Vivek M. Rangnekar *Editor*

Tumor Suppressor Par-4

Role in Cancer and Other Diseases

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Preface

Translational research on the tumor suppressor Par-4 has enabled fascinating insights into the multifaceted roles of Par-4 in diverse human diseases. This second volume of *Tumor Suppressor Par-4* builds on the background information on Par-4 presented in volume 1 to expound on the involvement of Par-4 in cancer, as well as neurodegenerative diseases, diabetes, renal and ocular diseases, and development of the salivary gland and the eye. Importantly, this volume describes the contribution of Par-4 to the downstream processes of epithelial-mesenchymal transition (EMT) critical for metastasis of tumor cells, inflammation that is causally linked to a broad range of cancers and other human disorders, and apoptosis, which is compromised in cancer but deregulated in neuronal diseases.

Most primary tumors, albeit not all, are efficiently treated by radiation, hormonal therapy, chemotherapy, or surgical resection. However, tumor recurrence is inevitable in most cases, and metastasis of the tumors at vital organs eventually impacts patient survival. Chapter 1 by Anindya Goswami and colleagues provides an overview of the pathways involved in the process of EMT and the integral role of Par-4 in regulating these pathways to modulate metastasis. This regulatory action of Par-4 is particularly intriguing as it occurs by downstream modulation of EMT-specific gene expression by a Par-4-dependent mechanism that is independent of its ability to induce apoptosis. As Par-4 is known to be a transcriptional regulator, this facet of Par-4 may be particularly effective in regulation of EMT and metastasis. This transcription regulatory function of Par-4 is also linked to suppression of inflammation. Chapter 2 by Nadia El-Guendy describes the induction of Par-4 by nonsteroidal anti-inflammatory drugs (NSAIDs) to inhibit key molecules that are involved in the process of inflammation. This chapter illustrates the role of NSAIDs and Par-4 in overcoming chronic inflammation and tumor survival, growth, and progression.

Par-4 plays a functional role in diverse solid tumors, as well as hematopoietic tumors. Chapters 3 through 10 exemplify the role of Par-4 in diverse cancers, including gynecological cancers, gastrointestinal cancers, prostate cancer, pancreatic cancer, breast cancer, gliomas, and chronic lymphocytic leukemia (CLL). These chapters also provide background information on the pathophysiology and molecular underpinnings of each type of cancer. Chapter 3 by Eric Asselin and colleagues discusses the histology and molecular features of ovarian and endometrial tumors and demonstrates the significance of Par-4 in regulating the signaling networks involved in chemoresistance of these cancers. Endometrial tumors are particularly noteworthy as Par-4 knockout mice show spontaneous endometrial tumors that are exacerbated by estrogen treatment. Importantly, endometrial tumors in humans exhibit Par-4 mutation in its obligatory SAC domain that generates a STOP codon, thereby truncating the protein to render it non-functional. Endometrial tumors also show methylation-induced suppression of Par-4 expression. Interestingly, a significant proportion of the endometrial tumors, as well as ovarian tumors show intact Par-4 that may be inactivated by posttranslational events, and Asselin and colleagues demonstrate that Par-4 can be readily cleaved by chemotherapeutic agents via the caspase pathway to generate a carboxyl-terminal fragment that translocates into the nucleus to induce cancer cell apoptosis.

Chapter 4 by Rosalyn Irby and colleagues provides a comprehensive view of Par-4 in various gastrointestinal (GI) tumors. This chapter describes the factors that influence Par-4 function

in GI cancer. Activated Par-4 can cause apoptosis in these cancer cells, and higher Par-4 activity indicates a better prognosis for GI cancer patients. Chapter 5 by Mansoor Ahmed and colleagues discusses the role of Par-4 as a radiosensitizer in cancer cells. They succinctly describe the role of Par-4 in suppressing cell survival pathways that are inadvertently activated by radiation and the function of Par-4 in supporting tumor cell death, most notably in prostate cancer. This chapter also presents evidence of Par-4 downregulation by oncogenic Ras in pancreatic tumors where KRAS is the key driver. In Chapter 6, Jeevan Ghosalkar and colleagues provide a comprehensive background on gliomas including their classification, etiology, epidemiology, and current approved treatment options for gliomas. They discuss the relevance of Par-4 in survival of glioma patients, emphasizing the differential effects of Par-4 in various brain tumors. They also illustrate the unmet need for the development of drugs, with insights on utilizing Par-4-based treatments for glioblastoma multiforme.

