a soluble cytoplasmic component in infected cells [24].

Although transcription of the early adenovirus genes decreases during the late phase of infection, the E4orf4 protein was detected late in the infectious cycle, albeit at low levels [25]. In this review I will discuss the group C (Ad2 and Ad5) E4orf4 protein.

4.2.2 E4orf4 Structure

The group C adenovirus E4orf4 protein is a 14 kDa polypeptide consisting of 114 amino acids. The Ad2 and Ad5 E4orf4 proteins differ by one residue: A67 in Ad2 and S67 in Ad5. This change has not been linked to functional differences. The E4orf4 primary protein sequence shares little homology with non-adenovirus proteins. Currently there is no solved structure of this polypeptide, and since there is no known E4orf4 homologue which could serve as a template for modeling the E4orf4 structure, ab-initio modeling of the 114-residues E4orf4 protein was carried out using the Quark computer algorithm, specifically designed to predict the structure of proteins smaller than 200 amino acids. The modeling predicted a protein structure consisting of three α-helical domains with no high-probability prediction for the C-terminus, which may be unstructured [26] (Fig. 4.1). This predicted E4orf4 structure is consistent with previous findings showing that even small deletions in the E4orf4 cDNA were unstable [1] or non-functional [27], conceivably due to disruption of the α helices. Furthermore, the largest dissimilarity between E4orf4 proteins of various human adenovirus phenotypes was observed in their carboxyl termini [27], possibly because the maintenance of the α-helical structures may be more sensitive to changes.

The E4orf4 polypeptide contains a highly basic sequence (residues 66–75), reported to be important for nuclear and nucleolar localization of this protein as well as for its interaction with Src kinases (see Sect. 4.3.3) [28, 29].

4.2.3 E4orf4 Functions During Viral Infection

The adenovirus E4orf4 protein performs multiple tasks during adenovirus infection, which contribute to efficient progression of viral infection from the early to the late phase. Initial studies showed that E4orf4 is part of a negative feedback loop controlling early virus gene expression. The adenovirus E1A proteins, expressed upon arrival of the viral genome in the nucleus, activate early viral transcription. In cells such as the mouse S49 T cell lymphoma cell line, E1A cooperates with cAMP to induce accumulation of the AP-1 transcription factor. Elevated AP-1 levels result from activated transcription of the cellular genes encoding the AP-1 components c-fos and junB. Increased AP-1 levels allow transcription of the early adenovirus genes which contain AP-1 and ATF binding sites in their promoters [30, 31]. One of these genes is the E4 transcription unit, containing E4orf4. Once the E4orf4 protein accumulates, it downregulates AP-1 expression as well as expression of early viral proteins. Work utilizing adenovirus mutants lacking E4orf4 revealed that AP-1 downregulation resulted from decreased junB transcription and reduced c-fos translation [32]. In addition to AP-1-mediated E4orf4 effects during virus infection, transfection experiments in HeLa cells suggested that reduced expression from the E4 promoter was possibly mediated by multiple transcription factors involved in E1A-induced transactivation, including the ATF-2 and E4F transcription factors, which are inhibited by E4orf4 [15]. E1A transcription itself was also inhibited by E4orf4 during virus infection [15]. Transfection experiments in HeLa and SAOS-2 cells further revealed that E4orf4 inhibited E1A-mediated activation of the E2 promoter mainly through E2F DNA binding sites, and could also inhibit E2F-1/DP-1-mediated transactivation. As E4orf4 did not inhibit the transactivation function of a Gal4-E2F hybrid protein, it was suggested that E4orf4 reduced the stability of E2F-DNA complexes [33]. Reduced E2 expression was also observed during infection with a mutant adenovirus that expressed E4orf4 in the absence of all other E4 orfs. Decreased E2 expression contributed to a marked defect in DNA synthesis [34, 35], suggesting an indirect role for E4orf4 in regulating replication.

Another contribution of E4orf4 to regulation of the progression of virus infection involves control of alternative splicing of the viral L1 RNA [36]. The mechanism responsible for the changes in splicing involves an interaction of E4orf4 with a subset of splicing SR proteins and their dephosphorylation, leading to diminished binding of the SR proteins to a repressor site controlling one of the L1 3' splice sites. The altered binding results in a shift from an early to late splicing pattern [36, 37].

A third contribution of E4orf4 to the adenovirus life cycle involves its ability to substitute for glucose-mediated activation of mTOR, a critical regulator of protein translation. Activation of mTOR in primary quiescent cells, bypassing the nutrient checkpoint for protein translation, allows the virus to activate translation of key proteins, such as cyclin D1, Myc, etc., which could contribute to S-phase entry, independently of E2F activation. This process is required for efficient viral DNA replication [38].

The combined contributions of E4orf4 to the adenovirus replication cycle were shown to modestly increase the efficiency of adenovirus replication in culture by at most a few fold [23, 38]. In addition E4orf4 was reported to repress the cytotoxicity of adenovirus in the non-permissive, non-transformed CREF cells, based on the finding that a mutant virus lacking only the E4orf4 region dramatically decreased the ability of CREF cells to form colonies as compared with the WT virus [32]. Moreover, E4orf4 was shown to provide potent protection against lysis of infected mouse fibroblasts by cytotoxic T lymphocytes [39]. The protective role of E4orf4 during adenovirus infection may have more significant consequences in natural infections, in which adenovirus maintains a long-term association with the infected host.

4.2.4 Protein Phosphatase 2A Is a Major E4orf4 Partner, Which Contributes to E4orf4 Functions During Adenovirus Infection

PP2A complexes comprise a large family of Ser/Thr phosphatases with diverse functions in the cell, which are involved in the regulation of most cellular processes. PP2A enzymes are usually found as heterotrimers consisting of a catalytic C subunit (PP2Ac), a structural A subunit (PR65), and a regulatory B subunit. There are at least two isoforms each of the A and C subunits, and several gene families encoding multiple isoforms of B-type subunits, facilitating the generation of numerous PP2A holoenzymes assembled combinatorially. The composition of the various holoenzymes dictates substrate specificity, cellular localization, and affects phosphatase activity (reviewed in [40]).

The human B-type regulatory subunits are encoded by at least four unrelated gene families containing 15 genes and giving rise to more than 23 isoforms by alternative splicing or translation [41]. These gene families include PPP2R2A to PPP2R2D encoding B55 subunits, also known as PR55/B; PPP2R5A to PPP2R5E encoding B56 subunits, also known as PR61/B′; PPP2R3A to PPP2R3C encoding PR72/B″ subunits; and STRN, STRN3 and STRN4 encoding the striatins, also known as PR93/PR110/B‴ [40]. Different B-type subunits interact with overlapping binding sites within the A subunit in a mutually exclusive manner [42, 43]. However, while the Aα structural subunit can interact with all regulatory B subunits,

Aβ is unable to interact with the B55 subunit family and shows a preference for binding PR72/B″ [44]. Holoenzyme assembly is regulated by post-translational modifications of the C-terminal tail of PP2Ac, and PP2A biogenesis is further controlled by regulators including PTPA, α4, and TIPRL1 [40]. Viral proteins were also reported to associate with PP2A and modify its function to benefit virus replication [45].

Studies aimed at identifying mechanisms underlying E4orf4 functions led to the discovery that E4orf4 physically associates with PP2A by binding directly to its regulatory Bα subunit in a complex that contains all three subunits, A, B, and C of PP2A. The E4orf4-associated PP2A complex has substantial phosphatase activity [46]. E4orf4 was also shown to interact with multiple isoforms of the PP2A-B56/B′ subunit [47]. The interaction with the B56 subunits may be sensitive to experimental conditions as one report described a failed attempt to reproduce it [48]. E4orf4 was also shown to bind Cdc55, the yeast PP2A-B55 subunit [49, 50], and recruitment of the PP2A holoenzyme by E4orf4 was shown to be dependent on Cdc55 [50].

Several reports over the years made it clear that all E4orf4 functions described above required an interaction with PP2A. Early studies of E4orf4 function revealed that expression of this viral protein was accompanied by hypophosphorylation of both viral and cellular proteins, including the adenovirus E1A proteins and c-fos [32]. Hypophosphorylation of E1A involved Ser-185 and Ser-188, which may be phosphorylated by mitogen-activated protein kinase (MAPK) [51]. However, whereas enhanced phosphorylation of these sites correlated in CHO cells with enhanced E4 promoter transactivation by E1A [51], no correlation was found in HeLa cells between the ability of E4orf4 to cause hypophosphorylation of E1A and its ability to repress transactivation [15]. Therefore it is not clear whether E1A hypophosphorylation is crucial for E4orf4 inhibition of gene expression. Nonetheless, repression of gene expression was shown to depend on the association of E4orf4 with PP2A by experiments demonstrating that addition of okadaic acid, a PP2A inhibitor, reversed downregulation of the junB, E2 and E4 promoters, and it was suggested that the E4orf4-PP2A complex may dephosphorylate multiple transcription factors involved in E1A-induced transactivation of early viral genes [15, 33, 46].

An interaction with PP2A also contributed to regulation of alternative splicing of adenovirus mRNAs by E4orf4. Okadaic acid was shown to reverse E4orf4-induced dephosphorylation of SR proteins, demonstrating that PP2A contributed to this process [36]. Since E4orf4 mutant proteins that failed to bind either SR proteins or PP2A were unable to alter splicing of the adenovirus L1 mRNAs, it was suggested that a ternary complex containing all three proteins was required for E4orf4-induced dephosphorylation of SR proteins and the resulting alternative splicing [37].

It was further suggested that the ability of E4orf4 to activate the mTOR pathway may be mediated through its binding to the B55 subunit of PP2A. This suggestion was based on the findings that an E4orf4 mutant, which failed to bind PP2A-B55, was unable to activate the mTOR pathway [38].

The interaction with PP2A is also an important determinant of E4orf4-induced cell death, as described below (Sect. 4.4.2).

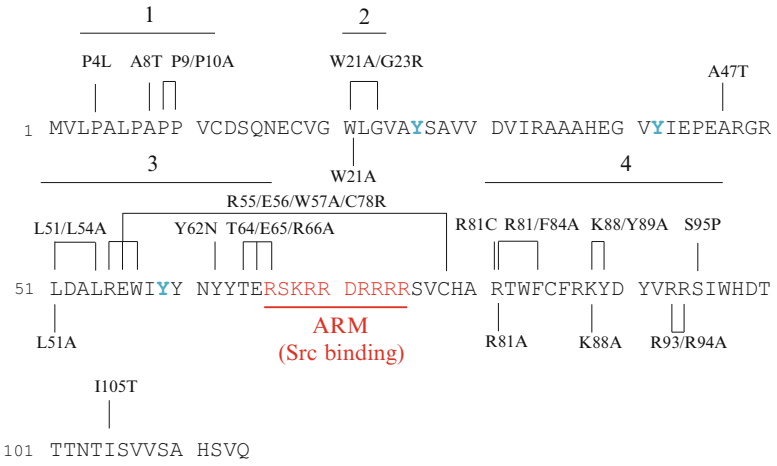

Fig. 4.2 PP2A and Src binding sites in E4orf4. The Ad5 E4orf4 protein sequence is shown. The basic E4ARM domain is marked in *red*. The tyrosines that are phosphorylated by Src kinases are in *bold cyan*. Mutations in the E4orf4 sequence that reduce association of E4orf4 with the PP2A phosphatase activity by at least twofold are shown above the sequence. Mutations that did not interfere more than twofold with PP2A association but were deficient in induction of cell death ("class II mutants", [27]) are shown below the sequence. The clusters of mutations affecting association with PP2A are numbered 1–4. Three more mutations were found to reduce PP2A binding: V19A/T102I, A25T/ΔD52/R87C and ΔV29/R81C, which are not shown for simplicity sake. The basic E4orf4 ARM domain is required for Src kinase binding, but has only a low effect on PP2A binding [1, 28]

4.2.5 Determination of E4orf4 and PP2A-B55 Binding Sites

The E4orf4 mutants that fail to bind PP2A contain mutations in many regions of E4orf4 (Fig. 4.2), however, a large percent of the mutations is found in the carboxyl half of the protein, up to residue 105 [1, 27, 52]. It is not known, however, which mutations affect the E4orf4-PP2A binding directly and which affect it indirectly by changing E4orf4 protein conformation. Many of the recent studies of E4orf4 used mutant R81F84A to represent E4orf4 mutants unable to bind PP2A [9, 27, 28, 53, 54].

A few studies aimed at delineating the E4orf4 binding site in the PP2A-B55 subunit have been described [26, 55, 56]. The first study [55] relied on the finding that an association of E4orf4 with the yeast PP2A-B55 subunit, Cdc55, was required for E4orf4-induced growth inhibition in yeast [49, 50]. A pool of randomly-mutagenized Cdc55-expressing plasmids was introduced into yeast together with E4orf4, and E4orf4-resistant colonies were selected. Mutant Cdc55 proteins unable to transduce the E4orf4 growth inhibitory signal were thus obtained, a few of which showed diminished binding to E4orf4 while still maintaining their interaction with the PP2A-A subunit, Tpd3. Four single point mutations emerged from this study, which reduced E4orf4 binding to Cdc55, two of

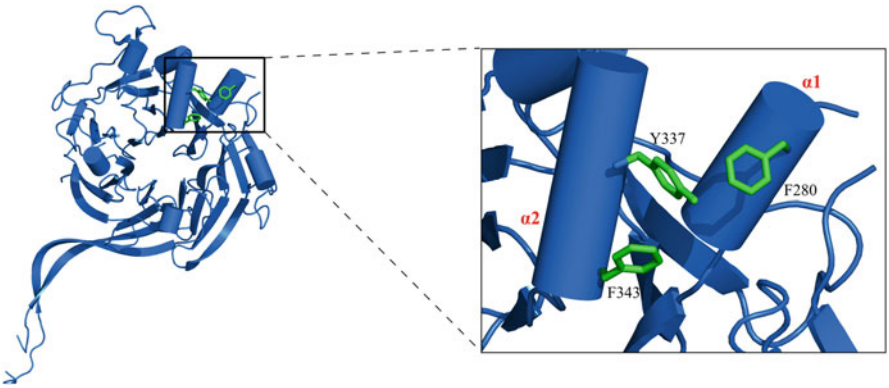

Fig. 4.3 The E4orf4 binding site in PP2A. The B55α structure including the core β-propeller (*ribbons*) and the α-helices (*cylinders*) is shown. Residues F280, Y337 and F343 are colored in *green*. A close-up of the α1,α2 helices is shown on the *right*, displaying residues F280, Y337 and F343 as they protrude from the α1 and α2 helices, facing each other (This research was originally published in J Biol Chem by Horowitz et al. [26]. © The American Society for Biochemistry and Molecular Biology)

which affected residue Y344. However, despite the powerful genetic approach revealing biologically-significant mutations in Cdc55, it was not known whether these mutations targeted the E4orf4 binding site directly or whether they altered the conformation of Cdc55, thus indirectly affecting E4orf4 binding. Once the PP2A holoenzyme containing the B55 subunit was crystallized and its structure was determined [57], it was possible to re-address the issue of the E4orf4 binding site in B55 [26]. A combination of bioinformatics analyses were utilized to model the E4orf4 structure and to predict E4orf4 docking sites in PP2A-B55α. A mutation analysis of two predicted sites revealed that the E4orf4 binding domain includes the α1 and α2 helices described in the B55α structure, which lie above the substrate binding site determined previously. Mutating three aromatic residues in these helices, F280A, Y337A and F343A (Fig. 4.3) inhibited E4orf4 binding to B55α, as determined by GST pull-down assays and co-immunoprecipitation experiments. Residue Y337 corresponds to residue Y344 in yeast Cdc55, which was implicated by the genetic screen to affect E4orf4 binding. Loss of E4orf4 binding was accompanied by reduced contribution of the B55α mutants to E4orf4-induced cell death [26]. Other mutations in PP2A-B55α which were shown by previous studies to influence E4orf4 binding were shown to reside in the B55α protein core, thus affecting the interaction with E4orf4 indirectly [55, 56].

4.3 E4orf4-Induced Cell Death

Whereas E4orf4 appeared to have a protective role during adenovirus infection of CREF cells [32], it was reported by several groups that when E4orf4 is expressed outside the normal context of virus infection it induces cell death [58–60]. E4orf4

proteins of several different adenovirus serotypes possess a similar ability to kill cells [27].

The findings that E4orf4 counteracts E1A-regulated signaling described above (Sect. 4.2.3), suggested the possibility that E4orf4 may interfere with E1A-induced cellular proliferation. Introduction of E4orf4 into 293 cells, which express the adenovirus E1A and E1B proteins, resulted in a strongly diminished ability of these cells to form colonies. The morphology of E4orf4-expressing cells suggested that they were undergoing apoptosis, and this was confirmed by several tests [60]. However, the ability of E4orf4 to induce cell death was not confined to cells expressing adenovirus proteins and was observed in several transformed cell lines [58–60]. E4orf4-induced cell death was shown to be p53-independent as it occurred in many cell lines lacking WT p53 and E4orf4 expression, unlike E1A expression, was not accompanied by accumulation of p53 [58–60]. These results are consistent with work showing that E1A proteins from viral mutants lacking the anti-apoptotic E1B proteins induced both p53-dependent and -independent apoptosis [61, 62]. The adenovirus E4 region, whose transcription is activated by E1A, was required for the p53-independent cell death induced by E1A [63]. E4orf4-induced cell death is dose-dependent and is apparent mostly at high E4orf4 levels [9, 48]. Because E4orf4 levels during virus infection are regulated by negative feedback, and due to the presence of viral cell death-inhibitory genes, E4orf4 may not contribute significantly to cell death during virus infection.

Studies of E4orf4-induced cell death in the past 16 years revealed several unique and interesting features of this form of cell killing, and several E4orf4 partners that contribute to its ability to induce cell death have been discovered. These studies will be summarized below.

4.3.1 Caspase-Independent Cell Death and Crosstalk with Other Forms of Cell Death

In mammalian cells, two main pathways of apoptosis exist, intrinsic and extrinsic, which are triggered by cell death stimuli from the intra- and extra-cellular environments, respectively (reviewed in [64]). Intracellular stimuli, such as DNA damage, activate the BH3-only Bcl-2 family of proteins, triggering the release of proapoptotic factors from the intermembrane space of the mitochondria into the cytoplasm [65, 66]. The release process is mediated by the Bcl-2 family members Bax and Bak and is antagonized by the antiapoptotic proteins Bcl-2 and Bcl-xL. One of the factors thus released, cytochrome c, activates Apaf-1 and, in the presence of dATP or ATP, induces the formation of a multimeric complex called the “apoptosome”. The apoptosome mediates activation of the initiator caspase, caspase-9, which subsequently activates the effector caspases, caspase-3 and caspase-7. The activity of these effector caspases contributes to the progression

of the apoptotic process. The active caspases are subject to inhibition by the inhibitor of apoptosis (IAP) family of proteins [67]. Smac/DIABLO, another protein released from mitochondria during apoptosis, interacts with multiple IAPs and counters IAP-mediated caspase inhibition. In *Drosophila*, the Reaper, Hid and Grim proteins function similarly to inhibit *Drosophila* IAPs (dIAP). In the extrinsic pathway, extracellular death molecules, such as the Fas ligand, bind and activate the death receptors by inducing assembly of a "death-inducing signaling complex" (DISC) at the plasma membrane [68, 69]. An adapter protein, Fas-associated death domain (FADD/MORT1), recruits the initiator caspases of this pathway, procaspase-8 or -10, to the DISC for activation. The activated caspase-8 then cleaves and activates caspase-3 and -7. Thus extrinsic and intrinsic apoptosis pathways both activate caspase-3 or -7. A crosstalk between the extrinsic and intrinsic forms of cell death can be mediated by the Bid protein. One of the caspase targets is DFF45/ICAD, which forms a tight inhibitory complex with DFF40/CAD, a potentially potent DNase. The cleavage of ICAD/DFF45 by caspase-3 or -7 releases free DFF40/CAD, which is responsible for the degradation of chromosomes into nucleosomal fragments during apoptosis. Another nuclease, endonuclease G, is also released from mitochondria and participates in DNA degradation during apoptosis. Morphological hallmarks of apoptosis include membrane blebbing, chromatin condensation, cell shrinkage, chromosomal DNA fragmentation, and formation of apoptotic bodies that can be engulfed by neighboring cells. In contrast, necrosis, which is triggered by major stress or trauma, is accompanied by cell swelling and membrane permeabilization leading to inflammation in the surrounding tissue [70].

Early work revealed that cell death induced by E4orf4 in CHO cells was not accompanied by activation of caspase-3, the effector caspase that plays an important role in classical apoptosis. Furthermore, a broad-range caspase inhibitor, zVAD-fmk, could not inhibit or slow down the appearance of apoptotic morphologies induced by E4orf4 in these cells [58]. It was later discovered that caspase-activation was induced by E4orf4 in other cell lines, such as H1299 and 293T [71] or C33A [72]. Interestingly, not only were caspases activated only in some cell lines, their contribution to cell death also differed in a cell line-dependent manner. Livne et al. found that in H1299 cells, caspase activation was required for accumulation of a sub-G1 cell population, representing cells with fragmented DNA, but was dispensable for nuclear condensation and for cell killing that was measured by colony formation. In contrast, in 293T cells, inhibition of caspase activation partially relieved nuclear condensation and cell death [71]. Robert et al. found no caspase-3 activation in 293T cells when using a specific antibody against the cleaved form of caspase-3, and two types of broad-spectrum caspase inhibitors (zVAD-fmk and BocD-fmk) did not inhibit nuclear condensation [72]. However, C33A cells in this case behaved similarly to H1299 in the first report. Li et al. reported that little caspase activation was detected in H1299 cells infected with an adenovirus expressing E4orf4 from a tetracycline-inducible promoter, and zVAD-fmk did not affect the mild induction of cell death assayed by trypan-blue staining [7].

Varying results were also presented regarding the pathway involved in caspase activation by E4orf4. Livne et al. [71] showed that the death receptor pathway was

activated in 293T cells. Co-transfection of E4orf4 with a dominant-negative mutant of caspase-8 or the death adaptor molecule FADD/MORT1, both downstream effectors of the death receptor pathway of apoptosis, resulted in inhibition of E4orf4-induced nuclear condensation and trypan blue uptake in 293T cells. In contrast, co-transfection of E4orf4 with a dominant-negative mutant of caspase-9, a downstream effector of the mitochondrial apoptosis pathway did not inhibit nuclear condensation, although E4orf4 expression was accompanied by cytochrome c release into the cytoplasm. Moreover, no increase in caspase-9 activity was detected in these cells. In addition, E4orf4 was shown to increase the levels of reactive oxygen species (ROS) in 293T cells, an effect that was inhibited by the caspase-8 inhibitor, CrmA, or by the dominant-negative FADD/MORT1 mutant. Reducing ROS levels using an antioxidant inhibited E4orf4-induced nuclear condensation in the 293T cells. In contrast, Robert et al. [72] showed that a dominant-negative mutant of caspase-9, as well as Bcl-2, but not the caspase-8 inhibitor zIETD-fmk, inhibited E4orf4-induced DNA fragmentation in C33A cells. These results indicated that the mitochondria-apoptosome pathway was involved in E4orf4-mediated DNA fragmentation in these cells. Bcl-2 did not inhibit caspase-independent nuclear condensation [72].

In addition to the caspase-dependent outcomes of E4orf4-induced cell death that were apparent in some cell lines and which indicate a crosstalk between E4orf4-induced cell death and classical apoptosis pathways, E4orf4-induced cell death was reported to culminate in mitotic catastrophe, leading to death with necrotic morphologies in H1299 cells [7]. In these cells E4orf4, which was expressed as a HA-tagged protein, did not cause mitochondrial dysfunction or ROS accumulation.

The apparently conflicting results obtained in various cell lines employed in different laboratories and while expressing E4orf4 alone or in the context of virus infection, can be interpreted to suggest that E4orf4 induces a caspase-independent cell death pathway that can connect downstream with other cell death pathways, such as classical apoptosis or necrotic-like cell death. The crosstalk with other cell death pathways may depend on the physiological state of the cells and their genetic or proteomic content. For example, unpublished results from our laboratory indicated that caspase-induced DNA fragmentation in 293T and H1299 cells varied with the serum in which the cells grew, suggesting a serum-dependent crosstalk with caspase-dependent apoptosis. The activation of classical, caspase-dependent pathways may facilitate amplification of E4orf4-induced cell death. A crosstalk between caspase-dependent and -independent cell death forms was also observed during E4orf4-induced cell death in *Drosophila melanogaster* in which both these types of cell death contributed to E4orf4-induced toxicity [9] (Sect. 4.5.2).

Whereas caspase activation was mostly not required for E4orf4-induced cell death, overexpression of calpastatin, an endogenous inhibitor of calpain proteases, dramatically inhibited the caspase-independent apoptosis induced by E4orf4 [72]. Calpains are Ca^{2+}-dependent intracellular proteases that cleave substrate proteins localized near the membranes and the cytoskeleton in a limited manner, and may thus act to regulate protein functions. The nature of the contribution of calpains to E4orf4-induced cell death is currently not understood.

4.3.2 Morphological Hallmarks of E4orf4-Induced Cell Death and Assays for Measuring E4orf4-Induced Cell Killing

Since upstream caspase-independent events in the E4orf4-induced cell death pathway can be linked downstream to various types of cell death, only few morphological hallmarks of E4orf4-induced cell killing can be consistently used to assay this process. It has been shown that the most typical morphologies associated with E4orf4-induced cell death include membrane blebbing, nuclear condensation, and cell detachment, whereas morphologies associated with classical apoptosis such as DNA fragmentation, caspase activation, phosphatidylserine externalization, or mitochondrial changes do not always accompany E4orf4-induced cell death [7, 58, 71]. Therefore, the assays commonly used to quantify E4orf4-induced cell death include determination of the percentage of cells with membrane blebbing or with nuclear condensation and clonogenic assays.

4.3.3 E4orf4 Cellular Localization: Nuclear vs. Cytoplasmic Cell Death

Cellular localization of E4orf4 was investigated in virus-infected cells or in cells expressing E4orf4 alone. Much of the work utilized epitope-tagged E4orf4, and only part of the conclusions were verified with the native protein. This review will specify which conclusions were based solely on work with epitope-tagged E4orf4.

During virus infection, cellular localization was investigated with a virus expressing GFP-E4orf4 from the endogenous E4 promoter [54]. GFP-E4orf4 localization was monitored during infection of H1299 cells at various times post infection. GFP-E4orf4 was detected throughout the infected cell nucleus between 8 and 18 hours post infection (hpi). It did not colocalize with viral replication centers stained with antibodies to the viral DNA-binding protein (DBP), but accumulated outside them. Between 8 and 14 hpi GFP-E4orf4 colocalized with nucleoli, as seen also in transfection experiments [29], and it localized with E1B55K aggresomes at early, but not late times. GFP-E4orf4 colocalized with late viral proteins first in the nucleus and then in small dots at the nuclear periphery, but it was not found to colocalize with PML bodies, Cajal bodies, or splicing speckles. Very little E4orf4 staining was observed in the cytoplasm during adenovirus infection. Because these studies were performed only with GFP-tagged E4orf4, and GFP itself is much larger than E4orf4, it cannot be ruled out that the large GFP tag altered native E4orf4 localization and that not all the cellular sites detected with GFP-E4orf4 indeed reflect localization of the native protein. For example, nucleolar E4orf4 localization was not prominent during infection with WT adenovirus: on the contrary, native E4orf4 staining excluded nucleoli (our unpublished results).

Analysis of E4orf4 cellular localization by transfection of E4orf4 cDNA into various cell lines revealed that the protein is first detected preferentially in the nucleus. However, it later accumulates in the cytoplasm, cytoskeleton, and membrane regions, with a high concentration in membrane blebs [5, 29, 53, 72]. The E4orf4 protein contains an arginine-rich motif (E4ARM, residues 66–75, Fig. 4.2). This motif was shown to be required for both nuclear and nucleolar localization of E4orf4, although it should be kept in mind that E4orf4 in these experiments was heavily tagged by GFP and an additional shorter tag [29]. The E4ARM motif was also shown to target heterologous proteins to the nucleus and the nucleoli. When basic residues in the E4ARM were mutated to alanines, or when ARM motifs from E4orf4 or other viral proteins were co-expressed with E4orf4, E4orf4-induced cell death declined [29]. These results suggest that targeting E4orf4 to the nucleus is required for the E4orf4 death function.

Interestingly, when E4orf4 was expressed alone outside the context of the virus, its association with one of its two major partners, Src kinase (see Sect. 4.4.1), and its phosphorylation on tyrosines by Src were shown to correlate with a shift from E4orf4 nuclear accumulation to cytoplasmic and membranal localization [5, 73]. Thus, coexpression of E4orf4 with Src, and even more so with constitutively active Src, led to a higher accumulation of E4orf4 in the cytoplasm and in membrane blebs. Biochemical analysis showed that in the presence of activated Src, E4orf4 accumulated in triton-insoluble fractions indicating cytoskeletal association [5]. Furthermore, when Tyr residues in E4orf4, shown to be phosphorylated by Src kinases, were mutated to phenylalanines, E4orf4 was preferentially distributed to the nucleus, whereas mutation of these residues to glutamic acid, mimicking constitutive phosphorylation, led to enhanced accumulation of E4orf4 in cytoplasmic-membrane regions [73]. These results indicate that phosphorylation of E4orf4 on specific tyrosines may induce a conformation change in the viral protein that promotes its retention in the cytoplasm, or inhibits its nuclear import. For example, it can be proposed that accessibility of the E4ARM to its associating proteins may be reduced by phosphorylation-induced conformation changes, which may thus inactivate it. However, cytoplasmic accumulation of E4orf4 was not affected by leptomycin B, an inhibitor of the CRM1-mediated nuclear export pathway, suggesting that E4orf4 was not actively exported from the nucleus [72]. Blebbistatin, an inhibitor of the myosin II motor, induced nuclear translocation and retention of E4orf4, suggesting that the myosin II motor promotes stable accumulation of E4orf4 in the cytoplasm [74].

Because E4orf4 cellular localization is altered during the cell death process from nuclear to cytoplasmic-membranal, investigations were performed to determine the relative contribution of nuclear, cytoplasmic, and membranal E4orf4 to cell death signaling [72]. GFP-tagged E4orf4 was targeted to cell membranes by tagging it with a CAAX-box or a myristylation consensus signal, or it was targeted to the nucleus using a nuclear localization sequence (NLS) or to the cytoplasm, using a nuclear export sequence (NES). Experiments with these constructs revealed that membrane or cytoplasmic targeting of E4orf4 mimicked faithfully the contribution of Src to E4orf4-induced cell death, inducing membrane blebbing and nuclear

condensation at least as efficiently as the WT E4orf4-GFP protein. Nuclear targeting of E4orf4 prevented the early appearance of membrane blebbing and delayed the appearance of nuclear condensation. It was further shown that E4orf4 phosphorylation by Src was required for the cytoplasmic cell death program initiated by E4orf4 but not for the nuclear program. Caspase activation occurred, but was dispensable for the execution of both cell death programs, whereas calpains were required for the Src-mediated cytoplasmic death signal. Based on these results it was suggested that E4orf4 induced two distinct cell death programs, nuclear and cytoplasmic, whose relative contribution to cell killing differs in various cell lines [72]. In support of this conclusion, analysis of E4orf4 mutants revealed that the major E4orf4 partners, PP2A and Src kinases, made different contributions to the nuclear and cytoplasmic forms of E4orf4-induced cell death [28]. One point should be kept in mind with regard to the conclusion postulating two independent cell death pathways, namely that artificial targeting of E4orf4 to various cellular sites was not absolute. The residual presence of E4orf4 in the nucleus may have been sufficient to perform the required preliminary nuclear functions that may potentially be needed to trigger both branches of the death program. Although mutants that did not bind PP2A were still able to induce the cytoplasmic cell death pathway [28], it is again possible that residual PP2A binding was enough for initiating this pathway. E4orf4-induced cell death was shown to be dose-dependent [9, 48], however, it was not determined yet whether both PP2A- and Src-dependent death programs required high E4orf4 levels. Thus it is not absolutely clear whether E4orf4 activates two independent cell death programs or whether initial E4orf4 functions in the nucleus contribute to activation of both pathways.

4.4 E4orf4 and Its Protein Partners

Whereas the cytoplasmic cell death pathway was tightly linked to the association of E4orf4 with Src kinases [5, 72, 73], the nuclear pathway may involve the interaction of E4orf4 with PP2A and its recruitment to nuclear targets including the ACF chromatin remodeling factor [75] and the anaphase promoting complex/cyclosome (APC/C) mitotic regulator [49]. The E4orf4 interactions with its partners and their consequences to induction of cell death are described below.

4.4.1 The E4orf4-Src Kinase Interaction and Its Contribution to Induction of Cell Death

E4orf4 was found to associate with Src family kinases (SFKs) [5, 28], although this interaction was never reported during virus infection. E4orf4 interacts with less than 1–5 % of endogenous Src proteins, depending on the cell type. This interaction

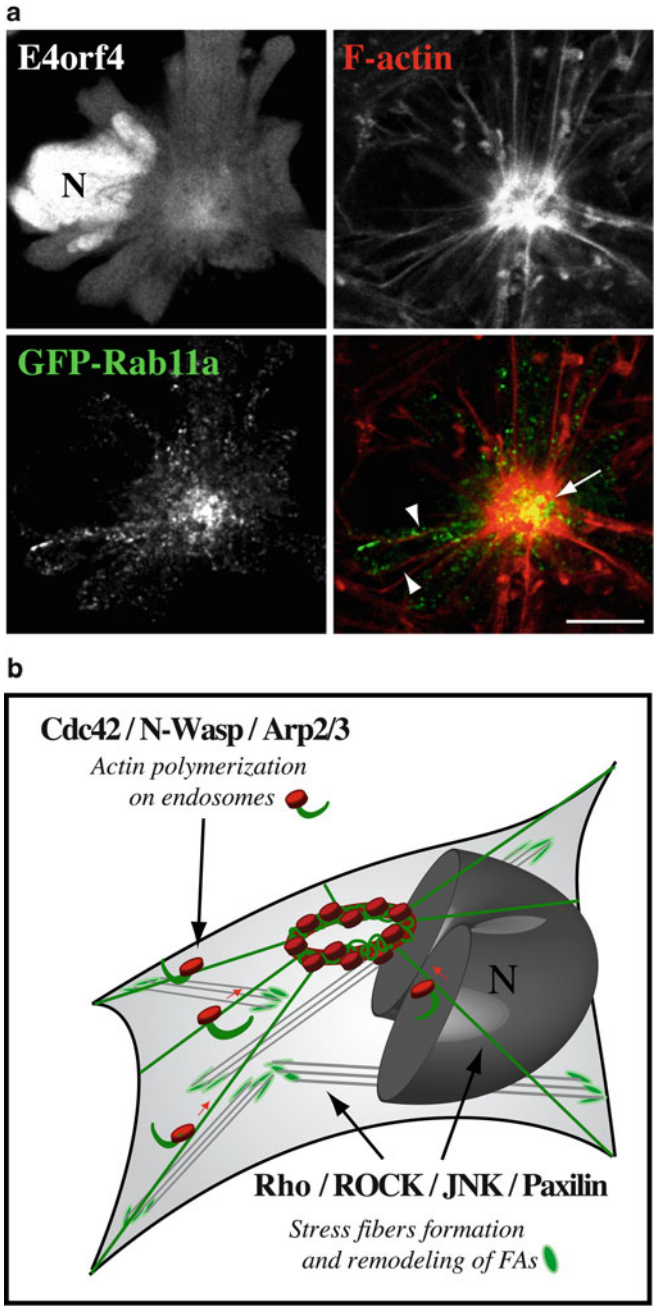

Fig. 4.4 Polarization of REs to the peri-Golgi region correlates with assembly of a juxtanuclear contractile network in response to E4orf4. (**a**) Single plane confocal images of a HeLa cell transfected with E4orf4-mRFP and GFP-Rab11a, showing a massive recruitment of REs labeled by GFP-Rab11a to the forming juxtanuclear actin-myosin ring (*arrow*) labeled by phalloidin-Alexa 647 (F-actin), and along actin cables (*arrowheads*). N, cell nucleus; Bar, 10 μM.

is mediated by the kinase domain of Src and the E4orf4 ARM domain [28]. The Src-binding domain of E4orf4 has partial overlap with the PP2A binding domain, but some E4orf4 complexes contain both PP2A and Src, demonstrating that their binding to E4orf4 is not mutually exclusive [28]. Src binding is required for the cytoplasmic cell death program induced by E4orf4 [28]. The E4orf4-SFKs interaction leads to several consequences for both protein partners. E4orf4 is phosphorylated on tyrosine residues found within motifs typical of SFK substrates including Tyr-26, 42, and 59 [73], and this phosphorylation facilitates the assembly of SFK signaling complexes. WT E4orf4 protein, but not E4orf4 mutants unable to bind SFKs, modulates SFK signaling by inhibiting the phosphorylation of substrates that are involved in SFK signaling to survival pathways, such as focal adhesion kinase (FAK) and ERK [5, 28], and promoting SFK phosphorylation of other substrates, involved in regulation of actin dynamics, such as the F-actin-binding protein cortactin, JNK, and myosin light chain [5, 28, 74]. As a result, E4orf4 is believed to recruit SFK signaling to promote actin-dependent membrane remodeling that leads to cell death through multiple mechanisms [76].

Following phosphorylation by SFKs, E4orf4 accumulates at cytoplasmic sites and induces the assembly of a juxtanuclear actin-myosin network, described as peculiar, in which the juxtanuclear actin-myosin structures are connected to the cell cortex by prominent actin cables converging to enlarged focal adhesions that reflect an increase in cell tension [74, 76] (Fig. 4.4). Activation of the myosin II motor and the juxtanuclear increase in actin-myosin-based contraction drives E4orf4-induced blebbing.

The vast actin remodeling induced by E4orf4 relies on two major pathways involving Rho GTPases. The first is a Src-Rho-Rho kinase (ROCK) signaling pathway that activates JNK to phosphorylate Paxillin. Paxillin phosphorylation leads to dysregulation of adhesion dynamics and perturbs tension homeostasis in the cell [77]. The second pathway includes Cdc42, N-Wasp, and the Arp2/3 complex, and promotes actin polymerization on internal membranes and changes in recycling endosome (RE) dynamics [74] (Fig. 4.4). REs are a heterogeneous population of tubulovesicular endosomes usually concentrated in the pericentriolar region, which form the endocytic recycling compartment (ERC). RE trafficking is regulated by the small GTPase Rab11 and is involved in retrieval of internalized membranes and signaling molecules to the plasma membrane or the trans-Golgi via retrograde membrane transport. Both pathways involving the Rho GTPases cooperate to transduce the death-promoting activity of E4orf4, which acts via actin remodeling. A Rac1-dependent pathway was also reported to participate in E4orf4-induced events in cooperation with RhoA, to promote formation of stress fibers and nucleation of perinuclear actin filaments anchored to the actin ring [74]. Interestingly, inhibition of

Fig. 4.4 (continued) (**b**) A scheme of the juxtanuclear actin-myosin network assembled as a result of E4orf4-induced changes to Src signaling. *Red circles*: recycling endosomes; *green tails*: actin patches nucleated at the surface of endosomes; *green lines*: actin cables and remodeled focal adhesions; *N* nucleus. See text for details (Sect. 4.4.1) (This figure was kindly contributed by Lavoie et al. [76]. © American Society for Cell Biology (2009) and © Elsevier (2010))

actin polymerization by low doses of drugs such as cytochalasin D reduced E4orf4-induced cell death, indicating a causal role for actin dynamics in this process [5, 74].

Investigation of how actin remodeling and RE trafficking can participate in E4orf4 death signaling revealed that in the early stages of E4orf4-induced cell death, some SFKs, Cdc42, and actin interfered with the organization of the endocytic recycling compartment and enhanced RE transport to the Golgi apparatus while decreasing recycling of protein cargos back to the plasma membrane. The ensuing changes in Golgi membrane dynamics required actin-regulated Rab11a membrane trafficking and triggered dissociation of Golgi membranes. This process functionally contributed to the progression of cell death as shown by the findings that Rab11 knockdown inhibited cell death as did knockdown of syntaxin-6, a TGN trafficking factor involved in fusion of RE with Golgi membranes, or overexpression of golgin-160, a Golgi matrix protein associated with Golgi dynamic changes during apoptosis. Thus, E4orf4 acts by recruiting SFK signaling to converge on REs and promoting RE transport to the Golgi where they may facilitate the dynamic rearrangement of membranes. This may be done by several cycles of interactions between REs and Golgi membranes observed at sites of Golgi membrane fission, possibly reflecting the delivery of proteins and lipids. It was suggested that the E4orf4-induced assembly of the "peculiar" perinuclear actin network may be the outcome of the polarized traffic of membranes, which may promote delivery of actin-remodeling factors [78]. Since golgin-160, a caspase target involved in apoptotic Golgi disassembly, was found to negatively regulate RE-Golgi interactions and dissociation of Golgi membranes, it was suggested that RE trafficking and caspases could act in overlapping pathways to promote death through changes in Golgi dynamics [78]. It is currently not known how fission of Golgi membranes brings about cell death, although it is appealing to consider a possibility whereby factors released from the Golgi may contribute to this process, similarly to factors released from the mitochondria [78]. It was also suggested (based on unpublished results quoted in [76]) that Rab11a-endosomes and Golgi-derived vesicles associate with mitochondria upon Golgi membrane scattering, and alter mitochondrial functional integrity, possibly contributing to cell death.

4.4.2 The Contribution of the E4orf4-PP2A Interaction to E4orf4-Induced Cell Death

4.4.2.1 Association of E4orf4 with PP2A Phosphatase Activity Correlates with E4orf4-Induced Cell Death

Some of the first papers reporting that E4orf4 induced cell death showed that an E4orf4 mutant which did not bind PP2A (S95P, known also as mutant "A3") lost the ability to kill cells, and even increased cell survival [47, 60]. More detailed

mutation analyses of the E4orf4 protein confirmed the hypothesis that an interaction with PP2A was required for E4orf4-induced cell death [1, 27]. These analyses probed the correlation between physical association of E4orf4 mutants with the B55 or C subunits of PP2A as well as their association with PP2A phosphatase activity and their ability to induce cell death. Some interesting results were obtained in these experiments. First, most of the mutants that lost part or all of the ability to bind PP2A, retained reduced cell killing activities. Second, a few mutants that bound an active PP2A at reduced levels possessed a somewhat higher than expected cell death activity. One of these mutants (F84A) was suggested to be a gain-of-function mutant in the Src pathway, possibly by increasing the association of E4orf4 with major targets in the cytoplasmic cell death pathway or their regulation [28]. A third interesting observation made in these experiments indicated that some E4orf4 mutants that bound PP2A at more than 50 % efficiency were greatly impaired in induction of cell death. This class of mutants was termed class II, as opposed to the so-called class I mutants which lost both PP2A binding and the ability to induce cell death [27] (Fig. 4.2). It was suggested that binding to PP2A by class II E4orf4 mutants may be altered compared with binding by WT E4orf4 and may thus fail to exert a biological effect [27]. For example, these mutants may fail to target PP2A to the relevant substrates.

More support for the conclusion that the B55 subunit of PP2A was required for E4orf4-induced cell death came from experiments showing that an antisense construct that could reduce B55 expression, could inhibit E4orf4- but not p53-induced cell death [1] and that overexpression of B55 enhanced E4orf4-induced cell death [47]. Furthermore, because all PP2A subunits are highly conserved in evolution, the investigation of the interaction between E4orf4 and PP2A was extended to yeast (see Sect. 4.5.1). It was shown in this organism that deletion of *cdc55*, the yeast gene encoding the B55 subunit ortholog, or deletion of *tpd3*, encoding the PP2A-A subunit, reduced significantly E4orf4 toxicity in this organism [49, 50].

Interestingly, the S95P E4orf4 mutant ("A3") was shown to bind the B subunits of PP2A, but not the A and C subunits, and it acted in a dominant-negative fashion to inhibit cell death induced by the WT protein [47]. This finding suggests that the whole PP2A complex, and not just the regulatory B subunit is required for E4orf4-induced cell death. On the other hand, work in yeast revealed that although the PP2A-C subunit contributes to E4orf4-mediated toxicity, the Cdc55 subunit may provide an additional contribution to E4orf4-mediated toxicity that is independent of stable complex formation with the PP2A-C subunit [79]. This conclusion was reached based on experiments in which PP2A heterotrimer formation was disrupted by mutations in the carboxyl terminus of the PP2A C subunit that prevent its association with the Cdc55 regulatory subunit, or by deletion of the Ppm1 methyltransferase required for C subunit methylation and subsequent association with Cdc55 [80, 81]. These perturbations of the PP2A heterotrimer only partially affected E4orf4 toxicity. In contrast, Cdc55 did not appear to mediate E4orf4 toxicity through a Tpd3-independent pathway, as deletion of either Cdc55 or Tpd3 caused similar levels of resistance to E4orf4-mediated growth inhibition,

and a double deletion was not more effective than a single Tpd3 deletion [79]. The nature of the PP2A-C-subunit-independent role of the Cdc55 subunit is not clear, although it was suggested that Cdc55 may form a heterotrimeric phosphatase with Tpd3 and with another PP2A-like catalytic subunit, Sit4, a yeast PP6 ortholog. Remarkably, deletion of Sit4 in yeast and knockdown of PP6 in mammalian cells increased E4orf4-mediated cell death. However, a physical interaction between E4orf4 and Sit4 was not detected [79].

4.4.2.2 How Does PP2A Contribute to E4orf4-Induced Cell Death?

Does E4orf4 Affect PP2A Activity?

E4orf4 was shown to associate with substantial levels of PP2A phosphatase activity, and experiments with the PP2A inhibitor okadaic acid revealed that phosphatase activity was required for E4orf4 functions including down-regulation of gene expression and regulation of alternative splicing [36, 46]. Moreover, E4orf4 expression was associated with hypophosphorylation of E1A, c-fos, and SR proteins during virus infection [32, 36]. On the other hand, a recent report suggested that inhibition of PP2A activity contributed to E4orf4-induced cell death [48]. In this work, PP2A activity associated with E4orf4 was compared with PP2A activity associated with B55 in the absence of E4orf4, using co-immunoprecipitation-phosphatase assays. Phosphatase activity was measured *in vitro* using various non-physiological substrates. Under these conditions, no difference in PP2A activity was recorded using a small universal phosphopeptide substrate recognized by many Ser/Thr phosphatases. When phosphorylated phosphorylase a or histone H1 were utilized, a two to fourfold reduced activity was observed in the E4orf4 immune complexes compared to B55-containing immune complexes from cells lacking E4orf4. However, this comparison is not direct, and as E4orf4 associates with various B55 and B56 subunits [47, 48] it is possible that the observed differences resulted from altered substrate specificity of the various holoenzymes. Two proteins that may be substrates of a PP2A holoenzyme containing the B55α subunit were found to be hyperphosphorylated in E4orf4-expressing cells, but as described above, other proteins were hypophosphorylated in the presence of E4orf4 [32], and it is not known which of these effects was direct. It was further shown that treatment of cells with the PP2A inhibitors okadaic acid and I_1^{PP2A} enhanced E4orf4 toxicity, leading the authors to conclude that E4orf4 must inhibit PP2A in order to induce cell death [48]. However, okadaic acid can inhibit other PP2A-like phosphatases at least as efficiently as PP2A itself [82], and the I_1^{PP2A} inhibitor was shown to associate with PP1 and modify its substrate specificity [83], indicating that these inhibitors may have off-target effects that are not dependent on PP2A. Indeed, reduced expression of PP6, a PP2A-like phosphatase, was shown to increase E4orf4-induced cell death [79], and PP6 is inhibited by okadaic acid even more efficiently than PP2A [84] suggesting that the increase in E4orf4 toxicity that was reported to be caused by okadaic acid treatment may stem from PP6 inhibition.

In addition, The E4orf4 binding site in PP2A lies above the substrate binding groove but is not adjacent to the catalytic site, suggesting that E4orf4 does not inhibit the catalytic activity directly, though it may influence substrate binding [26]. We propose that only when the physiological substrates of the E4orf4-PP2A complex are identified, it will be possible determine whether a change in the PP2A catalytic activity affecting these substrates may be required for E4orf4-induced cell death.

E4orf4 Recruits PP2A to Potential Substrates: ACF and APC/C

An alternative mechanism that could be employed by E4orf4 to affect PP2A function may involve recruitment of PP2A to novel substrates or alteration of the affinity of substrate binding to PP2A by E4orf4. Indeed, E4orf4 was reported to recruit PP2A to the ACF chromatin remodeling factor in mammalian cells [75] and to the mitotic regulator APC/C in yeast [49].

A protein interaction screen aimed at finding novel E4orf4 interactors revealed an interaction of E4orf4 with the Acf1 regulatory subunit of the ATP-dependent chromatin remodeling factor ACF [75]. This factor includes the SNF2h ATPase in complex with Acf1 [85]. Co-immunoprecipitation experiments confirmed that both Acf1 and SNF2h were found in E4orf4-containing complexes. These experiments further revealed that the PP2A C subunit co-immunoprecipitated with ACF in the presence of WT E4orf4 but not in its absence or in the presence of a mutant E4orf4 protein that cannot bind PP2A. Moreover, it was shown that when chromatin proteins were fractionated by extraction in increasing salt concentrations, the PP2A-B55 subunit was enriched in higher salt fractions in the presence of E4orf4 compared to its distribution in the absence of E4orf4 or in the presence of the mutant E4orf4 protein. These results suggest that E4orf4 alters the chromatin-binding properties of PP2A, likely by recruiting it to ACF. In addition, it was shown that the ACF complex was involved in E4orf4-induced cell death. Reduced SNF2h expression or expression of a dominant-negative catalytically-inactive SNF2h mutant inhibited cell death, whereas, surprisingly, knockdown of Acf1 increased cell death. Knockdown of an Acf1 homolog, WSTF, which also associates with SNF2h, inhibited E4orf4-induced cell death. These results suggested a scenario wherein the E4orf4-PP2A complex inhibits ACF and facilitates enhanced chromatin remodeling activities of other SNF2h-containing complexes, such as WSTF-SNF2h. The E4orf4-induced switch in chromatin remodeling may determine life *versus* death decisions [75] (Fig. 4.5). This scenario predicts phosphorylation changes in ACF or ACF-associating proteins that could be significant to E4orf4 function, however, such alterations have not been reported yet. It is not likely, however, that E4orf4 recruits PP2A to a new substrate, such as ACF, with the sole purpose of inhibiting PP2A activity towards this substrate.

A second potential E4orf4-PP2A target is the mitotic regulator APC/C. Work in yeast revealed that E4orf4 caused mitotic arrest in yeast by inhibiting APC/C

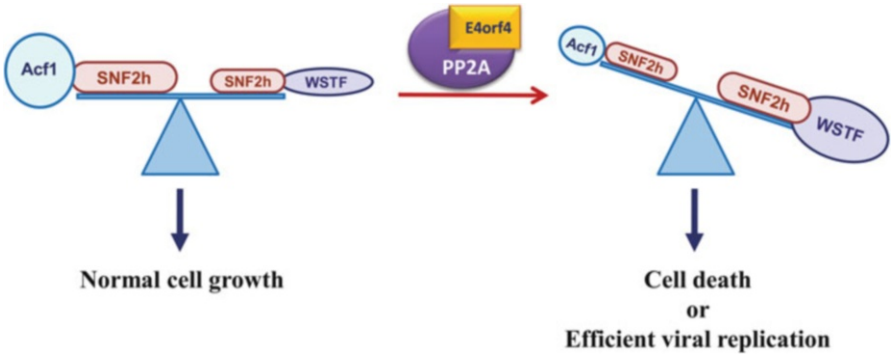

Fig. 4.5 A model for E4orf4 function in chromatin. Various SNF2h-containing complexes participate in chromatin remodeling and affect transcription, DNA replication, DNA repair, etc. The model proposes that E4orf4 targets PP2A to Acf1-containing chromatin remodelers and inhibits them. This inhibition leads to a shift in the balance between various SNF2h remodeling complexes, allowing, for example, more activity of a WSTF-SNF2h complex. The variation in chromatin remodeler activity alters chromatin remodeling and produces changes in transcription, DNA replication, DNA repair, or other processes that require remodeling. These events contribute to E4orf4 functions during virus infection and lead to cell death when E4orf4 is expressed alone (This figure is reprinted from Brestovitsky et al. [75] by permission of Oxford University Press)

complexes containing the Cdc20 or cdh1/Hct1 activating subunits (see Sect. 4.5.1.1, [49]). This finding led to examination of the possibility that E4orf4 acts directly on the APC/C. It was indeed shown that E4orf4 co-immunoprecipitated with the APC/C subunit Cdc16 (the yeast ortholog of mammalian APC6), and that PP2A was found in immune complexes precipitated with Cdc16, but only in the presence of E4orf4. Genetic experiments revealed a functional interaction between Cdc55 and the APC/C activating subunit Cdc20, as Cdc55 overexpression was highly toxic in a *cdc20* mutant background [49] and deletion of *cdc55* partially suppressed the temperature sensitivity of the *cdc20* mutant [86]. This data indicated that even in the absence of E4orf4, PP2A-Cdc55 acts as a negative regulator of the APC/C^{Cdc20}. Therefore, the results suggested that E4orf4 may be stabilizing an existing interaction between PP2A and the APC/C [49]. It was reported that E4orf4 increased nuclear accumulation of Cdc55, thus possibly enhancing the targeting of nuclear substrates, such as the APC/C by PP2A holoenzymes containing the B55/Cdc55 subunit [87]. However, phosphorylation sites in the APC/C complex or its activating subunits, which may be affected by the E4orf4-PP2A complex have not been identified to date.