In Chap. 7, Maria Nagai and her team provide a comprehensive review of the critical role of Par-4 downregulation in breast cancer resistance to therapy, recurrence of local and metastatic tumors, and patient survival. They describe the role of transcription factors that regulate Par-4 expression and function in breast cancer, as well as the downstream targets of Par-4. In particular, they cite clinical studies and meta-analyses that justify Par-4 as a prognostic indicator in breast cancer.

Chapter 8 by Subbarao Bondada and his colleagues, and Chap. 9 by Natarajan Muthusamy and team provide valuable foundational information on hematological malignancies and discuss the role of Par-4 in chronic lymphocytic leukemia (CLL). These two chapters are particularly noteworthy as they highlight the relationship between Par-4 expression in the tumor and its interplay with the microenvironment. Chapter 10 by Asfar Azmi, Ramzi Mohammad, and colleagues describe the functional significance of Par-4 in pancreatic tumors and outline strategies to restore and precisely utilize nuclear Par-4 for therapy of these tumors.

Unlike most tumor suppressors, Par-4 is secreted by normal cells, and this feature can be leveraged to target metastatic tumors. In Chap. 11, David Watt and colleagues take advantage of this attribute of Par-4, by generating small molecule secretagogues to induce robust secretion of the protein from normal cells. They describe the fascinating family of Arylquins that produce a two-pronged effect—these potent compounds induce the secretion of Par-4 from normal cells and also directly target cancer cells. Their observations imply that this dual targeting strategy should be effective against diverse primary and metastatic tumors as long as they express cell surface GRP78, the receptor for extracellular Par-4. These observations are corroborated by Chang-Guo Zhan and his team in Chap. 12. This group is implementing novel approaches to produce recombinant Par-4 derivatives in bacterial cultures. These derivatives tend to stabilize Par-4 in circulation in order to render it far more effective in inhibition of tumor metastasis.

On the one hand, restoration or induction of the apoptotic cell death program is an essential component of cancer therapy to overcome tumor growth and metastasis, and on the other hand, the undesirable association of cell death in neurons is noted in neurodegenerative diseases. Chapter 13 by Ahmed Elsherbini and Erhard Bieberich describes the involvement of Par-4 in ceramide-inducible effects in neurons. They discuss how Par-4 regulates neuronal apoptosis induced by intrinsic ceramide and further consider the paracrine effects of Par-4 in extracellular vesicles that impact ceramide neurotoxicity. Their observations are complemented by Qing Guo and colleagues in Chap. 14 with a description of how Par-4 sensitizes neurons to apoptosis or necroptosis in Alzheimer's and other neuronal diseases. Both cell culture and mouse models corroborate the association of Par-4 with neurodegeneration noted in these diseases. Chapter 14 illuminates the molecular link between Par-4 loss, impaired dopamine signaling, and depression.

In Chap. 15, Bing Chen and colleagues emphasize the involvement of endoplasmic reticulum stress and mitochondrial dysfunction in type 2 diabetes and discuss the mechanisms by which Par-4 sensitizes pancreatic islet of Langerhans β-cells to the action of apoptosis-inducing clues. It is particularly interesting that they speculate a link between single nucleotide

polymorphisms (SNPs) or mutations in Par-4 noted in schizophrenia and depression that interfere with Par-4 binding to the dopamine D2 receptor (DRD2) with diabetes, as dopamine/DRD2 signaling regulates appetite, obesity, and diabetes.

Par-4 is a regulator of gene transcription and Chap. 16 by Moulinath Acharya, Michael Walter, and colleagues describes the mechanisms activated by Par-4 in ocular development and diseases. They discuss the close relationship between Par-4 and the regulatory network of the transcription factors pituitary homeobox 2 (PITX2), forkhead box C1 (FOXC1), and FOXC2 that play critical roles in vasculogenesis and basement membrane formation during eye development. The critical role of this interaction is evident in the fact that disruption of this regulatory network can promote anterior segment dysgenesis and glaucoma pathogenesis. Apoptosis or programmed cell death is a naturally occurring process during the development of glands and organs, as cells whose function is obsolete need to be eliminated. Chapter 17 by Cláudia Malheiros Coutinho-Camillo draws attention to the role of Par-4 in apoptosis during human salivary gland development and sheds light on the relevance of nuclear versus cytoplasmic localization of Par-4 in prognosis and the treatment of salivary gland tumors. In Chap. 18, Shaolin Shi and his colleague describe the anatomy and physiology of the kidney and hone in on the relevance of Par-4 interactions with molecules that are implicated in kidney pathophysiology. Par-4 knockout mice have been generated by several laboratories, and in Chap. 19 Araujo and colleagues discuss the invaluable lessons learned from these and other mouse studies on the role of Par-4 in spontaneous and inducible tumorigenesis, role of secreted versus intracellular Par-4 in controlling tumor growth, and the unexpected phenotype associated with loss of the Par-4 leucine zipper domain in the brain.

Par-4 research has advanced from the bench to the bedside and two clinical trials have been completed and a third trial is ongoing with the Par-4 secretagogue hydroxychloroquine (HCQ), which functions to regulate tumor growth by a Par-4-dependent mechanism. In Chap. 20, Hao and Wang assess the results of the first clinical trial that indicated the safety and efficacy of secreted Par-4 induced in response to HCQ, and that led to the subsequent clinical trials for the benefit of cancer patients. Finally, Chap. 21 by Vivek Rangnekar provides an outlook on Par-4 as a therapeutic target in cancer and other diseases. Collectively, volumes 1 and 2 provide a comprehensive review of Par-4 and offer critical perspectives for basic mechanism-oriented research and clinical translation, with ample scope for population-based studies.

Lexington, KY, USA Vivek M. Rangnekar

Contents

Role of Par-4 in EMT

Mir Mohd Faheem, Archana Katoch, and Anindya Goswami

Abstract

The importance of Par-4 in apoptosis has been deciphered in depth. Interestingly, a paradigm shift is emerging with respect to the non-canonical roles of Par-4. The intricacy between Par-4 and EMT is significantly gaining traction, which is the main focus of this chapter. The chapter commences as we first delineate EMT's transitory and dynamic nature as opposed to the conventional view that portrays EMT as unidirectional and irreversible. We have emphasized EMT's culpability in the genesis of the metastatic program and how EMT-associated transcription factors (EMT-TFs) manipulate the cancer cells to acquire a motile phenotype suitable for intravasation, migration, and secondary metastasis. We as well discuss the molecular signaling pathways regulating EMT and the challenges rendered by the acquisition of EMT in cancer therapeutics. In the later sections, we have diligently highlighted the emergence of Par-4 as a prospective EMT nullifying candidate and therapeutic opportunities thus evolving around it. Particular emphasis is attributed to novel burgeoning role of Par-4-mediated negative regulation of the following anti-metastatic cascades; for example, modulation of β-catenin pathway, cytoskeletal rearrangements, and extracellular (ECM) remodeling and of course the anti-metastatic microRNAs. Lastly, we put forth innovative insights that link Par-4- and TGF-β-mediated lethal EMT.

Keywords

Epithelial to mesenchymal transition (EMT) · Cancer stem cells (CSCs) · Mesenchymal to epithelial transition (MET) · Extracellular matrix (ECM) degradation · Matrix metalloproteinases (MMPs) · E-cadherins · β-Catenin · Cytoskeletal rearrangements · Par-4 · SAC domain · Secretagogue · miR-200c · Vimentin · EMT-associated transcription factors (EMT-TFs) · Twist-1 · Zeb-1 · TGF-β · Lethal EMT · Chemoresistance