The functional consequences of the interaction between E4orf4 and the APC/C are discussed below (Sect. 4.5.1.1). Interestingly, the APC/C was found to be a target of many viral proteins and some of these viral proteins cause cytotoxicity specifically in tumor cells, providing evidence that targeting the APC/C could be exploited to selectively eliminate cancer cells [88].

4.5 The Mechanisms Underlying E4orf4-Induced Toxicity Are Highly Conserved in Evolution

Genetic model organisms can be very useful in revealing details of conserved cellular regulatory pathways.

The major E4orf4 protein partners, PP2A and Src kinases, are highly conserved proteins. In *S. cerevisiae*, redundant protein products of the *PPH21*, *PPH22*, and a related *PPH3* gene encode PP2A-C subunits [89], *TPD3* encodes the A subunit [90], and two B-type subunits exist, encoded by the *CDC55* (B55 ortholog) and *RTS1* (B56 ortholog) genes [91, 92]. Yeast and mammalian PP2A subunits share more than 53 % identity and 67 % similarity, depending on the specific subunit [91], with more than 75 % identity in the catalytic C subunits [93]. Therefore, the ability of yeast to serve as a candidate genetic model that could be useful in the analysis of the contribution of PP2A to E4orf4-induced toxicity was investigated [49, 50, 52]. However, the yeast system was not expected to reveal the full spectrum of E4orf4-induced events in mammalian cells, since yeasts do not contain genes encoding the Src kinases. Src orthologs exist from the unicellular choanoflagellate *Monosiga ovata*, through the primitive multicellular sponge *Ephydatia fluviatilis*, and up the evolutionary ladder, with more than 40 % amino acids identity [94]. The *Drosophila Src64B* gene encodes a 62 kDa protein that is remarkably similar to the Src proteins of higher eukaryotes, such as chicken *c-src*, and thus the *Drosophila* model system could potentially contribute to the investigation of the mechanisms underlying E4orf4-induced events. Therefore, to make use of the power of genetic tools in the analysis of E4orf4-induced cell death, E4orf4 was studied in the yeast *S. cerevisiae* and in the multicellular organism *Drosophila melanogaster* (see Fig. 4.6 for summary of the results).

4.5.1 Lessons from the Yeast S. cerevisiae *on E4orf4-Induced Toxicity*

When E4orf4 was expressed in yeast from an inducible galactose promoter (gal), growth inhibition was observed, which became irreversible after a few hours of E4orf4 expression. Much less growth inhibition was detected in yeast lacking the PP2A subunits Cdc55 and Tpd3, but loss of the Rts1 subunit did not prevent E4orf4 from inhibiting cell growth [49, 50]. These results were very similar to results obtained in mammalian cells showing that the PP2A-B55 subunit, but not B56 subunits were required for E4orf4-induced cell death [1, 27, 47]. It was further shown that E4orf4 enhanced cellular levels of reactive oxygen species (ROS) [49], as was shown also in mammalian cells [71].

To further confirm that yeast was an appropriate model for the study of E4orf4-induced cell death, yeast cells were utilized to select E4orf4 mutants that have lost their ability to induce toxicity. Plasmids containing E4orf4 cDNA expressed from a

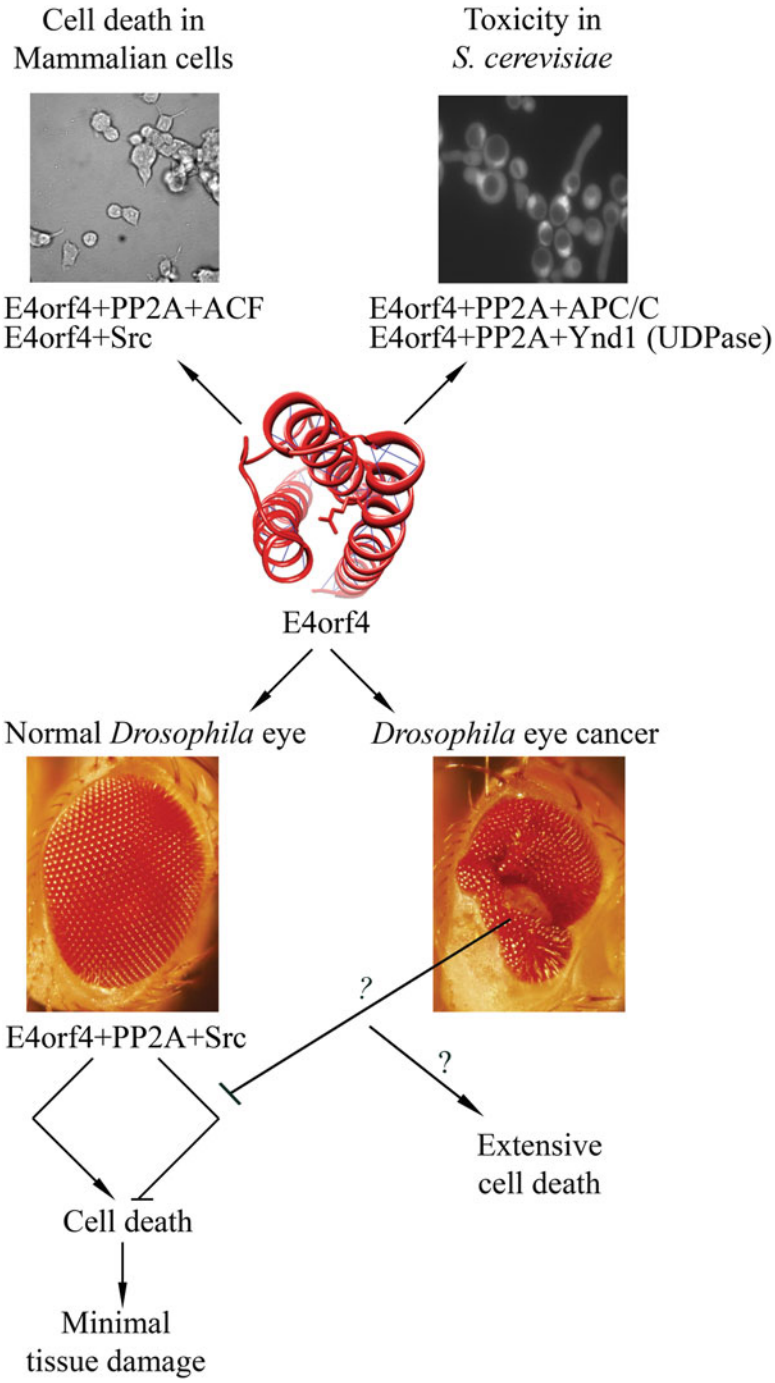

Fig. 4.6 Various model systems provided insights into E4orf4-induced cell death. Mechanisms underlying E4orf4-induced cell-death were investigated in various organisms. Work in mammalian cells in tissue culture revealed a role for PP2A, Src kinases and the ACF chromatin remodeling

galactose-inducible promoter were randomly mutagenized, transformed into yeast cells, and E4orf4-resistant colonies were selected on galactose. E4orf4 mutants thus obtained were expressed in mammalian cells, and were found to have a diminished cell killing activity as well as a reduced ability to bind PP2A [52]. These results suggested that E4orf4-induced toxicity in yeast reflects accurately E4orf4-induced cell death in mammalian cells and thus that the yeast system may serve as a powerful genetic model for the study of E4orf4-induced cell killing. In fact, several new insights into E4orf4-induced cell death were contributed by work in yeast. First, it was found that E4orf4 induced G2/M arrest in cells prior to induction of cell death (see Sect. 4.5.1.1). Second, it appears that not all of E4orf4-induced toxicity is generated via a Cdc55-dependent pathway. Although much of the E4orf4 toxicity was abrogated in yeast cells lacking Cdc55, some residual inhibitory effect of E4orf4 could be detected [50, 95]. Moreover, E4orf4 mutants that were deficient in PP2A binding could still elicit low levels of toxicity [50]. Indeed, further work in yeast demonstrated that a second E4orf4 partner, Ynd1, contributed to E4orf4 toxicity, and the contributions of Cdc55 and Ynd1 to E4orf4-induced toxicity were additive, indicating that they were not part of the same pathway [95] (Sect. 4.5.1.2). Third, work in yeast described above (Sect. 4.5.1), revealed that some E4orf4 toxicity was mediated by Cdc55 in a PP2A-C subunit-independent manner, possibly by inhibition of another PP2A-like phosphatase, Sit4 (PP6 in mammalian cells) [79]. Sit4 may form a heterotrimeric complex with Tpd3 and Cdc55, however, a physical association between E4orf4 and Sit4 was not detected. Nevertheless, Sit4 deletion in yeast or knockdown of its mammalian ortholog, PP6, enhanced E4orf4-induced cell death [79].

4.5.1.1 E4orf4-Induced Cell Cycle Arrest Leads to Cell Death in Yeast and Mammalian Cells

E4orf4-induced growth inhibition in yeast was caused by a G2/M arrest, as detected by FACS analysis [49, 50], and visualization of spindles with GFP-marked tubulin demonstrated that most cells were arrested either with short pre-anaphase spindles or with extended telophase spindles [49]. The use of several yeast cell cycle mutants revealed that E4orf4 expression was synthetically lethal with reduction in the activity of Cdc28, the yeast Cdk1 [49]. Thus, for example, cells lacking the Mih1

Fig. 4.6 (continued) factor in E4orf4-induced cell-death [1, 5, 75]. Work in yeast revealed the roles of PP2A, Golgi UDPase (Ynd1) and the anaphase-promoting-complex/cyclosome (APC/C) in E4orf4 toxicity [49, 95]. Work in *Drosophila* revealed that E4orf4 induced PP2A- and Src-dependent cell-death in normal tissues while inhibiting classical apoptosis [9]. The concomitant induction and inhibition of cell-death resulted in minor damage to normal tissues. We hypothesize that the more effective cell killing induced by E4orf4 in cancer cells may stem from reduced inhibition of classical apoptosis in these cells. This as yet untested hypothesis is represented by question marks. E4orf4 is represented by its structural model [26] (This figure is reprinted from Pechkovsky et al. [10])

phosphatase, which normally removes an inhibitory phosphorylation from the Cdc28 kinase and activates its kinase activity, were more sensitive to E4orf4-induced toxicity, as were cells defective in mitotic cyclins or cells over-expressing the Swe1 kinase that inhibits Cdc28 [49]. Furthermore, it was shown that E4orf4 increased phosphorylation of Histone H1 by the Cdc28-Clb2 kinase complex in a Cdc55- and Mih1-dependent manner [49, 50]. It was suggested that the hyperactivation of Cdc28 may be a secondary E4orf4 effect that partially counteracts the main E4orf4 inhibitory effect on cell cycle progression [49]. It was also observed that E4orf4-expressing cells were highly sensitive to the microtubule-depolymerizing drug, benomyl, similarly to previously reported mutations in the kinetochore/spindle checkpoint. Benomyl supersensitivity was not observed upon expression of an E4orf4 mutant that was unable to bind PP2A [49].

The spindle checkpoint detects improper kinetochore-microtubule attachments and consequently inhibits the mitotic E3 ubiquitin ligase complex known as the anaphase-promoting complex, or cyclosome (APC/C), resulting in delayed anaphase onset. This delay allows correction of the situation and prevents improper chromosome segregation during cell division [96]. The APC/C associates with several activating subunits during specific periods of the cell cycle. The best studied of these are Cdc20 and Cdh1 [97]. High Cdk1 activity in mitosis leads to APC/C phosphorylation, allowing the assembly of APC/C^{Cdc20}, which initiates cyclin proteolysis and decreases Cdk1 activity. This drop in Cdk1 activity promotes the formation of APC/C^{Cdh1}, which does not require APC/C phosphorylation, but which requires Cdh1 dephosphorylation by Cdc14. APC/C^{Cdh1} then maintains cyclin instability in G1 and enables a new round of DNA replication [97]. Another layer of phosphorylation-dependent regulation involves phosphorylation of Cdc20 itself by Cdk1 or mitogen-activated protein kinase (MAPK). This phosphorylation is inhibitory and leads to Cdc20 binding to inhibitory proteins, such as the Mad2 spindle checkpoint protein [98, 99]. It was recently shown that PP2A contributes to APC/C activation by dephosphorylating inhibitory phosphor-sites in Cdc20 and allowing its association with the APC/C and the consequent activation of the APC/C [100].

Some of the cell cycle mutants exhibiting synthetic lethality with E4orf4-induced growth inhibition in yeast contained mutations in the APC/C co-activators Cdc20 and Cdh1 as well as mutations in the APC/C Cdc16 subunit [49]. Biochemical analysis revealed that E4orf4 inhibited degradation of Ase1 and Pds1, substrates of the APC/C^{Cdh1} and APC/C^{Cdc20} respectively, possibly as a result of stabilization of an interaction between PP2A and APC/C, discussed above [49]. The supersensitivity of E4orf4-expressing cells to benomyl is also best explained by inhibition of APC/C^{Cdc20}. Benomyl, which transiently triggers the spindle assembly checkpoint by inhibiting APC/C^{Cdc20}, might cause permanent arrest when the APC/C is already partially inhibited.

In contrast to the study showing that E4orf4 inhibited both APC/C^{Cdc20} and APC/C^{Cdh1} during mitosis [49], it was reported that E4orf4 activated APC/C^{Cdc20} prematurely when cells were arrested in S phase by hydroxyurea (HU) treatment, but did not affect APC/C^{Cdh1} [87]. A possible explanation for these seemingly

contrasting results may be offered by the existence of different potential targets of PP2A in APC/C and its modulators that may be targeted differently by the E4orf4-PP2A complex in S-phase and mitosis. In addition to APC/C itself and Cdc20, the APC/C inhibitor Emi1 is also regulated by Cdk1 phosphorylation, which inhibits it, thus activating APC/C [101]. Although Emi1 is not normally phosphorylated during S-phase, the Cdc55-dependent E4orf4-induced increase in Cdk1 activity [49, 50] may lead to Emi1 phosphorylation in S-phase followed by APC/C activation. However, during mitosis, Cdc55-dependent inhibition of APC/C may be more prominent [49], while Emi1 is degraded at mitotic entry [102, 103]. Whereas inhibition of APC/C complexes may account at least in part for the E4orf4-induced G2/M arrest, it is not clear how premature activation of APC/C^{Cdc20} in S-phase assists in this process. Thus a better understanding of the interaction between E4orf4 and the cell cycle requires more work. In any event, the delay in the cell cycle and M-phase perturbations may result in cells with improper chromosome segregation returning to the cell cycle, thus leading to cell death.

Following the studies in yeast, it was shown that E4orf4 caused a G2/M arrest in mammalian cells before induction of cell death [49]. Indeed, several reports demonstrated that perturbations in the cell cycle can lead to cell death [104, 105], and a combination between APC/C inhibition and Cdk stimulation was shown to be linked to induction of apoptosis in other situations as well [106]. Experiments carried out in H1299 cells showed that E4orf4 expressed from an adenovirus vector induced cell morphologies typical of mitotic catastrophe. Mitotic catastrophe is a form of cell death that results from aberrant mitosis, leading to the formation of large non-viable cells with several micronuclei containing uncondensed chromosomes. Mitotic catastrophe can result from deficient mitotic checkpoints in tumor cells, anti-microtubular drugs, and premature mitosis. All these conditions may involve the unscheduled activation of Cdk1 [104, 107]. Furthermore, mitotic catastrophe can lead to various modes of cell death, such as apoptosis and necrosis, as well as to senescence, depending on the genetic identity of the cells and the physiological conditions [108]. In the H1299 cells infected with the E4orf4-expressing virus there was some increase in the number of cells with $\geq$4N DNA content, 30 % of these cells contained two or more nuclei, and 5 % of the cells contained micronuclei. Moreover, a large increase in Cyclin E levels was observed in these cells, suggesting a release of the cells from G2/M arrest into G1 without proper chromosome segregation [7]. Thus under some cellular circumstances, E4orf4-induced cell death can result from cell cycle perturbations and mitotic catastrophe.

4.5.1.2 A Genetic Screen in Yeast Revealed Another Contributor to E4orf4-Induced Toxicity: Ynd1/Golgi UDPase

A classical genetic screen in yeast was used to search for novel genes whose protein products are required for induction of toxicity by E4orf4. This screen revealed one such gene, *YND1*, encoding a Golgi apyrase [95]. Apyrases are nucleoside triphosphate diphosphohydrolases that hydrolyze a variety of nucleoside 5'-triphosphates

and 5' diphosphates with different nucleotide preferences. Most members of this family are integral membrane glycoproteins, and their catalytic sites face the extracellular medium or the lumen of intracellular organelles [109]. Ynd1 is a Golgi apyrase whose enzymatic activity is required for regulation of nucleotide-sugar import into the Golgi lumen. This protein is inserted in the Golgi membrane, its 500 N-terminal amino acids including its catalytic domain, are located in the Golgi lumen, whereas its 113 C-terminal residues are found at the cytoplasmic face of the Golgi membrane [110, 111]. It was shown that *CDC55* and *YND1* made an additive contribution to E4orf4 toxicity, since deletion of each one separately conferred incomplete resistance to E4orf4 in yeast, whereas a double deletion conferred full resistance. On the other hand, there appeared to be a functional interaction between the two genes, since deletion of *YND1* sensitized cells to killing by E4orf4 in the presence of over-expressed *CDC55* [95]. It was further shown that Ynd1 and Cdc55 interacted physically [95]. The physical and functional interactions between Ynd1 and Cdc55 together with their additive effects on E4orf4 toxicity may be compatible with the hypothesis that the Ynd1-mediated E4orf4 function has PP2A-dependent as well as PP2A-independent aspects. Recent results describing the role of mammalian Ynd1, Golgi UDPase, in E4orf4-induced cell death support this possibility [112]. A functional interaction between *YND1* and the APC/C co-activators, *CDC20* and *CDH1* was also observed, although the nature of this link is not currently understood.

The relevance of mammalian Ynd1 to E4orf4-induced cell death was demonstrated by the finding that this protein was part of a complex containing E4orf4 [95], and by results demonstrating that knockdown of mammalian Ynd1 reduced E4orf4-induced cell death [112].

Surprisingly, we found that the Ynd1 cytosolic tail mediated E4orf4-induced toxicity whereas its apyrase activity was dispensable for this process [95, 113]. The Ynd1 cytosolic tail could potentially mediate E4orf4 toxicity by acting as a scaffold for a multi-protein complex which is targeted by E4orf4. It was indeed shown that E4orf4 dissociates Cdc55 from Ynd1 [95, 113], possibly reflecting the disruption of a multi-protein complex which includes Cdc55. The *Saccharomyces* Genome Database cites at least 10 membrane proteins which physically associate with Ynd1, likely through its cytosolic tail. Six of these proteins associate with each other, possibly as part of a large protein complex. Some of these proteins participate in both early and late secretory pathways in yeast, and it is possible that E4orf4 interacts with a secretory protein complex anchored to the Ynd1 cytosolic tail. As it was shown that E4orf4 affected protein trafficking in mammalian cells through its association with Src kinases [78], it is possible that in yeast cells, which lack Src family members, E4orf4 may utilize a backup mechanism which allows it to interact directly with components of the secretory pathway to affect protein trafficking, thus further transducing its toxic signal [113]. This mechanism is likely used by E4orf4 in mammalian cells as well.

4.5.2 *Lessons from Drosophila melanogaster on E4orf4-Induced Toxicity*

A study of E4orf4 in *Drosophila* revealed that the characteristics of E4orf4-induced cell death in the fly were very similar to those in mammalian cells [9]. Thus, in normal *Drosophila* tissue E4orf4 induced low levels of cell killing, caused by both caspase-dependent and -independent mechanisms. *Drosophila* PP2A-B55 (*twins/abnormal anaphase resolution*) and Src64B contributed additively to cell-death. However, this study, which addressed for the first time the consequences of E4orf4 expression in a whole multicellular organism, revealed a surprising finding: E4orf4 not only induced cell death but also inhibited classical apoptosis induced by the fly proapoptotic genes *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*. E4orf4 also inhibited cell death induced by JNK signaling. However, whereas inhibition of *rpr*, *hid* and *grim* partially reduced cell killing, JNK inhibition did not impede E4orf4-induced toxicity and even enhanced it. These results indicated that E4orf4-induced cell killing is a distinctive form of cell death that differs from classical cell death pathways induced by *rpr/hid/grim* or JNK signaling. Although E4orf4 was reported to activate JNK signaling in certain transformed mammalian tissue culture cells [28, 77], JNK signaling was shown by many reports to be highly dependent on cellular context and on the nature of the stimulus, and this may also impact the interaction between E4orf4 and the JNK pathway [9]. Future studies will have to determine whether JNK inhibition or activation by E4orf4 depend on the tumorigenic state of the cells, on the cell environment (monolayer or tissue) or on the type of organism studied.

The combination of both induction and inhibition of cell death by E4orf4, observed in *Drosophila*, led to generation of minimal tissue damage. This finding may suggest a possible explanation for the differential effect of E4orf4 in normal and cancer cells. It may be hypothesized that E4orf4 does not inhibit cell death in cancer cells, thus inducing higher cell death levels [10] (Fig. 4.6). This hypothesis is one of several possible explanations for enhanced cell killing by E4orf4 in cancer cells, which will have to be tested in the future (see Sect. 4.1).

4.6 Preliminary Exploration of the Therapeutic Potential of E4orf4

As E4orf4 induces a p53-independent, non-canonical programmed cell death [58–60] and a large percent of human tumors are p53-deficient [114], study of the unique mode of E4orf4-induced cell death in cancer cells may have exciting implications for cancer therapy. Although understanding of the differential sensitivity of normal and cancer cells to E4orf4-induced cell death is still lacking, researchers were tempted to explore the feasibility of using E4orf4-based approaches for cancer therapy. To date, sporadic preliminary experiments have

been carried out to test the ability of E4orf4 to eliminate cancer cells in animals and in tissue culture.

In an initial experiment, plasmid DNA expressing E4orf4 or a control plasmid were administered by several successive electroporations into tumors generated in mice by subcutaneous inoculation of B16(F10) melanoma cells. The tumors receiving the E4orf4 genes were reported to be smaller than those receiving the control plasmid. However, this effect was maintained for a short time, after which the tumors grew back [115]. It should be noted that the electroporation efficiency in these experiments was low.

In another approach, an E4orf4 protein fused to epidermal growth factor (EGF) was expressed and purified from the yeast *P. pastoris* to evaluate its tumor inhibitory effects on tumors expressing the EGF receptor [116, 117]. The fusion protein was first added to cells in tissue culture and was shown to be internalized by the EGF receptor and to induce cell death. It was then tested in human carcinoma xenograft models [117]. Intraperitoneal administration of EGF-E4orf4 was initiated when the xenograft tumors in mice grew to 3–4 mm in diameter and tumor volume was monitored for 30–35 days thereafter. EGF-E4orf4 inhibited tumor growth in a dose-dependent manner, producing 79 % inhibition of tumor volume for xenografts derived from MDA-MB-231 cells and 49 % inhibition of xenografts derived from BGC823 cells. The liver and kidney from the EGF-E4orf4 treated mice showed no histopathological alterations and the mice did not suffer weight loss during this time, indicating lack of general toxicity associated with the EGF-E4orf4 protein.

A third approach for exploring the use of E4orf4-based technology for cancer therapy was described recently [118]. Peptides containing the PP2A-binding site of canine adenovirus E4orf4 protein linked to a cell penetrating peptide called DPT-sh1 were generated. These peptides were shown to have a predominantly α-helical structure and to penetrate the cells. They reduced cell survival of five transformed human cell lines in tissue culture, but not of human foreskin fibroblasts. Based on these results it was suggested that mimicking the anti-cancer effects resulting from the E4orf4-PP2A interaction may be a promising strategy for the development of new cancer therapies.

Another possibility of employing E4orf4 in cancer therapy involves its incorporation into oncolytic virus vectors [119]. An E1-deleted non-replicating adenoviral vector expressing the herpes simplex virus thymidine kinase gene was used in co-infection experiments with an E4-deleted adenovirus expressing E4orf4 only, providing trans-complementation which facilitated mutant virus replication. Human melanoma xenografts were generated in nude mice. When reaching a volume of around 400 mm^3 they were injected with several virus combinations. Tumor regression was observed when the trans-complementing viruses were introduced together, and they also significantly enhanced median survival. No viral DNA was detected in the liver of mice treated with the trans-complementing viruses, indicating low virus shedding.

These initial attempts to use E4orf4 to treat cancer cells *in vivo* provide further motivation to expand the basic research aiming to unravel the E4orf4 cell death network both in tissue culture cells and in animal models.

4.7 Future Directions

E4orf4 research has progressed significantly in the past several years, as summarized in Fig. 4.7, but many questions still await clarification. Two major E4orf4 partners, PP2A and Src kinases were identified and appear to provide a large portion of the contribution to E4orf4-induced cell death. However, several other E4orf4 partners may make additional contributions, like the Ynd1 Golgi UDPase. Downstream effectors of both PP2A and Src have also been identified but a detailed analysis of the connectivity of the E4orf4 network is still lacking. Thus, for example, what are the direct targets of the E4orf4-PP2A complex in chromatin and in the APC/C and how exactly do they influence E4orf4-induced cell death? What is the molecular nature of the contribution of alterations in protein trafficking and Golgi membrane disruption to nuclear condensation and cell death? Is there crosstalk between PP2A and Src kinases during the E4orf4-induced cell death process? What is the contribution of the E4orf4-Ynd1 interaction to induction of cell death? These are just a few examples of open questions. Once the molecular mechanisms are further elucidated, it will be easier to compare them in normal and cancer cells and find which differences between the two types of cells may account for their different sensitivity to E4orf4. Moreover, the E4orf4 cell death network

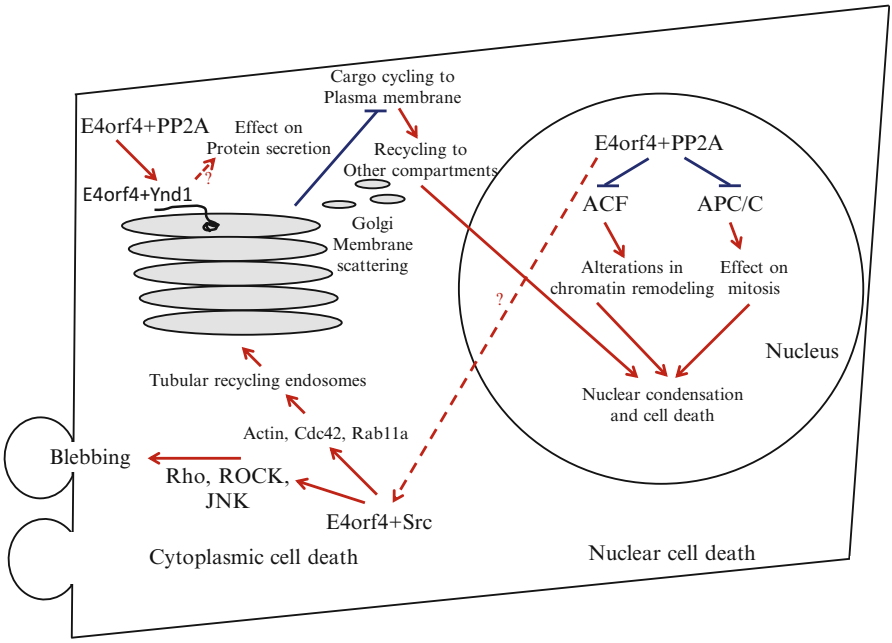

Fig. 4.7 A schematic summary of the mechanisms underlying E4orf4-induced cell death. E4orf4, in collaboration with its partners, induces several alterations in the nucleus and the cytoplasm, which result in nuclear condensation and cell death: see the text for details. Connections that were suggested but not proven yet are shown by *discontinuous arrows* and marked with *question marks*

should be probed not only in tissue culture cells but also in animal models to provide additional insights. The knowledge obtained in such experiments will further aid in design of novel E4orf4-based cancer therapeutics.

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Chapter 5
Tumor Suppressing Properties of Rodent Parvovirus NS1 Proteins and Their Derivatives

Jürg P.F. Nüesch and Jean Rommelaere

Contents

Abstract Cancer chemotherapy with monospecific agents is often hampered by the rapid development of tumor resistance to the drug used. Therefore, combination treatments aiming at several different targets are sought. Viral regulatory proteins, modified or not, appear ideal for this purpose because of their multimodal killing action against neoplastically transformed cells. The large nonstructural protein NS1of rodent parvoviruses is an excellent candidate for an anticancer agent, shown to interfere specifically with cancer cell growth and survival. The present review describes the structure, functions, and regulation of the multifunctional

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protein NS1, its specific interference with cell processes and cell protein activities, and what is known so far about the mechanisms underlying NS1 interference with cancer growth. It further outlines prospects for the development of new, multimodal cancer toxins and their potential applications.

Keywords Parvoviruses • Non-structural protein NS1 • Anti-cancer activities • Cellular targets • Signaling cascades • Phosphoinositide-dependent kinase 1 pathway • Post-translational modifications • Cytoskeleton dynamics • Cytolysis • DNA-repair • Host macromolecular synthesis shut-off • Gelsolin • ERM-family proteins • Tropomyosin • Actin

5.1 Introduction

Rodent parvoviruses (PVs) are known for their intrinsic oncotropism, oncolytic activity, and oncosuppressive properties [1–4]. This, together with their low pathogenicity towards humans [5], led in late 2011 to the launch of a first phase-I/IIa clinical trial of self-propagating oncolytic PVs for patients suffering from recurrent glioblastoma multiforma [6]. Parvoviruses are small, non-enveloped icosahedral particles approximately 25 nm in diameter, with a 5.1-kb single-stranded linear DNA genome. This small nucleic acid encodes two structural and at least six nonstructural proteins, of which only the 83-kDa NS1 protein is essential to virus propagation in all cell types [7].

Although rather small (only 672 amino acids), NS1 is highly multifunctional. It comprises at least five distinct, characteristic domains exerting diverse activities [8]. For particular functions (e.g. transcriptional modulation of viral and cellular promoters, viral DNA amplification), 'selected' domains act in concert to prime the polypeptide for the appropriate task(s). NS1 can also recruit cellular multiprotein complexes and machineries through specific protein/protein interactions. This allows high versatility while minimizing the required viral coding capacity and genome size [8].

To exert its many actions, NS1 is regulated by differential expression, post-translational modification, and multimerization [8]. This allows tight organization of NS1 functioning in the course of the viral life cycle. For example, coordinated timing [9] of changes in the NS1 phosphorylation state determines the functions this protein will perform [10–13]. NS1 phosphorylation is carried out notably by members of the PDK1 (phosphoinositide-dependent kinase 1)/PKC/PKB signaling cascade [13–15], a regulatory pathway stimulated upon neoplastic transformation [16].

The action of NS1 on various pathways and processes in the host cell eventually causes this cell to die [17], either by apoptotic [18–22] or lysosomal-like [23] programmed cell death, by necrosis [24] or culminating in cytolysis (see below,

NS1 cytotoxicity). Interestingly, the extent of cell death induced by ectopic expression of NS1 depends strongly on the host cell environment: neoplastically transformed cells are killed preferentially [25, 26].

The current review aims to offer insights into the mechanisms of parvovirus NS1 oncotoxic activities and a view of how its properties might be exploited in cancer therapy.

5.2 Actions of Parvovirus NS1

5.2.1 Properties and Functions of the Parvoviral NS1 Protein

As the major regulatory protein of rodent parvoviruses, NS1 plays multiple roles in progeny particle production and spread [7], interacting (specifically or not) with nucleic acids and proteins and exerting enzymatic activities [8]. It notably initiates and promotes viral DNA amplification [12, 27–32] and *trans*-activates the viral promoter P38 driving capsid gene expression [33–35]. Furthermore, as the main parvoviral factor interfering with cell processes, it exerts a range of cytopathic effects [10, 25, 33, 34] that appear essential for efficient progeny particle release and spread [24]. Beyond its participation in triggering stress responses leading to programmed cell death, NS1 induces severe cell disturbances [10, 25, 26, 36]. It notably causes cytoskeleton collapse [37] and permeabilization of the plasma membrane [20, 38]. As a key player in the virus life cycle with cytotoxicity preferentially targeting neoplastically transformed cells, the NS1 polypeptide of rodent PVs has been the focus of many studies and its action characterized in great detail.

5.2.2 NS1 Cytotoxicity – Multiple Functions, Different Manifestations, Gazillions of Targets

NS1 makes up for its small size by interacting/interfering with cell proteins and factories with activities complementing its own limited range [39–45]. This enables NS1 to perform or direct many, highly diverse functions in the course of a viral infection, with major consequences for the host cell. Figure 5.1 illustrates NS1 interference with cell processes and the potential impacts of this interference. These actions can be summarized as follows.

In the nucleus, NS1 controls viral genome replication, ensures capsid protein production by interference with mRNA processing/transport proteins and possibly assembly into progeny particles. Viral DNA amplification involves relocation of many components of the host cell's DNA replication and repair machineries (e. g., RPA, DNA polδ, RF-C, PCNA, polα, etc. for replication and RPA-P32, γH2AX,

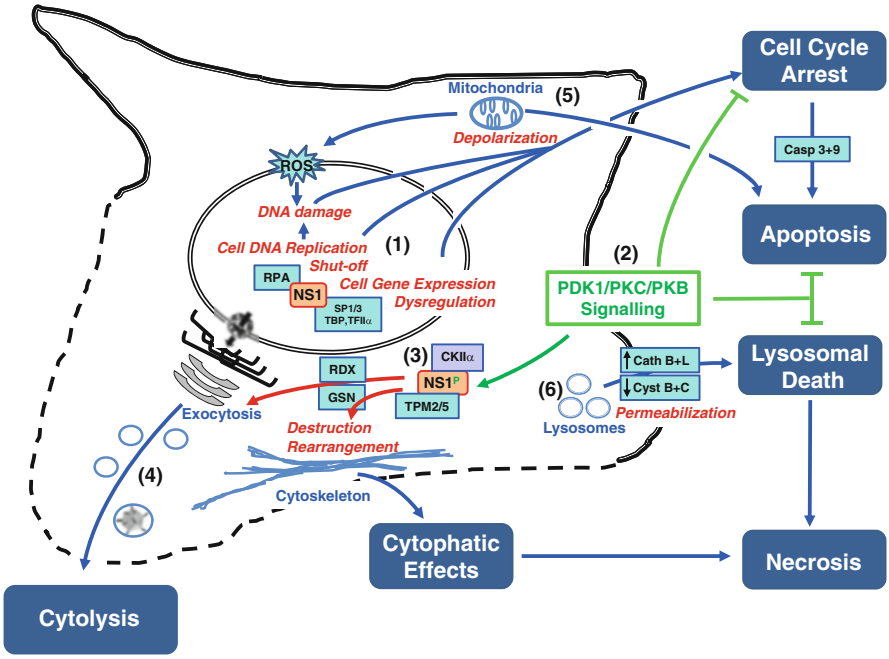

Fig. 5.1 NS1 targets and consequences for target cells. Important NS1 functions ensuring viral DNA replication, and protein production (e.g. viral mRNA processing and transfer to the cytoplasm) are performed in the nucleus and activated during the S-phase of the cell cycle (*1*). Although unable to induce target cells to enter S-phase, NS1 can prolong the S-phase by dysregulating factors involved in cell cycle control (e.g. cycB1, cdc2, p21, p27, p300). This supports NS1-mediated recruitment of DNA replication complexes to the viral origin of replication (NS1 interaction with RPA1-3). As a consequence of dislocation of the DNA replication machinery, cellular replication forks are stalled, and this leads to shut-off of host DNA replication, followed by induction of a DNA damage response and cell cycle arrest. Transcription control by NS1 is mediated through direct interactions with TBP, TFIIA, and SP1/3, mainly to induce the "late" P38 promoter driving capsid gene expression. In addition, these important interactions with the host cell's transcription machinery have a significant impact on the host cell transcriptome, as attested by up- and down-regulation of various target genes [46]. Moreover, NS1 interactions with mRNA-processing enzymes such as NSAP1 contribute to controlling host cell protein production. As a result of NS1-induced alteration of the host's replication, transcription, and mRNA-processing processes, the host cell undergoes stress and macromolecular synthesis shut-off. In response to stress signals, normal cells enter cytostasis and eventually undergo programed death through apoptosis mediated by caspase 3 and 9 activation. In PV-infected cancer cells, apoptosis is counteracted by activated PDK1/PKB/PKC signaling (*2*), but by modifying NS1 post-translationally, this pathway also promotes NS1 activities leading to cell death and lysis. In the cytoplasm, an NS1-CKIIα complex targets a variety of cellular proteins, phosphorylating them and modulating their activities and properties (*3*). Targeting of tropomyosin and of the actin-severing protein gelsolin for illegitimate phosphorylation causes the destruction of the cytoskeleton filaments and becomes apparent by NS1-induced cytopahtic effects. Cytoskeleton collapse is followed by necrosis. In addition, NS1/CKIIα-modified radixin participates in activating and modulating exocytosis (*4*). In productive viral infections, the exocytic pathway is hijacked to transport viral and cellular components to the plasma membrane and induces cytolysis. This process might contribute to raising an anti-tumor immune response. NS1 can also trigger depolarization of the mitochondrial membrane, causing surplus production of reactive oxygen species (*ROS*), which in return cause DNA damage and cell cycle arrest (*5*). Consequently, NS1-expressing cells undergo apoptosis. In addition, NS1-producing cells undergo lysosome permeabilization, causing cathepsin release and acidification of the cytoplasm (*6*). In conjunction with inactivation of cathepsin inhibitors, this leads to "lysosomal" cell death, which is followed by necrosis

NBS1-P, Chk2, P53, ATM for repair) to subnuclear structures termed autonomous parvovirus-associated replication bodies (APAR bodies) [47–49] Direct interaction of NS1 with certain components such as RPA1-3 [39] might explain the sequestering of whole replication complexes away from cellular DNA. This mislocation leads to stalled replication forks, known to activate a DNA damage response (DDR) [50]. Consequently, cell cycle arrest is observed at the S/G2 transition or the G2 phase [9, 18, 51, 52]. What's more, NS1 has been shown to interfere directly with cell cycle progression by targeting multiple factors involved in this process (e. g., cycB1, cdc2, p21, p27, p300) [18, 51, 53]. This NS1-induced cell cycle arrest in late S/G2 most likely provides a suitable environment for efficient viral DNA amplification [9, 18, 51, 52, 54]. For production of the protein shell, NS1 can modulate viral mRNA synthesis [33, 35, 55–57], processing, and nucleo/cytoplasmic transport. It notably binds key players in the transcription complex, such as the TBP, TFIIα, and SP1/3 transcription factors [42, 43] and mRNA-processing enzymes ([41], Nüesch unpublished results).

Ectopic expression of NS1, with concomitant usurpation of at least some cell products, is alone sufficient to disturb cell processes enough to trigger stress signals [18, 20] and shut-off of host macromolecular synthesis [7]. The multitude of initial targets makes it practically impossible for the cell to correct the disturbances in time to avoid committing suicide through induction of caspase-3/9-mediated programmed death by apoptosis [18, 21, 58, 59]. Both PV infection and ectopic NS1 expression have been found to trigger this pathway in a very statistically significant manner [17, 19, 21, 22].

Despite the above, it is noteworthy that stress-induced apoptosis often does not occur in a productive natural infection. This may be due to induction/activation of the PDK1/PCK/PKB signaling cascade [60, 61], a survival pathway with pivotal functions in virus propagation. In both PV-infected cells and cells producing NS1 ectopically, this pathway appears to play a dual role, at first counteracting stress-induced translation shut-off and apoptosis so as to allow sufficient time for progeny production, but at a later stage triggering NS1 functions that promote export of progeny virions and eventually lead to cytoskeleton collapse and cytolysis [10, 24, 37, 44, 45].

The main trigger of parvovirus-induced cytoskeleton alterations and cytopathic effects appears to be modification of NS1 by PKCλ/ι, enabling NS1 to interact with CKIIα, the catalytic subunit of casein kinase II [44]. By binding this enzyme, NS1 acquires the ability to act as a signaling molecule capable of modifying target proteins so as to meet viral needs.

NS1 targets microfilaments for rearrangement and destruction [37]. A direct target of the NS1/CKIIα complex is filamentous tropomyosin. Differential NS1/CKIIα -driven phosphorylation of tropomyosins 2 and 5 leads to perinuclear restructuring and decay of these filaments [45]. These alterations are likely to be especially deleterious to cancer cells, where tropomyosin 1 is underrepresented [62].

Another NS1/CKIIα target is the actin-severing protein gelsolin, which becomes activated upon phosphorylation and causes depolymerization of actin filaments.

This attack on actin filaments occurs in conjunction with the detargeting and repression of the polymerization factor N-WASP by an unknown mechanism. It might again have a greater impact on cancer cells, which lack the most rigid cytoskeletal fibers [63].

The intermediate filament vimentin also becomes reorganized and is eventually destroyed upon PV infection. The viral triggers leading to these events remain elusive, but this action on vimentin seems important for virus production and spread [64]. Since this filament structure is essential for cell shape, it seems likely that the destruction of vimentin filaments significantly contributes to cytoskeleton collapse, shrinkage of the cytoplasm, and rounding of NS1-producing cells [37].

Cytoskeletal filaments are not the only NS1 target in the cytoplasm: mitochondria are another. NS1 drains stored energy resources from the mitochondria [20] and causes depolarization of the inner mitochondrial membrane. In response to this attack, reactive oxygen species (ROS) are produced and released. The surplus ROS cause damage to cellular DNA, thus contributing to induction of a DDR [18]. Whether DDR activation serves a purpose beyond recruitment of repair enzymes to APAR bodies remains an open question. Interestingly, ectopic expression of NS1 is sufficient to cause a DDR and cell cycle arrest, in keeping with the role of this protein as the major effector of virus-induced cytostasis and cytotoxicity [18, 53].

Recently it was shown that progeny virions are actively transported to the cell periphery by vesicles [65]. Transport through the ER and Golgi seems important for inducing post-assembly modifications that confer infectivity and in triggering cytolysis after transport of progeny particles to the plasma membrane [24]. There is evidence suggesting that NS1 is involved in regulating exocytosis: on the one hand, NS1/CKIIα-modified radixin regulates generation/loading and targeting of cellular vesicles by the ER and Golgi [24, 66]; on the other hand, NS1 has been shown in parvovirus-infected glioblastoma cell lines to associate with cellular vesicles [23].

Interestingly, a ‘lysosomal’ mode of cell death has been evidenced in glioblastoma cell lines infected with the parvovirus H-1PV, involving multiplication and permeabilization of acidic vesicles. This, in conjunction with repression of cathepsin inhibitors, results in cytosolic accumulation of functional cathepsins. These findings prompt the intriguing speculation that NS1-induced acidification and/or processing by released cathepsins might contribute to the maturation of progeny virions before lysis occurs, and might thus be important in the virus life cycle. Whether this is true or not, accumulation of cathepsins and acidification of the cytoplasm induce the lysosomal death pathway, even in target cells that have become resistant to pro-apoptotic treatments [23]. Characterizing such alternative pathways in more detail is of prime importance for researchers aiming to develop alternative treatments targeting apoptosis-resistant cancer cells.

As mentioned above, the PDK1/PKC/PKB cascade plays a pivotal role in parvovirus-induced cell death. Aberrant up-regulation of this cascade in most cancers may contribute to explaining parvoviral oncotropism and oncotoxicity.

In conclusion, NS1 interferes with many cell processes to perform multiple tasks during virus replication and spread. To do this, it interacts with and/or modulates

the functioning of many host-cell proteins. This simultaneous attack on many different sites makes it very hard for a host cell to resist or correct the disturbances. Consequently, NS1-expressing cells are doomed to die, either directly from cytopathic changes induced by the viral protein or by suicide mechanisms such as apoptosis. Cancer cells are particularly vulnerable.

5.3 Molecular Determinants of Parvovirus NS1

5.3.1 NS1 Domain Structure

As described above, NS1 is highly multifunctional and capable of physically interacting with numerous cellular proteins [7, 8, 17, 67]. This suggests that the polypeptide combines a variety of features enabling it to perform specific tasks. Moreover, complex functions such as viral DNA amplification or *trans*-activation of the P38 promoter should require the intervention of several activities, enzymatic or otherwise, acting in concert. Studies over the past three decades have unraveled the properties of NS1 and led to a proposed domain structure.

The identification of distinct conserved motifs and comparisons with functionally related proteins have made it possible to deduce at least some of the processes in which NS1 is involved and, using the purified polypeptide and/or site-directed mutagenesis, to dissect its biochemical properties [33, 34, 57, 68–74]. It is striking that all the identified attributes and any as yet unidentified ones (such as interaction sites with cellular proteins) are combined in a stretch of only 672 amino acids. Evolution has achieved this by eliminating redundancies. As a consequence, certain motifs/domains are involved in multiple functions in the viral life cycle.

Figure 5.2 delineates the domains and motifs identified so far and their respective functional involvements. Characteristic structural motifs include a basic sequence acting as a Nuclear Localization Signal (NLS: site 7) [71] and a hydrophobic motif enabling the polypeptide to self-assemble into oligomers (Oli: site 6) [73]. Sequences typical of replicator proteins mediating rolling-circle-type replication are distinguished in the form of two conserved motifs necessary for site- and strand-specific nicking of the origin of replication [76]: a metal-coordination site (Me^+: site 1) and an active-site tyrosine mediating the covalent bond between the replicator protein and the 5′end of the nicked DNA strand (Y-link: site 2) [72]. To unwind double-stranded nucleic acids, helicases depend on ATPase activity as an energy source. Core elements of the NS1 helicase domain are the conserved Walker motif [77] with its nucleotide-binding sequence (NTP: site 3) [34, 57, 74], a conserved aspartic acid making contact with the nucleotide (site 5), and a magnesium-binding site for catalytic hydrolysis of nucleoside triphosphate (Mg^+: site 4) [69]. Striking sequence similarities exist with the helicase domain of SV40 large T-antigen and with the nicking domains of the adeno-associated virus (AAV) REP68/78 and HBoV NS1 proteins. Since the crystal structures of these related proteins have been solved, important structural information might already be

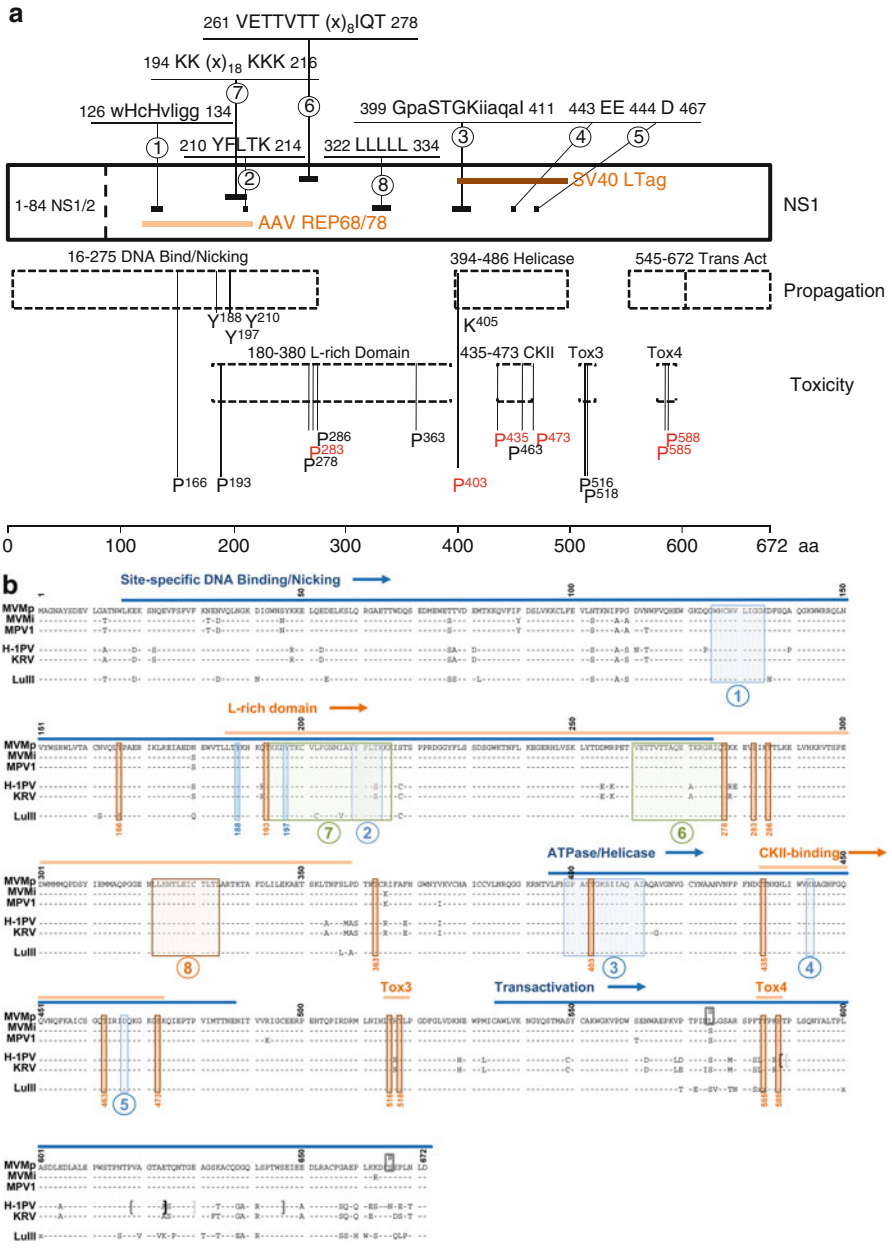

Fig. 5.2 Domain structure and variability of rodent parvovirus nonstructural NS1 proteins. (**a**) *Structural and functional motifs and domains present in NS1*. The common N-terminus of NS1 and of the small nonstructural protein NS2 (aa 1–84) is indicated on the *left* (NS1/2). Individual motifs identified by sequence alignments and site-directed mutagenesis are indicated on the *top* with the respective amino acid numbers. Conserved residues are in capital letters, variable amino

available for these parts of the NS1 protein [78, 79]. Besides the DNA nicking and helicase domains involved in viral DNA replication, there is a site-specific DNA-binding domain (aa 16–275) [70] recognizing an $[ACCA]_{2-3}$ repeat, present in both the viral origins of replication and the *tar* element of the P38 promoter [68, 80]. There is also a C-terminal acidic domain responsible for *trans* regulation of viral and cellular promoters (Trans Act, aa 545–672, of which the terminal 65 aa are essential) [33, 75]. Besides these domains involved in the production of viral DNA and proteins (Propagation), there are areas in NS1, which have been identified

Fig. 5.2 (continued) acids in small letters. The positions of motifs, which are scattered throughout the polypeptide chain, are indicated with *black bars*. Similarities in the nicking domain to the large AAV REP/human Bocavirus NS1 and in the helicase domain to SV40 LTag are indicated with *orange* and *brown bars*, respectively. Below the main body, the domains associated with distinct functions involved in production of progeny virions (Propagation) and the regions associated with toxic functions of NS1 (Toxicity) are specified. Potential regulatory sites (e.g. target PKC phosphorylation sites) modulating NS1 toxicity are indicated with "P" and their corresponding amino acid number, while known *in vivo* phosphorylation sites are presented in *red*. The site-specific DNA binding domain is located between aa 16 and 275 [70]. Within this domain are located a hydrophobic metal coordination site (*1*), 126- wHcHvligg-134, similar to the copper-binding site of cytochrome B, and an active site tyrosine (*2*), 210-YfltK-214. These two essential motifs are involved in site- and strand-specific nicking, while the two tyrosines, Y188 and Y197 (near Y210), play an important role in site-specific DNA binding [72]. The sequence and structure of the helicase domain, which spans aa 394–486, show remarkable similarities to those of SV40 LT. This domain harbors the NTP-binding pocket (*3*), 399–GpaSTGKiiaqaI-411 (where K405 interacts with the alpha-phosphate group), the nucleoside interaction site (*4*) around D467, and the two negative charges, EE443/444, thought to bind the Mg^{2+}cofactor (*5*) required to hydrolyze the nucleotide [34, 57, 69]. The 67 carboxy-terminal amino acids serve as a transactivator domain for P38 promoter activation. Full activity, however, requires additional motifs located between aa 545 and 605 [33, 75]. In addition, an interaction site, 261-VETTVT(X_9)IQT-278, which controls self-assembly into a dimer or higher-order oligomers (*6*), and a bipartite nuclear localization signal (*7*), 194-KK(X_{31}) KKK-218 [71], are mapped. Besides an L-rich area (*8*), which was shown to be crucial for NS1-mediated toxicity [45], additional areas in NS1 associated with CPE-induction were identified by site-directed mutagenesis of consensus PKC-phosphorylation sites [10] Grossen, Master's Thesis). These are in particular, the region binding CKIIα (CKII), the area between the helicase and Transactivation domain (Tox3) and a stretch within the extended TA-domain which becomes phosphorylated at late stages of infection (Tox4). Six *in vivo* target phosphorylation sites have been identified: S283 (Nüesch unpublished observations); T403, T435 [10], S473 [14], T585, and S588 [38]. T435 and S473 are known targets of PKCλ [12, 14]. (B) *Variations in the toxicity-domains of rodent PV NS1 proteins:* Many toxic activities of rodent PV NS1 proteins have been ascribed to protein/protein interactions with host cell factors rather than to intrinsic enzymatic functions of NS1 [10, 44, 45]. Such interactions may be host- and virus-type specific. Sequence alignments between NS1 proteins encoded by mouse PVs (MVMp/MVM_{CR}, MVMi; MPV-1), rat PVs (H-1PV/H-1derivatives, KRV), and a PV of unknown origin (LuIII) were produced in order to determine host-specific NS1 interaction sites with cell proteins mediating toxicity. Domains and motifs involved in virus propagation (DNA replication, *trans* activation of the capsid gene promoter) are indicated in *blue*, domains, motifs, and residues mediating toxicity in *orange*, structural motifs in *green*. Sequence motifs as shown in the graph (**a**) are labeled by numbers. Heterogeneities between MVMp and the parental MVM_{CR} are boxed on top of the MVMp sequence, variants of H-1PV harboring deletions in the C-terminal part of NS1 are indicated by brackets in the H-1PV sequence

as mediating cell disturbances (Toxicity). This includes a leucine-rich domain (aa 180–380) mediating interaction of NS1 with cellular proteins. At least some of the proteins interacting with this domain (e.g. tropomyosin) are targeted for phosphorylation by NS1-bound CKIIα, interacting with the viral protein in the helicase domain (CKII). Therefore, this L-rich domain is referred to as the "targeting domain". Although of unidentified function, there are 84 N-terminal amino acids shared with the small nonstructural protein NS2 (NS1/2). Each domain works alone or in concert with appropriate others to mediate distinct tasks such as DNA replication, promoter regulation, and modulation of cell processes. Coordination is thought to be achieved through post-translational modifications, homo-oligomerization, intercalation of small compounds, and interaction with host-cell proteins.