1 Introduction

1.1 Epithelial to Mesenchymal Transition (EMT)

For the first time in 1908, Frank Rattray Lillie described the interconversion between epithelial cells and mesenchymal cells [1]. However, much later, in a seminal finding, Greenburg and Hay, on their studies in the primitive streak of chick embryos, unveiled that EMT is an evolutionarily conserved distinct cellular process involving epithelial to mesenchymal phenotype changes [2]. EMT is a distinct physiological roadmap illustrating a trans-differentiation process that allows an epithelial cell to attain a mesenchymal phenotype as illustrated by the following features: (1) massive transcriptional reprogramming (2) loss of cell adhesions and the apical-basal polarity (3) extracellular matrix (ECM) remodeling (4) transitions in cellular morphology (from a cobblestone epithelial morphology to a spindle-shaped mesenchymal one) (5) alteration in the signaling pathways controlling cell shape as well as motility (6) reprogramming of the gene expression [3]. Owing to this chain of transforma-

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tion, EMT generates a mesenchymal cell with increased motility and invasiveness compared to its epithelial counterpart. Although this dynamic process was initially coined as epithelial to mesenchymal transformation, it is in this day and age precisely known as epithelial-mesenchymal transition (EMT) to highlight its transient nature. Pertinently, the morphological changes that a cell endures all through the EMT phenomena are neither single-step/unidirectional alterations (from epithelial to mesenchymal) nor a *fait accompli*. Rather, cells undergoing EMT are distinguished by multiple quasi-mesenchymal states of intermediary nature (Fig. 1). The capability of cells to transition between the epithelial and mesenchymal states, partly or completely, demonstrates the inherent plasticity of epithelial cells. EMT is a reversible process, and mesenchymal cells can experience reverse transition by a process known as mesenchymal to epithelial transition (MET), which is of utmost therapeutic significance.

The epithelial-mesenchymal transition (EMT) is a biological phenomenon in the course of physiological processes, for example, embryonic development, induction of pluripotency, embryonic stem cell differentiation, tissue repair, and wound healing, respectively [4]. Surprisingly, unlike true epithelial features, the mesenchymal cells are truly aggressive in terms of their massive invasive as well as migratory properties exercise through the extracellular matrix (ECM). Therefore, the extremely rigorous differentiation potential of mesenchymal cells is considered as a vital cog for normal embryonic development in various organisms. As a result, EMT not only succeeds in orchestrate cellular rearrangements but, at the same time, it also facilitates the organization of highly specialized tissues and organ systems [5]. This could explain why the essential molecular pathways regulating EMT, including TGF-β, Twist, Slug/Snail, Cripto, Six1, and Wnt/β-catenin, are highly conserved among mammalian systems [5]. Contextually, the convergence of all these pathways has empowered EMT in several pathophysiological conditions such as tissue/organ fibrosis, tumorigenesis, and metastasis as well as influencing the cancer stem cell behavior [6]. While the role of EMT during embryonic developmental stages, wound healing, and tissue remodeling processes is cumulatively beneficial for normal physiological events, contrast, the activation of EMT in cancer rather predispose the malignant cells more aggressive with acquired abilities of invasion, migration, stemness, and drug resistance. Albeit, these new postulates have driven the researchers to define EMT more explicitly so that we can more accurately distinguish between the physiological and pathological EMT processes and rationally discriminate the relationship between the two.

Although there is a substantial advancement in our understanding of the involvement of EMT in the invasion, migration, and metastasis of the tumor over the past decade, there