5.3.2 Functional Interplay Between Different NS1 Domains

To increase the relatively small size of its polypeptide chain, NS1 can self-assemble into oligomers in an ATP-dependent manner [71]. A crucial element for this self-assembly is the hydrophobic motif VETTVTT$(x)_8$IQT, site 6), but other regions might also contribute to oligomerization after becoming accessible through conformational changes [73]. The formation of dimers or higher-order multimers could be important in enabling the protein to interact simultaneously with multiple cell factors having affinity for identical, overlapping or distinct motifs within a short amino-acid stretch: the same stretch could appear on the multimer surface at several distant positions. This should enable NS1 oligomers to act as scaffolds for multi-protein complexes, despite the relatively small size of the polypeptide chain.

Using the same domain for multiple purposes is another way for a small protein to carry out numerous tasks. An example in NS1 is the DNA-binding domain, which interacts site-specifically with $[ACCA]_{2–3}$-motifs and participates in both viral DNA amplification and *trans* activation of the P38 promoter [68, 80]. This duality requires structural plasticity of the polypeptide chain and tight control of protein conformation in order to make appropriate other domains/sites accessible according to the task to be performed. Apparently, structural plasticity is controlled in part by the ATP-binding/ATPase domain, whose functional knock-out abolishes both viral DNA amplification and *trans* regulation of viral and cellular promoters [10, 33, 34, 57]. It is noteworthy in this regard that a T403A-substitution in this domain results in a NS1 protein, which is resolved as two distinct bands in SDS gels [10]. This suggests that NS1 can indeed adopt alternative conformations depending on the state of the NTP-binding pocket. Thus, it can be speculated that changes in the nucleotide occupancy of this pocket may lead to conformational modifications of the protein and, as a consequence, to the activation of one specific NS1 function over the others.

NS1 oligomerization is a dynamic process depending on NTP binding and hydrolysis [71]. NS1 multimers easily fall apart in solution unless they become

stabilized through antibody crosslinking or non-hydrolyzable nucleotide analogues [80]. Changes in the oligomerization state and dynamic energy transfer seem important for certain functions performed by NS1. This is best illustrated by NS1 involvement during rolling circle replication, which has been studied in detail. At first, NS1 binds site-specifically to its cognate recognition motif $[ACCA]_{2-3}$, a process requiring di(multi)merization of the polypeptide in the presence of NTP binding without NTP hydrolysis [80]. NS1 binding to the double-stranded DNA is stabilized by formation of a ternary complex with a cellular accessory protein, either the parvovirus initiation factor PIF at the left-end origin [27, 28] or HMG at the right-end origin [81]. NTP hydrolysis is then required to locally unwind the origin of replication, allowing site- and strand-specific nicking to occur [12]. This trans-esterification and covalent attachment of the polypeptide to the free 5′end of the nicked DNA occur at a specific single-stranded sequence and are energy neutral [12]. To accomplish the subsequent steps, the DNA-attached NS1 polypeptide oligomerizes again in the presence of ATP to form at the forking point, as does the SV40 LTantigen, a hexamer ring around the intact DNA strand [82]. It then associates with the host replication machinery (e.g. RPA1-3) [39]. Leading-strand-only, strand-displacement synthesis is driven by NS1 helicase activity upon rapid hydrolysis of ATP [39, 72, 76].

This whole sequence of events requires considerable changes in NS1 properties: the protein must switch from a molecule binding with high affinity to a specific duplex DNA sequence in the presence of NTP-binding without hydrolysis, to a protein that slides along a single-stranded DNA in the 3′ to 5′ direction, thereby unwinding very efficiently the duplex DNA provided sufficient energy is supplied by ATP hydrolysis [39, 68]. NTP-induced conformational alterations and multi-merization and interaction with accessory proteins (PIF, HMG) contribute to coordinating this switch. Additional features of this dynamic process include intercalation of metal ions at the metal-coordination site of the DNA-binding domain [72, 76] and at the Mg-binding site of the helicase domain [69].

When NS1 acts as a transcription factor to *trans*-activate the P38 promoter, the process again involves site-specific binding to an $[ACCA]_{2-3}$-repeat, this time in the *tar* element of the promoter [35, 68, 83, 84]. Although this resembles the interaction mediating viral DNA amplification and likewise requires NTP binding [68], at this site NS1 remains tightly associated with the duplex DNA to assemble the host transcription machinery. For this it interacts with components such as TBP, TFIIα [43], and the transcription factors SP1/SP3 [42]. With the cellular transcription machinery and the viral DNA, NS1 might form a stable ternary complex similar to that formed upon initial binding to the origins of replication. Once NS1 is in place, *trans*-activation is effected by the C-terminal acidic part of the polypeptide [75], a domain totally dispensable for viral DNA amplification [85].

To interfere with cytoskeleton dynamics and vesicular transport, NS1 associates with membrane and scaffold structures (Nüesch, unpublished observations). This interference appears to be controlled by NTP binding or hydrolysis, since mutant proteins with functional knock-out of the corresponding site (substitution of

the conserved lysine^{405} or glycine^{404}) render the polypeptide tolerable by target cells [10, 33, 34]. Yet interaction with NTP seems to be required only for oligomerization of the polypeptide chain, since in experiments where fusion proteins were generated by combining different toxicity domains (e. g., aa 235–379 with DLEPDEELED), the NTP-binding region proved not to be required to promote cell disturbances [45]. This region thus probably governs the exposure and/or conformation of toxicity-related domains, rather than having an intrinsic cytotoxic function.

In conclusion, most of the diverse functions of NS1 arise through cross-talk between specific regions. This is regulated, at least in part, by interaction with small compounds [69, 72], by post-translational modifications such as phosphorylation [11] or acetylation [86], by cellular structural elements (e.g. membranes and scaffolds) [23, 45], and by interaction with host-cell proteins.

5.3.3 NS1 Regulation – Dependence on the PDK/PKC/PKB Pathway

The diversity of NS1 activities and functions in the viral life cycle strongly suggests tight regulation in time and space through post-translational modifications. This idea is supported by analysis of the NS1 phosphorylation pattern, which changes in the course of a synchronized infection [9], and by identification of target phosphorylation sites regulating specific activities of the polypeptide [8, 10, 13, 14]. In addition to contributing to the regulation of domain cross-talk (discussed above), such modifications within contact sites for protein/protein interactions could directly control the ability of the polypeptide to bind certain host cell proteins selectively, thus controlling NS1 toxicity. This is clearly the case of the NS1 protein of minute virus of mice (MVM), whose ability to bind CKIIα in the helicase domain seems to require phosphorylation by PKCλ at S473 [13, 14, 44]. An S473A mutation causes NS1 to lose a major feature of its toxicity: its capacity to induce cytoskeleton alterations in the host cell [10, 44, 45]. Another potential phosphorylation site is T363, located in the "targeting domain" required for binding of tropomyosin [45] and a variety of other proteins ([44]; Nüesch, unpublished observations). A T363A substitution reduces NS1 toxicity without abolishing it [10], and the binding of several proteins has been found to depend on the proper sequence in the vicinity of this residue [44] Nüesch unpublished observations). In contrast to phosphorylated S473, which appears as an essential element in the interface of NS1 with a specific partner, CKIIα, and thus seems crucial for all NS1 functions requiring formation of the NS1/CKIIα complex, the site surrounding T363 would appear to govern accessibility of the whole "targeting domain" to cellular partner proteins. Additional post-translational modifications within this domain (at T278, S283, T286) have also been shown to modulate NS1 toxicity and are assumed to further modulate binding of certain proteins.

Regulation of NS1 functions by post-translational modification may be crucial for its specific functioning in certain cell types, and may explain at least in part why neoplastically transformed cell lines are more susceptible than their normal counterparts to NS1-triggered killing [4, 26, 61, 87]. Worth stressing is the importance of the PDK1/PKC/PKB signaling cascade in priming NS1 for specific tasks [10, 12–15, 38]. In 'normal' cells, the activity of this cascade is tightly controlled by extracellular growth factors. These interact with growth factor receptor kinases, which activate phosphoinositide-kinase 3, which provides the cofactor PIP3, which activates PDK1, PKB, and Akt1, and consequently the PKC isoforms [88–90]. It is noteworthy that in the 'normal' lung embryonic fibroblast cell line MRC-5, the controlled and thus limited activity of the PDK1/PKC/PKB signaling cascade seems insufficient to promote NS1-triggered cell killing by the rat parvovirus H-1PV, whereas in genetically engineered MRC-5 cells expressing a PIP3-independent, constitutive-active PDK1, NS1 gets activated and the cells become susceptible to H-1PV-induced necrosis [61].

The cell-disturbance-inducing activities of NS1 depend strongly on NS1 phosphorylation by PKCη, a short-lived kinase requiring permanent stimulation through PDK1 phosphorylation [60]. Furthermore, phosphorylation of NS1 by PKB/Akt1 is important for late functions of this parvoviral protein (Bär and Nüesch, unpublished observations). All these examples highlight the strong dependence of parvoviruses on this important signaling cascade. Yet conversely, PVs have been observed to target this signaling cascade in order to drive virus propagation and thus regulate NS1 function, including toxicity, in permissive cells [60, 61, 66]. In its natural host cell line (A9 mouse fibroblasts), the parvovirus MVMp is able to stimulate phosphorylation of PKCη, PDK1, and PKB [60, 61], and thereby promote its own propagation. This ability seems to be lost in 'normal' human cells correlating with the impairment of NS1-mediated viral DNA amplification [61]. Importantly, transformation favors PDK1/PKC/PKB activation through a variety of mechanisms [16] and this may contribute to make them prime targets for NS1-triggered cell killing, as opposed to the surrounding normal tissue.

NS1 is thought to be subject to other modifications besides phosphorylation [8]. It was recently shown that NS1 becomes acetylated upon infection of transformed human cells (e. g., HeLa or HEK293 cells). Interestingly, the amount of acetylated NS1 can be increased by treating infected cells with deacetylase inhibitors (HDI), which correlates with a marked stimulation of NS1 activities, including cytotoxicity [86]. This effect can be achieved at subtoxic HDI concentrations, resulting in a striking synergy between H-1PV and HDIs in the induction of oncotoxicity *in vitro* and tumor suppression *in vivo*. The combination of parvoviruses with HDIs is very promising in that it broadens the spectrum of tumor targets for virus-mediated suppression, and allows PVs to be effective at significantly lower doses. Since NS1 appears to be a major determinant of these synergistic effects, HDIs could also prove important in the application of NS1 as an anticancer agent.

In conclusion, the properties of NS1, and hence the oncolytic potential of this multifunctional protein, are modulated in response to post-translational modifications, some of which probably remain to be discovered. This regulation may notably explain both the oncotropism and the oncotoxicity of parvoviruses.

5.3.4 Differences Among Rodent PV NS1 Proteins

Selective killing of target cells is a prerequisite for an oncolytic agent. In the case of parvoviruses, this selectivity is likely to depend both on host-cell features (such as the ability to effect toxicity-promoting NS1 modifications, as discussed above), and on the characteristics of the parvoviral NS1 protein considered (e.g., its ability to bind the appropriate host-cell factors). The PVs of different rodent hosts encode highly homologous NS1 proteins, but these proteins show some differences that may determine whether the protein considered is able to induce oncolysis in a given cell type when ectopically expressed. In experiments comparing the toxicity of H-1PV NS1 versus MVM NS1 after site-directed mutagenesis of potential regulatory sites, the two related proteins showed significantly different behavior, and the toxicity of some mutants was found to depend on the target cell (Grossen, Master's Thesis). Figure 5.2b show alignments of rodent PV NS1 proteins, featuring known differences between them. Disparities can be seen within and in the vicinity of known toxicity determinants, supporting the view that their action depends on the host. Thus, different NS1 polypeptides could exert enhanced or reduced toxicity according to the target cell. This justifies present efforts to compare rodent PVs or their NS1 products for their relative efficiencies against distinct human tumors [91].

5.3.5 Producing Artificial NS1-Derived Polypeptides with Specific Functions

As mentioned above, NS1 is able to play its many roles in the viral life cycle and in killing host cells thanks to cross-talk between appropriate domains and to the fact that the same domain can contribute to more than one function. This hindered early efforts to identify regions involved in specific activities by site-directed mutagenesis of conserved motifs [10, 33, 34, 57, 69, 71, 72]. Subsequent targeting of phosphorylation sites potentially involved in regulating particular activities were more successful, enabling investigators to identify activities, to characterize the interplay between the domains, and particularly to identify domains involved in mediating toxicity [10, 12, 13, 38]. This led to proposing the domain structure shown in Fig. 5.2a [8]. The proposed structure was then put to the test by combining

NS1 fragments and studying the behavior of the fusion proteins obtained. Figure 5.3 presents these types of such proteins:

(i) **The NS1-Transactivator**. To *trans* activate the "late" parvoviral promoter P38, NS1 needs to bind to its cognate DNA-recognition sequence located in the *tar* element. This brings its C-terminal acidic domain in position to stimulate promoter activity [35, 68, 75]. An artificial "NS1-transactivator" was produced by combining the DNA binding domain [70, 72] with the extreme C-terminus [75] of NS1 via a spacer consisting of the green fluorescent protein (GFP). To ensure multimerization (essential to site-specific DNA-binding), glutathione-S-transferase (GST), known to dimerize in solution [11], was fused to the N-terminus. The resulting polypeptide efficiently *trans* activated the *tar* element-containing promoters, but unlike the genuine NS1 protein, had no impact on cell metabolic activity and did not trigger cytolysis [24].

(ii) **The NS1 replicator**. Initiating and promoting viral DNA replication is a complex, dynamic process in which NS1 plays multiple roles by promoting ATP binding and hydrolysis (aa 399–467), interacting site-specifically with the cognate DNA-recognition motif (aa 16–275), binding to the single-stranded nicking sequence, acting as an endonuclease and causing NS1 covalent attachment to DNA 5′ends (Me-coordination site aa 126–134; Linkage tyrosine aa 210–214), and exerting helicase activity (aa 394–486). Moreover, to interact with the origins of replication for initial nicking and to promote viral DNA amplification, NS1 must act in concert with certain components of the host-cell DNA replication machinery. NS1 sites involved in the interaction with cellular accessory proteins and replication factors remain to be identified, but the known NS1 elements involved in DNA replication are scattered over the whole polypeptide chain, with the exception of the extreme C-terminus. The fragment consisting of aa 1–604 thus constitutes a polypeptide competent for driving PV DNA replication, while being devoid of the capacity to *trans* regulate viral and cellular promoters [33, 85]. Interestingly, upon expression in target cells, this polypeptide proved to be significantly less toxic than wild type NS1 [33], although it still contains many elements involved in inducing NS1-mediated cell disturbances [10, 44, 45]. In contrast to wild-type NS1, a significant proportion of which (30 %) resides in the cytoplasm, the polypeptide containing only aa 1–608 was found almost exclusively (>95 %) in the nuclear compartment [71]. This localization presumably prevents the polypeptide from interacting efficiently with cytoplasmic host proteins that NS1 normally targets in order to rearrange and destroy cytoskeleton filaments [37, 45].

(iii) **The NS1 oncotoxins**. It initially came as a surprise that some of the most important determinants of NS1 cytotoxicity consist of structural features (e.g. oligomerization state), domain crosstalk, or partner protein binding, rather than of intrinsic NS1 enzymatic activities. Two discoveries contributed importantly to shaping the current view of NS1 cytotoxicity: (i) that NS1 forms a

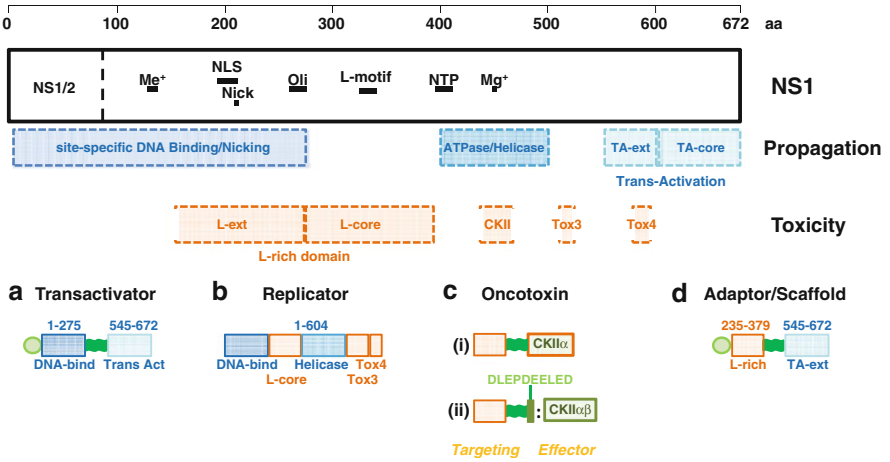

Fig. 5.3 NS1-related polypeptides with different functions. NS1 is a multifunctional protein exerting diverse activities necessary for virus propagation. Besides initiating and promoting viral DNA amplification through its site- and strand-specific nickase and helicase activities, it acts as a transcription factor to induce capsid gene expression and is able to modulate the cellular environment in order to recruit host cell machineries and mechanisms to promote production of viral components, their assembly, and virus spread. These multiple activities require different domains to work in concert, while other functions of NS1 are silenced. To generate NS1 derivatives with specific functional spectra, we fused different domains and deleted others. The *top* represents a simplified domain structure of NS1 with the domains involved in progeny particle production (Production) in *blue*, domains necessary to induce cytopathic effects (Toxicity) in *brown*. Positions of conserved motifs are indicated with *black bars*. Me^+, metal coordination site; NLS, nuclear localization signal; Nick, active site tyrosine; Oli, oligomerization site; L-motif, five consecutive leucines necessary for NS1-induced toxicity; NTP, ATP binding site with a conserved lysine interacting with the alpha-phosphate group of the nucleotide triphosphate; Mg^+, magnesium-binding site necessary for ATPase activity; Four types of NS1-derived polypeptides with selected activities are illustrated. (**a**) *Transactivator:* Composed of the site-specific DNA-binding domain (NS1 aa 1–275) and the *trans* activation domain (NS1 aa 545–672). The DNA-binding domain interacts specifically with the $[ACCA]_{2-3}$ motif of the *tar* element at the PV P38 promoter [35, 68]. This binding is essential to bringing the polypeptide into position to stimulate capsid gene promoter activity with the acidic C-terminal domain [75]. A potent "Transactivator" polypeptide was generated with these two NS1-domains, by adding a N-terminal GST-tag (*green circle*) generating constitutive dimers [11] and spacing the two NS1-domains with the *green* fluorescent proteins (GFP, *dark green wave*). This polypeptide is able to activate the PV P38 promoter without any measurable toxic effects [24]. (**b**) *Replicator:* Initiating and promoting viral DNA amplification by NS1 is a complex process requiring specific actions of and crosstalk between domains as well as interactions with host cell proteins. Besides DNA-binding/Nicking (aa 1–275) and Helicase (aa 395–490), areas mediating toxicity (i.e. L-core aa 278–379; Tox3, Tox4) are essential. The latter are thought to mediate interactions with cell proteins. The only domain not required for viral DNA amplification is the C-terminal *trans* activation domain [33, 85]. An NS1 polypeptide lacking the 65 C-terminal amino acids can promote, in vivo [33] and in vitro [85], rolling circle replication of a plasmid containing the MVM origin of replication. In in vitro replication assays, purified NS1 polypeptides have been shown to replicate and resolve dimer- and tetramer-bridge-containing plasmids as efficiently as wild-type NS1 [85]. In contrast to the full-length NS1 protein, NS1*dl*65 is incapable of *trans* regulating viral and cellular promoters and is significantly less toxic towards target cells [33].

complex with CKIIα capable of targeting cell components (e.g. tropomyosin, gelsolin, radixin) and altering their properties or functions, and (ii) that a region within the leucine-rich stretch can bind at least four proteins, including the cytoskeletal filament tropomyosin. The CKIIα –binding region (aa 435–473) was called the "effector domain", and the region binding the four proteins (aa 235–379) was named the "targeting domain", the hypothesis being that NS1 works as an adaptor protein, exerting multiple toxic effects by bringing together a modified protein kinase (CKIIα in the case of MVM NS1) and a number of illegitimate substrates (tropomyosin, gelsolin, radixin) attached to the targeting domain. This hypothesis was tested by fusing the targeting domain with either CKIIα itself or with a CKIIα -binding site (that of CKIIβ). The resulting polypeptides were found to severely jeopardize cell survival in colony formation inhibition assays, whereas each separate component was well tolerated [45].

(iv) **Potential NS1 adaptor/scaffold protein**. Other regions (besides those mediating NS1 ternary structure, e.g., the oligomerization signal and the NTP-binding site) might contribute to modulating cytotoxicity, notably the C-terminal part [38], which, along with the "targeting domain", becomes phosphorylated at a late stage of infection, when virus-induced cytopathic changes appear [9, 38]. Although no attempt has yet been made, it seems feasible to produce a combination likely to perform an adaptor function at this stage as proposed in the figure. It would also be exciting to design a construct displaying functions related to vesicular transport of progeny particles to the

Fig. 5.3 (continued) Failure of NS1*dl*65 to efficiently kill target cells was ascribed to its intracellular localization. While a significant proportion of wild-type NS1 (~30 %) is found in the cytoplasm after PV infection and ectopic expression, NS1*dl*65 remains more than 95 % nuclear [71]. Since multiple cellular targets of NS1-triggered cytopathic effects are located in the cytoplasm, this aberrant localization of the polypeptide explains the significantly reduced toxicity. (**c**) *Oncotoxin:* A main toxic function of MVM NS1 is the targeting of illegitimate substrates by the NS1-CKIIα complex. One of these targets is the cytoskeletal filament tropomyosin, which becomes phosphorylated at different isoforms in by CKII in the presence of NS1. Consequently, the filament structure becomes altered and depolymerized [44]. The effector protein CKIIα has been found to interact with NS1 in its helicase domain (CKII), the target tropomyosin associates within the L-core. Consequently, polypeptides were constructed by combining the NS1-targeting domain (L-core domain aa 278–379) directly with the active catalytic subunit of CKII, CKIIα (construct "i") or with a strong CKIIα-binding site such as the CKIIα-interaction site of CKIIβ (DLEPEELED) (construct "ii"), so as to bring NS1 targets to the protein kinase CKII for illegitimate phosphorylation. Although expression of each separate domain (the targeting domain alone or CKIIα alone) or of a targeted inactive CKIIαE81A had no impact on cell survival, a polypeptide where the effector (CKIIα) was physically linked to the NS1 targeting domain through GFP severely jeopardized cell survival in a cell-type-specific manner [45]. (**d**) *Adaptor/Scaffold:* Preliminary data suggest the involvement of two regions of in the regulation of progeny particle egress through the ER and Golgi. Adaptor or scaffold proteins are thought to coordinate the loading/targeting of PV virions for vesicular egress. NS1-related proteins combining the L-rich domain (aa 235–379) and the Transactivator domain (aa 545–672) are thought to serve as such adaptors/scaffold and are currently being tested for their role in/modulation of this cell process

cell surface and concomitant permeabilization of the plasma membrane [24], but proof-of-concept for this possibility is still lacking. Besides the targeting domain, additional cytotoxicity-mediating regions proposed to be interaction sites for cell proteins might be combined with CKIIα, to see if the resulting fusion proteins act as alternative oncotoxins and/or trigger alternative death pathways.

5.4 Parvovirus NS1 – An Oncolytic Agent for Cancer Therapy

5.4.1 Exploiting NS1 Toxicity Functions in Cancer Therapy

Using rodent parvoviruses for the oncolytic virotherapy of cancer seems promising because of their intrinsic oncotropism and oncotoxicity and their low pathogenicity towards humans [4, 5]. The potential of parvovirus H1-PV as an anticancer agent is currently being evaluated in a phase I/IIa clinical trial on patients suffering from recurrent glioblastoma multiforma [6]. A number of cell lines and short-term cultures derived from this type of cancer have proven vulnerable to H1-PV-induced cell killing. Glioblastoma multiforma as some other cancers, withstand conventional therapies such as surgery, radiation, and chemotherapy [92, 93]. This can be due to infiltration of healthy tissue, but also to rapid development of resistance to treatment. Pathway analyses have provided important information about resistance mechanisms, pointing to a network of signaling events enabling cancers to bypass chemotherapeutic intervention [94, 95]. Investigators therefore seek combination treatments that inactivate multiple targets and thereby block alternative routes of cancer progression. Thanks to their pivotal functions, regulatory proteins of (human) pathogens are excellent candidate anticancer agents.

The parvoviral protein NS1 is an example of such a regulatory protein, with the following potential advantages.

(i) The capacity for inducing apoptosis via multiple triggers [18].
(ii) The ability to trigger other cell death pathways besides apoptosis as inferred from the "lysosomal" type of death of various PV-infected cells [23] and the necrosis of NS1-transduced cells [61]. NS1 might thus be able to bypass acquired resistance to apoptosis inducers such as cisplatin or TRAIL [23], and also to PI3 kinase inhibitors such as wortmannin [61].
(iii) The strong dependence of NS1 functions, including cytotoxicity, on PDK1/PKC/PKB signaling. In consequence, this pathway plays an important role in cell permissiveness towards PV replication and NS1 toxicity [61]. As mutations activating the PDK1/PKC/PKB pathway are the second most frequent type of cancer-associated genetic alterations after P53-inactivating

mutations [16], NS1 should specifically target cancer cells. Some reports have indeed demonstrated the susceptibility of transformed or tumor cell lines to NS1-induced killing, in contrast to normal diploid cells [26, 61].

(iv) The interaction with the cell exocytic pathway. First evidenced by the association of NS1 with intracellular vesicles [23], this interaction appears to accelerate the vesicular egress of progeny viral particles through ER and Golgi ([24] and unpublished results). This NS1 function is important since PV egress was found to be coupled with cytolysis and transport of both cellular and viral antigens to the plasma membrane [24]. By stimulating the display of antigens on the cell surface, and/or the release of specific cellular or viral molecular patterns, NS1 may trigger antitumor immune reactions that are essential for the success of cancer therapy through their bystander effects. Adoptive transfer, rechallenge, and immunodepletion experiments have indeed shown that the host immune system takes part in PV-mediated oncosuppression [96, 97]. The view that PVs play an active role in inducing an anti-cancer immune response is further supported by the observation that PV infection of tumor cells enhances their ability to serve as an autologous vaccine [97, 98] and to activate both natural killer cells [99, 100] and dendritic cells [101] with which they come into contact. NS1 may thus contribute to unmask tumor cells so that the immune system can do its job. The immunogenic features of PV- and more particularly NS1-induced cell death are still ill-defined, and likely to result from additional NS1 activities besides the above-mentioned modulation of the exocytic pathway.

5.4.2 *Prospect: Generation of Chimeric Oncotropic Viruses Armed with PV NS1 or NS1-Related-Toxin(s)*

In some cancer models (e.g. pancreas carcinoma, Kaposi sarcoma, multiple melanoma) parvovirotherapy was found to be hampered by tumor cell resistance to H-1PV infection [102–104]. In natural PV infections, cells may resist viral attack because of various reasons, in particular inefficiency in the nuclear delivery of internalized viruses [102]. This limitations can be at least partly overcome by selecting PV fitness mutants showing higher infectivity, as exemplified by a recently described H-1PV variant that harbors a small, in-frame deletion in the NS gene and is endowed with enhanced oncolytic and oncosuppressive properties [105].

Another strategy consists of arming heterologous (viral) vectors with the PV NS1 toxin. Proof-of-concept of the above approach has been provided by generating an adeno-parvoviral chimera that proved able to attack cancer cell lines resisting both the adenovirus and the parvovirus used separately [106]. This approach might be extended to other viral vectors in order to use NS1 or NS1-derived toxins against PV-nonpermissive targets. Quiescent tumor stem cells are of particular interest. Their ability to survive in G0, in a dormant stage, for extended periods prevents efficient

killing of these pluripotent cells by autonomous PVs. Although susceptible to infection and uncoating, these cells resist PVs because they do not support conversion of the single-stranded virion DNA to a double-stranded transcription template and protein synthesis [107]. This latter process is strictly dependent on the S-phase of the cell cycle, and PVs, unlike some larger DNA viruses, are unable to induce cell cycle progression [7]: conversion of the single-stranded DNA genome to a double-stranded transcription template depends on cyclin A [107], and the P4 promoter driving nonstructural gene expression is stimulated by S-phase-dependent transcription factors [108]. Conversely, agents such as vectors based on herpes or adeno-associated viruses, capable of producing viral proteins in quiescent tumor cells, might require arming with a potent oncotoxin in order to kill these cells efficiently. In this system, synergistic oncolytic effects can thus be expected from the combination of PV NS1 with these agents in the form of chimeric viruses. Besides providing added value to oncolysis, NS1 may also boost the immunostimulating capacity of recombinant viral vectors. In clinical trials, some oncolytic viruses have shown great efficacy in the treatment of a variety of primary tumors. Yet although the tumor mass was rapidly eliminated, long-term efficacy in regard to disease recurrence remains open [109, 110]. In this respect additional efforts need to be undertaken to induce an anti-tumor immune response capable of taking over from the initial viral effect. One might speculate that arming these viruses with NS1 or a related toxin could help to overcome this limitation by stimulating the host immune system and inducing a bystander effect, as discussed in the previous section.

In conclusion, PV NS1 is a multimodal oncotoxin worthy of evaluation as an anticancer agent, provided a suitable vehicle can be found to transfer the toxin into a sufficient number of cancer cells.

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Part II
Cellular Anticancer Genes

Chapter 6
MDA-7/IL-24: Multifunctional Cancer Killing Cytokine

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Abstract First identified almost two decades ago as a novel gene differentially expressed in human melanoma cells induced to terminally differentiate, MDA-7/IL-24 has since shown great potential as an anti-cancer gene. MDA-7/IL24, a secreted protein of the IL-10 family, functions as a cytokine at normal physiological levels and is expressed in tissues of the immune system. At supra-physiological levels, MDA-7/IL-24 plays a prominent role in inhibiting tumor growth, invasion, metastasis and angiogenesis and was recently shown to target tumor stem/initiating cells for death. Much of the attention focused on MDA-7/IL-24 originated from the fact that it can selectively induce cell death in cancer cells without affecting normal cells. Thus, this gene originally shown to be associated with melanoma cell differentiation has now proven to be a multi-functional protein affecting a broad array of cancers. Moreover, MDA-7/IL-24 has proven efficacious in a Phase I/II clinical trial in humans with multiple advanced cancers. As research in the field progresses, we will unravel more of the functions of MDA-7/IL-24 and define novel ways to utilize MDA-7/IL-24 in the treatment of cancer.

Keywords MDA-7 • IL-24 • Cytokine • Cancer • Apoptosis • Autophagy • Bystander antitumor activity • Cancer terminator virus

6.1 Introduction

The growth and differentiation of an individual cell is controlled by the signals it receives from its surroundings. Based on the stimuli a cell receives, most cells will ultimately differentiate into a specific cell lineage type (besides stem cells). Experimentally, terminal differentiation in human melanoma can be achieved by treating cells with recombinant fibroblast interferon (IFN-β) and the protein kinase C activator, mezerein [26]. While performing subtraction hybridization and screening cDNA libraries for genes that were differentially expressed in melanoma cells before and after terminal differentiation, our laboratory identified MDA-7 (melanoma differentiation associated gene – 7) as one of the transcripts whose expression was induced in terminally differentiated cells [43]. This gene was later designated interleukin-24 (IL-24) by the Human Gene Organisation (HUGO) based on its conserved structure, chromosomal location and cytokine-like properties [9, 73, 81]. Upon examining various cancer cell lines, it was observed that MDA-7/IL-24 protein expression was low or absent in cancer cells as compared to normal

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tissues [24, 38]. Further studies revealed that MDA-7/IL-24 played a key role in tumor inhibition [44, 48, 56, 69, 92]. Extensive work carried out on MDA-7/IL-24 since then, both in melanoma and other cancers, has revealed several clues to the functioning of MDA-7/IL-24 as an anti-cancer gene. In this chapter, we will first discuss our current knowledge of the MDA-7/IL-24 gene, we will then look at the various ways in which MDA-7/IL-24 exerts its anti-cancer properties, followed by the application of MDA-7/IL-24 in the clinic and conclude with the future prospects of MDA-7/IL-24.

6.2 MDA-7/IL-24

Melanoma differentiation associated gene-7 (*mda*-7)/interleukin-24 (IL-24) is a secreted cytokine that belongs to the IL-10 gene family [9, 24, 38, 71, 73, 81]. Along with several other IL-10 cytokine family members, *mda*-7/IL-24 is located on chromosome 1q32-33 in humans and encompasses seven exons and six introns [38]. The *mda*-7/IL-24 cDNA is 1,718 base pairs in length and encodes a 206 amino acid protein with a predicted molecular weight of 23.8 kDa [38, 43, 44, 71]. *mda*-7/IL-24 open reading frame is flanked by a 274 bp 5′ untranslated region (UTR) and a 823 bp 3′ UTR. Three consensus elements (AUUUA) involved in mRNA stability and three polyadenylation signals (AAUAAA) are present in the 3′ UTR. A 49-amino acid N terminal hydrophobic signal peptide was identified by sequence analysis and allows the molecule to be cleaved and secreted. Secreted MDA-7/IL-24 protein can have several molecular sizes because of putative N- glycosylation sites [95] at amino acids 85, 99 and 126 (Fig. 6.1) [81]. *mda*-7/IL-24 sequence

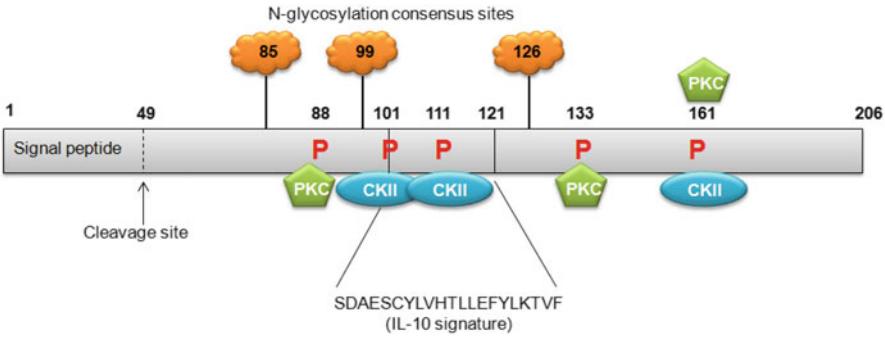

Fig. 6.1 Schematic representation of MDA-7/IL-24 protein with predicted and established domains and protein modification sites illustrated. MDA-7/IL-24 contains an IL-10 signature sequence between amino acids 101 and 121. The MDA-7/IL-24 signal peptide is cleaved to allow secretion of MDA-7/IL-24 protein. MDA-7/IL-24 is glycosylated at three sites at amino acid 85, 99 and 126. Protein kinase C consensus phosphorylation sites are present at amino acid 88, 133 and 161, while Casein kinase II (CKII) consensus phosphorylation sites are present at amino acid 101, 111 and 161. Numbers indicate amino acids. Not drawn to scale (Figure modified from Sauane et al. [81], Copyright 2003, with permission from Elsevier)

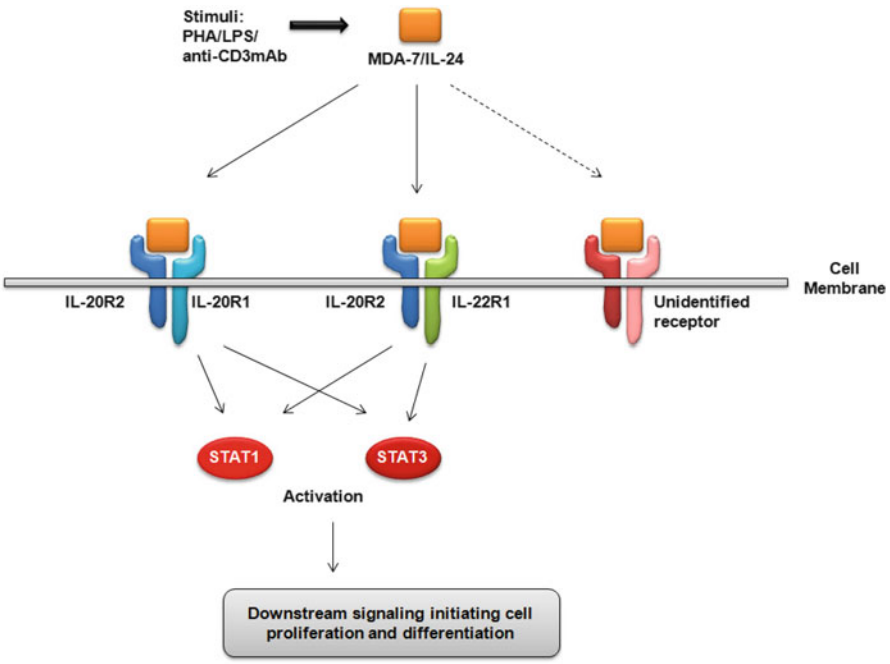

Fig. 6.2 Schematic representation of MDA-7/IL-24 action mediated through its cognate receptors. MDA-7/IL-24 can be induced by PHA, LPS and anti-CD3 monoclonal antibody. MDA-7/IL-24 then binds to its receptor dimers IL-20R1 and IL-20R2 or IL-22R1 and IL-20R2 or possibly an as-yet unidentified receptor to activate STAT1 or STAT3 downstream signaling

analysis also revealed a region of the gene encoding an IL-10 signature sequence from amino acid 101 to 121, three protein kinase C consensus phosphorylation sites at amino acid 88, 133 and 161 and three casein kinase II consensus phosphorylation sites at amino acid 101, 111 and 161 [81]. Structural analysis also revealed the possibility of dimerization of MDA-7/IL-24 due to a potential disulfide bond. MDA-7/IL-24 protein was found to be highly conserved throughout evolution with sequence homology with species such as yeast, cat, dog, cow and monkey.

Members of the IL-10 cytokine family signal through receptor dimers consisting of an R1 type receptor (with a long cytoplasmic domain) and an R2 type receptor (with a short cytoplasmic domain). MDA-7/IL-24 can bind to its cognate receptors – IL-20R1 and IL-20R2; or IL-22R1 and IL-20R2 in order to activate downstream STAT1 (signal transducer and activator of transcription 1) and STAT3 signaling (Fig. 6.2) [20, 73, 81, 95].

To gain insight into the regulation of *mda*-7/IL-24 expression, the *mda*-7/IL-24 promoter was isolated and characterized [54]. Several recognition binding sites for two transcription factor families, AP-1 and C/EBP, were identified within the *mda*-7/IL-24 promoter. Overexpression of c-Jun (a member of the AP-1 family), C/EBP-α or β caused an upregulation of *mda*-7/IL-24 promoter activity indicating their involvement in the transcriptional control of *mda*-7/IL-24 expression. Electrophoretic mobility shift assays (EMSA) further demonstrated that AP-1 and

C/EBP could bind to the *mda*-7/IL-24 promoter [54]. Since *mda*-7/IL-24 expression was induced upon treatment with IFN-β and mezerein, *mda*-7/IL-24 promoter activity was assessed in HO-1 and MeWo melanoma cells with and without the treatment [55]. *mda*-7/IL-24 promoter activity did not increase above basal levels with and without treatment with IFN-β and mezerein. However, *mda*-7/IL-24 mRNA was markedly increased in melanoma cells upon treatment with IFN-β and mezerein. Thus, *mda*-7/IL-24 expression appears to also be regulated at the post-transcriptional level during melanoma cell differentiation and IFN-β and mezerein might somehow be stabilizing *mda*-7/IL-24 mRNA levels [55]. As mentioned earlier, *mda*-7/IL-24, like other cytokines, contains AU-rich elements (ARE) in the 3′UTR region that correlate with rapid mRNA turnover and posttranscriptional control and might play a role in the post transcriptional regulation of *mda*-7/IL-24. *mda*-7/IL-24 expression is also regulated by p38 MAPK-mediated stabilization of *mda*-7/IL-24 3′UTR [60]. A recent study showed that IL-2 can upregulate the expression of *mda*-7/IL-24 [42].

At low concentrations, MDA-7/IL-24 predominantly functions as a cytokine. MDA-7/IL-24 is normally expressed in humans in tissues of the immune system such as the thymus, spleen, peripheral blood leukocytes (PBL) and normal melanocytes [38]. In fact, MDA-7/IL-24 transgenic mice (overexpressing MDA-7/IL-24 specifically in the skin) showed neonatal lethality within hours of birth, and displayed epidermal hyperplasia and abnormality in keratinocyte differentiation [36]. Several cells of the immune system can be induced to upregulate expression of *mda*-7/IL-24. Megakaryocyte differentiation induced in human hematopoietic cells upon treatment with TPA (12-O-tetradecanoyl phorbol-13-acetate) causes an increase in *mda*-7/IL-24 expression [38]. Stimulation of peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) or lipopolysaccharide (LPS) also caused induction of *mda*-7/IL-24 expression. *MDA*-7/IL-24 is upregulated in monocytes treated with LPS and in T cells, especially CD4+ naïve and memory cells activated by anti-CD3 monoclonal antibody. Assessment of the secretion profile of PBMC treated with MDA-7/IL-24 protein (purified from conditioned media obtained from HEK 293 cells infected with Ad.*mda*-7) showed increase in secretion of IL-6, TNF-α and INF-γ at high levels and IL-1β, IL-12 and GM-CSF at low levels within 48 h [9]. Simultaneous administration of IL-10 caused a partial or complete block in these changes mediated by MDA-7/IL-24, probably due to shared receptors with IL-10 having a ten-fold higher affinity for its receptor. However, PBMC proliferation was unaffected by treatment with MDA-7/IL-24. Of note, although IL-10 and MDA-7/IL-24 belong to the same cytokine family, IL-10 is a suppressor of immune response and inflammation while MDA-7/IL-24 is immunomodulatory.

MDA-7/IL-24 plays a role in wound healing [87], in autoimmune diseases such as psoriasis, rheumatoid arthritis and spondyloarthropathy [45], and protection against infectious diseases caused by bacteria such as *Pseudomonas aeruginosa* [3], *Salmonella typhimurium* [52] and *Mycobacterium tuberculosis* [53].

Besides the normal physiological role that MDA-7/IL-24 plays in the immune system, MDA-7/IL-24 has been studied in great detail for its role in cancer. The known functions of MDA-7/IL-24 are schematically represented in Fig. 6.3. The next section describes the role of MDA-7/IL-24 in cancer.

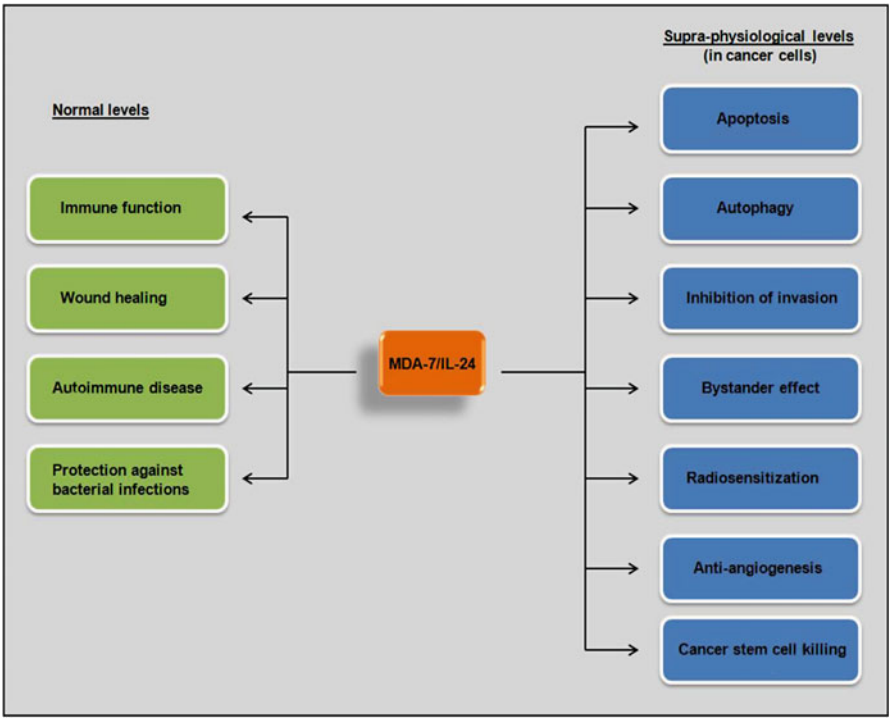

Fig. 6.3 Schematic representation of the currently known functions of MDA-7/IL-24. Under normal physiological conditions or when triggered by the appropriate signals, MDA-7/IL-24 can function in immune regulation, wound healing, autoimmune disease and in protection against bacterial infections. When expressed at supra-physiological levels in cancer cells, MDA-7/IL-24 exerts several anti-cancer activities

6.3 Role of MDA-7/IL-24 in Cancer

6.3.1 Apoptosis

Apoptosis, also known as programmed cell death, involves a sequential series of events that are responsible for the elimination of unwanted cells from the system. Aberrant apoptosis is considered to be a hallmark of cancer [35]. The ability of cancer cells to escape apoptosis is one of the major contributing factors to chemotherapeutic resistance. Much of the research over the past several years has been dedicated to understand the mechanisms adopted by tumor cells to evade cell death. This has led to the development of novel strategies and therapeutic agents that can modulate cell death pathways in a manner to selectively induce apoptosis in cancer cells.

There is abundant evidence in the literature that emphasizes the role of MDA-7/IL-24 as a selective anti-cancer agent. The tumor growth suppressing effects are contributed largely by apoptotic pathways that are triggered in response to MDA-7/

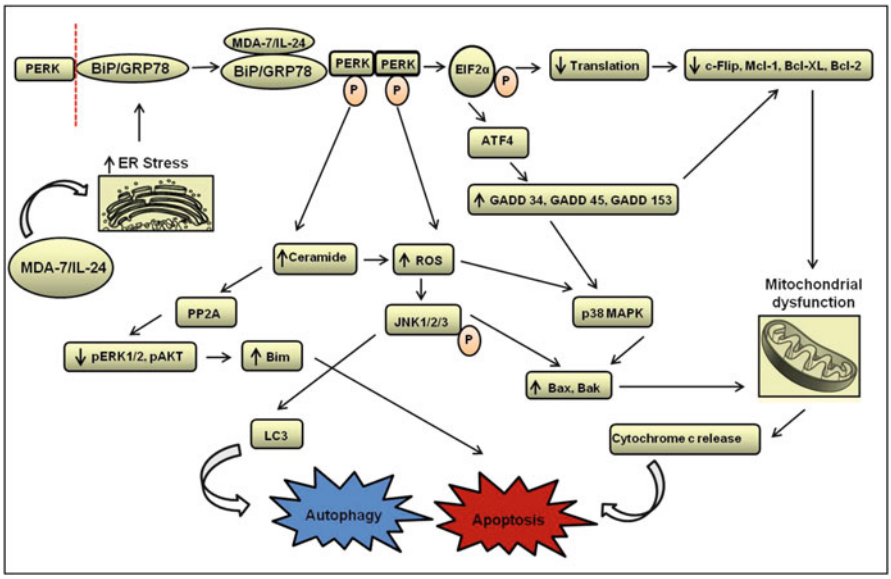

Fig. 6.4 Overview of the molecular pathways involved in MDA-7/IL-24-mediated regulation of cell growth. GRP-78/BiP chaperone protein represents one of the key molecules targeted by MDA-7/IL-24. The cytokine dissociates the complex formed between GRP-78/BiP and PERK allowing activation of PERK and EIF2α protein. This, in turn, affects the global translation of proteins resulting in loss of pro-survival proteins like c-Flip, Mcl-1, Bcl-XL, and Bcl-2. Phosphorylated PERK also enhances the transcription of certain DNA damage response genes (i.e., GADD 34, GADD 45, GADD 153) in a PERK-dependent manner. Activation of PERK also results in increased levels of ROS and ceramide that affects autophagy pathway and triggers JNK and p38 signaling. Overall, these events disrupt the mitochondrial integrity by affecting the balance between pro-survival and pro-apoptotic proteins and eventually lead to growth inhibition and cell death (Figure modified from Dent et al. [18], Copyright 2010, with permission from Elsevier)

IL-24. Previous and ongoing research efforts by our research group and others have shed some light on the key players that are involved in MDA-7/IL-24-mediated apoptosis (Fig. 6.4). Most of these proteins play an important role in the regulation of endoplasmic reticulum (ER) stress and mitochondrial function [16, 17, 32, 49, 50, 61, 73, 74, 80, 99–101]. In earlier reports from our laboratory, it was observed that MDA-7/IL-24 induces cell death by the activation of PKR-like endoplasmic reticulum kinase (PERK), an unfolded protein response (UPR) sensor. MDA-7/IL-24 prevents the interaction between the ER residing chaperone protein BiP/GRP78 and PERK by associating with BiP/GRP78 [33]. Dissociation of PERK from BiP/GRP78 results in the oligomerization and auto phosphorylation of PERK. Phosphorylated PERK in turn, leads to the activation and phosphorylation of EIF2α protein that shuts down the global translation of proteins. This leads to decreased expression of pro-survival proteins like Mcl-1, Bcl-XL, and c-Flip [23, 28, 68]. In other studies, treatment of cancer cells with MDA-7/IL-24 reduced the expression of pro-survival proteins and this reduction was found to be correlated

with the increased levels of pro-apoptotic markers like Bax and Bak [28, 91, 92]. Recent studies have also explored the role of second messenger molecules like ceramide and dihydroceramide (generated after PERK activation) upon MDA-7/IL-24 exposure [6, 84, 100]. It was hypothesized that MDA-7/IL-24 possibly functions by stabilizing ceramide synthase 6 protein thus increasing the levels of dihydroceramide in a PERK-dependent manner. Enhanced levels of ceramide result in calcium ion-dependent production of reactive oxygen species (ROS) that impacts diverse signaling pathways and alters mitochondrial integrity [49].