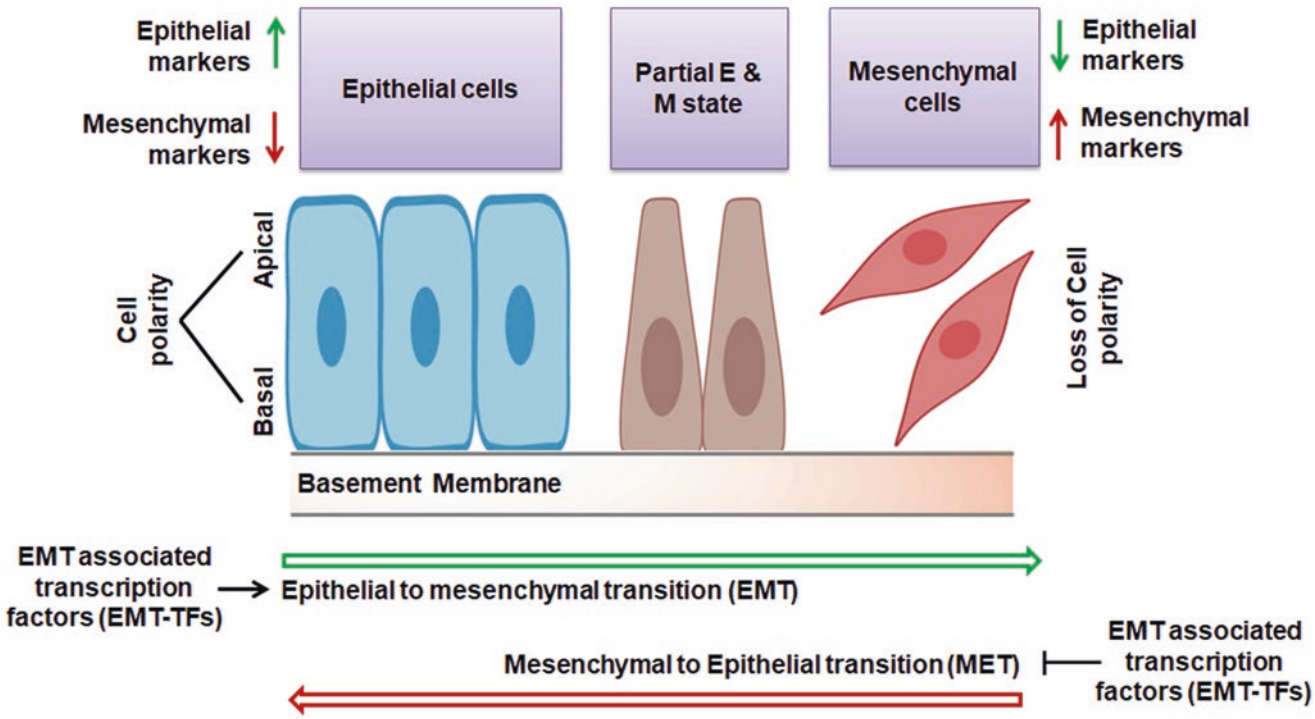

Fig. 1 Epithelial-mesenchymal transition (EMT). EMT encompasses a series of molecular events that result in the transition of a polarized epithelial cell into a mesenchymal cell. This transition is accompanied by a gradual loss of epithelial markers and a simultaneous gain of mesenchymal markers. The reverse process is known as mesenchymal-epithelial transition (MET)

are still large voids concerning the prognosis during the activated EMT state of cancer patients. Robert Weinberg and his colleagues have successfully defined "metastases" as one of the eight hallmarks of cancer. Metastasis truly empowers the terminally differentiated cancer cells to pierce surrounding matrix and distant sites, conferring the cascade as the most challenging aspect of cancer in regard to cancer therapeutics and clinical prognosis. Nonetheless, it is a well-established fact that EMT lies at the core of the metastatic cascade's genesis and, in this context, is of utmost therapeutic significance. As such, the successful treatment of many cancers may be considerably improved given our ability to prevent or even reverse the process of metastasis.

1.2 Regulators of EMT

In a devastating pathophysiology of cancer, a range of diverse players, including transcription factors, signaling intermediates, cytoskeleton proteins, are grossly involved in paving the smooth function of EMT cells. Indeed, activation of epithelial-mesenchymal transition (EMT) is not only responsible for the integration of signaling crosstalk engaged in proliferative pathways, but it also triggers cancer cells survival in the unfavorable catastrophic milieu. A diverse range of signaling pathways have been implicated in the modulation of EMT; among them, the most intensely studied being the TGF-β, Notch, and Wnt signaling pathways. Since the loss of adheren junction proteins, e.g., E-cadherin and Claudins, is a landmark event during EMT, most EMT-inducing signaling pathways are involved in the regulation of the repressors of E-cadherin and other adheren junction proteins. Of note, Zeb family, Snail family, and the Twist-1 are some of the critical EMT effectors which impede the E-cadherin expression [7–10]. Accordingly, in the following paragraphs, we recapitulate the handpick of vital effectors and regulators of EMT.