Over the past decade, it has become increasingly clear that MDA-7/IL-24 affects tumor cell viability by inducing cell death, but the exact mode of action varies to certain extents in different cancer types. For instance, upon infection of prostate carcinoma cells DU145, Ad.*mda*-7 (a non-replicating type 5 adenovirus expressing *mda*-7/IL-24) induced apoptosis in a Bax-independent manner [49, 50, 80]. In contrast, in primary human glioblastoma cells, the mitochondrial dysfunction and cytotoxicity was largely contributed by the cathepsin-B dependent cleavage of a pro-apoptotic family member Bid [101, 104]. When melanoma cells were infected with Ad.*mda*-7, a dramatic reduction was observed in the levels of anti-apoptotic proteins Bcl-2 and Bcl-XL, whereas pro-apoptotic proteins like Bax and Bak were upregulated to a modest degree [48]. This suggests that Ad.*mda*-7 stimulates the apoptotic pathway by affecting the ratio of pro-apoptotic to anti-apoptotic markers in cancer cells. In another study, Dash et al. documented that ER stress response triggered by MDA-7/IL-24 in prostate cancer cells resulted in apoptosis due to the inhibition of an anti-apoptotic myeloid cell leukemia- 1 (Mcl-1) protein [17]. Conversely, when Mcl-1 was overexpressed in transformed cells, a significant inhibition of MDA-7/IL-24-mediated toxicity was observed. This data emphasizes the role of Mcl-1 in MDA-7/IL-24-induced cell death.

Sarkar et al. first reported the involvement of p38 mitogen activated protein kinase (MAPK) pathway in cell cycle arrest and cell death in context of Ad.*mda*-7 or MDA-7/IL-24 [74]. In this study, MAPK activity was shown to be partly dependent on the activation of PERK and subsequent stimulation of a subset of growth arrest and DNA damage (GADD) genes. Other studies also found that in some of the cancer cell types, exposure to MDA-7/IL-24 resulted in the dephosphorylation and inactivation of ERK 1/2 [99]. In others, c-Jun NH_2-terminal kinase (JNK) signaling cascade was triggered following treatment with this multipurpose cytokine [99, 103]. This resulted in the stabilization of Bim and activation of Bax and Bak proteins, finally leading to mitochondrial dysfunction and cell death. Additionally, MDA-7/IL-24 also induces cell death *via* apoptosis selectively in cancer stem cell without affecting normal breast stem cell growth [5]. The underlying mechanism of the unique ability of MDA-7/IL-24 to induce selective apoptosis in cancer cells requires further clarification. Fundamental biochemical differences between normal and cancer cells might be a possible reason for MDA-7/IL-24-mediated selective apoptosis in cancer cells. One of the main mechanisms by which MDA-7/IL-24 induces cell death is by inducing ER stress. Although ER stress activates both pro-survival and pro-apoptotic pathways, a particularly strong ER stress response for prolonged periods could shift the balance toward apoptotic

pathways, attenuating tumor growth and metastasis. Since cancer cells have higher levels of ER stress as compared to normal cells, this renders them more susceptible to ER stress-mediated cell death triggered by MDA-7/IL-24. Another plausible explanation why MDA-7/IL-24 mediates differential cell killing effects between normal and cancer cells relies on reactive oxygen species (ROS), which is one of the key mediators of MDA-7/IL-24 toxic responses in cancer cells. Numerous studies have demonstrated that the basal level of ROS in cancer cells is higher than the normal cells [65]. As a consequence, agents like MDA-7/IL-24 that enhance ROS production, could easily overcome the natural antioxidant effects in a more efficient manner in cancer cells as compared to normal cells, resulting in cell death.

6.3.2 Autophagy

Autophagy is an evolutionarily conserved pathway involved in the recycling of cellular components including whole organelles [7, 8, 19, 51]. The process is regulated by a set of autophagy related (ATG) proteins arranged in three different complexes [88]. The autophagy pathway is initially triggered by unc-51 like kinase (ULK1). The kinase is responsible for further stimulation of a signaling pathway that results in the formation of a double membrane bound vesicle called autophagosome. Autophagosome consists of components that are destined for degradation and its formation requires a PI3 kinase complex. In the final step, the autophagosome membrane elongation occurs with the aid of other conjugation systems like LC3 and ATG12 and it fuses with the lysosome resulting in the degradation of its enclosed components. There are three different kinds of autophagy categorized as micro-autophagy, macro-autophagy and chaperone-mediated autophagy (CMA). Micro-autophagy degrades the enclosed components directly by lysosomal invagination [105]. Whereas macro-autophagy sequesters the proteins meant for degradation in autophagosome, which are later on degraded by the lysosomal proteases [19, 105]. CMA is the only form of autophagy that involves no vesicular trafficking. During the process of autophagy, particular proteins are tagged by the CMA substrate chaperone complex, which are then directed to the lysosomes or endosomes for degradation [19, 105].

Numerous studies suggest the dynamic role of autophagy in regulating tumorigenesis [7, 57, 96, 97]. Autophagy is known to suppress growth of tumors during the early developmental stages but promotes tumor growth in advanced tumors [7, 37]. Based on this, different therapeutic agents may modulate tumor growth and proliferation by affecting the autophagic process. In fact, autophagy-related protein markers have gained significant interest for cancer therapy. There are several proteins implicated in tumor growth and proliferation that also play a role in the regulation of autophagy. For instance, mTOR is reported to negatively regulate the ULK1 complex. Therefore, inhibitors of the mTOR pathway, like PTEN,

stimulate autophagy and the activators of mTOR, such as AKT, inhibit the process [51]. Studies from our laboratory and other research groups have demonstrated the involvement of MDA-7/IL-24 in autophagy in different cancer models (Fig. 6.4). In glioblastoma (GBM) and transformed fibroblasts, cytokine-induced ER stress results in the activation and phosphorylation of PERK. Activation of PERK and UPR is generally considered a protective response. However, prolonged PERK activity and stimulation of UPR signaling can lead to cell death. Previous studies have validated the involvement of PERK in MDA-7/IL-24-mediated autophagy [62]. $PERK^{-/-}$ cells or cells that were transformed to carry a dominant negative PERK gene displayed increased resistance to MDA-7/IL-24-induced autophagy and cell death [99, 104]. Likewise, overexpression of BiP/GRP78 blocked MDA-7/IL-24 induced autophagy and cell death by inhibiting PERK activation [104]. Conversely, activation of PERK triggered the vacuolization of LC3 protein and resulted in an increased expression of autophagy markers like ATG5 and Beclin1 [104]. Furthermore, inhibition of autophagy by suppression of important mediators like Beclin1 and ATG5 or treatment with a chemical inhibitor, 3-methyl adenine, dramatically attenuated MDA-7/IL-24-induced cytotoxicity [104].

In prostate cancer cells, autophagy plays a different role upon MDA-7/IL-24 treatment. Unlike GBM cells, blocking autophagy in prostate cancer cells by using 3-methyl adenine or by suppressing the expression of Beclin1 enhanced MDA-7/IL-24 cytotoxicity [6]. In contrast, knockdown of ATG5, an autophagy marker, resulted in decreased MDA-7/IL-24-mediated toxic autophagy and apoptosis [6]. This discrepancy could be explained by the differential role of ATG5 in programmed cell death. In prostate cancer cells, ATG5 is cleaved into a 25-kDa fragment in an autophagy-dependent manner [6]. This small fragment, in turn, induces mitochondrial dysfunction *via* activation of pro-apoptotic molecules like Bax and Bak leading to enhanced cell death.

In renal and ovarian cancer cells, MDA-7/IL-24-induced autophagy is mainly dependent on CD95 signaling. Treatment of renal carcinoma cells with GST-MDA-7 enhanced ER stress response *via* CD95 signaling eliciting autophagy and cell death [63]. Previous reports have shown that drug-induced activation of CD95 resulted in a protective form of autophagy that promoted survival in melanoma, hepatoma, renal, and pancreatic cancer cells [64]. Subsequent studies in renal carcinoma have found a direct correlation between suppression of ATG5 and/or Beclin1 expression and MDA-7/IL-24-mediated cell death [61]. Thus, MDA-7/IL-24 induces autophagy in a wide variety of cell types, but the precise function of autophagy in response to MDA-7/IL-24 varies greatly according to the tumor cell type. The differential response induced by MDA-7/IL-24 in a broad range of cancer cells versus normal cells could be attributed to the inherent complexities in these cells. ROS is known to be involved in the autophagic process. We speculate that MDA-7/IL-24-mediated PERK activation might lead to increased ROS production in cancer cells (that have higher basal levels of ROS as compared to normal cells), which leads to toxic autophagy and cell death in these transformed cells without affecting normal cells.

6.3.3 *Anti-angiogenesis*

Induction of angiogenesis is another hallmark of cancer [35]. Tumors rely on the (tumor) vasculature in order to maintain a source of nutrients and oxygen and to eliminate metabolic wastes and carbon dioxide. In addition to direct targeting of cancer cells, MDA-7/IL-24 also plays a role in inhibiting angiogenesis, thereby further inhibiting tumor growth.

Human umbilical vascular endothelial cells (HUVEC) do not endogenously express *mda*-7/IL-24. Infection of HUVEC cells with Ad.*mda*-7 induced sustained *mda*-7/IL-24 expression from day 1 to 4 with no sign of cytotoxicity. The ability of MDA-7/IL-24 to inhibit endothelial cell differentiation was assessed in HUVEC cells and compared to a known cell cycle regulator p16. Both MDA-7/IL-24 and p16 caused an inhibition of endothelial cell formation; however MDA-7/IL-24 showed a more profound effect with no evidence of tube formation in HUVEC as compared to p16, that allowed formation of full and hemi-tubes [70]. Injection of Ad.*mda*-7 into subcutaneous lung tumor xenografts in athymic mice caused an inhibition of angiogenesis (as evidenced by reduced tumor vasculature) [67] with tumors expressing low levels of CD31 expression, a protein highly expressed in neo-angiogenic endothelial cells. Simultaneously the expression of TRAIL, a promoter of apoptosis, was upregulated, indicating that Ad.*mda*-7 infection caused tumor inhibition by multiple mechanisms [70].

Inhibition of tumor angiogenesis can be brought about by directly suppressing tumor blood vessel formation or indirectly by suppressing production of tumor-derived growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and intereukin-8 (IL-8) [46]. Evidence of direct suppression of tumor blood vessel formation was demonstrated using secreted purified MDA-7/IL-24 protein [67]. MDA-7/IL-24 protein inhibited endothelial cell differentiation *in vitro* in a dose-dependent manner, without any cytotoxic effects. Induction of endothelial cell migration by VEGF and bFGF were also inhibited by MDA-7/IL-24 protein. Further analysis revealed an essential role of IL-22R1, one of the two MDA-7/IL-24 cognate receptors, in MDA-7/IL-24- mediated anti-angiogenic abilities. Introduction of an IL-22 receptor-blocking antibody along with MDA-7/IL-24 protein led to abrogation of inhibition of endothelial differentiation, indicating IL-22 receptors play an important role in MDA-7/IL-24 anti-angiogenesis abilities. The role of IL-20 receptors in MDA-7/IL-24-mediated anti-angiogenic abilities is currently unknown. MDA-7/IL-24-receptor-mediated downstream signaling (such as STAT-1) might also enhance MDA-7/IL-24 anti-angiogenic activity. MDA-7/IL-24 inhibits PI3K/AKT signaling in lung and breast cancer cells. PI3K and AKT have known roles in angiogenesis and their inhibition might further aid in MDA-7/IL-24-mediated anti-angiogenic abilities.

MDA-7/IL-24 also inhibits angiogenesis indirectly by suppressing angiogenic tumor-derived growth factors. Inhibition of tumor-derived growth factors will lead to further suppression of tumor growth by blocking neovascularization [46]. Infection of human lung cancer cells with Ad.*mda*-7 causes down regulation of the

expression of two key modulators of angiogenesis, vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β). Infection of human lung cancer xenografts with Ad.*mda*-7 caused an inhibition of VEGF, bFGF and IL-8 [59]. MDA-7/IL-24 was shown to regulate VEGF transcription resulting in reduced VEGF protein expression. Src kinase is known to regulate VEGF transcription and expression and MDA-7/IL-24 can directly inhibit Src kinase activity [41]. Thus, MDA-7/IL-24 inhibits tumor angiogenesis by multiple mechanisms.

6.3.4 Inhibition of Invasion and Metastasis

MDA-7/IL-24 also plays a role in inhibiting tumor invasion and metastasis, yet another hallmark of cancer. Treatment options and survival rates are adversely affected once a tumor has metastasized to a distant area in the body. MDA-7/IL-24 was shown to inhibit migration and invasion of several different types of cancer. Human non-small cell lung carcinoma cells (H1299 and A549) infected with Ad.*mda*-7 showed a decrease in *in vitro* migration and invasion by downregulating expression of PI3K (phosphatidylinositol 3-kinase)/PKB (protein kinase B), FAK (focal adhesion kinase), MMP-2 (matrix metalloproteinase-2) and MMP-9. Treatment using Ad.*mda*-7 in an *in vivo* experimental lung metastasis model also resulted in a decrease in the number of tumors [66]. Infection of human cervical cancer cells (CaSki) with Ad.*mda*-7 caused a decrease in *in vitro* cell migration and invasion that was attributed to downregulation of MMP-2 and upregulation of p38 MAPK (mitogen-activated protein kinase) [86]. Over expression of MDA-7/IL-24 in human hepatocellular carcinoma cells (HepG2 and BEL-7402) *in vitro* inhibited tumor cell adhesion and invasion and induced a G2/M cell cycle arrest [39], but not in normal liver cells (L02) [94]. MDA-7/IL-24 mediated inhibition of adhesion and invasion by inhibiting the expression of metastasis-related genes such as CD44, ICAM-1, MMP-2, MMP-9, Cyclin B1, Twist, Survivin, TGF-β and pAkt and downregulating NFκB transcriptional activity. On the other hand, expression of E-cadherin and p-ERK was increased along with an increase in transcriptional activity of AP-1 [39].

To determine whether MDA-7/IL-24-mediated inhibition of invasion was brought about *via* binding to its cognate receptors or in a manner independent of receptor signaling, a non-secretable version of MDA-7/IL-24 (lacking the secretory signal peptide) was generated. Infection of prostate cancer cells (C8161) with both secretable and non-secretable versions of Ad.*mda*-7 inhibited Matrigel invasion to a similar extent [79] suggesting that *mda*-7/IL-24 could inhibit invasion by both receptor-mediated and receptor-independent pathways.

An interesting discovery regarding MDA-7/IL-24 was made while examining its role in pancreatic cancer. Infection of Ad.*mda*-7 into a number of different cancer cell lines resulted in cancer cell growth suppression and apoptosis (as discussed in the apoptosis section); however Ad.*mda*-7 did not show a growth suppressive

or apoptotic effect in pancreatic cancer cells. Pancreatic cancer is often associated with genetic changes in a subset of genes and one of the most frequently altered genes in pancreatic cancer is the K-ras oncogene (85–95 %). Silencing the expression of K-ras in pancreatic cancer cells along with simultaneous infection with Ad.*mda*-7 caused growth suppression and apoptosis, suggesting K-ras mediated translational suppression of *mda*-7/IL-24 mRNA to protein [90]. Interestingly pancreatic cancer cells (MIA PaCA-2) infected with Ad.*mda*-7 and transfected with antisense K-ras plasmid before injecting into athymic mice resulted in complete tumor suppression as compared to rapidly growing control Ad.*mda*-7 infected tumors [90]. Thus, MDA-7/IL-24 secreted from a very small subset of tumor cells was able to inhibit growth of the entire tumor, an effect that is known as a '*bystander*' antitumor effect. Since then this '*bystander*' effect has been observed in several cancer types. It was determined that N-glycosylation of MDA-7/IL-24 was not essential for the '*bystander*' effect [82]. The ability of MDA-7/IL-24 secreted by normal cells to mediate '*bystander*' effect was also evaluated [89]. Normal cells (primary and immortalized human melanocytes FM516-SV, astrocytes IM-PHFA and prostate epithelial cells P69) infected with Ad.*mda*-7 were able to secrete MDA-7/IL-24 without adverse effects on normal growth. Secreted MDA-7/IL-24 from these three normal cell lines was able to inhibit anchorage-independent growth of DU-145 prostate cancer cells. To validate that the '*bystander*' effects were mediated by secreted MDA-7/IL-24, the ability of MDA-7/IL-24 to inhibit anchorage-independent growth and invasion was assessed in cells with and without functional IL-20/IL-22 receptors. MDA-7/IL-24 secreted from normal prostate epithelial cells (P69) inhibited anchorage-independent growth and invasion in prostate cancer cells (DU-145 and BxPC-3) with functional IL-20/IL-22 receptors, but not in lung cancer cells (A549) that lack a complete set of receptors [89]. Evidence for '*bystander*' activity *in vivo* was obtained using a xenograft mouse model. Tumors were formed by injecting breast cancer cells into the right and left flanks of athymic mice. Tumors on the left flank were injected with Ad.PEG-E1A-*mda*-7 (a Cancer Terminator Virus; *CTV*), a conditionally replication competent adenovirus controlling E1A expression in cancer cells and expressing *mda*-7/IL-24 when replicating in cancer cells, which eliminated tumors on both flanks of the mice [76]. Purging of tumors on the right untreated flank by MDA-7/IL-24 treatment of the left flank serves as a good model to assess systemic inhibitory potential of MDA-7/IL-24 *in vivo* on secondary tumors in distant regions of the animal. The *CTV*, first generation in a serotype 5 adenovirus and a second generation *CTV* in a tropism-modified Ad.5/3 virus, has also shown profound primary and '*bystander*' antitumor activity in additional tumor models including breast cancer [76], therapy resistant prostate cancer [2, 72], melanoma [75], glioblastoma multiforme [34], and pancreatic cancer [78, 77].

In conclusion, MDA-7/IL-24 exerts its anti-cancer activities by targeting multiple tumor growth mechanisms making it a very attractive translational molecule for treatment of human cancers.

6.4 Combination Effects of MDA-7/IL-24

Cancer is a multi-factorial disease and involves cross talk between complex molecular pathways [35]. Advances in the biomedical field have broadened our understanding regarding the key effector molecules and aberrant pathways involved but successful application of targeted therapies has been limited. This is due to the presence of alternate or compensatory pathways that cancer cells use to thrive and acquire enhanced resistance to drugs. To address this problem, a combination approach using multiple targeted agents has been successfully used in several pre-clinical and clinical studies. A combination strategy offers multiple advantages. Firstly, combination of therapeutic agents that target similar molecular pathways may provide synergistic effects. Secondly, agents that target different pathways may help to overcome compensatory pathways that are responsible for reducing the sensitivity toward drugs and providing low therapeutic benefit. Thirdly, combination treatments may also circumvent the tumor heterogeneity, particularly in advanced stage cancer patients, where genetic instability gives rise to aggressive variants, each with different characteristics and sensitivity to therapeutic regimens. Tumor heterogeneity may not only result in treatment failure but also increases the number of potential sites that need to be targeted to inhibit tumor growth and proliferation. In this scenario, combinatorial approaches may provide better therapeutic effectiveness as compared to single agents. Also, in the adjuvant setting, combination therapy is considered more favorable as compared to a single therapy due to the survival benefit from a clinical standpoint. MDA-7/IL-24 has been shown to induce synergistic effects in a wide spectrum of human cancers when used in combination with other agents. For example, McKenzie et al. demonstrated significant growth inhibitory effects in Her-2/neu-overexpressing breast cancer cells following treatment with Ad.*mda*-7 in combination with a monoclonal antibody targeting Her-2/neu receptor [58]. The inhibition in tumor growth was partly mediated by effect of combination agents on the β-catenin, and AKT survival pathways. In another study, *mda*-7/IL-24 incorporated in an adenoviral vector was used along with bevacizumab in a lung cancer model [40]. The combination treatment induced cell cycle arrest and apoptosis in the *in vitro* system. Similar effects were recapitulated in the xenograft lung cancer model. The subcutaneously implanted tumors receiving Ad.*mda*-7 plus bevacizumab showed improved survival rate as compared to their control counterparts with complete tumor regression observed at the completion of the study. Emdad et al. determined the efficacy of combining Ad.*mda*-7 with a selective EGFR inhibitor, gefitinib, in non-small cell lung cancer [22]. Combination treatment enhanced apoptotic cell death in the treated cells by increasing the expression of a downstream effector molecule, RNA-activated protein kinase (PKR). Zheng et al. combined Ad.*mda*-7 with an alkylating agent, temozolamide, to overcome the chemoresistance in human melanoma cells [106]. They observed increased cell death in melanoma cells that were treated with optimal doses of Ad.*mda*-7 and temozolamide. A combinatorial effect was also observed when a bacterially synthesized glutathione S-transferase (GST)-fusion protein of MDA-7/IL-24, GST-MDA-7, was combined with Tarceva in

non-small cell lung carcinoma cells [31]. Similarly combining GST-MDA-7 protein with arsenic trioxide effectively controlled growth and proliferation of renal carcinoma cells [102]. The free radicals generated by arsenic trioxide interacted with GST-MDA-7 and induced cell death in the treated cells by a non-apoptotic pathway. An oncolytic adenovirus incorporated with *mda*-7/IL-24 (ZD55-IL-24) was used in combination with cisplatin to analyze growth inhibitory effects on a panel of cancer cells [98]. The combination approach dramatically enhanced the cytotoxic and apoptotic effects in the tested cancer cells as compared to their normal counterparts. Chimeric adenoviral vector carrying *mda*-7/IL-24 gene (Ad5/3-MDA7) also mediated enhanced gene transduction to the otherwise refractory colorectal cancer and prostate cancer cells and when used in combination with an apogossypol derivative BI-97C1, Sabutoclax, toxicity to MDA-7/IL-24 was enhanced [2, 14].

In addition to chemotherapeutic agents, monoclonal antibodies, and chemical inhibitors, MDA-7/IL-24 has also been effectively used in conjunction with other conventional therapies. Nishikawa and colleagues observed that in non-small cell lung carcinoma, MDA-7/IL-24 enhanced the sensitization of resistant cancer cells to ionizing radiation and induced cell death by affecting angiogenesis in the supporting tumor endothelial cells [59]. In another study, Chada et al. compared the efficacy of gene transfer when MDA-7/IL-24 was combined with a range of chemotherapy agents, monoclonal antibodies, and radiation [10]. The authors found that combination approach effectively improved the overall sensitivity of the breast cancer cells toward most of the tested therapeutics in an additive or synergistic manner.

Another interesting study explored the idea of targeting both the tumor as well as immunologic components by administering Ad.*mda*-7 along with a secreted form of an ER resident chaperone protein Grp170 (Ad.sgrp170) [29]. Grp170 acts as a strong immunostimulatory agent and antigen carrier. Combined delivery of both the Ad vectors (Ad.*mda*-7 and Ad.sgrp170) effectively reduced growth of tumors in TRAMP-C2 prostate cancer model when compared with single agent. Furthermore, it enhanced the antigen and tumor-specific T cell response evident by increase in the production of interferon-gamma and cytolytic activity. The MDA-7/IL-24 and Grp170 combination also provided protection against subsequent tumor challenge and displayed '*bystander*' effect.

Collectively, these observations suggest that careful design and rational combination of traditional and novel therapeutic agents with MDA-7/IL-24 represents a viable strategy to induce multi-pronged attack on cancer cells and significantly enhance therapeutic outcome.

6.5 Phase I Clinical Trial with Ad.*mda*-7 (INGN-241)

The efficacy of MDA-7/IL-24 as a cancer therapeutic has been demonstrated in a wide range of tumor models [16, 32, 46]. Based on the unprecedented success in pre-clinical studies, a Phase I clinical trial was initiated to determine the therapeutic

potential of *mda*-7/IL-24 incorporated in an adenoviral vector (Ad.*mda*-7; INGN 241) [11, 21, 25, 46, 47, 71, 93]. The adenoviral vector used in this study had deletions in the E1 and E3 regions making it replication-incompetent. The human *mda*-7/IL-24 sequence was cloned into the vector along with the cytomegalovirus immediate-early promoter and SV40 polyadenylation sequence. The Phase I study evaluated the safety profile, pharmacodynamics, pharmacokinetics of vector-specific DNA, mRNA, MDA-7/IL-24 protein distribution and its biological effects, both locally and systemically. Twenty-eight patients diagnosed with squamous cell carcinoma of the head and neck (SCCHN), non-small cell lung carcinoma, melanoma, breast carcinoma, colorectal carcinoma, lymphoma, hepatoma, adenocarcinoma, sarcoma and carcinomas of the adrenal, bladder, penis, parotid, lip and kidney were enrolled in this dose-escalating study. All the patients included in the study received prior treatment with chemotherapeutic drugs, immunologic agents, radiation, and/or surgery. They were divided into eight cohorts based on the treatment dose of Ad.*mda*-7 (INGN 241) (ranging from 2×10^{10} vp (viral particles) to 2×10^{12} vp), time of post-treatment biopsy (24 h to 30 days), and frequency/mode of administration (single dose, divided dose or multiple doses) (Table 6.1).

Cohorts one to three were administered with single intratumoral injection of Ad.*mda*-7 (INGN 241) and tumors were resected 24 h post-injection. Cohorts four and five were treated with high dose of Ad.*mda*-7 and tumors were excised 48 h or 96 h after treatment, respectively. Subjects in cohort six received ten injections totaling 2×10^{12} vp and the tumors were resected 48 h post-administration. Cohort seven was injected with single dose of Ad.*mda*-7 (INGN 241) and biopsies were taken 30 days post-treatment. In cohort eight, patients received high dose of Ad.*mda*-7 (INGN 241) twice a week for a period of 3 weeks and tumors were biopsied at day 30 (Table 6.1). The biological effects in response to Ad.*mda*-7 (INGN 241) are summarized according to the tumor type in Table 6.2.

A comprehensive analysis was done to follow the vector biodistribution with time. To identify the injection site, Ad.*mda*-7 (INGN 241) was mixed with isosulfan blue dye before administration (Fig. 6.5). Maximal levels of vector-specific DNA and mRNA were detected at the site of injection (the center of the injected tumor averaged more than 1×10^8 vector DNA copies/μg) and it decreased in the peripheral areas. Low levels of vector DNA was detectable up to 3 cm from the site of injection. This could be due to the '*bystander*' activity induced by secreted MDA-7/IL-24 protein in the neighboring tumor cells [82, 83, 85, 89]. The overall levels of vector DNA and RNA varied in a dose- and time-dependent manner with high levels detected in tumors 24–48 h post-injection [11, 21, 71]. The levels dropped by nearly three logs of magnitude in tumors that were resected at day 4. However, vector DNA was present above baseline levels at 30 days.

Further, immunohistochemical analysis was done to analyze the expression of MDA-7/IL-24 protein (Fig. 6.6). On an average, 20 % of tumors that were treated with low dose of Ad.*mda*-7 (INGN 241) displayed positive staining for MDA-7/IL-24 protein [11]. The percentage of lesions showing positive MDA-7/IL-24 staining increased up to 53 % for cohorts that were administered with high dose of Ad.*mda*-7 (INGN 241). Tumor sections were also evaluated for the expression of β-catenin proto-oncogene [93]. It was found that the expression of β-catenin was dramatically

Table 6.1 Patients cohorts and treatment profile

Cohorts	No. of patients[a]	Diagnosis[b]	Prior treatments[c]	Dose (vp)	Biopsy time	Adverse events[d]
1	1 (1)	BrCa (1)	S, C, Ca, N, H, G	2×10^{10}	+24 h	2
2	1 (1)	CoCa (1)	S, F, I	2×10^{11}	+24 h	1
3	3 (3)	SCCHN (1), Mel (1), LCL (1)	S, RT, T, P, C, V, D, Fl, Cl	2×10^{12}	+24 h	0/1/1
4	3 (3)	BrCa (1), AdrCa (1), Hep (1)	S, A, C, T, Ta, N, P, RT, E	2×10^{12}	+48 h	0/1/0
5	4 (3)	BrCa (1), Mel (1), Mel (1)	S, Ta, RT, I, D	2×10^{12}	+96 h	0/2/2
6	1 (1)	BrCa (1)	S, A, T, Ta	2×10^{12}, divided dose	+48 h	0
7	7 (5)	TCC (1), Mel (1), CoCa (1), SCCHN (2)	S, M, V, A, P, T, G, RT, F, I	2×10^{12}	Day 30	2/0/0/2/2
8	8 (5)	Mel (3), SCCHN (2)	S, RT, IF, IT, P, T, F	2×10^{12}, twice weekly × 3	Day 30	2/2/2/1/1/[e]

[a]Number of patients enrolled per cohort is listed. The number of patients completing at least one round of treatment is indicated in parentheses

[b]Diagnosis: *BrCa* breast carcinoma, *CoCa* colon carcinoma, *SCCHN* squamous cell carcinoma of the head and neck, *Mel* melanoma, *LCL* large cell lymphoma, *AdrCa* Adrenal carcinoma, *Hep* hepatoma, *TCC* transitional cell carcinoma

[c]Prior treatments: *S* Surgery, *A* adriamycin, *C* cyclophosphamide, *Ca* capecitabine, *Cl* chlorambucil, *D* dacarbazine, *E* etoposide, *F* 5-fluorouracil, *Fl* fludarabine, *G* gemcitabine, *H* herceptin, *I* irinotecan, *IF* IFN-α, *IT* immunotherapy, *M* methotrexate, *N* navelbine, *P* platinum, *RT* radiotherapy, *T* taxane, *Ta* tamoxifen, *V* vincistine

[d]Adverse effects possibly due to Ad.MDA-7 (INGN 241) administration are indicated

[e]One patient from cohort 8 experienced a grade 3 SAE and withdrew from the study (Adapted by permission from Cunningham et al. [11], Copyright 2005)

Table 6.2 Ad.mda-7 (INGN 241) induces biological effects in different tumor types

Tumor type	Dose (vp)	Apoptosis at central region[a]	Apoptosis at peripheral regions	Biological response[b]
Breast carcinoma	2×10^{10}	++	+	T, b, K
Colorectal carcinoma	2×10^{11}	+++	+	T, b, I, K
Melanoma	2×10^{12}	++	–	T, b, I, C
Breast	2×10^{12}	+++	++	T, b, K
Squamous cell carcinoma of the head and neck	2×10^{12}	+++	++	T, b, C, K
Adrenal carcinoma	2×10^{12}	+++	+	T, b
Hepatoma	2×10^{12}	+	–	T, b, C

[a]TUNEL reactivity: –, <5 %; +, 5–19 %; ++, 20–49 %; +++, >50 %

[b]Biological response is mentioned as *T* TUNEL reactivity, *b* β-catenin decrease, *I* iNOS reduction, *C* CD31 reduction, *K* Ki-67 staining decrease (Adapted by permission from Tong et al. [93], Copyright 2005)

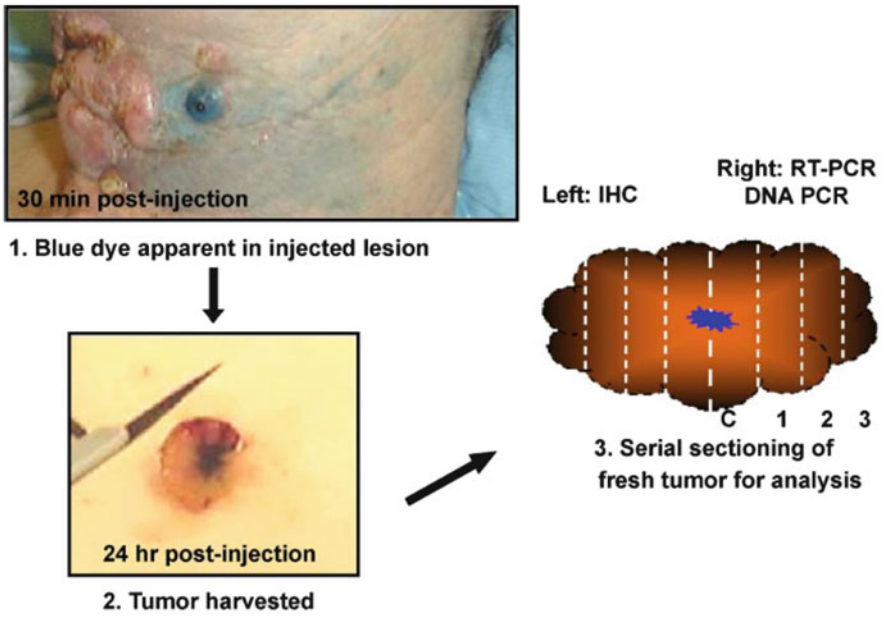

Fig. 6.5 Representation of excisional biopsy procedure. *1* Ad.*mda*-7/IL-24 (INGN 241) vector was mixed with isosulfan *blue* dye to identify the site of injection. *2* Lesion was resected 24 h post-injection. *3* The bisected lesion was processed into serial sections and the *left* portion is fixed and analyzed by immunohistochemistry. The *right* half was sectioned and frozen for quantitative PCR analyses (Reprinted by permission from Cunningham et al. [11], Copyright 2005)

reduced post-treatment with Ad.*mda-7* (INGN 241). In addition, the levels of iNOS (inducible nitric oxide synthase) were also decreased in four of the nine treatment subjects [93]. CD31 acts as a marker for angiogenesis. Upon analysis, it was found that CD31 levels decreased by 28 % in the treated lesions [11, 93]. TUNEL assays

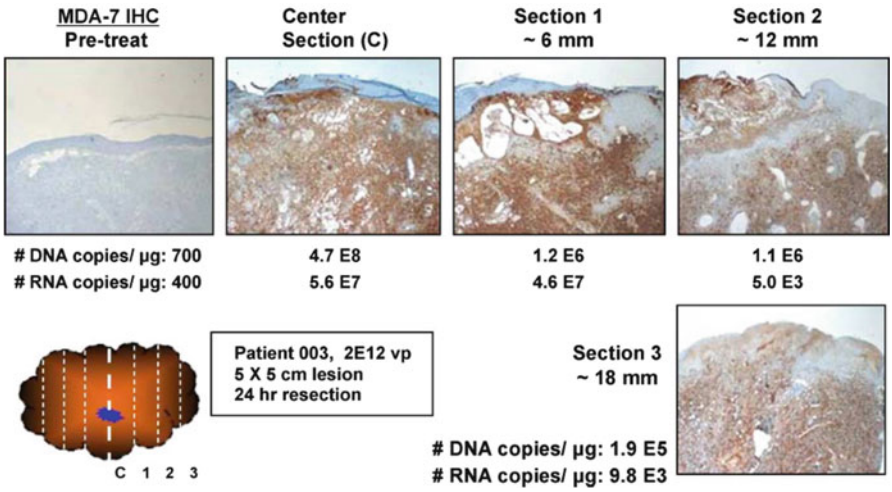

Fig. 6.6 MDA-7/IL-24 gene expression correlates with Ad vector distribution throughout the tumor. Half of the tumor was analyzed for MDA-7/IL-24 transgene expression and the remaining half to detect vector specific DNA and RNA levels. The number of DNA copies/µg genomic DNA and number of RNA copies/µg total RNA are indicated for each tumor section (Reprinted by permission from Cunningham et al. [11], Copyright 2005)

were performed to detect the apoptotic activity in the tumor sections. Up to 80 % of tumor cells at the injection site showed positive TUNEL staining. A good correlation was found between the areas of TUNEL reactivity and MDA-7/IL-24 staining [11, 93]. The MDA-7/IL-24 staining and TUNEL reactivity dropped down to baseline levels 30 days post treatment of tumor lesions. Ki-67 staining was performed as an indicator of tumor cell proliferation. After evaluation, 67 % of tumors showed reduced expression of Ki-67 following Ad.*mda*-7 (INGN 241) administration [93].

Activation of immune response by MDA-7/IL-24 was studied by analyzing the levels of inflammatory cytokines [93]. Most of the patients displayed enhanced serum levels of IL-6, IL-10, and TNF-α. A small number of patients showed a rise in the levels of GM-CSF, and IL-2 [71, 93]. This cytokine profile indicates stimulation of TH-1 type of immune response following Ad.*mda-7* (INGN 241) administration [71]. However, observed increase in the levels of different cytokines was a transient response, which subsided within 15–30 days post-injection. There were some mild side effects including pain and erythema at the site of injection. One of the patients suffered from grade 3 serious adverse event (SAE) and was discontinued from the trial.

At the completion of this study, three patients from cohort seven were followed up for clinical evaluation. No change was evident in tumor size in two patients with melanoma and colorectal carcinoma whereas about 23 % reduction in tumor size was observed in one of the patients in same group. Partial regression in tumors was observed in at least two of the five cohort eight patients who received bi-weekly

injections of Ad.*mda*-7 (INGN 241) for 3 weeks. On follow-up, a 64-old female patient with metastatic melanoma showed a dramatic reduction in tumor size after the sixth injection with continued regression for another 2 weeks. Similar response was observed following Ad.*mda*-7 (INGN 241) injection in the second lesion. Tumor size was decreased by 84 % by the fifth injection. Non-injected lesions also displayed erythema but there was no reduction in tumor size.

Overall, Ad.*mda*-7's (INGN 241) entry into the clinic in a Phase I trial employing intratumoral injection was exceptionally successful demonstrating that this therapy was well-tolerated in human subjects with an ~44 % clinical response in injected lesions [11, 21, 71, 93]. Future clinical trials with MDA-7/IL-24 employing improved delivery vectors, such as the *CTV* [72, 75, 76, 78, 77] and targeted delivery systemically, using ultrasound-targeted microbubble-destruction [14, 15], should elicit even more relevant and enduring clinical responses. The findings from these newer studies will expand our current knowledge and culminate in safer and even more efficient delivery of this therapeutic gene in cancer patients.

6.6 Conclusions and Future Prospectives

As described in this chapter, the encouraging results in the preclinical studies and Phase I clinical trial with MDA-7/IL-24 (INGN 241) highlights the potency of this multifunctional cytokine as an efficient anti-cancer agent. Over the past few years, numerous studies have provided insights into the mechanism of action and diverse signaling pathways that are triggered in response to MDA-7/IL-24. At present, research is focused on expanding our current understanding and enhancing the overall potential of MDA-7/IL-24. This involves development of novel strategies to transfer the therapeutic gene to cancer cells in a selective manner [4, 12, 13]. Our laboratory and others are finding solutions to efficiently deliver the conditionally replicating adenoviruses carrying MDA-7/IL-24 or purified protein using targeted microbubbles by the ultrasound-targeted microbubble-destruction (UTMD) approach [2, 13–15, 30, 1]. This would not only shield adenoviral vectors from neutralizing antibodies following systemic administration but also avoid its sequestration in the liver and other non-specific tissues thus, maximizing the biological outcome. Furthermore, studies involving high throughput screening approaches to develop the next generation of molecules that would augment the expression and/or activity of MDA-7/IL-24 in the target cell/tissue are currently underway. Rational combination of agents that would synergize with MDA-7/IL-24 would help to overcome the resistance of cancer cells toward conventional treatment regimens as well as generate the biological response at low doses. Additionally, novel strategies to define ways of selectively inducing *mda*-7/IL-24 expression, protein production and secretion using small molecules would also augment the applications of this novel cytokine for therapy of cancer. Accordingly, concerted research efforts in the context of MDA-7/IL-24 will not only augment our understanding in the related field of mechanisms of action of cytokines, but will also pave the way for future translational studies for therapeutic intervention of cancer.

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Chapter 7
Cancer-Selective Apoptosis by Tumor Suppressor Par-4

Nikhil Hebbar, Tripti Shrestha-Bhattarai, and Vivek M. Rangnekar

Contents

Abstract Tumor suppressor genes play an important role in preventing neoplastic transformation and maintaining normal tissue homeostasis. Par-4 is one such tumor suppressor which is unique in its ability to selectively induce apoptosis in cancer cells while leaving the normal cells unaffected. The cancer cell specific activity of Par-4 is elicited through intracellular as well as extracellular mechanisms. Intracellularly Par-4 acts through the inhibition of pro-survival pathways and activation of Fas mediated apoptosis whereas extracellular (secreted Par-4) acts by binding to cell surface GRP78 leading to activation of the extrinsic apoptotic pathway. Many studies

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have highlighted the importance of Par-4 not only in preventing cancer development/recurrence but also as a promising anticancer therapeutic agent.

Keywords Par-4/PAWR • Apoptosis • Tumor suppressor protein • Extracellular systemic effects • Prostate cancer

7.1 Introduction

Cancer, a leading cause of death worldwide, is one of the most imminent health problems faced by mankind today. According to WHO reports over seven million people across different countries died of cancer in 2008 alone [1]. Over the years there has been a lot of progress in cancer research and discovery which has led to improved overall survival times and quality of life for patients suffering from certain types of cancer. However, for the vast majority of tumor types, there remains a huge deficit in effective treatment strategies. Traditionally, the treatment of cancer has been a mutli-modal approach involving radiation and surgical resection to reduce the tumor burden wherever possible, followed by chemotherapy to eradicate the remaining tumor cells [2, 3].

These approaches have been met with limited success and are associated with significant toxicities leading to increased patient suffering and poor quality of life post therapy. In the more recent years, advances in the understanding of cancer biology and the mechanisms underlying therapeutic resistance have led to a surge in the development of targeted therapies. These agents target specific cellular markers that are preferentially expressed in cancer cells over normal cells. Neoadjuvant therapy which makes use of such agents has effectively lowered the required doses of radiation or conventional chemotherapeutic agents. Such treatment strategies have proven beneficial by increasing patient survival without debilitating side effects [4, 5].

The search for new targets has led to the discovery of a number of cancer-associated genes which can either promote cancer cell survival and tumor progression or can induce cancer cell death when expressed. One such anticancer gene called Prostate apoptosis response-4 (Par-4) was discovered by Sells et al. in 1994. Par-4 was first identified as one of the immediate early genes that were expressed in androgen independent AT-3 rat prostate cancer cells in response to increased Ca^{2+} influx due to treatment with the ionophore, Ionomycin. Further analysis revealed that Par-4 was up-regulated only in response to apoptosis but not in response to other processes such as necrosis or growth arrest. The association of Par-4 as an apoptosis related gene was further strengthened by the finding that upon castration, Par-4 was induced only in the apoptotic androgen dependent rat ventral prostate cells, but not in cells of the liver or kidney which were not affected by androgen ablation [6].

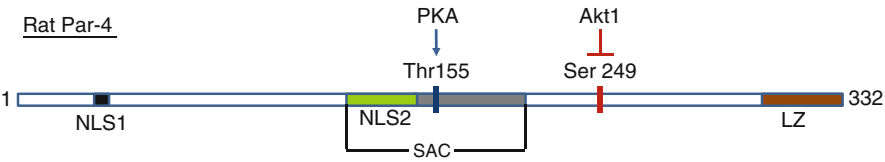

Fig. 7.1 Schematic representation of the functional domains of Par-4 which are conserved across rat, human and mouse species

Although Par-4 was discovered in prostate cancer cells, through a number of experiments in diverse cell types, it has been established that Par-4 is ubiquitously expressed throughout the body. Due to its role as an apoptosis related gene, Par-4 has been studied extensively in the field of cancer biology and it has been classified as a bona fide tumor suppressor. For a gene to be defined as a tumor suppressor it must satisfy some important functional criteria such as preservation of genomic integrity, regulation of cell proliferation and apoptosis. In practical terms the loss of function mutations or inactivation of a tumor suppressor should give rise to tumor initiation, development, and cancer progression. The *Par-4* gene is located on the unstable human chromosomal 12q21 region and is often found to be deleted or mutated in a number of different tumors [7]. Over 70 % of renal cancers exhibit loss or down-regulation of Par-4; similar observations have been made in breast cancers, neuroblastomas, acute and chronic leukemias [8–11]. Due to its unstable nature the region 12q21 is often deleted in gastric and pancreatic cancers. Over 30 % of endometrial tumors exhibit hypermethylation of the Par-4 promoter leading to gene silencing and in some cases show a single base mutation in exon-3 of Par-4 that inserts a STOP codon in the SAC (Selective for Apoptosis of Cancer Cells, Fig. 7.1) effector domain of Par-4 resulting in truncated non-functional Par-4 protein [12].

Oncogenes are known to promote cancer development and progression and gain of function mutations in oncogenes are frequently observed in a variety of cancers. One of the most commonly mutated oncogenes is Ras, which undergoes a mutation that renders it constitutively active allowing it to promote tumor initiation [13]. K-Ras mutations are a common feature in pancreatic cancer and they are also known to significantly downregulate Par-4 in these tumors. K-ras mutant tumors tend to be highly aggressive and therapy resistant, whereas tumors with higher Par-4 levels have better prognosis. Down-regulation of Par-4 has been shown to be a critical factor in Ras-induced transformation. Ras-mediated down-regulation of Par-4 has been shown to occur through the MEK-ERK pathway. Activation of this pathway leads to hypermethylation of the Par-4 promoter, resulting in long term silencing of the *Par-4* gene, thus allowing transformation of epithelial cells. Studies have shown that stably introducing ectopic Par-4 in these cells or inhibiting MEK using chemical inhibitors can successfully prevent the transformation of these cells [14].

PTEN is a tumor suppressor which is often inactivated in a number of tumors. The loss of PTEN leads to the constitutive activation of the cell survival protein AKT. The AKT1 gene encodes a serine/threonine (S/T) kinase that promotes cell

survival, growth and proliferation and is known to be up-regulated in a number of cancers. It is seen that loss of the *PTEN* gene leads to the development of prostate intraepithelial neoplasia (PIN) lesions and spontaneous tumors [15]. Studies using RNA interference to inhibit Par-4 production have shown that Par-4 is necessary for the proapoptotic activity of PTEN in prostate cancer. This has also been verified by comparing the role of Par-4 in PTEN wild type and knockout mouse models [16].

The loss of PTEN leads to AKT activation which has a profound biological effect on Par-4 activity. Using co-immunoprecipitation studies AKT has been shown to directly bind and phosphorylate Par-4. The binding of AKT leads to the phosphorylation of Par-4 at the critical S249 residue; this is an inactivating phosphorylation, which renders Par-4 unable to induce apoptosis. This was verified by using a S249A mutant of Par-4 which retained its apoptotic potential despite the presence of AKT. Further, inhibition of AKT using small molecules resulted in restoration of the pro-apoptotic potential of Par-4. The role of AKT-mediated inhibition of Par-4 is not limited to direct binding and subsequent phosphorylation. The phosphorylation of Par-4 by AKT at the S249 residue makes it target for 14-3-3 which retains it in the cytoplasm. The 14-3-3 proteins are chaperone proteins which are known to play a role in promoting cell survival. They are also known to bind to phosphoserine residues on target proteins. Sequestration of Par-4 by the 14-3-3 chaperones, and lack of its nuclear translocation thereof results in further inhibition of its activity as a tumor suppressor protein [16, 17].

c-Src is a proto-oncogene that is known to play an important role in the promotion and progression of colon cancer. It is a non-receptor tyrosine kinase which is implicated in tumorigenesis and metastatic progression, however its role in apoptosis inhibition was not well known [18]. A study by Kline et al. suggests that Par-4 is phosphorylated by c-Src at certain tyrosine residues which also promotes the binding of Par-4 to 14-3-3 and further preventing nuclear translocation of Par-4. Whether AKT plays a role in the interaction between c-Src and Par-4 is not completely understood. The pharmacological inhibition of Src kinase prevents the inhibitory phosphorylation and results in increased nuclear localization of Par-4. This has been in observed in a colon cancer background and suggests that inhibition of Src kinases in colon cancer might promote Par-4 induced apoptosis and improve therapeutic outcome [19].

More recently the role of Par-4 in breast cancer recurrence has been established. Through elegantly designed experiments Par-4 has been shown to be both necessary and sufficient to prevent the recurrence of breast cancer in women. Down-modulation of the tumor suppressor Par-4 is shown to be the primary factor in determining the recurrence of breast tumors [10]. The study demonstrates that tumor cells with high Par-4 levels respond well to chemotherapy and oncogene inhibition whereas those with low levels of Par-4 tend to be highly resistant to therapy and have an increased propensity to recur at local and distant sites [10]. The loss of Par-4 expression is most commonly associated with the most aggressive breast tumor types such as basal like, and triple negative breast cancers. These subtypes have the highest rates of recurrence and mortality. Breast cancers are often driven by the oncogenes HER2/neu, MYC or WNT1 and the study shows

that treatments that result in the inhibition of these oncogenes or the use of chemotherapy results in selection of tumor cells that inherently exhibit extremely low levels of Par-4. These surviving cells then give rise to a highly resistant population culminating in the formation of recurrent breast tumors. Interestingly the Par-4 levels do not seem to play in a role in the formation of primary breast cancers however Par-4 loss is strongly correlated to breast cancer recurrence [10].

The Par-4 gene encodes a protein that is 340 amino acids long and consists of a number of domains. The domains of Par-4 that are considered critical for functional activity are highly conserved across human, rat and mouse genomes (Fig. 7.1). Par-4 contains two nuclear localization sequence domains, NLS1 and NLS2. Although both domains are conserved across the human mouse and rat species, studies have shown that only the NLS2 domain, which is a bipartite sequence, is necessary for the translocation of Par-4 into the nucleus. Studies using the ΔNLS1 mutant of Par-4 suggest that the relatively shorter NLS1 domain does not play a role in the nuclear localization of Par-4. There is no known function for this domain much like some of the other putative domains such as the ATP/GTP binding domain, the phosphorylation sites for Casein Kinase 2 (CK2) and Protein Kinase C (PKC). The presence of such domains raises many challenging questions about the role of Par-4 in cellular functions that might be distinct from it pro-apoptotic function [20].

Par-4 protein contains multiple putative phosphorylation sites for CK2, a protein that is known to promote cell survival, tumorigenesis and metastasis. Drug trials aimed at inhibition of CK2 as a therapeutic option have been promising [20]. Although there is no evidence yet linking the inactivation of Par-4 via CK2 phosphorylation, based on the effect of other pro-survival kinases like AKT and c-Src, there could be a strong possibility that CK2 might play a role in inhibiting apoptosis by Par-4.

7.2 Apoptosis by Par-4

Many research studies have provided insight into the mechanisms of Par-4-induced apoptosis, and the reasons for its specificity towards cancer cells. Direct over-expression of Par-4 is sufficient to induce apoptosis in a wide variety of cancer cells [21] This action of Par-4 is independent of the presence of wild-type p53 or PTEN, and can override the cell-protective effects of anti-apoptotic molecules such as Bcl-2 and Bcl-xL [22]. On the other hand, ectopic over-expression of Par-4 does not induce apoptosis in normal or immortalized/non-transformed cells, but sensitizes these cells to other apoptotic insults, such as growth factor withdrawal, tumor necrosis factor-α, ionizing radiation, etc. [21, 23, 24]. Various studies in cell culture models suggest that, in susceptible cancer cells, Par-4 induces apoptosis by: (a) activation of the pro-apoptotic machinery, and (b) suppression of cell survival mechanisms. In these cells, Par-4 drives the trafficking of Fas and Fas ligand (Fas/FasL) to the cell membrane [23]. Fas receptor or Apo-1 (CD95) belongs to

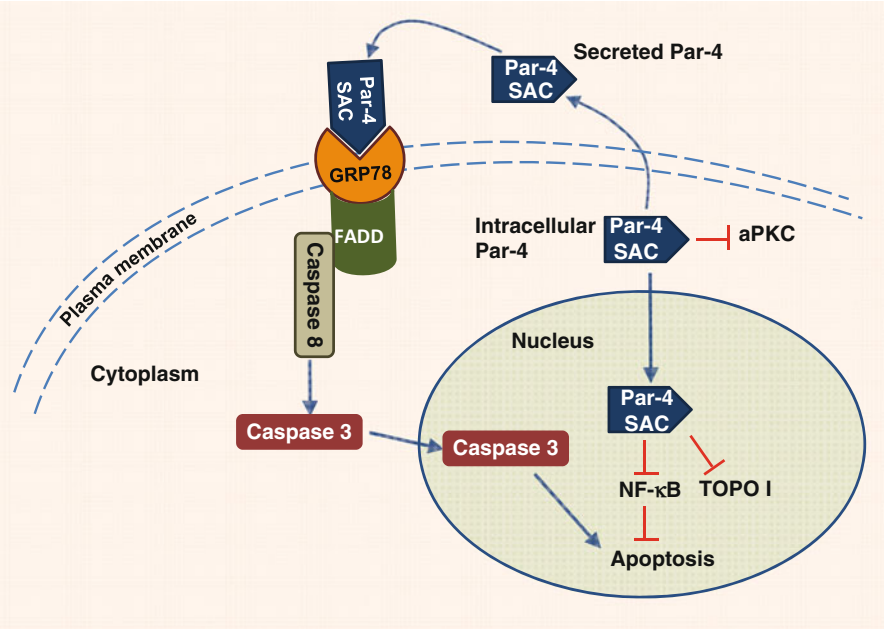

Fig. 7.2 Apoptosis by Par-4. In response to various apoptotic stimuli Par-4 translocates to the nucleus where it inhibits NF-kB and TOPO I thereby blocking the activation of cell survival pathways and inhibiting DNA relaxation which prevents erroneous DNA replication. Cytoplasmic Par-4 binds to aPKC and can inhibit NF-kB thereby inducing apoptosis. Secreted Par-4 binds to cell surface GRP78 resulting in the activation of the extrinsic apoptotic pathway. The resulting ER stress leads to the trafficking of more GRP78 from the ER to the cell membrane thus intensifying the pro-apoptotic effect of extracellular Par-4

the TNF-receptor (TNFR) family of type-I transmembrane proteins that possess a conserved C-terminal domain of approximately 80 amino acids- which represents an intracellular 'death domain'. Activation of Fas receptor, for example by binding to FasL, triggers trimerization of the receptor, and recruits an adapter protein called Fas-associating protein with Death Domain (FADD). FADD then engages procaspase-8 molecules through its Death Effector Domain, leading to the formation of a Death Inducing Signaling Complex (DISC). Induced proximity within the DISC enables the auto-proteolytic cleavage of procaspase-8 molecules, thereby unleashing the caspase cascade and resulting in cell death [25].