1.2.1 TGF-β Signaling Pathway

The overwhelming role of TGF-β signaling has been largely implicated in the context of dual purposes—physiological development as well as in promoting malignancy [3, 11, 12]. Not only it acts as a multifunctional and ubiquitously expressed cytokine, apart from EMT, TGF-β also regulates various cellular activities, including cell growth and tissue fibrosis [13, 14].. This multifaceted function of TGF-β primarily concedes its dual nature, i.e., a tumor-suppressive role in the early phases of tumor development but promoting metastasis in the later stages [15, 16]. Albeit, among all the TGF-βeta subtypes, TGF-β1 strongly adheres to the induction of EMT in tumor cells [17]. TGF-β regulates EMT through canonical Smad-dependent and Smad-independent manner. In the Smad-dependent signaling, binding of TGF-β to the TGF-β type II (TβRII) receptor trans-phosphorylates the TGF-β type I (TβRI) receptor which, in turn, activates the Receptor-Smads (R-Smads), Smad2, and Smad3. Following receptor activation, the R-Smads can regulate the gene expression by binding to the Common Smad (Co-Smad) - Smad4 and translocating into the nucleus [18–22]. Eventually, Zeb-1, Slug, Snail1, and Twist-1 transcription factors are examples of vital downstream targets of activated Smads, which predominantly alter the TME by triggering EMT-cascade [23, 24]. For generating a proof of concept, in an elegant experimental setup, using various mutant R-Smad constructs, Valcourt et al. have shown that a dominant-negative mutant of either Smad2 or Smad3 significantly abrogates the EMT induction in response to TGF-β [25]. Even though Smad3 is considered fundamental for EMT induction, the consequences of Smad2 on EMT induction are distinctly proven controversial [26–28].On the other hand, the Smad-independent signaling pathways are equally detrimental since they, too, elicit diverse cellular responses, including EMT induction [25, 29–31]. Intriguingly, TGF-β facilitates EMT, independent of Smads, by regulating the Ras, Rho-like GTPases, p38, Erk, and PI3K/Akt pathways and via extensive modulation of Notch, Wnt, and integrin signaling pathways [32].

1.2.2 Wnt Signaling

The canonical Wnt signaling pathway during embryonic development as well as tumorigenesis in the context of EMT induction has been well-documented in the literature [33–35]. β-catenin plays a significant role in the deregulated Wnt signaling pathway in a vast range of cancers. However, as far as its stability is concerned, β-catenin is phosphorylated by GSK-3β and degraded via the ubiquitin-dependent pathway in the absence of activated Wnt signaling, thereby maintaining lower cytoplasmic β-catenin levels. As soon as Wnt signaling pathway is activated, cytoplasmic β-catenin translocates to the nucleus, where it facilitates to the formation of a complex with TCF/LEF transcription factors and stimulates the expression of EMT-inducing target genes. Furthermore, β-catenin, together with TCF augments the expression of one of the central EMT-effector molecules, Slug [36] and prevents the degradation of Snail [37]. As mentioned above, Wnt signaling indirectly aids EMT by collaborating with the TGF-β and PI3K/Akt signaling pathways. In that context, one of the elegant examples is the stabilization of the β-catenin by PI3K/Akt signaling through Wnt ligands via the blockage of GSK-3β activation leading to promote spontaneous tumor formation [38]. In another classical experimental set up with palate medial-edge epithelial cells, Nawshad et al. have demonstrated a consistent suppression of E-cadherin protein levels due to the formation of stable complex, engaging LEF, Smad2, and Smad4 proteins [39]. Apart from its prominent role to curtail the cytoplasmic

E-cadherin levels, LEF, on the other hand, in alliance with Smad4 drastically augments the mesenchymal markers Vimentin, and fibronectin and thus facilitates cellular motility [39].