In addition, apoptosis in susceptible cells also require a co-parallel suppression of NF-κB-mediated prosurvival pathways. The transcription factor NF-κB positively regulates the expression of a number of prosurvival genes such as the X-linked Inhibitors of Apoptosis (XIAP), as well as the anti-apoptotic members of the Bcl-2 family, e.g. Bcl-XL, Al/Bfl1. Intracellular localization of Par-4 appears to be important in this context. As illustrated in Fig. 7.2 in sensitive cancer cells, Par-4 translocates into the nucleus and inhibits NF-κB-mediated transcriptional activity- an action essential for Par-4-induced apoptosis [21, 26]. Activation of the

pro-apoptotic Fas signaling pathway, and inhibition of pro-survival NF- κB pathway are both necessary and sufficient for Par-4 to induce apoptosis in susceptible cancer cells, such as PC3, DU145, H460, MDA MB231 etc. In normal cells, in immortalized but non-transformed cells; and in resistant cancer cell lines (e.g. LNCaP, A549, MCF-7) Par-4 does not translocate to the nucleus, and thus is unable to inhibit NF-κB transcriptional activity. In these cells, therefore, Par-4 does not induce direct apoptosis, but only serves a sensitizing function [21].

Research in a wide array of cell lines suggests a strong correlation between nuclear translocation of Par-4 and its ability to induce apoptosis. In some cancer cells, Par-4 is retained in the cytoplasm by the action of Akt1. In such scenarios, Par-4 cannot directly induce apoptosis, but serves to sensitize these cells to additional apoptotic stimulus [16]. It has been demonstrated that apoptotic action of Par-4 requires 'activation' of the Par-4 molecule by Protein Kinase A I (PKA I)-mediated phosphorylation. The amino acid sequence in Par-4 molecule [^{151}KRRST^{155}] matches the consensus phosphorylation sequence for PKA [RXXS/T], wherein threonine residue at position 155 (T155) is shown to be phosphorylated by PKA I. PKA I is a cyclic AMP (cAMP)-dependent serine/threonine (S/T) kinase that is involved in the regulation of cell growth, metabolism and differentiation. Cellular substrates of PKA I include transcription factors such as c-AMP Response Element-binding protein (CREB), NF-κB; pro-apoptotic proteins such as Bad etc. PKA I-mediated phosphorylation of T155 residue in Par-4 is necessary for nuclear entry, translocation of Fas/FasL to the cell membrane, inhibition of NF-κB activity, and induction of apoptosis. PKA I is reported to be over-expressed in various cancer cell lines and primary tumors, whereas normal or immortalized cells show a low basal PKA levels and activity. Many studies have correlated elevated PKA I expression or activity with tumorigenesis. Higher levels or activity of PKA I in cancer cells could be one of the key factors regulating the selective activation of Par-4 in cancer cells. However, it is important to note that phosphorylation of T155 is necessary but not sufficient to drive nuclear localization of Par-4, suggesting that other mechanisms also potentially regulate this process [26].

7.3 The Leucine Zipper Domain

The C-terminal leucine zipper domain of Par-4 has been shown to be involved in interactions with many other proteins in the cytoplasm and the nucleus to facilitate and/or to regulate Par-4 function. Wilm's tumor protein (WT1) is one of the earliest recognized partners of Par-4. WT1 is important in the development of the urogenital system, and its inactivation is associated with the etiology of Wilm's tumor. WT1, a zinc finger containing protein, can function both as a transcriptional activator and a repressor, depending upon the promoter and different physiological contexts; and it is a known inducer of the *Bcl-2* gene [27]. The expression patterns of the anti-apoptotic Bcl-2 and the pro-apoptotic Par-4 proteins show inverse

relation in prostate tumors [28]. Par-4, by binding to the zinc finger domain of WT-1 through its leucine zipper domain, regulates Bcl-2 expression by functionally inhibiting WT1 mediated transcription of Bcl-2 [29].

Par-4 is also known to interact with the regulatory zinc-finger domain of atypical protein kinases C (aPKCs)- PKCζ and PKCλ/ι- and inhibit their catalytic functions. These aPKCs are regulated by the lipid messenger ceramide, and are involved in cell survival mechanisms by mediating transactivation of genes regulated by NF-κB and AP-1. PKCζ phosphorylates IκB, thereby releasing NFκB from its inhibitory complex, and allowing for activation of NF-κB mediated transcriptional activation. Par-4 inhibition of PKCζ enzymatic activity and consequent loss of NF-κB and AP-1 mediated generation of cell survival signals constitutes one of the mechanisms of Par-4 induced apoptosis [30].

The leucine zipper domain of Par-4 is also deemed important in apoptosis induced by several partner proteins of Par-4, such as THAP1 [31] and ZIP-kinase (ZIPK or DAP-like kinase or Dlk) [32, 33]. Recent research has shown that Par-4 expression in breast cancer cells causes apoptosis via induction of ZIP kinase which leads to Myosin Light Chain 2 (MLC2) phosphorylation resulting in multinucleation and cytokinesis failure [10]. The leucine zipper is also involved in the interaction of Par-4 with topoisomerase I (TOPO-I). TOPO-I is an enzyme that relaxes the positive and negative supercoils in the DNA molecule, thus rendering it more amenable to fundamental biological processes of DNA transcription, replication and recombination. TOPO-I activity is increased in rapidly proliferating cells; consequently, various types of cancers exhibit elevated TOPO-I levels or activity. Par-4 binds to TOPO-I via its leucine zipper, thereby sequestering it from the DNA. Thus, Par-4 binding to TOPO-I inhibits its catalytic ability, creates a functional block in DNA relaxation, gene transcription, cell cycle progression, and suppresses cellular transformation [34].

7.4 The SAC Domain

Structure functional analysis of Par-4 protein has identified a unique 59-amino acid domain that is sufficient to induce apoptosis in a wide spectrum of cancer cells, but not in normal cells- and hence is aptly termed as ‘Selective for Apoptosis of Cancer Cells’ (SAC) domain. The SAC domain comprises amino acids 137–195, a segment of Par-4 that includes the NLS2 domain (which, as mentioned above, is essential for nuclear translocation) and the T155 site (which is important for Par-4 activation), and excludes the C-terminal leucine zipper domain. Interestingly, SAC domain is 100 % conserved among mammals (including humans, rodents and chimpanzee), chicken, frog and zebrafish. SAC is designated as the minimal core domain of Par-4 that is capable of nuclear entry, activation of Fas pro-apoptotic pathway, inhibition of NF-κB activity, and induction of apoptosis selectively in cancer cells. Characterization of the SAC domain in numerous cell types reveals that it enters the nucleus in cancer cells as well as in normal cells. The apoptotic action of SAC

appears to be broader and includes not only Par-4-sensitive cancer cells (e.g. PC3, DU145, H460, NIH/Ras), but also a wide array of cell lines resistant to apoptosis by full-length Par-4, such as LNCaP, MDA 2b, A549, MCF-7 etc. However, normal and/or immortalized but non-transformed cells such as PrE, PrS, HMEC, MCF10a, NIH/3 T3 remain unharmed by SAC over-expression [21].

Mechanistically, SAC induces apoptosis in cancer cells in the same manner as Par-4, i.e. by promoting Fas/FasL and FADD-dependent caspase activation and co-parallel inhibition of NF-κB activity. Since T155 site is retained in the SAC molecule, it is subject to enhanced phosphorylation at the T155 residue by PKA I specifically in cancer cells (which over-express PKA I). On the other hand, these studies demonstrate that the leucine zipper domain at the C-terminus of Par-4 is not required for its apoptotic function. Considering the role of this domain in protein-protein interactions, the leucine zipper appears more important for the regulation of Par-4 function by proteins such as WT-1, PKCζ, Akt etc. The loss of the leucine zipper means that the binding interaction with, and thereby, the negative regulation by Akt1 is lost, which enables the SAC domain to induce apoptosis in all types of cancer cells tested, irrespective of their sensitivity towards Par-4. Consistent with cell culture studies, transgenic animal models over-expressing the SAC domain show that ubiquitous and sustained expression of the SAC protein is harmless to normal development and physiological processes in these mice while demonstrating significantly higher resistance to the development of spontaneous as well as induced tumors.

7.5 SAC Transgenic Mice Show Tumor Resistance

A 174-bp DNA construct containing the GFP-tagged SAC domain of rat Par-4 protein cloned into a pCAGGS plasmid vector (which has a chicken β-actin promoter and a CMV enhancer) was used to generate transgenic mice that ubiquitously and constitutively express the SAC transgene. GFP transgenic mice were also generated as control. Both SAC-GFP and GFP transgenic mice were made using the B6C3F1 background. The SAC transgenic mice have proved to be physiologically and developmentally normal when scored in terms of fertility, gender ratio, viability, body weight and aging characteristics [35].

Murine embryonic fibroblasts (MEFs) derived from SAC mice and control littermates were tested for anti-tumor and apoptotic function of SAC under conditions of oncogenic transformation by Ras or c-myc, and the results revealed that overexpressing the SAC domain results in ten-fold higher rates of apoptosis in these MEFs, and also protects them against oncogene-induced transformation. *In vivo*, SAC transgenic mice show remarkable resistance towards the development of spontaneous tumors as well as towards oncogene-inducible tumor growth. At $\geq$18 months of age, spontaneous hepatocarcinomas and lymphomas were observed in the livers and spleens of about 50 % of B6C3F1 control mice. None of the SAC transgenic mice showed the presence of spontaneous tumors in any organs. Similarly, SAC transgenic mice, when crossed with transgenic TRAMP mice that

produce adenocarcinoma of the mouse prostate, showed conspicuously less (21.4 %) progression of the disease from prostate intra-epithelial neoplasia (PIN) to adenocarcinoma, as opposed to the control $GFP^{+/-}/TRAMP^{+/-}$ and $SAC^{-/-}/TRAMP^{+/-}$ mice that showed 100 % progression to adenocarcinoma at 6 months of age. Moreover, in $SAC^{+/-}/TRAMP^{+/-}$ mice, SAC transgene expression was observed to be down-regulated within the PIN lesions, and in all adenocarcinoma sections, indicating that loss of SAC is a pre-requisite for progression into malignant disease. Overall, these results reiterate the cancer cell-specific apoptotic action and tumor-suppressive potential of the SAC domain.

Following the discovery of Par-4 and its role as a tumor suppressor the majority of studies revolved around studying its functions as an intracellular protein. However in 2009 a seminal paper by Burikhanov et al. showed that Par-4 gets secreted out of the cells and available in systemic circulation. It was seen that both, full length Par-4 and the primary apoptotic domain SAC, are both secreted and by normal and immortalized cells alike. The secreted proteins retained their pro-apoptotic potential and were able to selectively target cancer cells when applied from the outside. The secreted Par-4 was found to induce apoptosis through its pro-apoptotic SAC domain and this was confirmed by using conditioned medium from cells in culture and also using recombinant Par-4 and SAC proteins. The secretion of Par-4 occurs, independent of apoptosis induction, through the classical secretory pathway involving the endoplasmic reticulum (ER) and golgi network and the secretion can be inhibited using an inhibitor called Brefeldin A (BFA) which prevents ER golgi trafficking and thus blocks the secretion of Par-4. Using chemical inducers of ER stress it has been shown that increased ER stress promotes the secretion of Par-4 [36].

Using a series of elegant experiments it has been shown that Par-4 binds to GRP78 at the cell surface, which acts a receptor for Par-4. GRP78 is a stress protein and the binding of GRP78 to Par-4 at the cell surface results in the initiation of robust feedback loop which involves the trafficking of more GRP78 to the cell surface in a Par-4 dependent manner. The binding of Par-4 to GRP78 results in elevated ER stress which causes upregulation of the ER stress response protein PERK (PKR-like ER kinase) which activates caspase-8. The induction of PERK leads to the activation caspase-8 and caspase-3 through a FADD dependent mechanism. The cancer specific activity of secreted Par-4 is due to differential expression of GRP78 in cancer and normal cells. Cancer cells tend to display a higher number of GRP78 receptors at the cell surface thereby making them more susceptible to apoptosis by secreted Par-4 compared to normal cells [36].

The clinical relevance of extracellular Par-4/SAC protein was underscored when the intravenous injection of recombinant Par-4/SAC protein inhibited the metastasis of the highly aggressive Lewis Lung Carcinoma 1 (LLC1) cells in mice [37]. Par-4/SAC transgenic mice produce elevated levels of systemic Par-4/SAC and are known to be resistant to non-autochthonous tumors [36, 37]. The sera from the SAC transgenic mice also exhibited potent anti-tumor activity when applied to cancer cells in culture *ex vivo* [37]. Furthermore, bone marrow transplantation studies have shown that extracellular Par-4/SAC activity can be transferred from

cancer resistant transgenic mice to genetically normal mice [37]. The secreted Par-4/SAC was stable for over 4 months in the mouse model [37] which is suggestive of its potential in treatment of cancer patients lacking the necessary circulating levels of Par-4.

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Chapter 8
Tumor-Necrosis-Factor-Related Apoptosis-Inducing Ligand (TRAIL)

Simone Fulda

Contents

Abstract The concept to exploit death receptors for cancer therapy is very attractive, since these cell surface receptors have a direct connection to the intracellular cell death machinery. Among the death receptor superfamily, the tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) receptor/ligand system is of special interest. TRAIL receptor agonists have recently entered the stage of clinical evaluation for the treatment of human cancers. Further insights into the regulatory mechanisms of TRAIL signaling will help to better understand the determinants of TRAIL sensitivity versus resistance of human cancers.

Keywords Cancer • Cell death • Apoptosis

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8.1 Introduction

Tissue homeostasis is the result of a delicate balance between cell proliferation on one side and cell death on the other side [1]. This general principle forms the basis of various physiological processes and is typically disturbed in human diseases. For example, too little cell death can contribute to the formation of cancer [1]. In addition, the inability of current anticancer therapies to suppress tumor growth or to cause regression of tumors is often caused by defects in cell death programs [2]. This implies that a better understanding of the regulatory mechanisms that govern this tight balance between cell survival and cell death will likely form the basis for the development of rational, molecularly defined treatment strategies. The enormous research efforts performed over the last two decades have resulted in the development of therapeutic strategies aiming at targeting the cell death machinery in cancer cells [3]. Some of these approaches have already been transferred into a clinical application. Among these, the concept to target death receptors on the cell surface to elicit cell death in tumor cells has attracted considerable attention [4]. In particular, the TRAIL receptor/ligand system that belongs to the death receptor family is considered among the death receptor family members as the most suitable target for therapeutic exploitation in human cancers [5]. The current review will provide an overview on the concept to target the TRAIL receptor/ligand system for the treatment of human cancers.

8.2 Apoptosis Signaling Pathways

Apoptosis represents one of the most common and best studied forms of programmed cell death that is evolutionary highly conserved across various species [6]. In a variety of physiological processes, apoptotic cell death plays an important regulatory role to ensure tissue homeostasis. Furthermore, the deregulation of apoptosis has been implicated as a causative event in a large variety of human diseases, including cancer, as too little apoptosis can promote tumorigenesis and treatment resistance [1]. In principle, two major apoptosis signaling pathways have been delineated, the death receptor (extrinsic) and the mitochondrial (intrinsic) pathways of apoptosis [6] (Fig. 8.1). In the death receptor signaling system, the binding of death receptor ligands to their cognate death receptors on the cell surface is the initial event that leads to the oligomerization of death receptors in the plasma membrane, thereby forming a cell death signaling platform that transmits the apoptotic signal from the cell surface into the intracellular signaling machinery of a cell [7]. More specifically, ligation of TRAIL receptors by TRAIL and the subsequent oligomerization of TRAIL receptors initiate the recruitment of adapter molecules such as FADD as well as signaling molecules such as caspase-8 to form the death-inducing signaling complex (DISC) [7]. This membrane-bound platform promotes activation of caspase-8 by induced proximity. Once activated,

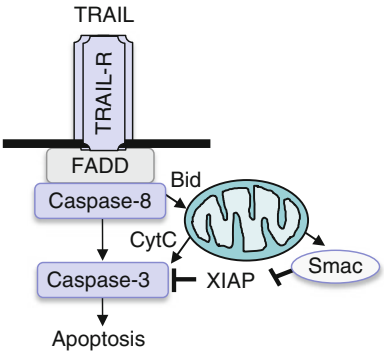

Fig. 8.1 Scheme of apoptosis pathways. There are two key apoptosis signaling pathways, i.e. the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. Stimulation of TRAIL receptors (TRAIL-R) by TRAIL leads to activation of caspase-8, which either directly activates downstream caspases or initiates the mitochondrial pathway by cleaving Bid. Engagement of the intrinsic pathway results in the release of mitochondrial proteins such as cytochrome c or Smac into the cytosol, which in turn promotes activation of caspase-3 and apoptosis. See text for more details

caspase-8 can transmit the cell death signal to downstream events either via cleavage of Bid or, alternatively, directly via cleavage of the effector caspase-3. In the first scenario, caspase-8-mediated proteolytic processing of Bid results in the generation of truncated Bid (tBid) which in turn translocates to mitochondrial membranes to engage the mitochondrial pathway of apoptosis. In the second scenario, proteolytic processing of caspase-3 by caspase-8 leads to caspase-3 activation which in turn cleaves a range of substrates, thereby culminating in the effector phase of apoptosis. In the mitochondrial pathway, a large variety of upstream signals, including proapoptotic proteins of the Bcl-2 family such as tBid, intracellular signaling molecules, endogenous stress stimuli or exogenous cytotoxic agents, including chemotherapeutic drugs, can all promote mitochondrial outer membrane permeabilization and the release of intermembrane proteins of the mitochondria into the cytosol, including cytochrome c and Smac [8]. Once in the cytosol, cytochrome c forms a multimeric complex together with Apaf-1 and caspase-9 which culminates in the activation of caspase-9 as the initiator caspase within the mitochondrial pathway of apoptosis. Furthermore, the release of Smac from the mitochondrial intermembrane space into the cytosol leads to the neutralization of Inhibitor of Apoptosis (IAP) proteins, a family of antiapoptotic proteins that block apoptosis by binding to and inhibiting effector caspases such as caspase-3 and -7. Since activation of apoptosis pathways can have detrimental effects on cellular integrity, several breaks are in place that control the activation status of the apoptotic signaling network at various levels to avoid accidental engagement of this signaling network [2]. In cancer cells, there is typically a dominance of such antiapoptotic programs which contributes to the inability of cancer cells to engage the apoptotic machinery [2].

8.3 The TRAIL Receptor/Ligand System

The TRAIL receptor/ligand system is part of the death receptor family that belongs to the tumor necrosis factor (TNF) receptor superfamily of receptor/ligand systems [7]. As reflected by the name, death receptors have been initially primarily implicated in the regulation of cell death. By the virtue of transmembrane receptors on the cell surface, death receptors can transmit extracellular signals from the exterior into the inside of the cell, thereby contributing to the communication of individual cells with their microenvironment. For the TRAIL system, four different transmembrane receptors have been identified, including two agonistic TRAIL receptors (TRAIL-R1, TRAIL-R2) as well as two antagonistic TRAIL receptors (TRAIL-R3, TRAIL-R4). This organization implies that there is already an inbuilt dichotomy in the TRAIL pathway. Since the natural ligand TRAIL can in principle bind to all these four receptors, TRAIL can not only engage cell death pathways but also contribute to cell survival via the engagement of antagonistic TRAIL receptors. While this simplistic model of agonistic and antagonistic TRAIL receptors leading to pro-death and pro-survival signals has initially been proposed to account for the differential sensitivity of tumor cells compared to non-malignant normal cells towards stimulation with TRAIL, subsequent studies revealed that the regulatory mechanisms of TRAIL resistance versus sensitivity are much more complex [7, 9, 10]. Thus, in addition to expression levels of pro- and antiapoptotic TRAIL receptors, various intracellular signaling events can contribute to the regulation of TRAIL signaling in cancer cells. A further level of complexity is due to the fact that TRAIL can interfere with signal transduction to apoptosis not only by binding to antagonistic TRAIL receptors but also by engaging cell survival pathways via one of the agonistic TRAIL receptors. Accordingly, TRAIL has been shown to stimulate activation of the transcription factor nuclear factor kappa B (NF-κB) upon binding to TRAIL-R1 or TRAIL-R2 [9]. Furthermore, kinase signaling cascades that eventually promote cell survival such as the PI3K/Akt/mTOR pathway or the MAK/ERK pathway can be engaged upon ligation of TRAIL receptors [9]. Thus, TRAIL cannot only signal for cell death but also for cell survival, in principle also in the same cell. In light of this dichotomy in the TRAIL receptor/ligand system, TRAIL can promote a variety of cell survival cascades leading, for example, to proliferation, migration, invasion and even metastasis, especially in cancers in which the cell death signaling part of the signaling network is impaired [9]. Accordingly, TRAIL has been reported to paradoxically stimulate proliferation in leukemia cells that are resistant to TRAIL-mediated apoptosis by engaging NF-κB-mediated signaling events [11]. Similarly, resistance to TRAIL-induced cell death shifted the TRAIL response towards an increased metastatic behavior of pancreatic carcinoma cells [12]. These reports underscore the importance to better understand the signaling events that are elicited in cancer cells in a context-dependent manner upon exposure to TRAIL.

8.4 Pro- and Antiapoptotic Mechanisms that Control Cancer Cells' Sensitivity Towards TRAIL

There is a large variety of different molecular mechanisms that are involved in the regulation of TRAIL-mediated cell death. Typically, these processes are altered in such a way in cancer cells that they eventually result in decreased susceptibility of cancer cells to undergo TRAIL-mediated cell death. This implies that proapoptotic events are typically downregulated, whereas antiapoptotic programs are aberrantly engaged. Finally, the deregulation of these regulatory events contributes to a decreased sensitivity or even complete resistance to TRAIL-induced apoptosis.

Signaling via the TRAIL receptor signaling cascade can already be disturbed at the receptor level. For example, downregulation of agonistic TRAIL receptors on the cell surface or mutations affecting TRAIL receptors have been reported in different cancer types and have been implicated to confer TRAIL resistance [13, 14]. In addition, epigenetic modifications, including hypermethylation, of TRAIL receptor promoters [15, 16] or impaired transport of TRAIL receptors from intracellular stores to the cell surface are possible causes for decreased TRAIL receptor surface expression [17]. Also, the antagonistic TRAIL receptors have been reported to interfere with signaling to cell death via agonistic TRAIL receptors [18–21]. Moreover, TRAIL receptors can be posttranslationally modified, for example by O-glycosylation [22]. In this respect, sensitivity to TRAIL-induced apoptosis has been linked to expression levels of enzymes that are involved in O-glycosylation, including GALNT-14 and fucosyltransferase-3/6 [23].

Besides deregulation of TRAIL receptors on the cell surface, receptor-proximal signaling events at the level of the DISC can be impaired by antiapoptotic proteins that interfere with the formation of the TRAIL DISC [10]. Cellular FLICE-like inhibitory protein (cFLIP) and phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes-15KDA (PED/PEA-15) are two antiapoptotic proteins that contain a death domain which allows them to interact with proteins of the TRAIL DISC, thereby blocking the transmission of the proapoptotic signal and preventing caspase-8 activation [24–30]. Overexpression of FLIP is a frequent event in human cancers and has been correlated with resistance to the induction of apoptosis, including TRAIL-mediated cell death [27, 28]. Vice versa, downregulation of cFLIP expression, for example by genetic knockdown or by pharmacological inhibition, has been shown to resensitize cancer cells to TRAIL-mediated apoptosis, demonstrating that cFLIP represents an important negative regulator of TRAIL-induced cell death [25, 27].

Another key regulator of TRAIL-induced apoptosis is caspase-8 which, besides caspase-10, represents the initiator caspase that is engaged during TRAIL-induced apoptosis [31]. While mutational events affecting caspase-8 have so far not commonly been detected in human cancers, epigenetic mechanisms have been implicated in suppressing caspase-8 levels in human cancers [31]. For example, hypermethylation of a regulatory motif that controls caspase-8 expression has been shown to be responsible for low or even absent caspase-8 expression in several

cancer entities, resulting in resistance or decreased sensitivity to TRAIL-induced apoptosis [32–38]. Accordingly, treatment with demethylating agents was able to restore caspase-8 expression levels, thus conferring sensitivity towards TRAIL [33, 34, 38]. Also, histone deacetylase inhibitors as well as certain cytokines such as interferons have been shown to upregulate caspase-8 expression levels, indicating that additional events contribute to the regulation of caspase-8 expression in human cancers [33]. For example, the caspase-8 promoter region contains interferon-responsive elements providing a molecular explanation for the interferon-mediated upregulation of caspase-8 expression [39]. Furthermore, caspase-8 function can be suppressed in a dominant-negative manner by aberrant expression of a splice variant of caspase-8, i.e. caspase-8 long (caspase-8L) [40–43]. This variant of caspase-8 was detected both in cancer cells such as leukemia or neuroblastoma cells as well as in non-malignant cells, i.e. CD34-positive hematopoietic progenitor cells, indicating that caspase-8 activity is fine-tuned by this variant in both normal and malignant cells. Caspase-8L interferes with caspase-8 activation by competing with wild-type caspase-8 for the recruitment into the TRAIL DISC. Additional regulatory mechanisms that control caspase-8 activity include posttranslational alterations of caspase-8, e.g. phosphorylation events. For example, kinase signaling networks can impinch on caspase-8 activation by inhibitory phosphorylation events. The tyrosine kinase Src has been reported to phosphorylate caspase-8 on one specific residue (tyrosine-308) which impairs the enzymatic function of caspase-8 [44].

8.5 Strategies to Target the TRAIL Receptor Ligand System for Therapeutic Purposes

Since TRAIL receptors are directly linked to the cell death machinery of cancer cells, the targeted activation of TRAIL receptors has attracted much attention over the last years. In principle, the TRAIL-mediated signal transduction pathway can be engaged by TRAIL ligand either in its soluble form or membrane-bound [5]. Alternatively, antibodies that bind to one of the two agonistic TRAIL receptors, i.e. TRAIL-R1 or TRAIL-R2, can be used to elicit cell death in cancer cells [5].

8.5.1 TRAIL Receptor Agonists

TRAIL receptors represent promising targets for therapeutic exploitation in the treatment of human cancers for the following reasons [5]. First, they are transmembrane cell surface receptors that can be engaged via their natural ligand TRAIL or, alternatively, by recombinant antibodies directed against one of the two agonistic TRAIL receptors [7]. In addition, the intracellular domains of TRAIL receptors are

directly connected to the cell death machinery inside the cell [7]. This implies that intracellular signaling cascades are immediately engaged upon TRAIL receptor ligation, which eventually leads to activation of caspases as cell death effector molecules. Second, TRAIL receptor engagement has been reported to preferentially and almost selectively trigger apoptosis in cancer cells whilst sparing non-malignant normal cells, although the molecular basis for this tumor cell selectivity of TRAIL receptor agonists has not yet been identified [5].

Recombinant soluble TRAIL or agonistic antibodies directed against TRAIL-R1 or TRAIL-R2 have been evaluated in numerous cancer types *in vitro* as well as *in vivo* and displayed considerable antitumor activities in a substantial proportion of cancers [5, 45, 46]. Notably, TRAIL-R2 antibodies were also described to mount a tumor-specific response via memory T-cells that protected from tumor relapse [47]. This implies that TRAIL receptor agonists may exert anticancer activities not only via their cancer cell-specific actions, but also via modulation of the immune response. However, a fraction of cancers did not fully respond to monotherapy with TRAIL, thus calling for TRAIL-based combination therapies. This aspect will be discussed in more detail in the next paragraph. Furthermore, TRAIL has been engineered by gene therapy approaches to be delivered to the tumor site either via a vector-based system or via the means of cell therapy [48–50]. For example, an adenoviral vector system was used to deliver TRAIL to tumor cells, where its expression was driven by an hTERT resulting in tumor cell apoptosis, inhibition of tumor growth *in vivo* and increased survival in this mouse model [48]. A cell therapy-based approach consisted of mesenchymal stem cells (MSCs) that were engineered to express TRAIL and that migrated to the primary tumor site as well as to distant metastases, where they delivered TRAIL [49]. In a mouse lung cancer model, TRAIL delivery via MSCs succeeded to suppress tumor growth via the induction of apoptotic cell death [50].

8.5.2 TRAIL-Based Combined Treatments

Although most cancers express one or the other agonistic TRAIL receptor, not all cancer types are susceptible to TRAIL-mediated apoptosis. This implies that intracellular regulators of TRAIL sensitivity may impinge on signal transduction pathways to apoptosis and can eventually even block cell death induction despite successful engagement of TRAIL receptors by TRAIL receptor agonists. Accordingly, there are many studies showing that surface expression levels of TRAIL receptors do not reflect the sensitivity of individual cancers towards TRAIL [51].

The recognition that a substantial proportion of cancers do not sufficiently respond to monotherapy with TRAIL receptor agonists has also led to the design of a large variety of combination therapies, using on one side a strategy to target TRAIL receptors and on the other side an approach to enhance cancer cells'

sensitivity towards TRAIL. This type of combination therapies ranges from cotreatment with chemotherapeutics, radiotherapy or immunotherapy to a multitude of signal transduction modulators and small-molecule inhibitors. As far as conventional chemotherapeutic agents are concerned, chemotherapeutic drugs of various classes, including e.g. topoisomerase-1 and -2 inhibitors, antracyclines, antimetabolites, microtubule-interfering agents and intercalating agents, just to name a few, have been shown to act together with TRAIL receptor agonists in an additive or synergistic manner in various cancer entities [52–58]. On mechanistic grounds, this additive or synergistic drug interaction has been linked to upregulation of TRAIL receptors by DNA-damaging drugs, enhanced oligomerization and aggregation of proapoptotic death receptors upon drug treatment or increased recruitment of adaptor or signal molecules to the activated TRAIL receptor complex at the cell membrane. In addition, chemotherapeutic drugs may also alter intracellular signaling events that facilitate signal transduction via the TRAIL receptor pathway.

In addition to classical chemotherapeutic drugs, inhibitors of the proteasome have also turned out to represent a promising strategy to enhance cancer cells' sensitivity towards TRAIL [59–66]. Bortezomib (Velcade, PS-341) represents one of the best-known proteasome inhibitors that reversibly block the proteolytic activity of the proteasome. Structurally, bortezomib represents a dipeptidyl boronic acid. In combination with TRAIL receptor agonists, bortezomib has been shown to synergistically trigger apoptotic cell death in various cancer entities, both *in vitro* as well as *in vivo* mouse models of human cancers. This bortezomib-mediated sensitization towards TRAIL has been linked to various cellular events that eventually lower the threshold for apoptosis induction. For example, bortezomib has been shown to increase expression levels of one of the two TRAIL receptors TRAIL-R1 or TRAIL-R2. Also, the formation of the TRAIL receptor-bound signaling platform to activate caspase-8, i.e. the TRAIL DISC, has been reported to be enhanced upon exposure to bortezomib. In addition, bortezomib has been described to change the ratio of various pro- and antiapoptotic proteins that are known to contribute to the regulation of apoptosis sensitivity, for example cFLIP, caspase-8, p53 as well as various pro- and antiapoptotic Bcl-2 family proteins. Furthermore, bortezomib is an important inhibitor of NF-κB activation, as it blocks the proteasomal degradation of IκBα proteins that require proteasomal degradation in order to allow IκBα to translocate to the nucleus and activate gene transcription.

Another class of targeted agents that prove to synergize together with TRAIL receptor agonists is represented by histone deacetylase inhibitors (HDACi) [67–72]. Again, various intracellular events have been shown to contribute to this HDACi-mediated sensitization towards TRAIL in a cell type- and context-dependent manner. Signal transduction via the TRAIL pathway can be modulated at various levels by HDAC inhibitors, for example, via upregulation of TRAIL-R1 or TRAIL-R2 on the cell membrane, enhanced recruitment of TRAIL receptors into lipid rafts and differential modulation of apoptosis-regulatory proteins towards an increased ratio of pro- to antiapoptotic proteins.

8.5.3 Clinical Studies with TRAIL Receptor Agonists

TRAIL receptor agonists have already entered the clinical stage and have been evaluated in a number of different clinical trials in the last years. These trials include studies using recombinant human TRAIL that binds to all TRAIL receptors [73–75]. In addition, agonistic monoclonal antibodies directed against one of the two agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2 were performed [73, 76–100]. Mapatumumab binds to TRAIL-R1, whereas lexatumumab, conatunumab, drozitumab, tigatuzumab and LBY135 bind to TRAIL-R2. After initial dose-response and toxicity studies, many of these early clinical trials were performed as combination protocols in order to exploit cooperative drug interactions. Such combination protocols comprise, besides TRAIL receptor agonists, conventional chemotherapeutic drugs or, alternatively, various kinds of signal transduction modulators, including kinase inhibitors, monoclonal antibodies or antiangiogenic drugs as well as proteasome inhibitors, histone deacetylase inhibitors or interferons.

8.6 Conclusions

The TRAIL receptor ligand system represents a promising signal transduction pathway that can be exploited for anticancer therapies. TRAIL-based combination therapies together with conventional chemotherapeutics or molecular-targeted agents will likely be superior to monotherapy with TRAIL or TRAIL receptor agonists to achieve maximal tumor growth inhibition. Although the cancer cell-selective killing properties of TRAIL have not yet been fully elucidated on a molecular basis, the preferential induction of cell death in tumor versus non-malignant normal cells by TRAIL receptor agonists represents a unique opportunity for cancer selectivity. One future challenge will be to select the population of patients that will most likely respond to TRAIL receptor agonists. To this end, suitable biomarkers need to be identified. TRAIL receptor expression levels in tumor tissue may not be helpful, since preclinical studies did not provide convincing evidence showing that the responsiveness to TRAIL receptor agonists is primarily dictated by the expression pattern of agonistic and antagonistic TRAIL receptors on the cell surface [51]. The engagement of the cell's intrinsic cell death program by ligation of TRAIL receptors will likely offer an exciting treatment option of cancers, once the full potential of this strategy will be harnessed.

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Chapter 9
SIRT6: A Promising Target for Cancer Prevention and Therapy

Michael Van Meter, Vera Gorbunova, and Andrei Seluanov

Contents

Abstract Many of the pathologies associated with the aging process also contribute to tumor initiation, growth or metastasis. Insights from biogerontology may be instrumental for developing new therapies for cancer. This chapter highlights the rationale for combining biogerontology and cancer research to generate new strategies for cancer treatment. In particular, this chapter focuses on one gene, SIRT6, which has emerged as an important regulator of longevity in mammals and appears to have multiple biochemical functions, which antagonize tumor development and may be useful in cancer prevention and treatment.

Keywords SIRT6 • Cancer • Longevity gene • Metabolism • Genome stability • Age-related disease • Warburg effect • Anti-cancer gene

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9.1 Introduction: Understanding Cancer as an Age-Related Disease

In mammals, increased age is the single greatest risk factor for the onset and development of cancer. Epidemiological studies indicate that, in humans, for adults, the odds of developing cancer increase exponentially during each year of life [1–4]. This is especially true for tumors of epithelial origin, such as breast and prostate carcinomas [5, 6]. Consequently, many of the pathologies canonically associated with the aging process also actively contribute to tumor development. For instance, decreased immune system efficiency, increased inflammation, irregular metabolism and the accumulation of senescent cells are all system-wide hallmarks of aging which also play an important role in the initiation and development of tumors [7, 8]. Additionally, age-related changes at the cellular level are also understood to predispose cells to tumorigenic transformation [9–11]. Most notably, during aging, cells accumulate disruptive mutations in key cellular pathways, including: apoptosis, cell cycle control, DNA repair, cytokine production and energy metabolism. As these pathways become perturbed, they lose their regulatory power and spontaneous neoplasms become more likely to emerge. This link between aging and cancer has prompted many researchers to investigate the cellular pathways which contribute to aging phenotypes in order to gain insight into the molecular mechanisms driving oncogenesis [3, 12]. Moreover, it is hoped that understanding the shared etiology of cancer and aging will assist in developing therapies that may prove useful in forestalling and treating this age-related disease [13].

One of the key findings from aging research in the context of cancer and cancer therapy is the observation that environmental, pharmaceutical and genetic manipulations which extend lifespan also tend to reduce the incidence and delay the onset of cancer. Perhaps the best characterized example of this phenomenon is caloric restriction. Multiple studies in a variety of different model organisms have demonstrated that when animals are fed a reduced calorie diet, without malnutrition, that the animals typically exhibit increased mean and maximum lifespans, concomitant with a delayed onset and reduced incidence of cancer [14, 15]. Similar observations have been made for pharmaceutical compounds, such as rapamycin, which extends lifespan and simultaneously delays and antagonizes tumorigenesis in multiple model systems [16–19]. Finally, a variety of genetic manipulations are known to extend lifespan in different model systems, including: overexpression of Catalase, Klotho and Sir2, or downregulation of FOXO, Growth Hormone and TOR; in accordance with dietary and pharmaceutical modulations, most of these genetic manipulations also result in a reduced risk of tumorigenesis [20–25]. Importantly, many of these studies have served as a foundation for further preclinical and clinical trials in the context of human cancer therapy. For example, there are currently over a dozen rapamycin-family compounds in clinical oncology trials for use as a single agent or in conjunction with other anti-cancer therapy regimens [26].

Mechanistically, these "longevity genes", or genes whose expression regulate lifespan, appear to impact on carcinogenesis through cross-talk with oncogenic and tumor suppressive signaling pathways [27–29]. Klotho, for instance, is a transmembrane protein which modulates the activity of several signaling pathways relevant to breast cancer tumorigenesis, including Insulin Receptor, IGF-1 Receptor and Fibroblast Growth Factor Receptor signal transduction [30]. Other longevity genes impact on, or are targets of, core tumor suppressor genes such as p53 and PTEN, while other longevity genes inhibit the activity of canonical oncogenes such as Ras and Myc. The identification of molecular pathways through which longevity genes antagonize cancer development has made many of these genes prime targets for pharmaceutical intervention [31, 32].

9.2 Sirtuins: A Family of Longevity Genes

One class of promising targets are the mammalian homologues of the yeast gene, Silent Information Regulator 2 (Sir2). Sir2 is a well-conserved gene, and Sir2 homologs, dubbed "sirtuins", can be found in nearly all organisms, where they catalyze NAD+ dependent protein deacylation and mono-ADP ribosylation reactions [33, 34]. Initial experiments in yeast indicated that overexpression of Sir2 extended replicative lifespan by approximately 30 %, whereas deletion of the gene reduced replicative lifespan by nearly 50 % [21]. Subsequent experiments found that Sir2 homologs positively regulated lifespan and protected against age-related pathologies in nematode and drosophila experimental systems as well [33]. Over the past decade, dietary modulations including mild caloric restriction and decreased glucose consumption as well as the application of pharmaceutical products, including resveratrol, a natural compound found in red wine, and synthetic sirtuin-activating compounds (STACs) have been applied to activate sirtuins and mimic the effects of experimentally manipulating these genes [35–39].

These findings spurred research geared toward understanding whether sirtuins could modulate aging and age-related disease in mammals. There are seven Sir2 homologs (SIRT1-7) in mammals; of these, SIRT1 shares the most homology with Sir2 and is the best characterized. Interestingly, while SIRT1 overexpression does not extend lifespan in murine systems, overexpression of the gene does confer numerous other health-benefits and does delay the onset and protect against many age-related pathologies, including cardiac hypertrophy and neuropathies such as Alzheimer's disease and amyotrophic lateral sclerosis [40–43].

Less is currently known about the remaining mammalian sirtuin genes. SIRT2 is predominantly localized in the cytoplasm, but can shuttle to the nucleus under stress. In the cytoplasm SIRT2 deacetylates RIP1 to promote programmed necrosis among other known functions [44]. SIRTs 3, 4 and 5 localize to the mitochondria. Here, these proteins regulate acetyl-CoA synthesis, fatty acid oxidation and insulin secretion in pancreatic β-cells [45–47]. Of the mitochondrial sirtuins, SIRT3 appears most relevant in the context of aging, as the protein protects against the accumulation of oxidative damage, attenuates age-associated hearing loss and also has a tumor

suppressive function – it does not regulate lifespan, however [48, 49]. Recent studies into the function of SIRT6 indicate that this sirtuin meets many of the criteria of a longevity gene, and appears to function as a powerful tumor suppressor. SIRT6 will be discussed in greater detail below. SIRT7 is the least studied of the sirtuins. It appears to function as a positive regulator of RNA polymerase I transcription and may promote oncogenesis in certain contexts [50, 51].

9.3 SIRT6

Knockout mouse models have been generated for each of the seven sirtuins. Of these, the SIRT6 knockout mouse exhibits the strongest phenotype in the context of aging. Specifically, SIRT6 null mice develop a premature aging disorder with complete penetrance. This progeroid syndrome is characterized by genomic instability, spinal curvature, decreased bone mineral density, hypoglycemia and a severely shortened lifespan [52]. In contrast, mice overexpressing SIRT6 exhibit extended mean and median lifespans [53]. Cumulatively, these mouse models provide strong evidence that SIRT6 regulates lifespan in mammalian systems.

Follow-up cellular studies designed to understand the molecular mechanisms through which SIRT6 operates to promote longevity revealed that SIRT6 is a multi-faceted protein which functions in a diverse set of pathways. The cellular functions of SIRT6 include the stabilization of telomeres, coordination of DNA double strand break repair, regulation of cellular metabolic pathways, and suppression of NF-κB hyperactivity [54–59]. Intriguingly, each of these pathways has also been independently identified as critical regulators of longevity in multiple model organisms, although it is unclear if SIRT6 signaling through each of these pathways or only a subset of these pathways is required to regulate lifespan [60–62]. Mechanistically, SIRT6 mediates these diverse functions through its NAD^+ dependent deacylase and mono-ADP ribosyltransferase activities. SIRT6 is known to deacetylate H3K9, H3K56, and CtIP. Additionally, SIRT6 is known to mono-ADP ribosylate itself and PARP1, while also demyristoylating TNFα. Finally, SIRT6 impacts on gene expression by functioning as a co-repressor protein; for example, SIRT6 binds to Hif-1α, co-localizes with Hif-1α to its target promoters, where SIRT6 deacetylates H3K9, thereby downregulating expression of Hif-1α target genes.

Further cementing the role of SIRT6 as a longevity gene, several studies have observed that SIRT6 protein levels decline during aging – although the exact mechanism underlying this change remains unclear [63, 64]. Telomeric instability, inefficient double strand break repair, aberrant metabolic processes and diminished stress responses are, however, well-characterized pathologies associated with cellular aging. Given the important role that SIRT6 plays in regulating each of these pathways, it seems likely that the depletion of SIRT6 in old cells is contributing to the aging phenotype. Excitingly, several reports have now demonstrated that restoring SIRT6 levels in these cells reverses many of these age-related pathologies and rejuvenates the aged cells. For instance, during replicative aging, cells become

much less efficient at repairing DNA double strand breaks – a cytotoxic DNA lesion which contributes to genomic instability and can potentially lead to tumorigenic recombination of the genome; when SIRT6 is expressed in old cells, however, the homologous recombination efficiency of the aged cells increases three-fold and is nearly restored to youthful levels [63]. Increased SIRT6 expression appears to be sufficient to reverse system-wide age-related pathologies as well. For example, aging and failing hearts typically have lower levels of SIRT6. This depletion of SIRT6 leads to hyperactivation of AKT-IGF signaling and leads to cardiac hypertrophy and heart failure. When SIRT6 is overexpressed in cardiomyocytes, it dampens AKT-IGF signaling and protects against cardiac hypertrophy; similar results are observed in transgenic mice overexpressing SIRT6 in the heart [65].

Cumulatively, SIRT6 has emerged as a powerful longevity gene. In the absence of SIRT6 mice have a shortened lifespan, while overexpression of SIRT6 extends lifespan. Importantly, SIRT6 also protects against and forestalls the onset of many age-related pathologies, both at the cellular and the organism level. Given the core role that SIRT6 plays in regulating longevity-related pathways, many researchers hypothesized that SIRT6 may function to suppress tumorigenesis. In particular, the role that SIRT6 plays in regulating energy metabolism and promoting genome stability suggested that SIRT6 may function as a tumor suppressor and have therapeutic applications in cancer therapy.

9.4 SIRT6 and Cancer

The severely shortened lifespan of the SIRT6 knockout mouse made it impossible to use that particular animal model to test whether SIRT6 impacted on tumorigenesis. Several preliminary pieces of evidence, however, provided strong support for the hypothesis that SIRT6 functions as a tumor suppressor gene. First, the SIRT6 chromosomal locus is mutated in a large number of acute myeloid leukemias [66], liver, breast and lung cancers [67]. Additionally, in the absence of SIRT6, cells exhibit global hyperacetylation of H3K56, a chromatin modification that is well-correlated with tumorigenicity and tumor grade [68]. Lastly, SIRT6 overexpression delayed the onset of lung tumor development in the whole-body overexpression mouse [53].

Pathway analysis also suggested that SIRT6 may function as a tumor suppressor gene. A common feature of nearly all cancer cells is the ability to sustain uncontrolled proliferation. Achieving this proliferative state typically requires the cells to acquire a series of mutations in multiple regulatory pathways. While there is no single recipe for mutations that confer tumorigenicity to normal cells, there are underlying commonalities between many cancer cells that enabled the acquisition of transforming mutations, namely: genome instability, inflammation, metabolic reprogramming and evasion of the host immune system. SIRT6 has been implicated as a negative regulator of three of these pathways – genome instability, inflammation and metabolic reprogramming – suggesting that the protein might play an

important role in protecting against tumor formation. Several recent studies have more closely examined the role that SIRT6 plays in protecting against tumor formation. These studies will be discussed in more detail below.

9.4.1 SIRT6 Suppresses Tumor Formation by Regulating Cellular Metabolism

The metabolic profile and requirements of cancer cells differ sharply from those of non-transformed cells [69]. While normal cells primarily use mitochondrial oxidative phosphorylation to generate the ATP energy needed for cellular processes, cancer cells instead tend to catabolize glucose via glycolysis and lactic acid fermentation to generate the ATP needed to meet their energy requirements. It is unclear exactly why cancer cells utilize the less-ATP-efficient method for generating energy, but exploiting this phenomenon – termed the Warburg Effect – has proven useful in the diagnosis, detection and treatment of cancer.

The first hints that SIRT6 may be relevant in the context of the Warburg Effect and metabolic reprogramming (the transition from aerobic to anaerobic energy production, which appears to be a requirement for the initiation of many tumors) came from the observation that SIRT6 deficient cells exhibited abnormally high levels of glucose in the cytosol [59]. Interestingly, plasma glucose levels in the SIRT6 knockout mouse are actually quite low, and the mouse is characterized as hypoglycemic on this basis – what appears to be happening is that cells are importing glucose faster than the bloodstream can supply it, so even though glucose intake is normal at the organism level, at the cellular level the glucose profile of the animal is quite perturbed. Subsequent studies revealed that SIRT6 tightly regulates glucose homeostasis and catabolism by controlling the expression of a broad panel of glycolytic genes. Specifically, SIRT6 functions as a co-repressor of the Hif-1α transcription factor, deacetylating H3K9 at the promoters of many glycolytic genes, including GLUT1, PFK1, PDK1 and LDHA thereby suppressing their transcription. In the absence of SIRT6, Hif1-α signaling is upregulated, leading to increased glucose uptake (through increased expression of glucose transporters) and glycolysis as well as diminished oxidative respiration (through increased expression of glycolytic enzymes) (Fig. 9.1) [59].

To more directly assess whether SIRT6 functions as a tumor suppressor gene, researchers injected embryonic fibroblasts from wild type and SIRT6 knockout mice into immunodeficient mice [70]. Typically wild type embryonic fibroblasts will not form tumors unless they have been transformed with oncogenes. The researchers found, however, that SIRT6 knockout embryonic fibroblasts did form tumors in the immunodeficient mice, even in the absence of transforming mutations. Moreover, when SIRT6 was re-expressed in the knockout embryonic fibroblasts, the cells lost their ability to form tumors in the immunodeficient mice. The researchers then used an animal model to confirm these cell-based assays which

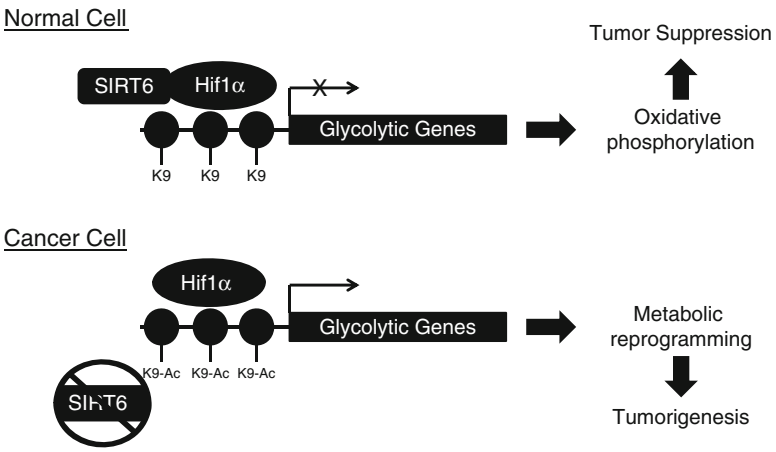

Fig. 9.1 SIRT6 suppresses metabolic reprogramming by repressing transcription of core glycolytic genes. SIRT6 functions as a co-repressor of Hif-1α; SIRT6 binds to Hif-1α and co-localizes with the protein to its target promoters. There SIRT6 deacetylates H3K9, thereby repressing transcription of Hif-1α target genes

suggested that SIRT6 functions as a tumor suppressor gene. Because SIRT6 knockout mice die young, the researchers instead created a mouse model where SIRT6 was specifically deleted in the intestines. When these mice were paired with APC knockout mice – an mouse model of accelerated intestinal tumorigenesis, the resulting $APC^{-/-}$;$SIRT6^{intestine-/-}$animals exhibited colorectal tumors at a much higher rate than $APC^{-/-}$controls. Collectively these experiments provided strong support for the hypothesis that SIRT6 is a tumor suppressor gene [70].

The researchers then sought to determine whether deletion of SIRT6 precipitated tumor development through the activation of oncogene signaling or through metabolic reprogramming. Interestingly, it was reported that while transformed SIRT6 knockout cells did not appear to have any distinct changes in their oncogenic signaling pathways, compared to transformed wild-type cells, the transformed knockout cells did exhibit a stark activation of glycolytic signaling pathways. Because transformed SIRT6 knockout cells were more tumorigenic than transformed wild type cells, the researchers reasoned that metabolic reprogramming was driving the observed tumorigenicity of the SIRT6 knockout cells. To confirm this, the researchers used shRNA targeting key glycolytic enzymes, including PDK1 to suppress glycolysis in the SIRT6 knockout cells. When glycolytic signaling was abolished using this approach, deletion of SIRT6 was no longer able to drive tumor formation, suggesting that SIRT6 suppresses tumorigenesis by regulating energy metabolism.

These results, based on mouse animal models, appear to be directly relevant to human cancer as well. Analysis of human cancer databanks reveals that the SIRT6 locus is deleted in nearly 35 % of over a thousand cancer cell lines analyzed (Fig. 9.2) [70, 71]. In particular, SIRT6 appears to play an important role in the manifestation of pancreatic and colon cancers, where the locus is deleted in 69.5 % and 29 % of those cancer cell lines, respectively [71]. Importantly, no

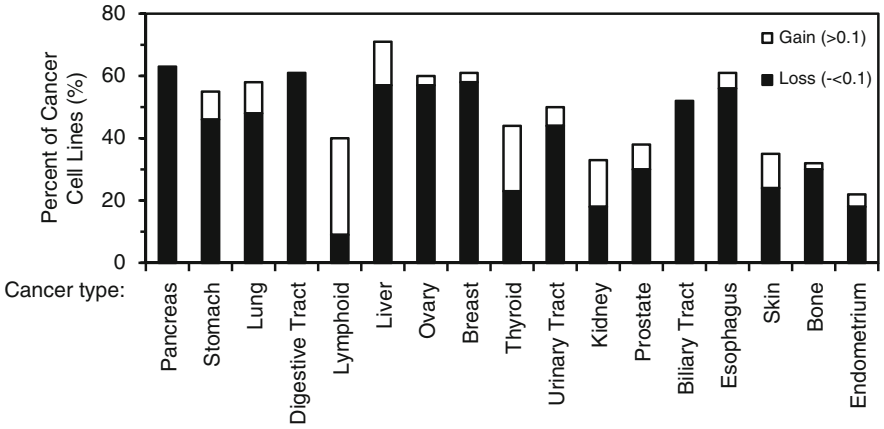

Fig. 9.2 Searching the Genome Cancer Atlas Portal, a copy number analysis dataset for human tumor cell lines, reveals that the SIRT6 locus is lost or perturbed in a wide array of cancer cell lines. Importantly, while SIRT6 is lost with great frequency in a wide range of human tumor samples, very rarely is the gene amplified

amplifications of the SIRT6 locus were observed in any of the pancreatic cancer cell lines and in only 4 % of the colon cancer cell lines [71]. Moreover, human cancer patients whose tumors expressed higher levels of SIRT6 were 2.2 times less likely to relapse than those patients whose tumors expressed low levels of SIRT6 [70]. In these human studies, activation of glycolytic signaling was also observed in conjunction with loss of SIRT6 [70]. Collectively, these experiments demonstrate that SIRT6 is a tumor suppressor gene that protects against carcinogenesis in animal models and in human patients.

Despite the strong evidence that SIRT6 antagonizes tumor formation by suppressing metabolic reprogramming, researchers have also suggested that SIRT6 may protect against tumor formation in metabolic-independent pathways. If true, this would broaden the importance of SIRT6 in protecting against tumor formation and enlarge the scope of therapeutic applications for activating SIRT6 in the context of cancer biology. Another such model of SIRT6 and tumor suppression is discussed below.

9.4.2 SIRT6 Suppresses Tumor Initiation by Mediating c-FOS Dependent Apoptosis

The canonical model of tumor initiation is that over time, cells acquire a series of mutations in a step-wise fashion, which may culminate in the transformation of the cell. Typically, however, the tumor suppressive machinery in the cell can detect pathway perturbations and permanently halt the cell cycle or initiate programmed

cell death before the aberrant cell acquires a sufficient number of transforming mutations. Suppression of this response is usually required for the successful transition for pre-cancerous legion to malignancy. Understanding the molecular mechanisms which drive the early stages of tumor initiation has long been a goal of cancer research because it has important implications in cancer prevention and therapy. Research in the context of hepatocellular carcinoma, a disease which causes over 500,000 deaths per year worldwide, indicates that SIRT6 is a critical component of the cellular machinery that initiates apoptosis in pre-malignant cells [72]. Typically, when liver cells begin to acquire transforming mutations, cell death is induced by activation of c-FOS mediated apoptosis. Specifically, activation of c-FOS in turn induces SIRT6 transcription which, in turn, represses transcription of Survivin by deacetylating H3K9 residues in the Survivin promoter. Survivin functions to inhibit caspase activation, thereby repressing apoptosis; many tumors express high levels of Survivin. As such, SIRT6 mediated silencing of Survivin leads to the initiation of apoptosis in pre-cancerous cells. These researchers found, however, that pre-malignant cancer cells were sometimes able to evade this response by suppressing c-FOS signaling. When c-FOS was suppressed, SIRT6 was unable to silence Survivin and the pre-cancerous cells progressed to a malignant state. Importantly, this transition from pre-cancerous legion to malignancy was independent of metabolic reprogramming. Moreover, using an epistasis approach, the researchers demonstrated that ectopic expression of SIRT6 was sufficient to reverse the deleterious effects of c-FOS silencing in the pre-malignant cells and prevented the transition of these cells to malignancy [72]. Using an animal model, it was also demonstrated that using adenoviruses to transiently overexpress SIRT6 in the livers of wild type mice during chemically induced tumor initiation markedly reduced liver tumorigenesis, suggesting that activating SIRT6 may be an important clinical tool for preventing hepatocellular carcinoma in at risk patients, such as patients with inflammatory liver disease, fatty livers or chronic hepatitis.

9.4.3 SIRT6 May Suppress Tumorigenesis by Promoting Genome Stability

While SIRT6 has clearly emerged as a potent tumor suppressor gene, the complete array of mechanisms by which it prevents tumorigenesis are still being elucidated. The abilities of SIRT6 to prevent the metabolic reprogramming of cancer cells and to initiate apoptosis in pre-malignant cells clearly are important mechanisms by which SIRT6 attenuates tumor growth and development. It is likely, however, that SIRT6 forestalls tumor formation through other, more subtle mechanisms as well. In particular, the role that SIRT6 plays in suppressing genome instability is likely to be important in this context.

Because pre-cancerous cells need to acquire multiple mutations in order to become malignant, cells which have higher levels of genomic instability are

predisposed to become tumorigenic. As such, genes which regulate DNA repair are often considered to be tumor suppressor genes. Indeed, animal models and human patients with loss-of-function mutations in genes encoding components of the DNA repair machinery typically exhibit higher incidences and an earlier onset of tumorigenesis [12, 62]. SIRT6 plays an important role in regulating DNA repair – especially, DNA double strand break repair via non-homologous end joining and homologous recombination. SIRT6 promotes DNA repair through both of these pathways by activating the upstream repair factor, PARP1 [56]. Additionally, SIRT6 facilitates homologous recombination by deacetylating and thereby activating the end resection protein, CtIP [54]. Importantly, in the absence of SIRT6, cells become extremely sensitive to DNA damage and exhibit abnormal levels of genomic instability. While the acute animal models described above did not observe a role for SIRT6 mediated preservation of genomic stability in tumor prevention, it is possible that other models, in which tumors are initiated at a slower, more natural pace, may find that the DNA repair function of SIRT6 is an important safeguard against tumorigenesis.

9.4.4 SIRT6: A Potential Role in Controlling Tumorigenesis by Suppressing Inflammation

Chronic inflammation is another hallmark of cancer. Several studies have indicated that inflammation is often initiated in cancer cells by activation of NF-κB signaling. Moreover, several cancer therapies which function to inhibit NF-κB have had modest clinical success – although broadly inhibiting NF-κB often has multiple, unintended side-effects, thus limiting the potential of this therapeutic strategy [73]. SIRT6 has emerged as an important inhibitor of chronic NF-κB signaling by deacetylating H3K9 at NF-κB target genes [55]. Moreover, the early lethality of the SIRT6 knockout mouse is in part due to chronic inflammation driven by constitutively active NF-κB signaling in the absence of SIRT6 [55]. Because SIRT6 appears to preferentially attenuate chronic NF-κB activity, activation of SIRT6 may provide an alternative route for silencing NF-κB in tumor cells addicted to its oncogenic signaling, thereby avoiding unwanted side-effects from more broadly suppressing basal NF-κB activity in non-transformed, healthy cells. It should also be noted that the role that SIRT6 plays in inflammation is not entirely understood. While SIRT6 suppresses NF-κB signaling, it also promotes the secretion of the cytokine, TNF-α, in certain contexts. The relevance of this in the context of cancer biology remains unclear, although several reports have indicated that in a narrow subset of conditions SIRT6 mediated secretion of TNF-α had an oncogenic effect [74]. Clearly more studies are needed to understand the regulatory role that SIRT6 plays in inflammatory pathways and how these pathways impact on tumorigenesis.

9.5 Therapeutic Activation of SIRT6

Given the broad role that SIRT6 plays in suppressing tumor formation (Fig. 9.3), there is considerable interest in developing methods for activating the protein in a clinical context. Excitingly, there is emerging evidence that SIRT6 can be activated through dietary, and pharmaceutical interventions. Moreover, there is good evidence that therapeutic activation of SIRT6 will have important clinical ramifications, not just in cancer therapy, but for other age-related diseases as well.

Because, Sir2 was activated by caloric restriction in yeast, nematode and fly systems, many researchers speculated that SIRT6 would also be activated by caloric restriction. Direct tests of this hypothesis revealed that animals fed a calorically restricted diet expressed nearly three times as much SIRT6 protein as control animals. Similarly, when human cells were cultured in low glucose and low serum conditions that mimic caloric restriction, SIRT6 was also upregulated by approximately three-fold [75]. This research suggests that SIRT6 may be activated by caloric restriction or by drugs which mimic the process. It is unclear, as yet, whether three-fold overexpression of SIRT6 is sufficient to confer significant protection against oncogenesis, but there is considerable evidence that caloric restriction does delay the onset of tumorigenesis in mammalian systems and it is entirely possible that increased levels of SIRT6 contributes to this phenotype [14, 76].

Resveratrol and its chemical derivatives were reported to enhance the enzymatic activity of SIRT1 by nearly a hundred-fold. While these chemicals do not appear to activate SIRT6, the shared homology of SIRT1 and SIRT6 suggests that it will be possible to generate pharmaceutical compounds which can enhance the activity of SIRT6. Moreover, there is good evidence that doing so would confer numerous health benefits to the patient. As previously, mentioned, transient overexpression of SIRT6, delivered by adenovirus, in pre-cancerous livers markedly impaired tumorigenesis in mice [72]. A study performed in our laboratory has shown that

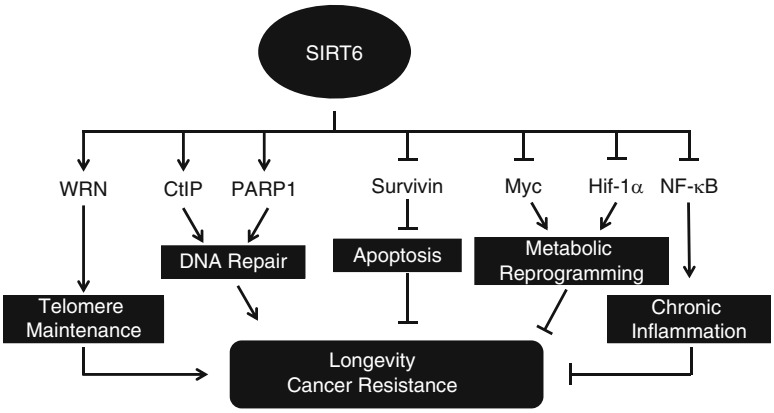

Fig. 9.3 SIRT6 functions in a wide-array of pathways relevant to longevity and cancer resistance. This figure summarizes those pathways and indicates how, collectively, SIRT6 signaling appears to converge on lifespan extension and cancer resistance

overexpression of SIRT6 in human cancer cell lines had an apparent therapeutic benefit [77]. In this study, overexpression of SIRT6 in a panel of cancer cells, including breast cancer cells, fibrosarcoma cells and cervical carcinoma cells drove massive apoptosis in these cancerous cell lines, suggesting that SIRT6 functions as an anticancer gene. Importantly, when SIRT6 was overexpressed in non-transformed control cell lines, no apoptosis was induced. Although the precise molecular mechanism of this effect remains elusive, apoptosis was driven redundantly though activation of both p53- and p73-dependent signaling pathways. Because either p53 or p73 are often mutated in cancer cells, the redundancy of this effect suggests that activation of SIRT6 may have broad therapeutic implications in a diverse array of cancer types. In addition to having a powerful role in suppressing and antagonizing tumor development and growth, SIRT6 activation appears to be a desirable strategy for several other diseases as well, most notably diet induced obesity and cardiac hypertrophy. In each case, animal models overexpressing SIRT6 were resistant to these pathologies, suggesting that pharmacological activation of SIRT6 may provide a similar protective effect. Additionally, SIRT6 overexpressing mice exhibited an increased lifespan, suggesting that SIRT6 activators may have long-term implications for human health.

9.6 Conclusion

Cumulatively, SIRT6 has emerged as an important tumor suppressor and anticancer gene. There is considerable evidence indicating that activation of SIRT6 is beneficial in numerous disease contexts, including cancer. Moreover, the ability of SIRT6 to delay tumorigenesis suggests that long-term activation of the gene could serve as a powerful preventative strategy for delaying the onset of carcinogenesis. Clearly more studies in animal models are required to fully validate and understand the extent to which SIRT6 represents an anticancer drug target and what the long-term effects of activating SIRT6 would be. Given the evidence that has accumulated so far, however, there is good reason to believe that activation of SIRT6 will be an important lynchpin of preventative and acute cancer therapies in the coming years.

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Chapter 10
An Overview of Brevinin Superfamily: Structure, Function and Clinical Perspectives

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Abstract Antimicrobial peptides are the backbone of first-line defense against various microorganisms in the animal kingdom. Thus, not surprisingly, they are gaining attention in the science and medical fields as a rich repository of new pro-drugs. Below, we focus our attention on the Brevinin family of anuran peptides. While most of them show strong antibacterial activities, some, e.g. Brevinin-2R, appear to be promising anticancer molecules, exhibiting better a therapeutic window than widely-use anticancer drugs like doxorubicin. We briefly introduce the field, followed by highlighting the promising therapeutic properties of Brevinins. Next, we provide information about the cloning and phylogenetic aspects of Brevinin genes. In the final paragraphs of this chapter, we discuss possible large-scale production methods of Brevinins, giving examples of some systems that are already in use. Towards the end, we discuss various means of modification of biologic properties of Brevinins, either by chemical modifications or by amino acid substitution and sequence rearrangements. In this context, also other unique properties of Brevinins are briefly mentioned. Finally, we discuss the future of the Brevinin field, particularly highlighting yet to be answered biologic questions, like for example presumed anti-viral and antitumor activities of Brevinin family members.

Keywords Amidophosphoribosyltransferase • Bionanomaterials • Brevinin • Bufodienolides • Bufogenines • Cathepsin • Esculentin • Hypoglycemia • Japonicin • Magainins • Nanocarrier • Nigrocin • Palustrin • Peptidomimetica • Phosphatidylserines • Rana box • Ranacyclin • Ranalexin • Ranateurin • Ranid frogs • Temporin

Abbreviations

AMPs	Antimicrobial peptides
ConA	Concanavalin A
IFN-γ	Interferon-γ
IL	Interleukin
PBM	Peripheral blood mononuclear cells
TLR-2	Toll-like receptor-2
TNF	Tumor necrosis factor-alpha

10.1 Introduction

The discovery of new therapeutic tools is one of the priority areas of biomedical scientific research. Billions of US dollars are spent on the search and development of new medicines every year. Pharmaceutical companies spend an average of $4 billion on the placing of one new drug unit in the market. In some cases, the cost reaches $11 billion (Sources: InnoThink Center for Research in Biomedical Innovation and Thomson Reuters Fundamentals via FactSet Research Systems).

Protection mechanisms of multicellular organisms from an aggressive environment, including bacterial and viral pathogens, have been evolving and improving for millions of years. That is why animal- and plant-derived materials have remained the main sources of leads for new drug development. Plants are the most popular source for searching of new biologically active compounds. Thus, the animal kingdom is actively "mined" for new generations of potentially more effective therapeutics [1].

Members of the *Ranidae* family of amphibians reside in a wide range of habitats (from tropical to subarctic regions) [2]. The ability of amphibians to survive in such different conditions may be attributed to the evolution of many different morphological, physiological, biochemical and behavioral adaptations. Like for other species, skin is one of the most important organs for amphibians, as it fulfills many functions such as *i*) respiration, (*ii*) water regulation and (*iii*) defense (barrier). Glands are major functional components of the skin of amphibians. Three types of glands are widely distributed in amphibians' skin: mucus glands, granular glands and the tubulosaccular or alveolar glands [1]. Mucus glands help to maintain a moisture and slippery skin surface. Granular glands are the place of a wide range of chemical compounds synthesis. Their secretions have a protective function against bacterial and fungal infection as well as predators [3]. Main types of amphibian skin biologically active compounds are: biogenic amines, bufodienolides (bufogenines and bufotoxins = steroids), alkaloids, peptides and proteins [4, 5].

Amphibian skin, especially granular gland secretions, is a rich source of novel therapeutic agents such as antimicrobial peptides (AMPs), polypeptides and proteins. An important event in this area was the discovery of magainins peptides isolated from the skin of *Xenopus laevis* [6]. Magainins exhibit broad-spectrum antimicrobial activity, inhibiting the growth of both Gram positive and Gram negative bacteria, *Candida albicans*, *Cryptococcus neoformansand*, *Saccharomyces cerevisiae* and also demonstrated to induce lysis in several protozoan species [6]. Those discoveries stimulated great interest in amphibian skin peptides [7, 8]. These peptides are stored in granular glands, which are localized mostly in the skin of dorsal area and are surrounded by myocytes and innervated by sympathetic fibers [9, 10]. Adrenergic stimulation of myocytes leads to compression of serous cells, which discharge their contents by a holocrine-like mechanism. As a result, secretions contain not only antimicrobial peptides and other biologically-active agents, but also cytosolic components and cells' genetic material [11]. The main advantage of using amphibian skin as the object of investigation is the use of gentle methods (e.g. skin stimulation by norepinephrine [12] or gentle electrical stimulation [13] for sample preparation. Hence, there is no need to harm or kill animals for this work.

The ranid frogs synthesize and secrete multiple active components. Skin secretions of the *R. palustris* contain at least 22 antimicrobial peptides [14]. On the basis of amino acid sequence similarity, antimicrobial peptides from ranid frogs may be divided into 14 families: Brevinin-1, Brevinin-2, Esculentin-1, Esculentin-2, Japonicin-1, Japonicin-2, Nigrocin-2, Palustrin-1, Palustrin-2,

Ranacyclin, Ranalexin, Ranateurin-1, Ranateurin-2, and Temporin [15]. In this chapter, we use the modified Simmaco nomenclature [10]. Peptides belonging to a species are named by the initial letter in capitals (or more than one letter in case of uncertainty) of the species to indicate their origin. Lower case letters are used to designate isoforms, e.g. Brevinin-1Ea and Brevinin-1Eb from *R. esculenta* [15]. In 2012, a new nomenclature for amphibian skin peptides was proposed [16].

Brevinins are among the most ubiquitous antibacterial peptides, which consist of two families: Brevinin-1 (approximately length of 24 residues) and Brevinin-2 (approximately length of 33–34 residues). The first members (and protoplasts) of the Brevinin superfamily peptides were discovered in 1992. They were isolated from *Rana brevipoda porsa* and called Brevinin-1 (FLPVLAGIAAKVVPALFCKITKKC) and Brevinin-2 (GLLDSLKGFAATAGKGVLQSLLSTASCKLAKTC), respectively. These proteins demonstrated microbicidal activity against a wide range of Gram-positive, Gram-negative bacteria and strains of pathogenic fungi [17]. Since that time about 350 types of Brevinins have been discovered (according to DADP database [18]. Skin secretions of the marsh frog *Rana ridibunda* exhibited significant healing effects on wound treatment process [19]. Furthermore, the antibacterial properties of two peptides named Temporin-Ra and Temporin-Rb isolated from the aforementioned frog species have justified the potential therapeutic application of AMPs [20].

Specimens of the Brevinin superfamily share some common features. These peptides are linear, amphipathic and cationic. Most of them have a C-terminal disulfide-bridged cyclic heptapeptide (Cys^{18}-$(Xaa)_4$-Lys-Cys^{24}), also called «Rana box» [21]. This sequence was thought to play a crucial role for antibacterial activity of those peptides. However, this hypothesis was refuted after discovery of C-terminal truncated Brevinin-1 and Brevinin-2 family peptides from *Rana okinavana* [22] and *R. septentrionalis*, respectively [23]. Those peptides did not have the characteristic C-terminal cyclic heptapeptide domain, but instead contained a C-terminally-amidated residue [22, 23].

The amino acid sequence of Brevinin-1 is poorly conserved across species and has four invariant residues (Ala^9, Cys^{18}, Lys^{23}, Cys^{24}) [24]. Pro^{14} is often present in Brevinin-1 peptides, and it was shown that this residue produces a stable kink in the molecule [25]. Functional activities of antibacterial peptides are largely determined by their structural features. The presence of cationic amino acids facilitates the interaction of Brevinins with the anionic phospholipids of the bacterial membranes and with negatively charged eukaryotic cell membranes (cancer cells, erythrocytes). In aqueous solution, Brevinin-1 exists predominantly as a random coil but adopts an amphipathic α-helical structure in hydrophobic membrane-mimetic environment such as 50 % trifluoroethanol [26]. It has been postulated that the α-helical structure in such an environment leads to perturbation of the phospholipid bilayer of targeted membranes. Such membrane function disturbances lead to growth inhibition or death of the targeted microorganisms. This hypothesis correlates with experimental results performed with synthetic D-amino acids peptides [27]. Biological activity of such analogs were similar to

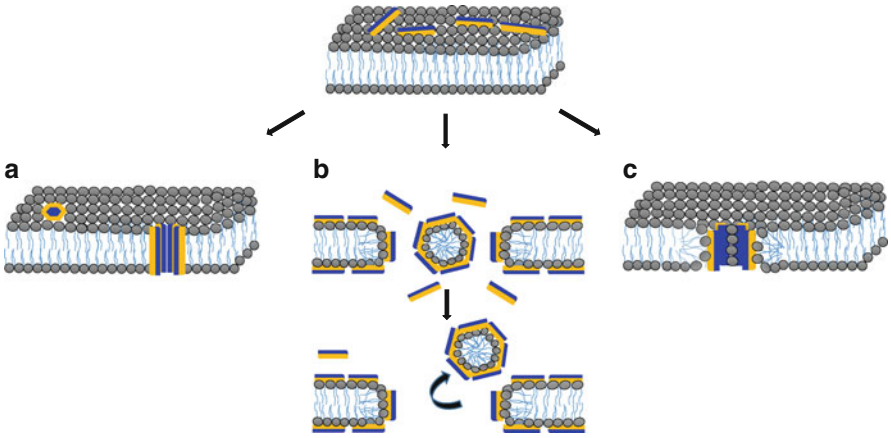

Fig. 10.1 Three basic models of Brevinins' antimicrobial and antibacterial activity, based on their mode of interaction with cellular membranes. The channel (barrel-stave) model (**a**) suggests that antimicrobial peptides form a typical pore. Inner/channel side of such pores would be made of polar residues (*blue*) of the peptides, whereas the hydrophobic ones (*yellow*) are in contact with the membrane phospholipids. The "carpet-like" model (**b**), predicts that peptides accumulate massively at the membrane interphase. Such sequestration of the membrane would lead to the disruption of the membrane integrity. The "two-states" (toroidal) model (**c**) could be interpreted as a variant of the "carpet-like" model, however with a different final outcome. The massive peptide accumulation creates mechanical tension. To relieve that tension, some peptides are forced to adopt a transmembrane orientation, forming a mixed phospholipid-peptide pore spanning the membrane. In a further step, the pore undergoes a stochastic disruption (loses its wall-integrity, with relocation of the monomers at both sides of the membrane), and thus membrane destabilization leading to the loss of its integrity

their corresponding native peptides, so a mechanism based on an interaction with chiral binding sites of receptors, enzymes, or other membrane proteins can be ruled out. The number and distribution of positive charges could be the cause of selectivity for some of these peptides to bacterial membranes [9]. Two main mechanisms of amphipathica-helical peptides interactions with membranes were suggested: the "barrel-stave" (Fig. 10.1a) and the "carpet-like" models (Fig. 10.1b) [28, 29]. The primary structure of Brevinin-2 is also poorly conserved with the invariant amino acid residues in the peptide being Lys^{7}, Cys^{27}, Lys^{28}, Cys^{33} [23].

10.2 Gene Organization and cDNA Cloning

A similar structural organization is being observed in biosynthetic precursors of AMPs including a signal peptide strongly conserved among different AMP families, an intervening region enriched with aspartic and glutamic residues, and an AMP region at the C-terminus [30]. While being well conserved among the

members of an AMP family in different frog species, the intervening sequence regions represent considerable variation among peptides of different AMP families. Sequence hyper-variability is observed in the C-terminal AMP coding regions not only in peptides of different families but also in peptides belonging to the same AMP family [31].

Studies on frog skin secretion revealed a huge structural diversity of the antimicrobial peptides from ranid frogs. Using neighbor joining method it was found that peptides from the closely related species segregate together, forming different clades. This suggests that these peptides formed as a consequence of relatively recent gene duplication events after the species diverged from each other. For example, it was found that *R. sphenocephala* was morphologically and genetically classified as being a close relative of *R. pipens*. Brevinin-1Sc is found in the *R. pipiens* clade, but other Brevinin-1Sa and-1Sb are found in the *R. berlandieri* clade, which suggested unknown phylogenetical relationships [24].

Nowadays, by the use of cDNA cloning technology, the precursors of several AMPs belonging to the Brevinin family have been studied. Protein sequence analysis of cloned cDNA led to the identification of the peptides. On the other hand, the deduced amino acid sequences can then be used as a guide for reverse-phase-chromatography purification of the individual peptides and their amino acid sequences can be uncovered by mass-spectrometry.

Through 'shotgun' cloning, AMPs such as Brevinin-1P, Brevinin-1S and Brevinin-1V have been identified from three species of Chinese frogs, including *Odorrana schmackeri*, *Odorrana versabilis* and *Pelophylax plancyi fukienensis* [32]. Wang and colleagues reported the deduced sequences of Brevinin-1LT1 and Brevinin-1LT2 from the skin of *Hylarana latouchii* using molecular cloning technique. Precursors of Brevinin-1RTa, Brevinin-1RTb, Brevinin-1RTc, Brevinin-2RTa, and Brevinin-2RTb have been identified from the skin-derived cDNA library of *Amolops ricketti* [33]. After isolating AMPs from skin secretion/extract of amphibians, a number of investigators have been interested in analyzing the expression of dermal peptides using semi-quantitative RT-PCR system. Ohnuma and Conlon have investigated the differential expression of some AMPs such as Preprobrevinin-2 in developing larvae and adult tissues of *Rana ornativentris*, which highlighted that the expression of amphibian AMP genes is correlated with metamorphosis but is subjected to differential regulation [34].

10.3 The Recombinant Expression of AMPs

Large quantities of AMPs are needed to meet the requirement for studies in basic science as well as clinical trials. Procuring the peptides from natural sources and chemical syntheses are not cost-effective. The most attractive tool for large-scale production of antimicrobial peptides is the recombinant approach.

Various AMPs belonging to different families and their cDNAs have been cloned. A prokaryotic expression system such as *Escherichia coli* is commonly applied. AMPs are expressed in *E. coli* as fusion proteins to protect the bacterial

host from the lethal effect of AMP and the peptide from proteolytic degradation. Several major fusion-protein systems for the expression of AMPs in *E. coli* have been reported which are summarized here:

- Thioredoxin as a low-molecular weight protein (~12 kDa) has been frequently exploited as carrier protein of antimicrobial peptides. This protein exhibits a chaperon activity that can promote the expression of recombinant peptides in *E. coli*.
- GST (glutathione S-transferase) is a commonly used carrier protein for fusion expression of antimicrobial peptides in *E. coli*. GST fusion proteins can be quickly purified from crude lysate by glutathione-affinity chromatography. The commercial GST-fusion plasmids usually contain a specific protease recognition site releasing the desired peptide from the fusion protein. Due to the relatively large size (~26 kDa) of GST, the efficiency of the system decreases and makes the fusion highly susceptible to proteolytic degradation as well.
- PurF fusion, the protein fragment containing the N-terminal 152 amino acids of PurF (amidophosphoribosyltransferase) is widely used as a carrier for the expression of antimicrobial peptides. Insoluble expression of AMP PurF fusions can not only protect the host cell from the peptides' intrinsic toxic effects but also effectively protect the peptides from proteolytic digestion. The inclusion bodies of PurF fusions can be easily removed from the cell lysate by centrifugation.
- Inteins chitin-binding domain: upon applying intein system, the usage of exogenous proteases or chemicals is eliminated to remove the carrier protein. Consequently, the downstream process of expression is simplified, and the target protein can be obtained at high purity in a one-step purification employing a single column.
- Npro fusion technology, which benefits from autoproteolytic function of N-terminal autoprotease, while Npro is originally extracted from classical swine fever virus (CSFV). The target protein/peptide is fused to the C-terminus of Npro and is expressed in inclusion bodies in *E. coli*. The expressed fusion protein must be dissolved under chaotropic condition. Upon switching to cosmotropic in vitro refolding conditions, the fused partner with an authentic N-terminus is released from the C-terminal end of the autoprotease by self-cleavage. A special Npro mutant called EDDIE has been designed for preparative application, which possesses a better solubility and cleavage rates [35–38].

A few peptides of the Brevinin family have been purified through the thioredoxin fusion system so far. The synthetic gene of Brevinin-2R has been also cloned into the pET32a (+) vector to allow the expression of Brevinin-2R as a Trx fusion protein in *E. coli* [39]. Brevinin-2GU, an antimicrobial peptide isolated from skin secretion of the Asian frog *Hylarana guntheri* possesses insulin-releasing activity. The coding sequence of Brevinin-2GU gene has been expressed using pET32a (+) vector as a Trx fusion protein in *E. coli* to produce over a 45 % yield of the total cell proteins. After purifying the soluble fusion protein by Ni^{2+}-chelating chromatography, the fusion partner was cleaved by Factor Xa protease to release mature Brevinin-2GU [40].

10.4 Anti-pathogen Activity of Brevinins

All peptides belonging to the Brevinin superfamily show high potency against a wide range of Gram-positive and Gram-negative bacteria, and against strains of pathogenic fungi (Table 10.1). Also, it was found that a carboxamidomethylated linearized derivative of Brevinin-1 (CAM-Brevinin) displayed antiviral activity against HSV-1M (35.0 ± 2.8 % protection; ID_{50} could not be determined; $c = 100$ mg/ml) and against HSV-2G (71.6 ± 1.8 % protection; $ID_{50} = 75$ mg/ml) [41].

Unfortunately most Brevinins, perhaps with the exception of Brevinin-2R, have strong hemolytic properties that impede their application as antimicrobial agents. However, some experiments indicate that this negative effect could be decreased by certain structural modifications. It was shown, for example, that transposition of brevinin-1E (FLPLLAGLAANFLPKIFCKITRKC), which was isolated from the skin secretion of *Rana Esculenta*, C-terminal sequence CKITRKC to central position (FLPLLAGLCKITRKCAANFLPKIF) leads to considerable reduction of its hemolytic activity without loss of antibacterial activity [42]. Replacement of Leu^{18} to Lys in the Brevinin-2-related peptide GIWDTIKSMGKVFAGKILQNL-NH_2 from *Lithobates septentrionalis* resulted in a higher level of erythrocyte integrity. It was also shown that the analogs of GIWDTIKSMGKVFAGKILQNL-NH_2: (Lys^4, Lys^{18}) and (Lys^4, Ala^{16}, Lys^{18}) retained activity against *Acinetobacter baumannii* (MIC = 3–6 μM) and had very low hemolytic activity ($LC_{50} > 200$ μM) [43]. Structure–activity studies also revealed that a linear acetamidomethylcysteinyl analog of Brevinin-1E had appreciably less hemolytic activity in comparison with the native peptide [26].

10.5 Effect of Brevinins on Cytokine Release

Activation of innate immunity system results in the stimulation pro-inflammatory cytokines release, including interferon-γ (IFN-γ), tumor necrosis factor-alpha (TNF), and interleukin (IL)-8 by mononuclear cells via Toll-like receptor-2 (TLR-2) pathway [44]. The effect of two AMPs belonging to the Brevinin family (Brevinin-2GU, and B2RP-ERa) on the release of pro-inflammatory and anti-inflammatory cytokines from peripheral blood mononuclear cells (PBM) have been assessed in the presence of 1 and 20 μg/ml of AMPs. Brevinin-2GU, and B2RP-ERa significantly reduced release of TNF from concanavalin A (ConA)-stimulated PBM cells while Brevinin-2GU reduced IFN-γ release from unstimulated PBM cells [44]. On the other hand, secretion of the anti-inflammatory cytokines including TGF-β, IL-4, and IL-10 from both control- and ConA-treated PBM cells was significantly increased by B2RP-ERa [44]. The potent activities of AMPs in the regulation of anti-inflammatory cytokines release suggest a possible therapeutic role of these peptides.

Table 10.1 Sequences and minimal inhibitory concentrations (MICs) of peptides isolated from different Rana species

MIC (minimal inhibitory concentration), μM						
Origin	Name	Sequence	*S. aureus*	*E. coli*	*C. albicans*	Source
Rana esculenta	brevinin-1E	FLPLLAGLAANFLPKIFCKITRKC	0.6	1.8	NA[a]	[10]
	brevinin-2E	GIMDTLKNLAKTAGKGALQSLLNK-ASCKLSGQC	2	0>5	NA	
Rana luteiventris	Brevinin-1Lb	FLPMLAGLAASMVPKFVCLITKKC	8	16	ND[b]	[60]
Rana berlandieri	Brevinin-1Ba	FLPFIAGMAAKFLPKIFCAISKKC	2	ND	ND	[60]
	Brevinin-1Bb	FLPAIAGMAAKFLPKIFCAISKKC	1	3	10	
Rana pipiens	Brevinin-1Pa	FLPIIAGVAAKVFPKIFCAISKKC	7	14	5	[60]
	Brevinin-1Pb	FLPIIAGIAAKVFPKIFCAISKKC	5	14	7	
	Brevinin-1Pc	FLPIIASVAAKVFSKIFCAISKKC	7	5	7	
	Brevinin-1Pd	FLPIIASVAANVFSKIFCAISKKC	27	78	29	
Rana pirica	Brevinin-2PRa	GLMSLFKGVLKTAGKHIFKNVGGSLLD-QAKCKITGEC	25	6	NA	[61]
Hylarana guntheri	brevinin-2GUb	GVIIDTLKGAAKTVAAELLRKAHCKLTNSC	64	32	64	[49]

[a]not attested
[b]not determined

10.6 Anticancer Activity of Brevinins

A unique peptide Brevinin-2R (KLKNFAKGVAQSLLNKASCKLSGQC) was isolated from *Rana ridibunda*. This peptide consists of 25 amino acids and has strong homology with Brevinin-2Ej and -2Ee. The antimicrobial spectrum of Brevinin-2R displayed activities against: *S. aureus*, *M. luteus*, *Bacillus spKR-8104*, *E. coli*, *S. typhimurium*, *P. aeruginosa*, *K. pneumonidae* and fungi, such as *C. albicans* and *C. tropicalis*. The most important property of Brevinin-2R peptide is low hemolytic activity (no more than 2.5 % of dead cells at up to 200 μg/ml of the peptide) [45]. This fact allowed the researchers to consider Brevinin-2R as a new potential therapeutic agent. Brevinin-2R kills different tumor cells (Jurkat, BJAB, MCF-7, L929, A549) at 1–10 μg/ml concentration, and exerts higher cytotoxicity in comparison with commercial doxorubicin and cisplatin drugs ($P < 0.0001$). In experiments with normal cell lines (CD3+ T cells from human donor and lung fibroblast), the level of cytotoxicity was approximately two times lower [45]. - Brevinin-2 kills cells in a caspase-independent manner, implying cell death mechanisms other than classical apoptosis. After treatment with Brevinin-2R, a decrease of both the mitochondrial membrane potential ($\Delta\Psi m$) and the ATP level was observed [45].

The main mechanism of its anticancer action is most likely the same as for pathogens namely the modification of membrane properties, especially membrane permeability. Brevinins preferentially interact with cancer cells because the outer membrane surface of these cells has an additional negative charge due to the presence of higher levels of O-glycosylated mucines [46], negatively charged phosphatidylserines [47] or higher number of microvilli, which leads to increasing of membrane surface area [48]. Also it was found that Brevinin-2R interacts with the lysosomal compartment, initiating lysosomal damage and cathepsin leakage into the cytosol, which leads to cell damage. These data suggest that Brevinin-2R-induced cell death also involves autophagy processes [45].

10.7 Other Activities of Brevinins

Experiments carried out on the rat BRIN-BD11 clonal β-cell line revealed a novel activity of several Brevinins: the stimulation of insulin release. This new function may give an additional protection for frogs by stimulating insulin secretion and causing hypoglycemia in attacking predators [13]. Examples of such insulin-releasing peptides belonging to the Brevinin family include: Brevinin-2GUb from *Hylarana güntheri* [49], Brevinin-2-related peptide (B2RP) from *Lithobates septentrionalis* [50], Brevinin-1 peptides from *Lithobates palustris* [13], *Pelophylax saharicus* [51] and *Glandirana emeljanovi* frog (insulin releasing stimulatory effect was shown on RINm5F insulinoma derived cells) [52].

Brevinin-1CBb (FLPFIARLAAKVFPSIICSVTKKC) provided a significant ($p < 0.05$) stimulation of insulin release (269 % of basal rate at a concentration of

1 μM with a maximum response of 285 % of basal rate at a concentration of 3 μM) from BRIN-BD11 clonal β-cells [12]. At the same condition, B2RP (Brevinin-2-related peptide GIWDTIKSMGKVFAGKILQNL-NH_2) showed 148 % of basal rate at a concentration of 1 μM with a maximum response of 222 % of basal rate at a concentration of 3 μM [50]. These values were comparable to those produced by insulinotropic peptides, GLP-1 and GIP (under the same experimental conditions). Unfortunately, however, the peptides were cytotoxic at the tested concentrations [53]. Also, it was shown that increasing the cationicity of B2RP ($Asp^4 \rightarrow$ Lys) enhanced the insulin-releasing potency (137 % of basal rate at a concentration of 0.3 μM; $p < 0.05$), while increasing amphipathicity and hydrophobicity showed reduced insulin-releasing potency of analog [50]. Those proteins might represent promising candidates for the development of therapeutically valuable agents for the treatment of patients with type 2 diabetes. Most investigators assumed that stimulation of insulin release was caused not only by the capacity of Brevinins to destabilize cell membranes but rather via other, as yet unidentified, mechanisms.

About 2,000 biologically active anuran peptides have been found and characterized (according to DADP database [18]). Due to the high sequence variability and a wide range of functional activities, these proteins constituted a strong basis for theoretical and experimental research leading to the design of new biologically active peptides and peptidomimetica.

10.8 Functionalization of Nanostructures with Peptides

Functionalization of nanostructures with various biomolecules including DNA, Herceptin, carbohydrates, lipids, peptides and proteins has multiple potential applications in biomedical imaging, clinical diagnosis, antimicrobial therapy, drug delivery and cancer treatment [54]. Several researches have been developing/discovering novel effective antimicrobial reagents to fight the increase of antibiotic-resistant in microorganisms [55]. Liu and colleagues introduced core-shell nanoparticles formed by self-assembly of amphiphilic peptide with potential antimicrobial activity against a broad spectrum of pathogens including bacteria and fungi [56]. Peptides, particularly cationic peptides, belonging to the Brevinin family represented antimicrobial effect against several multi-drug resistant microorganisms [45]. Recent reports clearly demonstrated that peptide-functionalized nanoparticles can considerably enhance the antibacterial activity of biomolecules [54]. Thus, a Brevinin functionalized nanostructure would be of great importance from the objective of developing advanced functional bionanomaterials with antimicrobial properties. Researchers reported the functionalization of a novel gold-based nanocarrier with a therapeutic application (PMI (p12)) as well as a receptor-targeted (CRGDK) peptide to investigate the biological and medicinal effects of conjugated gold nanoparticles on breast cancer cells [57]. Lia et al. have developed AuNPs (gold nanoparticles) to make not only hybrid model system for selective target binding along with cancer therapeutic effects but also

sensitive probes for sensing/imaging various analytes/targets such as ions and molecules [58].

Biomedical imaging is another field of application of peptide-functionalized nanoparticles. Synthesis of water-soluble gold nanoparticles functionalized with a Tat protein-derived peptide sequence facilitates the transfer of nanoparticles across the cell membrane, and therefore simplify the visualization of cellular or tissue components as well as nuclear targeting by electron microscopy [59]. The combined results of these studies have implications for functionalizing or decorating Brevinin-2R as an antimicrobial peptide onto nanostructure surfaces to create a hybrid model system for biological purposes.

10.9 Closing Remarks

Anuran bioactive peptides show great medical potential and will undoubtedly enter the clinic in a not so distant future. Major challenges to their large scale production, and also to research in this area as a whole, are fixed secondary structures achieved by those peptides, when secreted naturally. These secondary structures (i.e. cyclization) are often difficult to mimic, when peptides are produced in procaryotic expression system. These problems are, however, possible to overcome using current biochemical methods.

While most AMPs exhibit strong antibacterial activities, few of them (i.e. Brevinin-2R) show anticancer properties and low hemolytic activity, thus making them potentially compatible with an in vivo use. Noticeable hemolytic activity of most AMPs may be overcome either by structural modifications or simply by applying such drugs externally, directly on the site of infection, thus minimizing systemic load.

We have summarized the typical antibacterial activities of various AMPs (see Table 10.1). Interestingly, virtually no research has so far been done on antiviral activity of AMPs. With the growing demand for effective antiviral drugs (i.e. SARS, HIV, West Nile Virus, Ebola-virus), lipid-membrane-directed activities of AMPs may prove an effective antiviral drugs. Thus, the authors predict strong scientific and commercial interest in antiviral testing of AMPS.

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Chapter 11
Isolation and Characterization of the Anticancer Gene Organic Cation Transporter Like-3 (ORCTL3)

Ghada AbuAli and Stefan Grimm

Contents

Abstract ORCTL3, an organic cation/anion transporter expressed in various tissue types, was isolated in a genome-wide cDNA screen as a gene with a tumor-specific apoptosis activity. When overexpressed it elicits an apoptosis response in many transformed cells, while normal cells remain unaffected. It can be activated for apoptosis induction by individual tumorigenic mutations in renal cells. This effect is independent of the tumor cells' proliferation status and mediated by an incomplete ER stress response, characterized by the accumulation of the endoplasmic reticulum-stress marker ATF4, but not BiP. Recent studies show that for its apoptosis induction activity ORCTL3 targets the enzyme stearoyl-CoA desaturase-1 (SCD-1) that is involved in the fatty acid metabolism. This is evidenced by the inhibition of apoptosis induced through ORCTL3 when the SCD-1 product oleic acid is exogenously supplemented or when SCD-1 is co-transfected in the transformed cells. ORCTL3's activity to specifically target tumor cells is caused by the transmembrane domains 3 and 4 of the mouse, but not the human, gene. In an *in vivo* model ORCTL3 shows a significant shrinkage in the

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size of xenograft tumors when injected with an adenoviral carrier carrying the mouse ORCTL3 gene. An *ex vivo* study using human renal cancer cells confirmed the promising tumor-specific apoptosis effect of ORCTL3. Since ORCTL3 targets fatty acid metabolism in transformed cells and induces an ER stress specifically in these cells, it reveals a novel therapeutic interference option for tumor cells.

Keywords ORCTL3 • Organic cation/anion transporter • Genome-wide cDNA screen • ER stress • Stearoyl-CoA desaturase-1 • *In vivo* model • *Ex vivo* model • Domain mapping

11.1 ORCTL3 – Cloning and Mapping

Organic Cation Transporter Like-3 (ORCTL3) was originally cloned as a result of a large-scale DNA sequencing project of chromosome 3p22 to p21.3, a DNA segment that is homozygously deleted in a lung cancer cell line [1]. In this effort to identify tumor-associated genes, ORCTL3 was detected as one of nine genes in this 1,200-kb region. It was called ORCTL3 because of its structural homology to proteins belonging to the family of organic cation transporters, especially rat OCT-1r, rat NLT, mouse NKT, and a group of sugar transporting proteins including human glucose transporters [2].

Its predicted peptide sequence was 33.9 % identical to another protein that was mapped in the same study to a proximate region of genomic DNA and designated ORCTL4 to indicate a high conservation of transporter-specific amino-acid motifs and a similarity in transmembrane-domain profiles. Hence, it was predicted that ORCTL3 and ORCTL4 are likely to play a role in transporting organic cations in tissues, although functional studies were not carried out at that time [2].

It is generally known that organic anion and cation transporters play an important and multi-specific role in moving organic anions and organic cations across the plasma membrane. These molecules are the hydrophilic, charged metabolites of many lipid-soluble compounds and are generally difficult to be transported through the plasma membrane without the help of transporters such as ORCTL3 and ORCTL4 [3].

11.2 Structure and Tissue Distribution

The orphan transporter protein ORCTL3 belongs to the solute carrier family 22 (SLC22) but has not been thoroughly studied thus far [4]. ORCTL3 gene spans around 12 kb of genomic DNA and consists of ten exons. It was shown that the 2.4 kb transcript of this gene is universally expressed in all human tissues with some preference for kidneys, small intestine, and colon [2]. This can be explained

by the active transport and secretion of metabolites in these organs. The same northern blot analysis also showed, in addition to the 2.4 kb transcript, a larger 2.6 kb transcript that contained a longer 3′ polyA non-coding sequence specifically in the kidneys [2]. However, another, more recent, study that evaluated the tissue distribution of ORCTL3 by RT-PCR suggests that ORCTL3 is a renal-specific transport protein due to its strong expression in the apical side of renal proximal tubule cells in the kidney, and weaker expressions of other splice variants of its open reading frame in other tissues such as brain, heart, and colon [4]. This study also suggests that ORCTL3 is expressed in a gender-dependent manner with a dominant female over male expression, which might result in gender-specific reabsorption and secretion of its substrates.

ORCTL3 encodes a 551-amino acid protein [4] that shows a 90 kDa band on western blot when using an antibody against mouse ORCTL3 [3]. A hydropathy plot of the predicted polypeptide sequence of ORCTL3 performed by the program from Kyte and Doolittle indicated ten hydrophobic segments, suggesting that the protein spans the cell membrane several times [5]. When the membrane topology was analyzed by Lee et al., mouse ORCTL3 showed 12 putative transmembrane domains with both N- and C-termini located in the intracellular space. It also showed four N-glycosylation sites in the extracellular loop between the first and the second membrane-spanning domain, which explains the large protein size on the western blot. In addition, mouse ORCTL3 contained six intracellular PKC phosphorylation sites [3].

11.3 Physiological Function

When ORCTL3 was first isolated by Nishiwaki et al. in 1998, very little was known about its function and it was classified and referred to solely on the basis of its structure. The only study that was conducted to further understand the physiological function of this protein, however, suggests a different function than that of a cation transporter. In 2008, Bahn et al. aligned protein sequences of all the known organic cation transporters (OCTs) and organic anion transporters (OATs) and, based on comparisons to identify highly conserved amino acids, they hypothesized that ORCTL3 is rather an organic anion transporter [4]. Indeed, after examining the properties of ORCTL3, it was revealed that ORCTL3 is a transporter for urate, the end product of purine metabolism and nicotinate; this identified ORCTL3 as the first high-affinity nicotinate exchanger in kidneys and intestine. Nicotinate is an essential vitamin (Vitamin B_3) that is involved in NAD^+ synthesis, which in turn is important for energetic processes, signal transduction pathways, and the activation of the NAD^+-dependent histone deacetylase SIRT1 and hence, life extension [4, 6]. Therefore, ORCTL3 was suggested to be renamed as OAT10 (Organic Anion Transporter 10). Moreover, succinate and glutathione were proposed as two other substrates (counter ions) of ORCTL3 in the same study [4].

11.4 Anticancer Function

The anticancer activity of ORCTL3 was discovered in a genome-wide screen for tumor-specific apoptosis-inducing genes from a mouse cDNA library [7]. The screen was performed by searching for pro-apoptotic DNA plasmids in the kidney cell line HEK293T; cells that display characteristic features of tumor cells such as tumor formation in nude mice and growth in soft agar [8]. This was followed by screening for those genes that induce apoptosis in H-Ras-transformed normal rat kidney (NRK) cells but not in wild-type, parental NRK cells. Both screens used kidney cells but two completely different transformation scenarios. This meant that the synthetic lethality of the active gene(s) would not be confined to E1A/E1B transformed cells but target changes common to transformed cells. This was done as E1A/E1B expression is not found in naturally occurring kidney cancers. This set a very demanding criterion for the hits from the screen as the two oncogenic mutations (H-ras and E1A/E1B) elicit different signaling cascades. In fact, the sequencing of kidney cancer genomes has revealed considerable genetic inter- and intra-tumor heterogeneity of renal tumors, which would make such a broad-ranging agent necessary [9, 10]. Hence the question was whether there is a feature common to cancer cells that can be targeted with anticancer genes. Reflecting the disparate transformation events in HEK293 and H-ras NRK cells only one gene, ORCTL3, was isolated from this screen (Fig. 11.1). Apoptotic cell death was confirmed by an apoptosis-specific quantitative assay for DNA cleavage

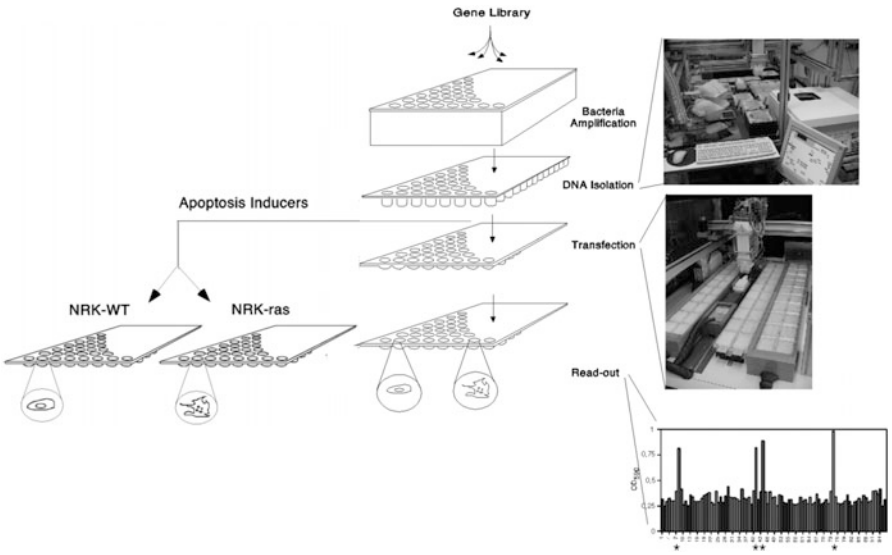

Fig. 11.1 A schematic outline of the robotic screen for tumor-specific apoptosis genes that lead to the isolation of ORCTL3 by Irshad et al. [7]

and cell death inhibition by the pan-caspase inhibitor z-VAD [7]. This apoptosis induction activity was specific to ORCTL3 and not observed with two other related gene family members of the organic cation and anion transporter family [7].

11.5 Universal Anticancer Activity?

The anticancer effect of mouse ORCTL3 indicated that it is not restricted to certain tumor cells. In fact, ORCTL3 induced apoptosis in many transformed cells, such as HeLa, LNCap, PC3, and 293T, and remained inactive in many non-transformed cells, such as MDCK, HUVEC, and RPT cells [7]. The targeted activity was not a consequence of different expression levels as a fusion with GFP and FACS quantification of the expression signals indicated. Moreover, this effect was not species-specific. Mouse ORCTL3 exerted its anticancer effect when overexpressed in human, rat and monkey transformed cells [7, 11]. Introduction of various individual tumorigenic mutations in normal cells was sufficient to activate ORCTL3 for apoptosis induction [11]. When the normal primate renal CV1 fibroblast cells were transformed by overexpressing the oncogene Myc, an activated allele of H-Ras, and an inactive dominant-negative-p53, all of these cells underwent apoptosis upon ORCTL3 overexpression. Non-transformed CV1 cells, on the other hand, remained resistant to ORCTL3's apoptosis induction (Fig. 11.2) [11]. This indicates that ORCTL3 is more likely to target a pathway that is generally disturbed in many transformed cells (in these settings) regardless of the transformation-causative mutation. This CV1 isogenic model was also used to compare the efficiencies of apoptosis induction of various known anticancer genes. It was found that, at least under the experimental settings in this study, also other anticancer genes (MDA7, IL-24 and Apoptin) were activated upon oncogenic transformation [11].

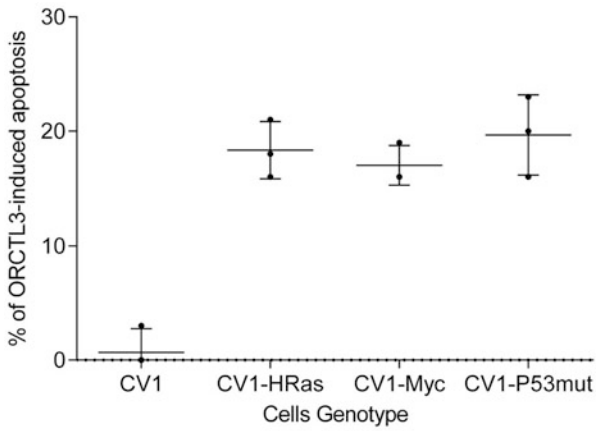

Fig. 11.2 ORCTL3 induces apoptosis in transformed but not in normal CV-1 kidney cells [11]

11.6 Structure and Mechanism of the Anticancer Protein ORCTL3

The apoptosis induction activity of ORCTL3 is found to be exclusive to the mouse gene and not the human homolog [11]. It was hypothesized that ORCTL3's apoptosis induction activity is independent from its transporter activity [7] as progressive deletions of the C-terminal half of the protein did not significantly disturb its apoptosis activity [7, 11]. In fact, a construct that consisted of only the first four transmembrane domains of the protein was sufficient to induce a significant level of apoptosis in the tumor cells [11]. Furthermore, it was observed that the third and the fourth transmembrane domains of the mouse protein are the only fragments of the entire protein sufficient to induce apoptosis, when expressed in the optimal protein context [11], but the precise structure-function connection has yet to be established.

The overexpressed ORCTL3 is localized to the endoplasmic reticulum (ER), Golgi and the plasma membrane but not to mitochondria. The cellular locale of ORCTL3 did not differ between normal and transformed cells [7]. When ORCTL3 was retained in the ER, the toxicity of the overexpressed ORCTL3 was increased compared to the control protein. This led to the hypothesis that ORCTL3 exerts its apoptosis activity through the ER [7]. This organelle is known to be involved in apoptosis; most established is the ER stress response of cells, which is caused by a variety of signals that lead to the accumulation of unfolded proteins in the ER. The initial part of the cell's ER stress response is trying to remedy this condition, which, when unsuccessful, leads to the active induction of apoptosis by the cell. The role of this response was supported by the finding that ORCTL3 overexpression results in the up-regulation of ATF3 and ATF4 as well as the splicing of XBP1, all of which are ER stress markers, in transformed cells that undergo apoptosis [7, 11]. However, ORCTL3's overexpression does not result in the up-regulation of Bip/Grp78 or the pro-apoptotic transcription factor CHOP, two other hallmarks of ER stress.

This is an unusual ER stress response. The lack of CHOP activation suggests that ORCTL3 induces apoptosis via another mechanism where the ER stress is only a secondary and incomplete response and hence incapable of triggering the full apoptosis cascade.

In a recent study, ORCTL3's role in tumor-specific apoptosis induction was linked to the enzymatic activity of the enzyme stearoyl-CoA desaturase-1 (SCD1) [11]. The connection between the two proteins was first suspected when Minville-Walz et al. and Masuda et al. showed ATF4 up-regulation, XBP-1 splicing but no BiP activation, markers of partially induced ER unfolded protein response (UPR), when down-regulating or inhibiting the ER resident enzyme SCD-1 [12, 13]. The same partial UPR was detected when ORCTL3 was overexpressed in the cells [7, 11].

SCD1, also known as fatty acyl-CoA delta-9 desaturase, is an ER-resident enzyme that catalyzes the introduction of the first double bond in the cis-delta-9 position of many saturated fatty acyl-CoAs (SFAs), mainly palmitoyl-CoA and

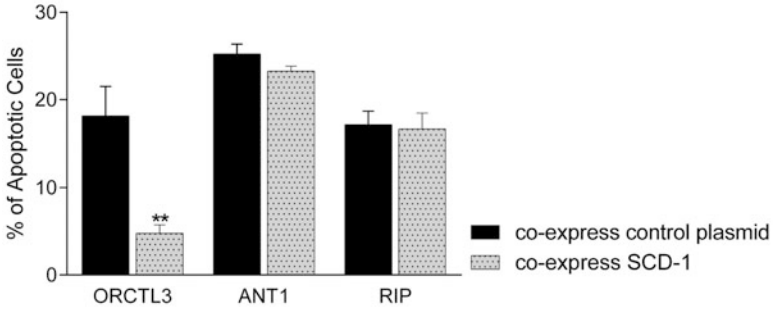

Fig. 11.3 Overexpressing SCD1 protects transformed 293t cells from apoptosis induced by ORCTL3 [11]

stearoyl-CoA, to yield the monounsaturated fatty acids (MUFAs) palmitoleoyl- and oleoyl-CoA, respectively [14, 15]. It was reported that this enzyme is highly expressed in cancer cells and oncogene-transformed lung fibroblasts [16–18]. In addition to cancer, SCD1 is found to be elevated in metabolic diseases, such as diabetes, obesity and atherosclerosis, suggesting a common molecular association between these diseases and cancer. In both there is a dominant metabolic shift towards biosynthetic reactions that result in the overproduction of lipids for different reasons. In cancer cells, the newly produced lipids are used in the formation of new phospholipids to be utilized in membrane biosynthesis [19]. The alterations of lipid metabolism and the elevation of SCD1 in cancer cells suggest an important role of SCD1 in proliferation, cancer development, and perhaps apoptosis in these cells, making it a potential target for cancer therapy.

Morgan-Lappe et al. showed in an RNAi screen that SCD1 down-regulation causes apoptosis in tumor cells but not in normal cells [20]. As mentioned above, Minville-Walz et al. showed that the apoptosis induced by down-regulated SCD-1 in cancer cells is mediated by an ER stress response that is, interestingly, similar to the one triggered by ORCTL3 and leads to splicing of XBP-1 and no BiP up-regulation [12]. Moreover, Masuda et al. showed an up-regulation of ATF4 and also splicing of XBP-1 upon chemical inhibition of SCD-1 [13]. Because of the similarity of the ER stress responses induced by ORCTL3 and SCD-1, and since both proteins reside in the ER, an interaction between them was assumed. Recent unpublished data show a physical interaction between the endogenous SCD1 and the overexpressed mouse ORCTL3 in transformed cells. This interaction is thought to be functional i.e., ORCTL3 is believed to inhibit the enzymatic activity of SCD1 in transformed cells. This inhibitory effect of ORCTL3 on SCD1 was further supported when it was noticed that the exogenous supplementation of oleic acid, one of the enzymatic products of SCD1, can rescue the transformed cells and inhibit the toxic effect induced by ORCTL3 overexpression. Moreover, the overexpression of both proteins, ORCTL3 and SCD1, results in less apoptosis induction compared to the overexpression of ORCTL3 alone (Fig. 11.3) [11].

The up-regulation of SCD-1 in cancer cells can cause one of the many metabolic changes that cancer cells adopt during the transformation process. Alterations in the metabolism of cancer cells was first reported over 80 years ago when Otto Warburg observed an elevated uptake of glucose and an increased glycolytic rate in cancer cells, which results in aerobic glycolysis and an increase in the production of lactate, a phenomenon known as the Warburg effect [21]. Aberrations in metabolism in cancer has recently gained more appreciation on the basis that the increase in proliferation makes it necessary for the malignant cells to increase their ATP production, macromolecule synthesis, and redox status [22]. One of these metabolic changes is the increased synthesis of mono-unsaturated fatty acids (MUFA) that are required for the augmented biosynthesis of membrane phospholipids due to the high proliferation rate in these cells. This alteration requires a continuous upshift in the activity of SCD-1 in these cells because of their dependency on (or addiction to) its products, the MUFAs. Inhibiting this enzyme, even though it is expressed at a higher level in cancer cells than in normal cells, results in a shortage in MUFAs supply that cannot be tolerated by the dependent cancer cells, and hence, cause cell death. Normal cells are usually more adaptive to this subtle change in metabolism since the existence of high amount of MUFAs is not a necessity for survival.

The unique and partial ER-stress response induced by SCD-1 down-regulation and ORCTL3 overexpression was not the only reason why a connection between the two proteins was suggested. As mentioned above the supplementation of oleic acid, a product of the SCD-1 enzyme, could partially rescue the transformed cells from apoptosis induced by ORCTL3. This indicated a cytotoxicity that results from the depletion of the MUFAs produced by SCD-1 rather than the build-up of the potential toxic pathway intermediates or SCD-1 substrates when SCD-1 is inhibited [23].

The fact that SCD-1 is commonly overexpressed in cancer and oncogene-transformed cells might explain the tumor-specificity of ORCTL3 to some extent. Different studies revealed an important role of SCD-1 in various cellular processes [15]. SCD-1 is a known key role player in membrane synthesis and fluidity. Interestingly, involvement of this enzyme's function was evidenced in cancer development [20, 24], neural regeneration [25] and brain development [26], and in particular obesity. SCD-1′s role in obesity has been the focus of hundreds of studies that looked at the enzymatic activity of SCD-1 as a therapeutic target [27]. Most of these studies concluded that targeted inhibition of SCD-1 is extremely effective in preventing diet-induced obesity [5]. This finding suggests an interesting connection between obesity and cancer through SCD-1. Such a link has been established through various studies that concluded that obesity was associated with an increased risk of developing cancer [28, 29], and in particular an increased risk of death due to prostate cancer metastases [30]. Due to the abnormally elevated levels of SCD-1, which is significantly linked to the metabolic alterations found in obesity, as well as diabetes, and the up-regulated levels of SCD-1 evidenced in cancer cells, SCD-1 has been suggested to be the mutual molecular link between cancer and obesity [31]. Its inhibition is positively correlated with preventing obesity and more recently to inducing apoptosis specifically in tumor cells through an interaction with the overexpressed ORCTL3. Although the

interaction between ORCTL3 and SCD-1 was clearly established and might explain the specificity of ORCTL3's induced toxicity [11], the existence of other additional targets of ORCTL3 cannot formally be ruled out.

What is more, the overexpression of ORCTL3 might affect the ER of the transformed cells via a different mechanism than the classical unfolded protein response. Newer studies have implied the ER-mitochondria interface for apoptosis induction, which is potentially independent of ER stress. These findings have linked ORCTL3 to Bap31, another resident protein of the ER membrane that together with Fis1 forms the ARCosome [32]. This complex is activated by the recruitment of caspase-8 and the cleavage of Bap31 into the p20 fragment, which then releases Ca^{2+} from the ER to activate mitochondria for apoptosis. ORCTL3 was indeed found to induce the cleavage of Bap31 to the pro-apoptotic fragment p20Bap31, which thereafter stimulated Ca^{2+}-dependent mitochondrial changes that eventually led to the release of cytochrome c and cleavage of PARP. Both ORCTL3 and Bap31 are transmembrane proteins that reside in the ER membrane and are involved in apoptosis responses. The cleavage of Bap31 takes place before the cells undergo apoptosis and the amount of the cleaved protein was found to be significantly higher in transformed cells compared to non-transformed cells upon ORCTL3 transfection [11]. These results suggest a role of ORCTL3 in the cross-talk between the ER and the mitochondria for apoptosis induction.

The involvement of Bap-31 in the lethal signal induced by ORCTL3 suggests an activation of a complex downstream in the apoptosis signaling cascade, but the co-localization of SCD-1, ORCTL3 and Bap31 in the ER membrane can also mean that an indirect interaction between ORCTL3 and Bap31 is responsible for SCD-1 inhibition. While highly specific compounds against SCD-1 have already been developed, ORCTL3 caused higher apoptosis in transformed CV1 and lower background in normal CV1 cells compared to a chemical SCD-1 inhibitor [11], indicating a more precise targeting by ORCTL3 and/or less accumulation of SCD-1 substrates that might be poorly tolerated by normal as well as transformed cells [27]. In fact, SCD-1 inhibitors have been investigated for a long time in connection with obesity and diabetes. However, in recent studies, SCD-1 inhibition has uncovered some side effects due to the accumulation of SCD-1 substrates (SFAs), which are largely evidenced to be potent pro-inflammatory molecules linked to a number of inflammatory diseases such as atherosclerosis [33], cell dysfunction [34–40, 53], steatohepatitis [41, 42] and colitis [43]. These side effects should be looked into when considering ORCTL3 for any future *in vivo* study. The lower toxicity levels observed in normal cells upon ORCTL3 expression compared to the treatment with the SCD-1 inhibitor, and the high concentration of this compound that was needed to achieve a similar level of apoptosis induced by ORCTL3, make it sensible to anticipate that ORCTL3 might not cause the same level of adverse side effects experienced when using chemical inhibitors of SCD-1 [11]. Does ORCTL3 trigger another tumor-specific signal? Is it tumor-specific because it does not cause too high an accumulation of SFAs that are generally toxic to cells? The latter question can be addressed by comparing the SCD-1's substrate/product ratios upon ORCTL3 expression and SCD-1 chemical inhibition.

Many cancer cells show increased proliferation rates compared to non-transformed cells, and hence several anti-tumor agents rely for their efficacy on targeting proliferation. This strategy might have been proven effective in some cases in which the proliferation status of the normal cells and their malignant counterparts differ by a wide margin. Nevertheless, the absence of high tumor-specificity hinders the development of drugs that target proliferation for suitable therapeutic indices and wide therapeutic window [37]. There is so far no evidence that suggests that ORCTL3 acts via a similar mechanism based on the proliferation differences of malignant cells. In fact, ORCTL3 was shown to be active for apoptosis induction in transformed cells where proliferation was comparable to the non-transformed counterpart cells [11].

11.7 *In Vivo* Applications

ORCTL3 is a novel anticancer gene based on its apoptosis induction activity. Its efficient toxicity for transformed cells and its equally notable absence in normal cells make it a promising anticancer agent. Despite the lack of a complete understanding of its exact mode of action as an anticancer gene and the potential issues to efficiently transport genes into targeted cells, ORCTL3 has been further investigated for being utilized as an anticancer agent. When ORCTL3 was expressed by an adenoviral vehicle in non-transformed and transformed cells, it maintained its tumor-specific and efficient apoptosis induction [11]. The ORCTL3-adenovirus was also effective to reduce the size of tumors of renal carcinoma cells (Caki-2) grown as xenograft in mice when compared to those tumors injected with control virus [11]. These findings were extended in an *ex vivo* approach to primary renal clear carcinoma cells when compared to normal renal cells from the same patients [11].

11.8 The Future of ORCTL3 as an Anticancer Gene

One of the most noticeable outcomes of the genome-wide screening carried out by Irshad et al. was the isolation of the anticancer gene, ORCTL3. This screen was performed to identify genes that are synthetic lethal with oncogenic E1A/E1B and Ras, of which the latter is frequently mutated in many cancers but difficult to target directly using conventional small molecule inhibitors [44, 45]. High-throughput screening of RNAi constructs and chemical compounds has become a widely used experimental approach that allows the interrogation of many potential biological modulators against a chosen set of defined targets. The idea of using a cDNA screen for the discovery of anticancer genes was realized for the first time by Irshad et al.

Nevertheless, such an approach has its limits: One common limitation of such screens is the use of artificially generated isogenic cell lines where the genetic background and cellular state do not strictly reflect “naturally occurring” cancer

cells. Therefore, the molecular interactions in these cells and in primary cancer cells that harbor the genetic aberrations are not necessarily equivalent and have to be validated.

Moreover, its clinical application by introducing an external gene into cells still appears to be challenging. Eradicating transformed cells with ORCTL3 would involve the expression of an exogenous gene in target cells but delivering DNA that carries a therapeutic genetic sequence into the cells in an *in vivo* system is still a challenging undertaking. The DNA has to overcome uptake by non-targeted cells, cross the plasma membrane barrier, avoid degradation by cellular nucleases, and finally reach the nucleus to be expressed in the target cell [46]. Nevertheless, novel technologies and vehicles, some of which are covered in this book, could make this safe and efficient in the near future. Three vehicles are currently available to deliver genes into cells. The first gene delivery method used in clinical trials was a viral vector in 1990 [47]. Such vectors are genetically modified viruses whose replication ability has been abolished. They are known for their high delivery efficiency to many cells and are usually the preferred approach for gene delivery. This is evidenced by the fact that 67 % of all clinical trials conducted so far employed viral vectors [46]. The other two methods are non-viral vectors such as synthetic and natural agents, which are less toxic than the viral vectors and more flexible for the size of genes they can carry. These include bacteriophage-, liposome- and polymer-based vectors and were employed in around 24 % of clinical trials. The third and last approach are methods where physical force is used to transfer the gene into the cells [46], for example, by electroporation or sonoporation [46]. Thus far, there is no universally preferred gene delivery method for all applications; instead, for each specific case the most suitable carrier has to be developed to target the desired cells with high delivery efficiency and to produce the appropriate amount of gene product for the required period of time [46]. For ORCTL3, an adenoviral vector seems to be a suitable delivery carrier due to its known high delivery efficiency and the finding that ORCTL3 needs to be expressed to a high level for its apoptosis effect [11].

Renal cancer is the sixth leading cause of cancer-related death in the USA. With over 30,000 new cases diagnosed every year, it is responsible for around 12,000 deaths annually [48, 49]. Renal cell carcinoma (RCC) comprises 85 % of all renal cancers in adults and more than 3 % of all adult malignancies [50]. Clear cell renal cell carcinoma (ccRCC) is the most common form of renal cancer accounting for 80–85 % of all renal tumors [48, 51]. So far, there is barely any systematic treatment for dispersed renal tumors and the therapy relies primarily on surgery. Although previously published data showed that ORCTL3 is active against a wide range of cancer cells [7], it was, thus far, mainly investigated as a treatment agent for renal cancer models [11]. The increased apoptosis exerted by the adenovirus-expressing ORCTL3 and the apoptosis effect that is seen by the mouse but not the human ORCTL3, seemingly caused by the increased stability of the mouse protein in the cells, strongly indicate that it is essential to efficiently introduce ORCTL3 gene into the target cells, should it be used for therapeutic purposes. Primary human cell cultures might be the closest in vitro model to what precisely happens in these

tumors *in vivo*, especially if cells were minimally passaged. The results obtained by using such cells to examine the effect of ORCTL3 promise a very specific effect to tumor cells [11].

The interaction between ORCTL3 and SCD-1 established by Abuali et al. might constitute the basis of the tumor specific effect of ORCTL3, especially based on the current understanding of the role and significance of SCD-1 in tumors and the differences of SCD-1 regulation and function in tumor versus normal cells. The physical interaction shown in immuno-precipitation experiments, the rescue of ORCTL3-transfected cells with oleic acid supplementation, and the detected SCD-1 up-regulation in transformed cells are all strong indicators that ORCTL3 and SCD-1 interact to induce apoptosis and this effect is likely based on the inhibition of the enzymatic function of SCD-1 by ORCTL3 [11]. However, more functional studies are necessary to support this finding. For example, although the differences in SCD-1 expression and function in normal versus transformed cells were addressed thoroughly in many studies, it would be interesting to study how these differences change the response of the cells to ORCTL3. This can be achieved, for example, by examining whether ORCTL3 and SCD-1 physically interact also in normal cells compared to transformed cells. Such experiments will allow identifying the exact difference between normal and transformed cells that activates ORCTL3 for apoptosis induction. So far it is not clear whether it is the dependence of only the transformed cells on high SCD-1 activity, or whether it is an aberration or a mutation in, for example, SCD-1 in transformed cells that allows ORCTL3 to exert its inhibition, or whether it is an indirect interaction through another molecule that is specific for transformed cells.

All these questions can be asked at this stage because SCD-1's role in cancer development is not entirely clear. Even though SCD-1 has been linked to increased neoplastic cell transformation and to a protection process of the neoplastic cells against apoptosis [15, 18], human prostate carcinoma, for example, often show strongly down-regulated or even diminished SCD-1 expression [52] and more recent data indicated down-regulation of SCD-1 in some tumor cell lines [20]. Moreover, the survival of human breast carcinoma cell line MDA-MB-468 was not affected by SCD-1 knock-down [20]. As it is still unclear whether and how SCD-1 plays a key role in tumorigenesis, studies that look into this question are necessary because of the therapeutic potential this protein might possess.

Even though the phenomena of the tumor-specific apoptosis induction discussed in this chapter is an exclusive feature of the mouse ORCTL3 and not the human gene, according to the Oncomine database this gene is repressed in human tumors of the adrenal gland and bladder. Furthermore, the Cancer Profiling Array blot that compares tumor versus normal tissues of the same patients shows that ORCTL3 is significantly down-regulated in human renal carcinomas [7].

In conclusion, the anticancer effect of ORCTL3 was determined in a systematic screen for anticancer genes and further studied in an effort to understand the molecular mechanism behind its tumor-specificity. This unique function is shared with other known anticancer genes discussed in various chapters of this book. More in-depth work is needed to identify all of its targets and characterize where exactly this gene interferes in a signaling cascade that is tumor-specific. Despite being

discovered as an anticancer gene only around 5 years ago, the work on ORCTL3 already identified one target and the results on the *in vivo* xenograft models predict a promising application of this gene.

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Part III
Anticancer Gene Therapy

Chapter 12
Introduction of Genes via Sonoporation and Electroporation

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Contents

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Abstract Gene therapy delivery using viral vectors has demonstrated efficient transfection but has safety issues. The need for safer yet effective delivery systems has led to the active development of non-viral techniques. In this chapter, we will discuss two evolving techniques – sonoporation with microbubble contrast agents and electroporation with focus on their basic principle, parameters affecting delivery efficiency, current evolving techniques and future prospects.

Keywords Sonoporation • Electroporation • Gene delivery

12.1 Introduction

The challenge for Gene therapy, in treating solid cancers and hereditary diseases lies in its delivery clinically. While viral vectors have demonstrated efficient transfection in vivo and in vitro, it is marred by key issues such as immunogenicity, cytotoxicity, non-specific gene delivery and mutagenesis.

The need for safer and effective delivery systems has led to the active development of non-viral techniques. Emerging prime candidates are sonoporation with microbubble contrast agents and electroporation. While transfection efficiencies and cell viabilities had been questioned in previous studies, sonoporation and electroporation possess significant advantages to be used in routine clinical practice such as lower cost and toxicity, safer for repeated treatments and an acceptable immunogenicity profile.

12.2 Sonoporation-Definition

Sonoporation is the use of ultrasound ("sonar") to induce a non–invasive increase in cell membrane permeability ("poration"). Sonoporation may be classified into reversible or irreversible sonoporation. In reversible sonoporation, transient pore formation is observed, followed by pore resealing and maintaining cell viability. Reversible sonoporation allows the uptake of drugs, therapeutic substances and genetic material from an extracellular environment [1, 2]. As cell membrane alteration is transient, targeted local drug/gene delivery is enhanced as they remain within the cell after ultrasound exposure [3, 4]. In irreversible sonoporation, pore formation is permanent; leading to cell lysis, disintegration and eventual cell death, which is more suited for treating cancers.

Sonoporation can be restricted to specific target cells over a desired time, limiting treatment to just the region of interest. The non-invasive nature of this method will allow the treatment of diseased organs located deep within the body cavity, (e.g. liver, prostrate, pancreas, kidney) as long as they are penetrable by the applied ultrasound beam. Potential clinical applications currently in pre-clinical studies include cancer therapy of solid tumors [5–7] as well as in the treatment of

cardiovascular and brain diseases [8]. Successful sonoporation of genetic material into the macrovasculature [4, 9] and skeletal muscles have also been demonstrated in vitro and in vivo [1, 10–13].

12.3 Ultrasound Field-Unfocused and Focused

Sonoporation is based on ultrasonic wave energy. Ultrasound propagates from a piezoelectric transducer as an oscillating pressure wave at frequencies in a range of 20 kHz–200 MHz. Piezoelectric transducers are devices that operate based on the piezoelectric effect i.e. converting electrical signals into mechanical vibrations and vice versa. Ultrasound beams can be divided into two categories: unfocused and focused.

12.3.1 Unfocused Beams

An unfocused transducer generates an acoustic field divided into the nearfield and farfield (Fig. 12.1). A characteristic quantity of interest in the farfield is the Rayleigh distance, which is the boundary point that marks the beginning of the farfield and the end of the nearfield. This is expressed as:

$$R_0 = \frac{ka^2}{2}, \tag{12.1}$$

where k is the wavenumber ($k = \omega/c_0 = 2\pi f/c_0, f$ being the frequency, ω the circular frequency of the source and c_0 the speed of sound) and a the radius of the source.

Unlike the nearfield, where ultrasound intensity is largely stable and varies between fixed maximum to minimum, ultrasound intensity in the farfield decreases monotonically as the distance from the source increases. In the nearfield, the sound is confined to a cylinder with a radius equal to the radius of the transducer [14]. Unfocused transducers can hence produce a large area of uniform pressure in the nearfield and this is ideal for treating superficial tumors/tissues.

12.3.2 Focused Beams

Focused transducers are widely used in various medical applications. They typically operate at frequencies from 0.5 to 10 MHz, have a diameter of 1–10 cm and a focal zone from 1 to 20 cm [15]. As focused transducers come in a variety of frequencies and sizes, they can produce a range of acoustic intensities that are suitable for both diagnostic imaging and therapy.

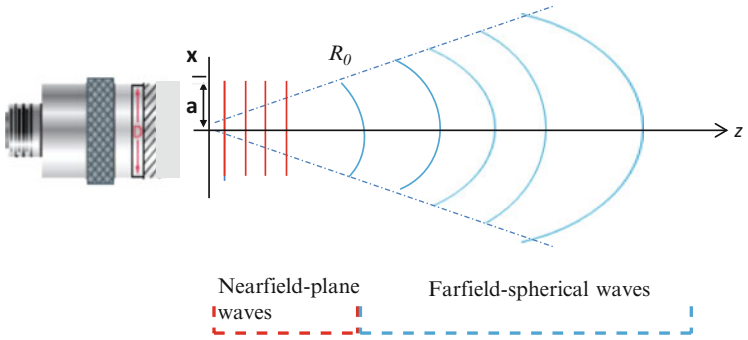
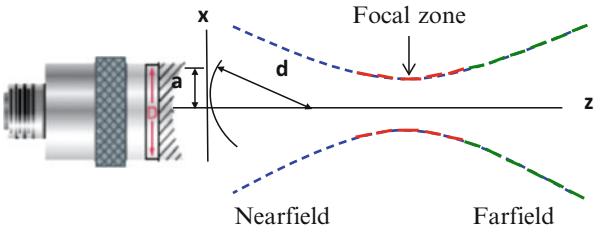


Fig. 12.1 Unfocused ultrasound beam transmission

Fig. 12.2 Focused ultrasound beam transmission



The acoustic field produced by a focused transducer is divided into three zones: the pre-focal zone (also known as nearfield), the focal zone, and the post-focal zone (also known as farfield). The nearfield is located just beyond the transducer's surface, while the farfield begins after the focal zone (Fig. 12.2). Sound beams converge from the source to the focus and then diverge to the post-focal region. Along the transducer axis, the acoustic pressure alternates into a series of maxima and minima.

At the focal zone, the acoustic pressure is equal to the initial acoustic source pressure multiplied by the transducer gain G:

$$p = p_0 G, \tag{12.2}$$

where G is equal to:

$$G = \frac{p(x = d)}{p(x = 0)} = k\frac{a^2}{2d}, \tag{12.3}$$

with p the acoustic pressure and d the focal distance of the transducer [16].

Focused transducers can thus concentrate energy deep inside the body within small tissue volumes. This will allow controlled, non-invasive and precise treatment of targeted diseased areas [17, 18].

Commercial and laboratory ultrasound machines used for in vitro and in vivo sonoporation studies have since been developed. Sonoporation specific devices typically comprise of transducers with a function generator and amplifier. They have a range of settings that can be adjusted, depending on the application.

12.4 Properties of Microbubbles

While the exact physical and biological mechanisms of sonoporation have not yet been fully understood, it is known that microbubble interactions with cell membranes under ultrasound exposure initiate the cellular and molecular mechanisms that are necessary to induce this phenomenon [3, 19, 20].

Gene delivery via sonoporation was first described using first generation air – filled microbubble agents encapsulated in albumin or galactose shells. Examples include Albunex® (Molecular Biosystems, Inc, San Diego, CA), Levovist® (SH U 508A, Schering AG) and Echovist® (SH U 454; Schering AG, Berlin, Germany) [3, 20]. As air is highly soluble, the first generation agents had a short longevity within the vasculature, limiting their efficiency.

Second generation ultrasound contrast agents such as Optison® (FS-069, perflutren protein type A microspheres for injection; GE Healthcare, Princeton, NJ), Definity® (MRX-115, perflutren lipid microspheres; Bristol-Myers Squibb Medical Imaging, North Billerica, MA) and Sonovue® (BR-1, sulfur hexafluoride; Bracco International BV, Amsterdam, the Netherlands) are widely used clinically across Europe, China, Japan, USA and Australia as intravascular tracers for imaging of the micro and microvasculature in organs such as the heart and liver. These agents increase the backscatter of ultrasound beams, producing highly echogenic signals. The contrast specific mode of the ultrasound machine will use the non-linear responses from microbubbles to form images while suppressing the linear response from tissue.

The second generation agents have stable shells that comprise of a thin (between 10 and 200 nm thick) biocompatible material (e.g. phospholipids [21]). The hydrophobic gases (e.g. perfluorocarbon, sulfur hexafluoride, nitrogen) within the core are characterized by high molecular weight, reduced solubility and diffusivity. These properties increase their resistance to arterial pressure, preventing them from dissolving in the blood stream.

The diameter of a microbubble ranges between 1 and 10 μm [22], which is close to the size of a normal human red blood cell (6–8 μm). They are much larger than the molecules of computed tomography (CT) and magnetic resonance (MR) contrast agents. This prevents their passage through the vascular endothelium, thus confining them within the blood pool. Their small size also allows movement into the microcirculation of the pulmonary capillaries for safe excretion [23, 24].

Besides facilitating sonoporation, microbubbles also act as “micro-vehicles” carriers, carrying and delivering high concentrations of drugs or gene material to specific diseased tissue. The types of micro-vehicles may be divided into two

broad categories: pressure or temperature sensitive systems. Pressure sensitive micro-vehicles such as lipid and polymer shelled microbubbles are activated by the applied acoustic pressure [25, 26], leading to the release of their contents. Temperature sensitive systems such as thermosensitive liposomes undergo phase transition, become more permeable and release their content under the influence of ultrasound- induced hyperthermia. (As tissue is exposed to ultrasound, energy is absorbed and converted to heat. The temperature elevation in the tissue depends on the removal of heat as a result of diffusion by the tissue and convection by the vasculature [27]. The temperature required in these instances is at a range of 39–41 °C [28–30]).

12.5 Microbubble Interaction Leading to Sonoporation

While the exact physical and biological mechanisms of sonoporation are not yet fully understood, the addition of microbubble contrast hastens this process. The microbubble interactions with cell membranes under ultrasound exposure underline the basic hypotheses that can potentially explain sonoporation as well as its cellular and molecular mechanisms [3, 19]. In the presence of an ultrasound field, the microbubble undergoes oscillations interacting with nearby cells or tissues. The acoustic waves whilst undergoing phases of negative and positive pressure volumetrically expand and contract the compressible microbubble [31]. At low-pressure amplitudes, microbubbles respond by decreasing and increasing their radius successively; undergoing linear oscillations (stable linear cavitation). When the microbubble expands, it pushes apart the surface of the cell membrane (Fig. 12.3a). During contraction, the cell membrane pulls towards the microbubble [32] (Fig. 12.3b). At the same time, ultrasonic radiation force (i.e. radiation force exerted by an acoustic wave on an object) is shown to initiate an imparting motion and a translational displacement upon the oscillating microbubble [33, 34]. Upon the microbubble translational force the cell membrane undergoes disruption which may enable drug delivery into the intracellular compartment [35] (Fig. 12.3c).

At high-pressure amplitudes, the microbubble grows in successive cycles, undergoing asymmetric nonlinear oscillation. It eventually reaches an unstable size at which it collapses violently and asymmetrically (inertial cavitation). This results in the production of liquid jets, which contain energy concentrated into small volumes. The liquid jets move with sonic speeds, penetrating the cell membrane wall.

When the microbubble collapses, it also produces local steady flows known as micro streaming [36–38] (Fig. 12.3e), shock waves, shear stresses and free radicals. During inertial cavitation, extremely high pressures and temperatures can be reached within the microbubble [39]. These phenomena change the microbubble – cell environment, increasing heat transfer at the microbubble – cell interface. The resulting friction creates pores in the cell membrane, enhancing the permeability to extracellular molecules and substances.

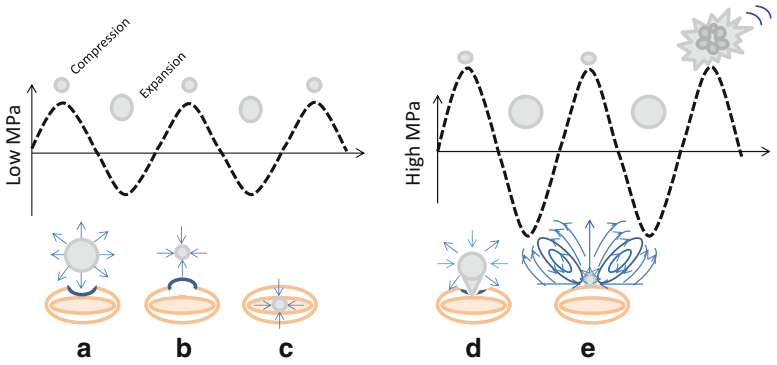


Fig. 12.3 Microbubble and cell interactions during ultrasound exposure

12.6 Sonoporation Pore Size

Evidence of pore formation on cell membranes was observed during microbubble-cell interaction by using scanning electron microscopy (SEM) [40, 41], optical microscopy and electrophysiology methods. Sonoporation pore size depends directly on the acoustic pressure and exposure duration of the cells to ultrasound and microbubbles. Various studies have suggested that the pore sizes are within a specific range. Taniyama et al. [4] demonstrated a maximum pore size of 100 nm in cultured human aortic endothelial cells and vascular muscle cells which were exposed to a high frequency, low intensity ultrasound beam in an experiment using Optison® microbubbles. Zhou et al. [42] noted a pore size of 110 ± 40 nm on Xenopus laevis oocytes which were exposed to ultrasound pulses (f: 1.075 MHz, p: 0.3 MPa, t: 0.2 s) and Definity® microbubbles. Qiu et al. reported sonoporation pores with mean size ranging from ~100 nm to 1.25 mm on human breast cancer cells MCF-7 which were mixed with polyethylenimine, DNA and microbubble components. Low acoustic pressures (0.05–0.3 MPa) and a frequency of 1-MHz [43] were used in their series. Zhao et al. [44] showed pores ranging from 500 to 2,500 μm on normal breast cancer cell lines (SK-BR-3) after induced sonoporation.

12.7 Ultrasound Parameters Affecting Sonoporation and Delivery Efficiency

The efficiency of transfection and cell viability are directly affected by the applied acoustic parameters. These parameters include frequency, acoustic pressure and amplitude, duration of ultrasound exposure, pulse length, interval and duty cycle of the transmitted wave [8, 40].

Transmitted frequencies correlate with the resonance frequency of the microbubbles (3–5 MHz) [45] and its size. The majority of in vivo gene delivery studies have been reported at 1 MHz, though frequencies between a range of 0.3–14 MHz [21, 46, 47] have also been used. For in vitro studies, the reported frequencies range from 1 to 10 MHz [48].

One of the most important parameters during sonoporation is the applied acoustic pressure. Acoustic pressure is expressed either as amplitude or intensity in laboratory setups. When diagnostic machines are used, it is expressed as the mechanical index [MI: ratio of peak negative pressure (MPa) over the square root of the transmitted frequency (MHz)]. In order for cell membrane permeabilization to occur, a minimum acoustic pressure threshold is required. For in vivo studies the boundary is set at MI $\leq$0.4 while for in vitro studies, it is set at 0.06 MPa/MHz [48].

Ultrasound exposure time is another critical component that affects sonoporation. Together with microbubble flow rate and microbubble concentration at the region of interest, it accounts for the rate of cell permeabilization [49]. In most sonoporation in vivo protocols, ultrasound exposure was less than 10 min in order to minimize tissue damage. Prolonged ultrasound exposure (20–30 min) during gene delivery had resulted in tissue damage and a decreased level of gene expression [50]. In vitro protocols have ultrasound exposure times, which are much shorter, being in the order of seconds [42–44, 51, 52].

The ultrasound pulses used in sonoporation are intermittent rather than continuous. They are represented by the pulse interval and pulse length (Pulse Repetition Frequency: PRF), which can be adjusted to control the duty cycle. For example, a 50 % duty cycle can have a pulse length of 5 ms (“on”) and a pulse interval of 5 ms (“off”). Both in vitro and in vivo studies had shown that pulse lengths between a micro to millisecond could result in a uniform distribution of extracellular molecules within the targeted regions [53]. It was also demonstrated that when longer pulses are applied the pressure threshold to cause permeability in the vasculature is decreased, resulting in a higher level of transfection [54, 55].

12.8 Mechanism of Gene Delivery via Sonoporation

Over the years, sonoporation has demonstrated great potential in the fields of gene therapy and drug delivery. Initially, macromolecules and small particles were delivered into cells [56–58]. With increasing knowledge of cellular and molecular biology along with advances in ultrasound technology, the delivery of nano and micro-vehicles carrying high concentrations of biological material such as monoclonal antibodies [59], therapeutic nucleic acids (pDNA, mRNA, siRNA, etc.) [60, 61] and anticancer drugs [62] into tissue and organs became possible.

Different aspects of gene delivery have been thoroughly studied. Caskey et al. [63] and Chen et al. [64] studied the interaction of microbubbles with blood vessels during cavitation, observing the disruption of vascular integrity. It was shown that the expansion of microbubbles resulted in the longitudinal strain on

blood vessels, while compression caused invagination of the vessels. Interestingly, the microbubbles continued to oscillate even when partially embedded into the endothelium [65].

The mechanisms of gene delivery beyond the vasculature via sonoporation was studied by both Caskey et al. [66] and Arvanitis et al. [67]. Both authors used tissue mimicking gel phantoms. During ultrasound activation, microbubbles were observed to form tunnels into the phantom, following the direction of ultrasound wave propagation.

Microbubbles were also thought to enhance endocytotic uptake during gene delivery. Meijering et al. [68] examined the endocytotic activity of cultured cells under the influence of stable cavitation. It was noted that microbubble endocytosis during stable cavitation depended upon the molecular size of the drug within the core of the bubble and that intracellular drug uptake was reduced to a large extent with the inhibition of clatherin-mediated endocytosis. Hauser et al. [69], further reinforced this idea, through the observation that the number of endocytotic vesicles and clatherin coated pits increased with the influence of stable cavitation.

12.9 In Vitro Studies of Sonoporation in Cell Cultures

In vitro studies of sonoporation generally involve the investigation of ultrasound bioeffects on cell cultures, cell suspensions or cell monolayers with the aim to optimize the minimum ultrasound exposure conditions required to achieve successful gene transfection or drug delivery. Two types of experimental setups have been developed for sonoporation on cells. In the first set up, the transducer is placed in direct contact with the cell culture. In the second set up, the cell culture is placed in a water bath while the transducer is placed at a distance. The ultrasound waves are made to propagate through the water bath, which will diffuse any resultant thermal energy [70]. Different cell lines had been investigated; with common examples including mammalian cells, breast cancer cells, prostrate cells, blood cells.

Cochran et al. [71] investigated in vitro gene delivery transfection into MCF 7 breast cancer cells by exposing a suspension of polymer microbubbles (poly lactid acid (PLA)) and naked plasmid DNA to ultrasound. Transfection efficacy and cell viability were examined based on the effect of frequency (1–5 MHz), pressure amplitude (0–2 MPa), pulse repetition frequency (5–20,000 Hz), pulse length (3–12,000 ms), and exposure time (0–30 s). Cell transfection was reported with sonoporation conditions at pressure threshold of $\geq$250 kPa, low frequency of 1 MHz, pulse length of 12 ms and exposure time of $\leq$2 s. A transfection efficiency of 24.2 ± 2.0 % was achieved at 1 MHz frequency, 1 MPa pressure amplitude, 12,000 ms pulse length and pulse repetition frequency of 5 Hz. This study represents a possibility of using polymer microbubbles for sonoporation to deliver genetic material into tumor cells.

MCF7 breast cancer cells were also studied by Qui et al. [72] under various sonoporation conditions. They hypothesized that inertial cavitation (IC) activities

accumulated during US exposure could be quantified as an IC dose (ICD) based on passive cavitation detection (PCD). The assessment of the sonoporation outcome could thus be correlated with ICD measurements. Ultrasound-mediated DNA transfection of plasmid vector pIRES2-EGFP with Polyethylenimine (PEI) was performed with a custom made 1 MHz-focused transducer. Stable 20 cycle pulses with various settings for PRF, acoustic peak negative pressure and total treatment time were used. ICD was directly affected by the applied ultrasound conditions and was highly related to sonoporation pore size and cell viability. Results also indicated an initial linear increase in DNA transfection efficacy, which was proportional to ICD increase before reaching saturation at higher dose. DNA transfection efficacy up to 44.7 ± 5.8 % was achieved. The authors concluded that inertial cavitation has a major impact on DNA transfection during sonoporation and ICD can be applied as a control parameter during ultrasound drug/gene delivery.

Local delivery of chemotherapeutic cytotoxic drugs to human gingival squamous carcinoma cells (Ca9-22) was observed by Iwanaga et al. [73]. The cells were exposed to sonoporation for 20 s at 1 MHz, at an intensity of 1 W/cm^2 and a 10 % duty cycle. Sonoporation was performed in the presence of Optison® contrast agents, delivering Bleomycin (BLM) into Ca9-22 cells. Flow cytometry after sonoporation determined the percentage of apoptotic Ca9-22 cells based on the presence of hypodiploid DNA. 17.7 % of the cells exposed to a combination of sonoporation and BML were apoptotic compared to 8.4 % and 8.5 % when exposed to sonoporation or BML alone respectively. Results suggest the potential use of cytotoxic chemotherapeutic drugs with sonoporation for molecular cancer therapy.

In vitro experiments have also been carried out using diagnostic ultrasound scanners. For example, DNA plasmid transfer in epidermoid cell monolayers with Optison® microbubbles had been conducted using a low power diagnostic scanner (1.5-MHz) [74]. Induced gene transfer following ultrasound exposure at 2.3 MPa with 2 % Optison® concentration was determined based on GFP (green fluorescent protein) levels. GFP expression of 3.7 % (1.2 % SD) was noted for the exposed group as compared to 0.4 % (0.7 % SD) for the non-exposed group ($P < 0.01$). The percentage of dead cells was greater at 28.6 % (6.3 % SD) for the exposed group compared to the control group at 3.4 % (1.7 % SD). These results represent a potential for gene transfer and clinical therapeutic applications with diagnostic ultrasound scanners and ultrasound contrast agents.

12.10 In Vivo Studies of Sonoporation

Most in vivo studies of sonoporation involve mainly small animal research, to demonstrate the therapeutic potential of this approach. In vivo sonoporation settings varying from intense conditions at 1–5 MHz, 1.2–4.6 MPa [75] to low settings at 1 MHz, 0.1–0.5 MPa [43] have been used. Skeletal and cardiac muscles of mice or rats have been used to a great extent and some work has been carried out

on brain, intestines, liver, kidney, lungs, knee joints, glands of small animals as well as different tumor types.

Enhanced plasmid DNA transfection into tibialis anterior skeletal muscle of mice has been reported using a P85 pluronic block copolymer. A 1 MHz, 1.3 cm radius transducer was used. Settings are set at a PRF of 100 Hz, a 20 % DC and a 3 W/cm^2 intensity. Exposure timing was set at 60 s. The use of ultrasound significantly enhanced the plasmid DNA transfection efficiency and expression to 304.9 ± 17.4 compared with the 52.88 ± 9.69 of the control group. Muscle damage was limited in these settings, suggesting the potential use of certain pluronics and ultrasound conditions for gene delivery in clinical applications [76].

In vivo siRNA targeting rat Glyceraldehyde-3-phosphatedehydrogenas (GAPDH) in the parotid glands by transdermal and directly exposed (via an incision on the lateral neck to expose the parotid glands) sonoporation had been evaluated [77]. A 1 MHZ transducer with a 6 mm radius was set at an intensity between 0.5 and 4 W/cm^2 and a 50 % DC for 2 min. Varying Optison® concentrations were used. SiRNA induced gene silencing of GAPDH was demonstrated with no significant tissue damage after 48 h, at 1 W/cm^2 (75 % silencing) and 2 W/cm^2 (80 % silencing); at an optimal Optison® microbubble concentration of 20–50 %. As ultrasound intensity was increased, (4 W/cm^2: 30 % silencing) tissue damage (cell apoptosis) occurred. Direct exposed sonoporation, which was expected to be more efficient compared to the transdermal approach showed no significant difference in gene expression. Results are encouraging and will contribute to the future development of siRNA-based gene therapy.

Mehier-Humbert et al. [78] exposed male BALB/c [Bagg Albino (Inbred strain of mouse)] mice muscles to ultrasound pulses (1 MHz, 3 mm radius, 20 % DC, 0.4 W/cm^2, PRF 200 Hz, 5 min) in the presence of polyethylenimine (PEI) nanoparticles combined with contrast agents (Sonovue®). Enhanced transfection efficiency and prolonged expression of internalized plasmid DNA were reported, while the applied ultrasound parameters achieved high levels of gene expression with low cytotoxicity. The amount of intracellular uptake of plasmid DNA by PEI and US mediated transfection was monitored through flow cytometry after 3 and 24 h, and compared to a control group (DNA + US). Cells uptake of 19.1 ± 1.3 % vs. 0.7 ± 0.4 % and 74.1 ± 2.7 vs. 55.8 ± 0.6 % were noted respectively.

Sheyn et al. [79] used thigh muscle from female wild type C3H/HeN mice to demonstrate in vivo bone formation via ultrasound-based non-viral osteogenic gene delivery. Optison® microbubbles were mixed with plasmid DNA encoding for the osteogenic gene, recombinant human bone morphogenetic protein-9 (rhBMP-9). The expression of luciferase plasmid (Luc), encoding rhBMP-9 was monitored after sonoporation was applied (frequency 1 MHz, duty cycle 50 %, intensity 5 W/cm^2, exposure duration 10 min, experiment repeated three times separated by a day of rest). After sonoporation, the volume of newly formed bone reached 0.9 ± 0.17 mm^3, bone volume density of ectopic bone formation was 0.7 ± 0.09 BV while the bone mineral density was equal to 793.51 ± 68.44 mg HA per cm^3. No indication of osteogenic tissue formation was present in the control group (empty plasmid vector + pcDNA3 + US).

Recently, Kotopoulis et al. [80] reported a case study evaluating the treatment of human pancreatic cancer using a combination of ultrasound exposure, Sonovue® microbubbles and intravenous chemotherapy treatment with gemcitabine. A total of five patients with pancreatic adenocarcinoma were recruited, each receiving an average of 9 ± 6 chemotherapy treatment cycles. Comparison was made to a control group of 80 patients receiving an average of 16 ± 7 cycles. A commercial clinical GE LOGIQ 9 scanner and 4C curvilinear probe (GE Healthcare, Waukesha, WI) were used with acoustic settings for sonoporation initially generated in water (frequency 1 MHz, duty cycle 1 %, MI 0.49) and then translated for clinical use (frequency 1 MHz, duty cycle 1 %, MI 0.2). Sonoporation was induced over a period of 31.5 min. Results showed a reduction in tumor size for two patients, while in three other patients, reduced growth was observed. This case report demonstrated the potential synergism of sonoporation and intravenous chemotherapeutic drugs in a clinical setting.

12.11 Future Prospects and Development

Sonoporation provides a novel and promising strategy for efficient and specific gene/drug delivery applications with desired therapeutic effects. Although tremendous progress has been made over the years, further technological, biological and medical studies are still needed before sonoporation can be translated into routine clinical practice.

Future designs of microbubble contrast agents will have to take into account its function as a gene/drug material carrier. Its safety profile, stability in the vasculature and echogenicity will have to be considered [81]. Focus should be placed upon microbubble architecture and shell composition since these two components directly affect the transfection efficiency under ultrasound application [78]. The loading capacity of the bubble, its circulation life, biodistribution and drug release mechanisms are areas for research and development. The optimal microbubble size should be reviewed as size is proportionally correlated to circulation life. Smaller microbubbles stay longer in the circulation and can potentially deliver more drugs/gene material to the treatment site.

Organ or tumor specific microbubbles can be designed to improve specific-localized drug/gene delivery. The idea is to restrict molecular uptake and treatment to the region of interest, minimizing toxic effects on healthy tissue. Active targeting may be achieved by loading specific antibodies or ligands on the surface of the microbubbles.

Ultrasound devices and transducers for sonoporation with optimized ultrasound parameters are currently being developed for clinical applications. Ideally, they should produce a homogeneous ultrasound field to enable safe and efficient delivery into large volumes. Such devices maybe coupled with commercially available diagnostic ultrasound machines to provide simultaneous imaging and therapy.

12.12 Electroporation: Definition

Electroporation (EP) is a non-thermal technique, which results in the structural rearrangement of bilayer cell membranes [82, 83]. During electroporation, cells and tissues are exposed to an electrical field using high intensity electrical pulses. This results in multiple nano – scale aqueous pores in the cell membrane.

Electroporation is divided into irreversible (IRE) and reversible electroporation. During irreversible electroporation, the permanent permeabilization process of the cell membrane when subjected to high electrical pulses will initiate apoptosis leading to permanent cell death. Irreversible electroporation is used in cancer treatment for uniform and complete tumor destruction [84, 85]. In comparison, reversible electroporation creates transient pores in cell membranes, which enable exogenous materials (Fig. 12.4) [40, 86, 87] present in the extracellular environment to penetrate cells. The delivery of chemotherapy drugs and genetic material via electroporation are described as electro- chemotherapy (ECT) [88–90] and electro-gene therapy (GET), respectively [91].

12.13 Pore Formation and Cell Membrane Permeability

When cells undergo electroporation, they go through a dielectric membrane breakdown and pore formation is initiated [92]. Pore formation is dependent on electro- mechanical and thermodynamic parameters, which include the strength of the electric field, temperature and membrane rigidity [93].

Pore formation though is only initiated when a specific voltage threshold reaches its critical value (1–2 V) [94, 95], causing an increase in membrane conductivity. The critical threshold value for membrane cell permeabilization depends largely on the energy potential of the cell before it is subjected to an electric field. Prior to electroporation, the energy potential within the cell is relatively negative when compared to its surrounding environment.

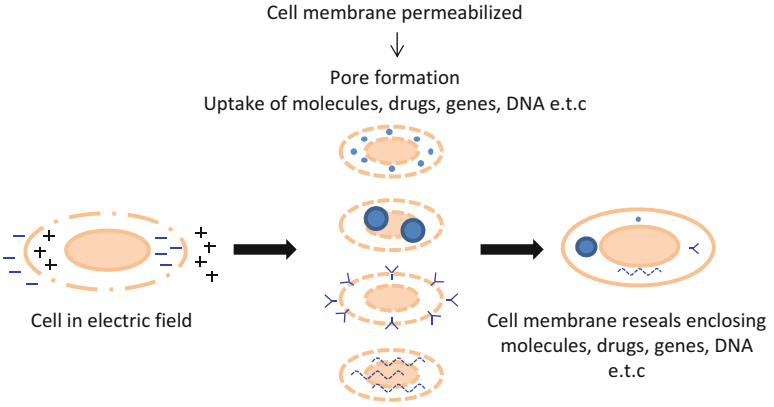


Fig. 12.4 Electroporation phenomenon

Applied electrical field induces a rapid increase in the trans-membrane voltage, with the membrane attracting small conducting ions (e.g. Na^+ and Cl^-) from the surrounding environment. This suggests a transition from an insulating to conductive state, in an effort to protect the membrane from destruction [86]. The negative potential within the cell will cause the capacitance of the membrane to be exceeded, resulting in pore formation [96, 97]. The lipid morphology of the membrane bilayer is rearranged, increasing the surface tension to generate hydrophilic pores [13, 98].

The resultant mechanical action leads to an expansion of membrane pores in an effort to induce intracellular molecular transport [86, 94, 99, 100]. Pore formation is observed over a time range of ns to μs while membrane resealing happens in a course of minutes [101–103]. Pore sizes ranging between 20 and 120 nm are reported in red blood cells [104].

12.14 Parameters Affecting Electroporation and Delivery Efficiency

Gene/drug delivery efficacy during electroporation relies heavily on cell membrane permeability [105]. A general consideration is that the smaller the cell radius, the larger the external field needed to achieve permeabilization.

Membrane permeabilization is largely controlled by the electric field strength, with electric field intensity varying from 200 V/cm for large cells to 1–2 kV/cm when applied to bacteria cells [106]. The area of permeabilization is controlled by the electric pulse amplitude, while the degree of permeabilization is controlled by the pulse duration and number of cycles [107, 108].

Various studies had reported that the use of low electric field strength (100–200 V/cm) with long pulses (20–60 ms) will facilitate gene material transfer, while high electric field strengths (>700 V/cm) with short pulses (100 μs) are preferred for drug delivery [109, 110].

Additional factors that affect gene/drug delivery efficiency are the size and number of pores. Of particular note, an optimum minimal number of pores are desired in order to avoid toxicity. Last but not least, the composition of the DNA vectors (e.g. polymeric forms), the electrode design and neoadjuvant therapy of the targeted region are also contributing factors [111, 112].

12.15 Electroporation: Instrumentation

The basic instrumentation for electroporation is a pulse generator and applicator that consists of the electrodes. In order to obtain successful targeted electroporation it is important to consider the shape, size and number of the electrodes as they will influence the strength, homogeneity, shape and total energy distributed from the

electric field to the targeted region. The effectiveness of electroporation and the desired distribution of the electric field are dependent on the distance between the electrodes, the depth at which they are placed within the medium of interest and the amplitude voltage applied [113].

Different types of electrodes have been developed. Needles, plates and catheter-based electrodes are the most frequently used in both in vitro and in vivo applications.

Needle electrodes are parallel arrays, which are flexible in placement, allowing treatment of both surface and deep tumors. Their precise placement (which sometimes requires imaging guidance) is important to predict the electric field distribution. This is crucial, to avoid extensive damage to surrounding normal tissue [94, 113]. Plate electrodes are used for surface tumors and are especially suitable for electroporation on small animals as the plates provide a more uniform electric field than the needle electrodes. They are not yet used on larger animals as greater electric fields will be required to achieve local targeting [114]. Catheter-based electrodes can be inserted into blood vessels and hollow organs [115, 116].

12.16 In Vitro Electroporation

The actual size of the applied electrical field has to be carefully calibrated to ensure both cell permeability and survivability. The composition of electrodes used is important in order to decrease ionic release from electrophoresis, while controlled low temperatures are needed to induce membrane resealing. A number of in vitro studies have been performed using square wave pulse generators, which allow independent control of amplitude and pulse length, leading to high cell viability with high percentages of permeabilized cells and efficient gene transfection [94].

Zorec et al. [117] investigated on how different square wave electric pulses influence transdermal drug delivery. It is thought that local transport regions (LTR) evolve on cell membranes during applications of an electric pulse on the skin to allow molecular and ionic transport. In their experimentations, the authors used short high voltage square pulses (HV) (amplitude 500 V, duration 500 μs, 500 μs pulse spacing) and long low voltage pulses (LV) (amplitude 45 V, duration 250 ms, pulse spacing 100 ms) pulses individually as well as in different combinations and orders. The general expert opinions are that short HV pulses create small, high conductivity pathways across the stratum corneum (SC), while longer LV pulses create long-lasting and larger pathways. Longer LV pulses significantly increased passive transport of calcein (a fluorescent dye) through dermatomed pig skin, while short HV pulses alone produced negligible transport. Interestingly, calcein transport was also reduced when a combination of HV pulses followed by LV pulses were used. The authors thus postulated that when HV pulses are first applied, they altered the structure of the SC in such a way that subsequent applied LV pulses were insufficient to initiate LTR expansion. They concluded that total pulse energy alone could not account for total solute transport and that the order of type of

pulse administered must also be taken into consideration when performing skin electroporation.

Labanauskienė et al. [118] assessed whether electroporation (1,200 V/cm, 0.1 ms, 1 Hz, 8 electric pulses) could enhance photodynamic tumor therapy (PDT- LED array UNIMELA-1 light exposure (7.6 W/m^2)) effectiveness in murine hepatoma MH22A cells. Photodynamic tumor therapy (PDT) is based on photosensitizers, which induced oxidative damage on cellular organelles when excited by light of a visible wavelength. Various concentrations of photosensitizers (chlorin e6 or aluminum phthalocyanine tetrasulfonate) were added to MH22A cells and exposed to electroporation. Fluorescence microscopy detected photosensitizers in the MH22A cells after electroporation, while cells not exposed to electroporation had insignificant or non-evident levels. Cell viability levels were consequently lower for cultures, which underwent PDT and electroporation than PDT alone. The authors concluded that in an in vitro environment, PDT was more effective with electroporation, even at low doses of photosensitizers.

Gene-electro transfer of siRNAs against CD146, which inhibited the migration and invasion of human malignant melanoma cells SK-MEL28 was studied by Todorovic et al. [119]. Compared to a control siRNAs group, down regulation of CD146 mRNA after GET was higher when compared to lipid-mediated transfer, in a range of 29–35 %. In addition, reduced cell migration (44.2 %), reduced invasion of melanoma cells (47.3 %) and reduced cell survival (76 %) were observed. Cell proliferation and viability were not affected. These results suggest that GET of siRNAs that target CD146 expression in melanoma cells may reduce disease progression and prevent metastatic potential.

12.17 In Vivo Electroporation

In vivo gene transfer via electroporation with high transfection rates is achievable [94]. Successful in vivo electroporation of genes/drugs have been reported in a wide range of tissues such as skin, liver, kidney, brain, lung, skeletal and cardiac muscle, joints, vasculature etc. [94, 120–122].

Yianli et al. [123] used a syringe electrode to transfect hNEP plasmid (PCSC-SP-PW-hNEP) to the Hind limb of KunMing male mice using square wave electric pulses (50 V/cm, 6 pulses, 20 ms per pulse). hNEP is an enzyme implicated in the degradation of toxic amyloid-beta (Abeta) peptide, which has potential application in Alzheimer's disease therapy. Increased levels of therapeutic hNEP protein were detected in the Hind limb muscle, serum and brain at a time frame of 7, 14 and 30 days after electroporation when compared to a control group, which had only systemic injection of hNEP plasmid. Only a low level of muscle damage was noted after DNA transfer and electroporation. The results demonstrated the necessity of an electric field to improve plasmid DNA uptake and expression.

Liu and Huang [124] were the first to report enhanced in vivo electroporation gene transfer to mice livers though vascular delivery. Systemic administration of

plasmid DNA (80 μg) via the tail vein of plasmid DNA was compared to direct local injection to the left median lode. In both instances, electroporation (250 V/cm, 20 ms per pulse, 8 pulses) was performed on the liver after administration of gene material. Broader transfection of reporter genes was noted in the liver with systemic delivery while direct injection confined the delivery to only the local injection site and along the needle track. In the former group (systemic administration), high levels of gene expression were reported in four divided sections of the left median lobe ($1 \times 10^8 - 8 \times 10^8$ RLU/per mg protein) after 8 h of electroporation. The later group (Direct injection) showed the highest level of gene expression in the injected site (1×10^9 RLU/per mg protein) while expression decreased 20–1,000 times in the three other sections. This study demonstrated the difference between local and systemic gene delivery to the liver under the influence of electroporation, implying that the mode of delivery should be determined by the desired effect.

Dean et al. [125] focused on establishing the potential of applying gene electroporation to the lungs. Female Balb/c mice were injected intra-tracheally with plasmid DNA (pCMV-Lux-DTS) expressing the luciferase gene. They were then subjected to an electric field (200 V/cm, eight 10 ms square pulses, 1 s pulse interval), with caliper electrodes placed on both sides of the thorax. Gene expression was seen 1 day after electroporation in the alveolar type I and type II epithelial cells, with maximum expression indicated between 2 and 5 days after induced doses of 20 and 100 mg of DNA. A survival of 88 % was achieved with mortality occurring due to fluid delivered into the lungs. Histological analysis showed no indication of damage secondary to electroporation, paving the possibility of its application on humans to treat local lung tumors.

Electroporation has also been extensively studied for gene transfer or chemotherapy into tumors. Tamura et al. [126] performed electro-gene therapy with interleukin expressing gene plasmids, which induce anti-tumor immunity. Increased local anti-tumor effects were observed in the treated area, while simultaneously affecting untreated tumors (immuno-effect) in other areas of the animal. Ramirez et al. [47] conducted an earlier study on electro-chemotherapy of liver tumors in rabbits, which showed a reduction in the number of liver metastases.

Animal studies have also indicated a synergistic effect for combined electrochemo gene therapy. For example, Kishida et al. [127] performed intra tumor delivery of IL-12 gene and Bleomycin alone and in combination via electroporation to subcutaneous melanomas in mice. While either chemotherapy or gene therapy alone suppressed subcutaneous and metastatic melanomas, the group receiving combination therapy showed the best therapeutic outcome with prolonged mean survival.

Daud et al. [128] were the first to conduct a Phase I trial utilizing in vivo DNA electroporation. Twenty-four patients with metastatic melanoma were treated with varying concentrations of interleukin-12 plasmid. A MedPulser DNA EPT System Generator (Inovio Biomedical Inc, San Diego, CA) incorporating a six needle electrode applicator was used. An electric field of 1,300 V/cm and pulse duration of 100 μs was applied during days 1, 5, and 8 of a 39-day cycle. Results on post-treatment biopsies indicated an increase in IL-12 expression levels as dosage

increased, while tumor necrosis and lymphocytic infiltration were observed. Distant metastatic lesions that were not electroporated and did not receive any other treatment showed complete regression in 2 out of 19 patients, while a further 8 patients (42 %) showed partial response or stable disease. There was minimal systemic toxicity, with some minor complaints of transient pain or minimal bleeding reported over the treatment site. This landmark trial has established electrogene therapy as a safe, effective, reproducible and titrable mode of therapy with good potential.

Irreversible electroporation with needle electrodes [NanoKnife (AngioDynamics, Latham, NY)] is currently routinely used in ablation of various malignant tumors from different organs such as liver, lung, pancreas and kidney. Retrospective reviews and phase 1 trials are now being carried out to determine its long-term efficacy [129–133]. Around the zone of irreversible electroporation is a wider volume of reversible electroporation. Hence the combination of IRE with chemotherapy potentially could limit the local recurrence rate.

12.18 Future Prospects and Development

Early in-vitro experiments and in-vivo trials have shown that electroporation can deliver gene/drug therapy. As IRE is already available as a clinical tool for treating malignancies, it offers great potential to facilitate gene therapy research in humans. Studies are still required in establishing the electroporation parameters that will deliver optimal levels of gene/drug therapy, while at the same time ensuring safety and minimizing side effects such as potential irreversible heat shock or burn injuries [134].

From a design and engineering as well as clinical practical perspective, further development of devices, to create a homogeneous electric field to increase target specificity and efficiency of therapeutic effects is needed. The material of the electrodes is also important to decrease the probability of systemic toxicity from metal release.

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Chapter 13
Anticancer Gene Transfer for Cancer Gene Therapy

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Contents

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Abstract Gene therapy vectors are among the treatments currently used to treat malignant tumors. Gene therapy vectors use a specific therapeutic transgene that causes death in cancer cells. In early attempts at gene therapy, therapeutic transgenes were driven by non-specific vectors which induced toxicity to normal cells in addition to the cancer cells. Recently, novel cancer specific viral vectors have been developed that target cancer cells leaving normal cells unharmed. Here we review such cancer specific gene therapy systems currently used in the treatment of cancer and discuss the major challenges and future directions in this field.

Keywords Cancer • Virotherapy • Gene therapy • Targeting

13.1 Introduction

13.1.1 Cancer Gene Therapy – Oncolysis

Viruses were discovered more than a century ago but from early times diseases like cancer and especially leukemia were attempted to be treated with viruses. Throughout the recorded history of diseases, there have been observations of cancer regression upon natural infection with viruses [1, 2]. During the early twentieth century, based on these observations, several clinical trials were conducted via fluid transfer from animal or human bodies that were infected with viruses to treat patients with cancer [3]. The immune responses of those infected patients were most of the times active and so only limited efficacy was observed but in immunocompromised individuals, on many occasions, the tumor regressed. However, even after obtaining positive results from the tumor shrinkage the morbidity from the infection was unacceptably high. During those desperate times for science and patients, ethical issues were of lower importance, but nowadays those techniques would not have met the current ethical standards.

Initially introduced as a revolutionary biomolecular technology with an unlimited potential for curing almost any disease, gene therapy has passed its three initial decades in turbulence. The initial concept of using genetic material and introducing it into cells to correct defective genes has broadened significantly but in contrast to the large number of clinical trials (more than 1,000) that have been conducted or are in process right now, the efficacy of gene therapy for cancer therapy has been limited. The lack of alternative treatment options in some terminal cancers gave gene therapy the opportunity to prove its efficacy and in combination with its lower cost compared to conventional therapies sometimes appears as the sole option for some patients. The advances in tissue culture in the second half of the twentieth century allowed the production and amplification of viruses in a more controlled environment compared to the usage of bodily fluids. Also the development of rodent-based models of cancers allowed pre-clinical experimentation using a variety of viruses. The possibility to force viruses to grow only in specific cancer cells

in vitro and subsequently use these viruses in the equivalent human tumors was utilized early on, but did not yield significant advances in virotherapy since the tools that were available to improve efficacy, safety and potency were very limited.

Oncolytic virotherapy is now a very promising treatment option that uses the replication inclination of some viruses in specific cancer cells only. By definition, oncolytic viral replication leads to intratumoral viral amplification, which ultimately leads to tumor destruction with minimal or non-existent damage to nearby non-tumor tissue. Many efforts concentrated to the viral tropism and constituted the first generation of oncolytics which was based on engineering the virus in such way that genes responsible for its replication in normal tissue were removed but were dispensable for the replication in tumors [4]. Generating viruses that were more specific for cancer cells was the ultimate goal since the "lysis" of the infected cell was a native characteristic of the virus. It was postulated and later proven that tumor cell environment was more suitable than the normal cell environment for viral replication. The latter finding was the core of subsequent efforts towards reprogramming viruses to become cancer-specific and thus safer. These efforts resulted in the first generation of viruses that have been used extensively by utilizing the native ability of the virus to enter a lytic cycle and also to be specifically targeted to cancer cells using advanced molecular engineering. We are now able to visualize the spread of the viruses using reporter genes and evaluate the efficacy of each virus in a specific biological system [5–8].

13.1.2 Viral Tropism – Viral Arming

In recent years the demand for specificity for any aspect of cancer therapy to avoid any type of toxicity, resulted in the advancement of another approach. Genes that can specifically induce programmed cell death (apoptosis) only in cancer cells but not in normal tissue appeared to be a very promising approach for cancer therapy since apoptosis evasion is a characteristic of cancer cells and the anticancer genes were able to induce it. However, significant pitfalls appeared for this approach since the delivery of these genes was an issue that could not be resolved with the means of delivery that were available. Our understanding of cancer has dramatically improved recently and allowed the generation of viruses encompassing highly sophisticated molecular characteristics that are specific for each cancer type. The first generation of viruses is currently being tested in patients for efficacy and concurrently new approaches are being explored for the enhancement of their therapeutic potency [9]. Currently clinical and pre-clinical studies are testing combination of viruses with pharmacological drugs in order to enhance the efficacy of treatment [10, 11]. These new studies utilized tumor-specific promoters in order to increase efficacy and specificity since the viruses are only able to replicate and lyse specifically in a tumor environment. Tumor epitopes are currently being used as targets of newly engineered viruses that initiated a revolution in tumor tropism [12, 13].

A new era for virotherapy research began when scientists realized that apart from the lytic potential, viruses could also be used as gene delivery vehicles. Those viruses were named "armed" since they have the ability to lyse the transduced cells and express proteins that would enhance the tumor-killing efficiency. The genes that commonly arm oncolytic viruses fall under four categories: (1) Pro-drug activating enzymes, which transform a non-toxic chemotherapeutic to an extremely toxic agent that kills tumor and non-tumor nearby cells, (2) Reporter genes that are normally used for *in vivo* monitoring of viral distribution, (3) Tumor microenvironment-modulating genes that alter significantly the tumor microenvironment so after the lysis of the initial tumor the microenvironment would become unsuitable for tumor regrowth and (4) Apoptosis-inducing genes that can potentially directly enhance the oncolysis by inducing a self- suicidal program [4].

Over the years advances in the field of apoptosis and cell death allowed the identification of specific pathways and genes that cancer cells use to escape apoptosis. This book deals with the exciting finding that some genes can induce apoptosis specifically in tumors cells without affecting nearby or normal tissue [14]. The findings are of great importance since specificity is attributed to the gene itself and not to the delivery system. One can realize the potential of combining the knowledge of viral tropism and specificity with the potency and specificity of the anticancer genes. Arming an oncolytic virus that is engineered in a way that it can transduce a certain tumor type with a tumor specific apoptosis gene is the direction that all the current efforts in the field of anticancer genes are focused on.

13.1.3 Gene Delivery – Viral and Non-viral Systems

There are many ways of delivering genetic material to a tissue but in general there are two approaches that include viral and non-viral means. Both have advantages and disadvantages and the majority are developed specifically for a narrow application system. Briefly, non-viral gene delivery can be achieved with physical methods which include naked DNA, gene gun particle bombardment, electroporation, ultrasound, magnetofection and the in rodents (so far) highly efficient hydrodynamic gene delivery [15]. Generally, while the non-viral physical methods are cost effective and less invasive than a viral approach the efficiency of delivery is extremely low and so far there only few examples of clinical use of these methods. Most significantly exon skipping approaches using oligonucleotides have been used successfully in the clinic for Duchenne Muscular Dystrophy [16–18] and antisense oligonucleotides for Spinal Muscular Atrophy are in clinical development at present [19]. Another method of non-viral gene delivery utilizes advanced nanochemistry and nanoparticles that are analyzed extensively in another chapter of this book and constitute an attractive approach when compared only with the more efficient viral systems. The delivery of the genes for cancer gene therapy is of extreme importance since the advances in the anticancer gene identification cannot be realized if they cannot lead to a translational approach for patients. There are

some key steps that the field has to take in order to improve or perfect the current viral or non-viral systems. Targeting and delivery are important determinants of the success of cancer gene therapy as is the enhancement of the intracellular delivery, the duration of the transgene expression and the local or systemic toxicity. Clinical successes in the last 5 years have breathed new air in the field of gene therapy like i.e. the treatment of X-linked SCID, ADA-SCID [20, 21], X-adrenoleukodystrophy [22] and Wiskott-Aldrich Syndrome (WAS) [23, 24] metachromatic leukodystrophy [25] by using *ex vivo* gene transfer into bone marrow hematopoietic stem cells and autologous transplantation or by direct gene transfer *in vivo* as in the cases of Leber congenital amaurosis [26, 27] and Parkinson's disease [28, 29].

13.1.4 Oncolytic Viruses and Vector Systems for Gene Delivery

There are several virus families and representatives of each family that are currently used in the battle against cancer. Oncolytic viruses have a native capacity of cell lysis and so an innate ability of killing. Other viruses do not have the ability of lysing the infected cells but have useful other characteristics such as transgene capacity and ability to express high amounts of the transgene. Viral vectors with backbones from arming-able oncolytics or other viruses are of particular interest for this chapter since anticancer genes can be used as arming agents for these vectors. Figure 13.1 shows the oncolytic viruses that are currently under clinical trials and viral vectors that are commonly used in gene therapy.

Successful delivery systems today include retroviruses, adenoviruses (types 2 and 5), adeno-associated viruses (AAV), herpes simplex viruses (HSV), pox viruses, lentiviruses and human foamy viruses (HFV). Adenoviruses, AAVs and HSVs have innate oncolytic capacity and constitute the most widely used oncolytics. Moreover, they can be efficiently armed and used as delivery systems. The engineering of viral vectors include the removal of some areas of their genome to manipulate the replication ability, the tropism and safety.

13.1.5 Viral Tropism Manipulation

Many types of viruses that contain a high oncolytic potential with low toxicity but with no tropism can be manipulated with precise molecular engineering to infect only a tumor derived from a specific tissue. Some viruses have natural tropism for a specific cell type and this property is extensively used after re-programing the tropism only to cancer cells while maintaining the oncolytic efficacy. An example for this class of viruses is the Herpes Simplex Virus (HSV), which has natural

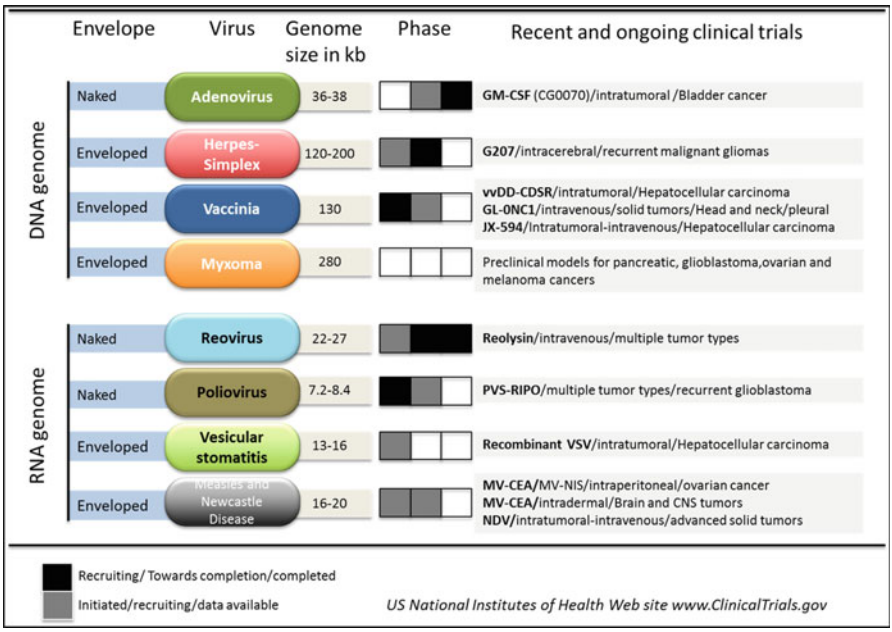

Fig. 13.1 Currently ongoing clinical trials utilizing oncolytic viral vectors

tropism for neuronal cells but is engineered in a way that it will only infect neuronal cancer cells leaving healthy neurons unharmed.

13.1.6 Cancer Specific Proteases and Oncolytic Activation

Cancer cells have deregulated proteome profile and events of protein overexpression are very common. Identification of such proteins that are overexpressed only in cancer cells and have minimal or no expression in normal cells has attracted the interest of many cancer biologists because each of these proteins could be a potential target for cancer therapy. However, virologists effectively used this characteristic of cancer cells in order to manipulate viral tropism. They exploited the innate need of viruses to use proteases of the host cells in order to be activated and exert any effect in the target cell. In detail, viral attachment and activation requires cleavage of viral proteins. Viral proteins contain specific sequences that are cleavable by proteases that are ubiquitously expressed in cancer cells. Viruses with envelopes such as HIV-1, influenza and paramyxoviruses recognize the receptor on a target cell but require the cleavage of their viral glycoproteins to allow entry and activation [30]. Insertion of a cleavage site in the virus genome and disruption of the native recognition site for ubiquitously expressed peptidase is the strategy that is followed to manipulate viral tropism.

Among proteins that are highly overexpressed in most types of cancers cells are the Matrix Metalloproteinase Proteins (MMPs). They have been associated with high metastatic potential, and enhanced cell invasion. Rationale-based therapies targeting those proteases with specific inhibitors were developed but with disappointing results due to the wide range of substrates and unknown mechanisms of actions of the MMP superfamily [31]. Additional functions are now attributed to MMPs that could explain the failure of MMP inhibitors to provided beneficial outcome for cancer patients. However, in cancer gene therapy MMPs' overexpression is utilized for viral tropism reprogramming without the need of pharmacological inhibition. Essentially, a harmful cancer-cell characteristic is used to destroy the tumor itself. Retroviruses and Measles viruses have recently been used in cancer-specific retargeting through the use of MMPs [32, 33]. Viral DNA or RNA was manipulated so genes that contained protease recognition sequences were disrupted and fused with linkers containing sequences that are recognized by MMPs. Surprisingly, the modified viruses could only be activated and exert oncolysis preferentially in cells that express MMPs. Similar results were obtained from experiments in mice in which modified viruses were injected in a background of MMP absence and so no damage (cell death) or infection were observed after viral distribution [34–36]. Importantly the safety of the viruses was greatly enhanced compared with the wild type counterparts through viral tropism re-targeting so the manipulation was at the level of particle activation.

13.1.7 Cancer Specific Transcription and Replication

Human cancers originate and evolve through mutations on several genes controlling cell cycle control, cell death, metabolism, adaptation to extreme environmental conditions, cessation of the DNA repair machinery or tumor suppressors and oncogenes. A widely accepted overview of cancer cells characterizes the hallmarks of cancer that are defined by at least six categories [37]. Changes occurring through detrimental alterations on cell homeostasis, orchestrate the malignant transformation of these cells. Self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, sustained angiogenesis, limitless replication potential and tissue invasion and metastasis are the hallmarks of cancer cells [37]. Interestingly, an alignment in the biological dependence of tumor cells on those altered pathways was found with viruses. Essentially, viruses exploit for their replication those pathways of the target cells. The recent findings about those similarities or dependences of viruses on biological pathways of cancer cells were utilized by scientists to alter the viral tropism and manipulate their replication in a cancer-specific way. Two main approaches have been taken in order to create advanced oncolytic viruses with specific tropism. The first approach includes deletion of viral genes that are critical for replication of viruses in normal or cancer cells. Essentially, specific pathways are required for the activation of the replication cycle, which are extensively de-regulated in cancer cells. These attempts to alter the viral tropism using pathway dependencies for viral replication have been utilized in the first generation of pre-clinical or

clinically approved oncolytic viruses like Adenovirus (Ad): ONYX-015, dl922-947, Herpes Simplex Virus (HSV): G207, R3616, R1716, bM24-TE, Newcastle Disease Virus (NDV), Influenza Virus (IFA), Vesicular Stomatitis Virus (VSV) [38–47]. The second approach for manipulating viral tropism and selective replication is to engineer viruses with genes responsible for replication controlled by tumor or tissue specific promoters.

The retinoblastoma tumor suppressor protein (pRB) is significantly altered in many human cancers [48]. The same protein is the target of the Conserved Region 2 (CR2) of the Adenovirus protein E1A. Adenoviruses express genes that inhibit apoptosis and confer limitless replication potential to quiescent cells. Oncolytics based on adenoviruses have been carefully manipulated for these genes with advanced molecular engineering and mutations in the E1A genes conferred tumor specificity. Dl922-947 is an example of an adenovirus with a mutated E1A gene that causes adenoviral-based lysis of cells with deregulated cell cycle control while other mutants of adenoviruses target specifically the pRB pathway that is altered in many cancers.

After infection, the early gene products of the virus progeny are extremely important for viral replication and oncolysis. Another example for tumor-specific replication comes from adenoviruses with deleted E1B-55 kDa gene. The latter binds to p53 protein and induces its degradation, which essentially leads to apoptosis inhibition that would allow viral replication. Deletion of E1B would allow the virus to enter the cell but since there is no inhibitory effect on p53 since E1B is deleted, the p53 will induce apoptosis and kill the target cell not allowing viral replication. In that scenario tumor specific replication is achieved using those specific oncolytics in tumors with defective p53 pathway that is a very common genetic abnormality in a wide range of human cancers. The idea was ingenious and the first oncolytic to utilize that strategy was ONYX-015 [38]. However, despite such a simple rational biology it was not able to generate what was thought to be a tumor-specific replication-competent oncolytic. It was noted later that the biology of viruses is more complex than initially anticipated and ONYX-015 could also replicate in cells with wild type p53 [39, 49, 50].

As findings in tumor biology advance, so does the utilization of tumor defects for selective replication of the viruses. The defective interferon response pathway that is normally responsible to initiate apoptosis due to viral infection is commonly attenuated in cancer. Extensive activation of Ras/Raf1/MEK/ERK pathway is also another common genetic abnormality. VSV or NDV oncolytics are usually sensitive to interferon cytokines. However, due to the attenuation of this antiviral response in host cells the viruses are able to replicate selectively in tumors [44, 46, 51, 52]. Activation of the Ras/MEK pathway in human cancers results in the inhibition of the protein kinase R (PKR) pathway. The latter is a host defense pathway, which halts protein synthesis of virally-infected cells and induces their death. Since activated Ras leads to inactivation of PKR, oncolytics like HSV-1 or Influenza virus A (IFA) are able to conditionally replicate only in cancer cells using this common defect of protein synthesis inhibition in tumors [47, 53–55]. The Ras pathway has a central role in tumor initiation, progression and sensitivity to treatment. Being so central in the biological processes that govern tumor cells makes it prone to activation by many different signals stemming from upstream

tyrosine kinase receptors or regulatory oncoproteins. Examples of proteins that potently activate Ras are the Epidermal Growth Factor Receptor (EGFR), or BRAF or many other tyrosine kinases that require Ras activation for downstream signaling. In that context Ras can potentially have additional abilities that are utilized by oncolytics for conditional replication in tumors.

Cancer-specific replication is a central component of oncolytic virotherapy. Although it is a major field of research, it constitutes an autonomous level towards improving viral safety and specificity. This chapter aims to determine the methods for tumor-specific destruction that can be integrated into a new generation of viruses that have several levels of controls from virus targeting, replication and induction of apoptosis. Particularly the latter is essential.

13.1.8 Cancer-Specific Promoters

The generation of disease models through transgenic animals was made possible by the identification and use of tissue- or organ-specific promoters. Specificity of protein expression allows controlling toxicity and off-target effects to non-target cells. The ability to direct the level of gene expression at a specific site was utilized by virologists in order to generate oncolytics that contained genes responsible for viral replication under the control of a tissue-specific promoter. Although the approach could not be applied to all the available oncolytic viruses due to restrictions in their biology (i.e. RNA viruses), it could potentially lead to effective agents for directed oncolysis. There are two ways for utilizing promoter specific expression of viral genes. The first is to use promoter sequences from genes that are tumor-specific. A representative example of such promoters is the hTERT gene promoter [56]. Human Telomerase Reverse Transcriptase promoter is highly expressed in tumors with minimal or no expression in normal tissues. The high expression in tumors makes it an attractive tool for cancer- specific replication of viral genes. An alternative method of using a promoter to confer replication specificity is to use tissue-specific promoters. Characteristic examples of such cases are the PSA (prostate specific antigen) gene promoter for prostate cancers replication, the tyrosinase gene promoter for skin cancers, or alpha-fetoprotein for liver cancers [57]. Although the second approach is rationale-driven and could potentially revolutionize tumor selective replication of viruses, the strength of each promoter system needs to be investigated extensively since a weak promoter would probably diminish the oncolytic potential of a virus. In turn combinatorial approaches could be employed in order to achieve high specificity and increased potency [58].

13.1.9 Viral Binding Through Tumor-Specific Receptors

The binding and the entry of an oncolytic virus onto and into a cancer cell is a pivotal step for successful tumor destruction. There are many factors that can

determine the specificity of each oncolytic to bind to a specific receptor. The complexity that governs the specificity of binding determines the feasibility of molecular engineering of recombinant viruses to target cancer-specific receptors. Adenoviruses enter the cells through endocytosis [59] after the binding of the fiber knob of the proteins on the viral capsid to the Coxsackie-Adenovirus-Receptor (CAR) on the cell surface. Subsequently it gets internalized through clathrin pits via interaction of integrins on the host cell with the RGD motif on the virus [12]. HSV based oncolytics use a different set of receptors [60] for viral entry. The tumor necrosis factor family members, proteoglycans and nectin-1 [61] as well as nectin-2 are the host-cell surface receptors that are preferred by HSV without being the only ones. Multiple ligands are also available on the surface of the HSV like glycoproteins gB, gC or gD. In contrast to adenoviruses, HSVs are enveloped viruses, which adds an additional step to the entry of the virus into the host cell or another level of complexity. Endocytosis like the adenovirus or fusion with the plasma membrane are the two different routes required for viral entry. However, the choice of the route that the infection is going to follow is not determined by the receptor but rather on which cells this receptor is expressed on. Some viruses such as vaccinia have a very broad spectrum of cells that they could infect and so far there are no exclusive receptors that these viruses utilize for viral entry [62]. CCR5 receptor has been recently shown to allow viral entry to previously non-permissive cells but there is limited specificity by this family of viruses in comparison to adenoviruses or HSVs.

The hypothesis is that if the viruses are engineered in a way that they can express ligands for specific receptors on the host cell membrane that would allow specific entry to the cell of interest. Researchers are now utilizing bi-specific ligands (binding both the receptor on the host cell and receptors on the virus) that are expressed through engineered adenoviruses in order to allow entry through a cancer cell receptor. Likewise there have been attempts to specifically target HSV to EGFR-expressing cancer cells through a gD-specific bridging molecule [63]. However, since HSVs require an additional step, namely fusion with the plasma membrane or endocytosis, efforts have now shifted to the separation of those two events in order to generate a more specific strategy for viral entry.

The latest advancements in receptor targeting report the use of domains that present antibody-like characteristics and specificity. However, antibodies are extremely difficult to be engineered for viral proteins due to their size and their tetrameric form. Also they contain disulphide bonds, which increase the complexity and make it extremely difficult to be utilized for vector retargeting. In order to fill this gap in this technical conundrum single-chain Fragment variables (scFv) were developed, which contain the variable region of the heavy and light chains. Proof of principle was obtained using enveloped viruses such as the Measles Virus (MV) for CD20 binding in Hodgkin's lymphoma [64] or CD38 in myeloma [65]. HSVs were also engineered to express scFvs as well as in other enveloped viruses that are generally easier to be genetically manipulated.

13.1.10 Viral Vectors for Transgene Delivery

There are multiple RNA and DNA viruses that have been or are being used in cancer gene therapy. However, several of them are being used as tools for gene therapy in other diseases like neurodegenerative diseases or diseases with known gene defects. As it was previously mentioned in this chapter Fig. 13.1 shows a virus classification according to their fundamental characteristics. Below, the main classes of viruses that are used as gene delivery vehicles are described. Anticancer gene transfer can be utilized with any of the vectors described but with some restrictions in size of the cancer-specific apoptosis genes. Since apoptosis is induced in a tumor-cell-specific manner, vectors with low oncolytic activity can also be used. Arming oncolytics with cancer-specific apoptosis genes might prove a better approach combining all the levels of specificity that we described above with the potency of an apoptosis-inducing gene.

13.1.11 Adenoviruses

More than 100 types of adenoviral serotypes have been described so far and have been derived from a wide range of species. Types 2 or 5 are common adenoviruses that most humans have been exposed to. Although natural infection by an adenovirus or gene therapy application would probably be acute and limited adenoviruses have been used in gene therapy applications mostly due to their capacity for the gene of interest (~36 kb), because of the high expression level of the transgene and because of their safety profile [66]. Adenoviruses do not integrate into the host's genome and can transduce dividing and non-dividing cells. Having all these advantageous attributes, adenoviruses became one of the most widely used vectors for cancer gene therapy [67]. Although superior in many characteristics than other vectors, adenoviruses initiate a rapid and strong immune response, which limits the level of transgene expression while being completely eliminated from the host cells [68]. Strong immunogenic response might benefit tumor destruction but is at the same time extremely dangerous for the patient [69]. A common drawback for using adenoviruses as gene therapy vectors is the availability of CAR, which varies in expression on the membrane of cancer cells [70]. In that sense efforts have been focused on the reduction of the immunogenic response by removing nearly the complete viral genome coding sequences. The latter acquired the name "gutless adenovirus" and requires the help of a helper virus in order to produce viral particles [71, 72]. Although deficient in the ability of replication as standalone vector, it maintains high infectivity rates and wide tropism. This new generation of adenovirus has been tested in preclinical and clinical models of cancer with promising results but the toxicity of the helper virus remains too high and at a level that is not acceptable for use in human clinical trials. There are many examples of use of adenoviruses for cancer gene therapy mainly serotypes 2 and 5 which were

engineered ("armed") to express suicidal genes, tumor suppressors, immune boosters, oncogenic inhibitors, antigens for tumor vaccines, antiangiogenic factors, prodrug activating genes and many others. Further details will be discussed later about "armed" oncolytics.

13.1.12 Adeno-Associated Vectors (AAVs)

As their name imply, AAVs are similar to adenoviruses in many ways, however, they contain a few but fundamental differences which differentiate them from the common adenoviruses. They have a deficiency in replication and pathogenicity that enhances their safety profile and show superiority against adenoviral vectors [73]. Moreover, AAV infection in humans does not associate with any diseases or specific immunogenic responses. Another fundamental difference, but in favor of the adenoviruses this time, is the capacity of AAVs. Gene inserts bigger than 4.8 kb cannot be inserted into an AAV vector. Examples of successful use for AAVs in the clinic include hemophilia B, Leber congenital amaurosis, PD and alpha-1 antitrypsin deficiency [26, 27, 74, 75]. The use of AAVs was extended to cancer with many successful pre-clinical models of many types of cancers using AAV vectors and recently an AAV vector expressing the anticancer genes IL-24 and apoptin was used in a pre-clinical model of hepatocellular carcinoma [76]. AAV vectors are currently under pre-clinical development for use in clinical trials while oncolytic Adeno-associated viruses currying the CEA gene are currently being tested for efficacy and safety in stage IV gastric cancer (www.clinicaltrials.gov).

13.1.13 Herpes Simplex Viruses

Herpes simplex virus can cause severe lethal encephalitis and it occurs naturally as a pathogen as an enveloped virus. HSV has been extensively used as a viral vector for virotherapy as an oncolytic or as a carrier vector to induce cytotoxic effects. HSV-1 in comparison with other viral vectors, especially compared to adenoviral vectors have superior characteristics for cancer virotherapy [77]. It contains a large genome that allows deletion of non-essential genes and insertion of therapeutic genes of interest (153 kb is the genome while around 30 kb can be entirely deleted). Only minimal infectious virions are needed for a large scale and effective cell killing since it is exceptionally cytolytic. HSV is not an integrating virus so it does not affect the host genome stability and it has itself a very stable DNA genome. It can infect most tumor types and has been extensively investigated in neuronal cells, which are normally difficult to transduce and so brain tumor therapy can prove specifically effective using HSV [42, 78]. In case of adverse effects in a patient, there are many available anti-virals for HSV that can prevent or calm negative effects of infection.

HSV delivery systems include the DISC system that is named from Disable Infectious Single Copy virus. DISC viruses contain a mutant glycoprotein H and are replication defective [79]. HSV-G207 was the first HSV-1 based virus that was used in a clinical trial in the United States [80] and the results have proved the efficacy and potency of the virus. So far the third generation of HSV oncolytics is being developed to improve specificity, tropism and safety. To accomplish that, scientists are identifying non-essential genes and delete them (alpha47 gene – to create the HSV-G47delta 3rd generation) [81, 82] in order to induce further an immune response to attack the tumor and better cytotoxic effects [15].

Epstein-Bar viruses (EBVs) are a class of herpes viruses that retain some of the attractive characteristics of the HSVs. EBVs can accommodate large DNA fragments and when inserted into the nucleus they stay as circular episomes allowing long term, non-invasive gene expression. EBVs have natural tropism for B-cells and have been used before to treat B-cell lymphoma and immunotherapy for cancer.

13.1.14 Pox Viruses

Vectors derived from poxviruses are used for extremely high cytoplasmic expression of the engineered transgene. The capacity of that vector is relatively big being able to accommodate fragments of 25 kb. The high expression and the high capacity are the two major characteristics of the poxviridae family vectors [83]. Gene delivery with Poxviruses has not advanced so far as expected. The complexity of the viruses and the complicated molecular engineering that is required to achieve cloning, production and expression presents as a major barrier for the advancement of this vector as an effective gene delivery option [84, 85]. For transgene insertion a step of homologous recombination or *in vitro* ligation is required in order to produce recombinant vaccinia virus [85]. There have been some successful attempts to use poxviruses in cancer gene therapy and especially in breast, prostate, colorectal and lung cancer.

13.1.15 Retroviruses-Lentiviruses

Transduction of dividing cells and stable integration of the virus into the host genome are the main characteristics of retroviruses. They are one of the most frequently used delivery vectors for somatic or germline gene therapies. Retroviruses can pass through the nuclear pores of mitotic cells and so they are ideal for *in situ* treatment. The capacity of retroviruses is relatively restricted (~8 kb) after the removal of all the viral genes. They have been used as gene therapy options of X-SCID with great success but with a major fatal disadvantage. They specifically integrated upstream of the lymphoproliferative LMO2 gene and activated this gene with leukemia being the clinical outcome in some patients. This has now been bypassed by use of SIN LTRs

or by using lentiviral vectors. Familiar hyperlipidemia was another example of use for the retroviruses however their uses remain extremely restricted in the clinic. Their ability to integrate into the host genome raises safety issues since they can potentially inactivate tumor suppressors or cause oncogene activation.

Lentiviruses are a subclass of retroviruses. They possess the characteristic of the integration into the host genome but they have the superior ability to transduce non-dividing cells making them ideal for gene therapy of neurological diseases. They can accommodate transgenes up to 8 kb and can transduce a large number of cells without immunological responses or toxic side effects. Their natural tropism is for neuronal stem cells and they have been extensively used for *ex vivo* gene delivery in the central nervous system. The integrated transgenes are stably expressed for long periods without having any immunogenicity. Successful use of these vectors has been achieved in PD, metachromatic leukodystrophy, X-Adrenoleukodysrtophy and several immunodeficiencies (WAS, ADA-X SCID). Lentiviruses have also been used for cancer immunotherapy (through enhancement of dendritic cell antigen presentation) of T-cell leukemia or prostate cancer when bound to trastuzumab [86, 87].

13.1.16 Arming Viruses for Cytotoxic Virotherapy

Per definition, cancer is the irregular and unrestrained proliferation and multiplication of cells that are able to invade adjacent tissues and are able to metastasize to proxy regions of the body through the bloodstream [88]. While chemotherapy and cytotoxic therapy have brought tremendous progress to cancer therapeutics they cause detrimental side effects that hamper the benefits of the tumor shrinkage or destruction. Common practice so far has been the systemic administration of cytotoxic therapies that instead of affecting the tumor specifically they affect all the human tissues non-specifically and uncontrollably [89]. A new era for cancer therapeutics has emerged through the use of targeted and rationale-based therapies using small molecular inhibitors of proteins or pathways that are specifically deregulated in certain cancers. Such examples are the use of tyrosine kinase inhibitors for Epidermal Growth Factor Receptor (EGFR) mutations or amplifications, for Anaplastic lymphoma kinase (ALK) and many other common genetic alterations in cancers. Although initially effective these therapies are not long-lasting since resistance almost always occurs and tumors re-grow and they require more effective treatments like chemotherapy or radiation [90–93]. Distinguished among these research endeavors is the concept of oncolytic virotherapy that utilizes wild-type or recombinant viruses to selectively infect and kill cancer cells while leaving normal tissues viable or not affected. There has been an era of rapid development of oncolytics that changed the way we thought of cancer therapy. Specificity and effectiveness are the main challenges that scientists face in order to generate new recombinant viruses but tremendous progress has been achieved through the strategies that were discussed before for viral tropism and specificity. In addition to targeting, strategies are often employed to amplify the cytolytic capabilities of oncolytic viruses to increase their efficacy.

This process was named "oncolytic arming" since a transgene is helping the innate cytotoxic ability of a virus [8]. Arming of a virus requires a careful selection of a viral vector that will be used as oncolytic agent and can be achieved through three major strategies that are discussed below and are: pro-drug oncolytic arming, pro-apoptotic gene arming and microenvironment-regulation-able gene arming.

13.1.17 Pro-drug Arming

Expression of so-called prodrug convertases by the virus is an approach where a non-harmful enzyme is engineered to be expressed as the oncolytic infects specifically the cancer cell. Subsequently, systemic delivery of a pro-drug follows and in cancer cells that are infected with the oncolytic expressing the enzyme, a chain reaction of cell suicide is initiated. This strategy is named gene-directed pro-drug activation therapy and has many examples where it was used with very promising results. One characteristic class of pro-drug arming is the expression of the thymidine kinase gene in HSV vector that is able to monophosphorylate ganciclovir, which in turn is converted to triphosphorylated forms that induce cell death by blocking DNA synthesis [94, 95]. Several other systems have been described such as the nitroreductase in combination with the pro-drug CB1954 or the cytosine deaminase (CD) with the pro-drug 5-fluorocytosine, which is forming the chemotherapeutic 5-fluorouracil (5-FU) [96, 97]. Moreover, fusion of two different enzymes has been employed for increased killing efficacy, in difficult to target cancers. The majority of the viruses that have been used to deliver these genes were non-replicative but recently replication-able oncolytics have been employed for pro-drug suicidal strategy [98].

13.1.18 Microenvironment Gene Manipulation Arming

The microenvironment of tumors is a very complex intercommunicating cell network that supports the solid tumor for its growth and expansion. It consists of host fibroblasts, immune cells, endothelial cells, immune cells and pericyte cells. The tumor is supported by a vast amount of signals that constitute the extracellular matrix (ECM), which dominantly controls tumor vascularization, tumor growth and expansion, invasion and metastasis. The network of signals is a complicated mix of secreted proteins that provide structural support for the tumor and its stroma but is also a biological barrier that prevents administration of therapies to the tumor core. The barrier is not only effective against chemical therapeutics. It is preventing the host's immune cells to attack the tumor protecting it from the most clever and efficacious defense system in nature (immune system). Therefore, the need for agents that would manipulate the tumor microenvironment and convert it to a more permissive barrier became imperative. To this end oncolytics armed with genes that

can modify or destroy the tumor microenvironment were generated. The viruses that were employed to carry the microenvironment disseminating genes were armed with anti-angiogenic factors, with chemokine/cytokine that mediate tumor microenvironment or proteases or glycosidases that have a direct effect on stroma stability and structure. Adenoviruses or HSV were armed with inactivating Vascular Endothelial Growth Factor Ligands (VEGF) which stopped the intracellular signaling and reduced the rate of angiogenesis (reviewed in [4]). Inhibitors of matrix metalloproteinases also achieved modification of angiogenesis where oncolytics carrying the gene Tissue Inhibitor of Metalloproteinase 3, was expressed [99–102]. Numerous examples of viruses armed with anti-angiogenic peptides, with shRNAs that downregulate fibroblast growth factor receptor (FGFR) signaling, antibodies that would affect interleukin 8 signaling were used with satisfactory results in preclinical and clinical models of many cancers. The network of the proteins that comprise the tumor microenvironment is so complicated that it requires extensive knowledge of the signaling networks that are activated due to their expression. The main approach is to identify a way to disseminate the tumor microenvironment and allow the immune system or the oncolytic virus to attack the tumor. For a detailed and comprehensive review about the tumor microenvironment modulating oncolysis please refer to the work of Chiocca and colleagues [4].

13.1.19 Pro-apoptotic Genes Arming

One of the cancer hallmarks, as discussed in a previous paragraph, is the inhibition of programmed cell death (apoptosis) whereby tumor cells are unable to commit to their innate suicidal program even in the presence of extensive deregulation of their homeostasis. Research in the apoptosis field has revealed a well-defined network of genes that can induce apoptosis and are inactivated in cancer or genes that can inhibit apoptosis and they are aberrantly expressed in human malignancies. Viral vectors as powerful delivery systems were employed to deliver pro-apototic genes or gene such as tumor suppressors that can deploy the apoptotic response. An early example of the use of viral vectors to deliver pro-apoptotic genes in cancer gene therapy was the use of an adenoviral vector for the expression of the TP53 gene. The China State Drug and Food Administration approved this vector (Gendicine) for the treatment of head and neck cancer but its efficacy was limited due to the non-replicative phenotype (E1A gene deletion) [103]. On the other hand oncolytics, which are replication-competent appear as more effective delivery systems especially when armed with apoptosis-inducing genes. One example of an effective oncolytic armed with a tumor suppressor is the generation of an oncolytic adenovirus with E1A under the hTERT and hypoxia response element promoter and the TP53 gene under a cytomegalovirus promoter. This virus combines tumor selectivity, p53 tumor suppressor expression and oncolysis [104]. Cancer is a multifactorial disease and many signaling pathways are altered during its initiation and progression. An effective strategy would target pathways and avoid single genes in order to induce the apoptosis cascade [105]. A good example of

such approach is the use of TNF-Related Apoptosis-Inducing Ligand (TRAIL). TRAIL oncolytic gene therapy utilized the chimeric Adenovirus AD5/35 which is able to transduce cancer cells without the use of a receptor, replicate in cancer cells and allow TRAIL expression which lead to apoptosis induction [106]. Efficacy of this vector was shown in leukemia, gastric cancer and pancreatic cancer *in vivo* [107, 108]. TRAIL has been extensively analyzed in a previous chapter of this book since it is one of the "anticancer genes" which are able to promote tumor-specific apoptosis initiation when overexpressed without being toxic in normal cells. MDA7 or NOXA also utilized an adenoviral delivery system to be delivered intratumorally and induced apoptosis in xenograft models of cancers [109, 110]. The use of adenoviruses to deliver anticancer genes is based on an obvious scientific rationale. Adenoviruses (as described before) are able to stay as episomes around the host DNA and drive really high expression of the gene of interest. This is particularly important since anticancer genes are able to induce their tumor-killing effects only when they are overexpressed and the level of expression correlates with the apoptotic phenotype. However, extremely high expression of apoptotic gene can impede the advantageous effects of a combinatorial strategy with oncolytics. Premature apoptosis in the infected cells can reduce the virus progeny yields and counteract the oncolytic activity of the virus. An efficacious approach would be to induce apoptosis in a delayed manner in order to allow viral replication, apoptosis induction and lysis of the cells. The principle of these types of genes is that they can be overexpressed but their effect on cell viability is only exerted in cancer cells and not normal cells due to extensive deregulation of pathways that control these genes in human cancers. Most of the "anticancer genes" have been used to arm viruses and have proven their efficacy both *in vitro* and *in vivo*. Some of them (HAMLET, TRAIL or MDA7) entered or are entering clinical trials [111–114]. A novel approach using the unique characteristics of oncolytic viruses and tumor-specific apoptosis genes can open a new window in cancer therapeutics since the use of anticancer genes introduce an additional step in specificity and efficacy.

13.1.20 Hurdles for the Use of Viruses as Delivery Vectors

While oncolytic virotherapy has emerged rapidly as a treatment option and distinguished itself from cytotoxic therapies, it has not yet reached its full potential due to several limitations or further research that is needed in certain areas. As discussed earlier in this chapter the level of expression of each gene plays an important role for the determination of the therapeutic window. There is an urgent need for the regulation of transgene expression which can be achieved either with the use of specific promoters or with the utilization of regulatory elements that can be controlled exogenously through systemic drug administration [115]. The expression of the transgene can be coupled with advance *in vivo* monitoring of expression levels in order to be able to detect signaling pathway alteration after viral administration and in combination with a controllable expression system to manipulate the course of the tumor progression [116].

There is currently an emerging field of research that is focusing on the use of carrier cells for viral delivery to tumor sites. The reason that this approach is particularly significant is the possibility of pre-existing immunity [117]. The idea stems from the fact that certain cell types have natural tropism for tumor cells so in principle a cell infected with an oncolytic ex vivo can be injected in order to reach the tumor site and so the oncolytic will lyse this cell and infect the main tumor site. Circumventing pre-existing immunity is a major hurdle that oncolytic virotherapy faces. The majority of human population has previously been exposed to viruses that are used as oncolytics and so the administration of a viral population will be effective only in one round of injection [117]. Part of the immune response that is initiated after viral infection is the complement activation. Some viruses like vaccinia or HSV produce specific proteins that can neutralize the complement activation [118]. However, almost certainly a viral infection will lead to an immune reaction that needs to be suppressed or bypassed for effective oncolytic virotherapy. The process of protecting the viruses from pre-existing immunity or acute immune response is called viral shielding and has the same principle as the "Trojan horse". Briefly, the immune system is bypassed by viruses that are camouflaged under a different capsid or shielded with chemical compounds like polyethylene glycol in order to "trick" the immune system and be delivered to the tumor site. Stem cells, cancer stem cells, endothelial cells and progenitors, immune cells and even cancer cells as carriers have been, or are being tested for their efficacy and proof of principle [119–121]. Another approach to overcome the pre-existing immunity is the temporal immunosuppression using pharmacologic interference to bypass the adaptive and innate immune response. The latter would allow opening of the therapeutic window until the virus reaches the tumor site and starts the destruction of tumor cells.

Another obstacle for effective oncolytic virotherapy is the unspecific uptake of the virus by some organs or tissues like lungs, blood or spleen. Vaccinia or adenoviral vectors have been extensively studied for identifying ways to reduce viral uptake by the organs. These ways include pharmacological inhibition of virus-organ interaction or molecular ways by mutating molecules on the surface of the virus to prevent crosstalk with unspecific tissue [118].

While scientists have taken large steps in understanding the viral biology there are significant gaps in the mosaic of viridae family. One of the major limitations that scientists face is the partial or incomplete knowledge of viruses and that became apparent with the use of ONYX-015, which was found to replicate in cells that also had wild type p53 protein. Furthermore, understanding the viral biology is only part of the story. Our detailed knowledge should also be applied to the cancer itself. Current comprehensive approaches to characterize the genetic profile of all the tumors (i.e. The Cancer Genome Atlas-TCGA) provide invaluable information about the molecular basis of each cancer. This information can be used in order to generate new strategies that target several other molecular pathways that are affected in the tumor milieu.

13.2 Conclusions-Future of the Field

Oncolytic virotherapy with viruses armed with anticancer genes is inevitably going to be a new big player in the field of virotherapy. The ultimate goal for each medical filed is to be able to apply the scientific finding and the years of research in pre-clinical and clinical models to human patients. To achieve that, three major milestones need to be accomplished: increased safety, enhanced efficiency, and improved knowledge of the tumors. Infectious agents as are the viruses need to be carefully handled and primarily tightly controlled since apart from only being cleverly engineered to be immunoinvasive for the tumors they have to be carefully controlled to protect the patient and the patient environment. Arming oncolytics should be the future directions of the field. Above discussed are the advantages of oncolytic arming. However, careful consideration should be given to the choice of transgenes that will be used for arming. Pro-apoptotic genes that have no other control features are obviously dangerous and unacceptable. Fortunately, oncolytics have their own control systems as they were discussed in the previous paragraphs so arming them with genes that when overexpressed can induce apoptosis only in cancer cells can be a clever strategy to move forward. A fundamental paradigm of cancer treatment is that there is no unique and stand-alone therapeutic regiment that will cure cancer. This relatively de-motivating statement stems from the fact that cancer is based on its nature a multifactorial disease. Combinatorial approaches that have already been taken proved more efficient that mono-therapies and when oncolytics where combined with cytotoxic therapies the disease progression was the majority of the times improved. Cancer therapeutics were recently improved by the utilization of rationale-based therapies using small molecule inhibitors targeted against specific molecular pathways that are deregulated in cancer cells [122]. Non-small cell lung adenocarcinomas are great examples of a lung cancer type that has benefited from the use of targeted therapies with unprecedented initial responses and limited side effects [123]. However, acquired resistance to the drug almost always occurs, so oncolytics armed with anticancer genes should be utilized to enhance the effects of small molecule inhibitors and to prevent development of drug resistance, which will ultimately lead to disease improvement and amended clinical outcome. Research is already directed towards the use of combinatorial approaches of targeted therapies and oncolytics as clinical successes in melanoma and glioblastoma multiforme denote [124–127]. We are entering an exciting era where we possess the tools and use new technologies against cancer. The time has come to use them to the best effect.

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