1.2.3 Notch Signaling

Notch signaling is a vital signaling arm that controls cell fate through regulating essential cellular functions, including cell proliferation and apoptosis [40]. However, the enigma of Notch signaling in EMT has been extensively studied in relevance to cancer progression [41, 42]. The promising outcome of some classical researches unearths that constitutive activation of Notch signaling governs the binding of Notch ligands to the transmembrane receptors, Jagged or Delta Like Ligands (DLL) of adjacent cells (DLL) [40], leading to the activation of the Notch pathway and subsequent cleavage of Notch to release its intracellular domain. As a consequence, Notch intracellular domain translocates to the nucleus and sequesters CSL (CBF-1-Suppressor of Hairless/Lag1), resulting in the transcription of Notch-target proteins, Hey1, Snail, Cyclin D, and c-Myc [43–45]. Apart from triggering EMT and therapeutic resistance, aberrant Notch signaling is widely prevalent in many cancers [46, 47]. However, crosstalk between the Notch and TGF-β signaling pathways are considered imperative for the TGF-β-induced EMT and migration [40, 44].

1.2.4 HIF-1α Signaling

A low oxygen level, also known as hypoxia, is a frequently observed phenomenon in primary tumors. What are the vital consequences when cancer cells undergo prolonged hypoxic stress? Hypoxic stress diligently fuels up in the accumulation of hypoxia-inducible factors (HIFs), which are known to induce EMT via Twist and Snail [48–50]. While HIFs are the major effectors of hypoxia, ERK, PI3K/AKT/mTOR, and NF-κB are vital pathways found to be extensively regulated by hypoxia-induced EMT [48, 51–53]. Burgeoning pieces of evidence show that loss of E-cadherin and augmented expression of Vimentin, N-cadherin, CXCR4, and SMA are prominent hallmarks of hypoxia-induced EMT [54]. While considering a major basic helix-loop-helix (bHLH) transcription factor Twist-1 in EMT activation, HIF-1α directly regulates Twist-1 by binding to the HRE elements in the Twist-1 promoter. Moreover, from a mechanism of action perspective, Twist-1 is found to be indispensable for HIF-1α-mediated EMT and metastasis [50, 55]. Despite its versatile role in the regulation of EMT, hypoxia indirectly potentiates the TGF-β-induced EMT via steadily augmenting Slug and Snail expression with concurrent inhibition E-cadherin [54]. Additionally, in pancreatic cancer cells, the Hedgehog signaling also regulates hypoxia-induced EMT and invasion [56].

1.2.5 Integrin Signaling

Integrins, a family of transmembrane receptors comprising α and β subunit, are extensively corroborated in the process of building an intracellular network through cell attachment between the neighboring cells or ECM. Notably, this meshwork is an essential component pertinent to cell proliferation, differentiation, adhesion, and migration [57, 58]. However, perturbed integrin signaling is deeply associated as a core mechanism in EMT / chemoresistance [57]. The mechanism which would explain the role of integrins in ECM destruction underscores the deliberate involvement of receptor tyrosine kinases (RTKs) to amplify pro-survival signals via ERK and PI3K/AKT axis [59]. As a part of this comprehensive mechanism, integrin signaling, possibly via integrin αv, could augment the TGF-β1- mediated downregulation of E-Cadherin, facilitating the EMT cascade [60]. Although cancer cells are adequately equipped with their own intrinsic survival and proliferation signals compared to non-cancer cells, specific integrins family members, even on top of that, further exaggerate the tumorigenesis. In contrast, some other integrins may either inhibit or confer negligible impetus on tumor promotion [58]. Nevertheless, the tumor-promoting integrins, αvβ3 and α6β4, seemingly work together with other RTKs for ECM degradation [61, 62]. Therefore, integrin signaling represents a potential target that may yield better clinical outcomes in anti-metastatic therapeutic development.

1.2.6 microRNAs

microRNAs (miRNAs) are small non-coding RNAs, approximately 20 to 22 nucleotides in length, overtly facilitate transcriptional and post-transcriptional gene regulation. miRNAs bind to the '3'untranslated region (UTR) of their target mRNA resulting in gene silencing via target degradation or translational repression. Interestingly, miRNAs are severely implicated in the pathogenesis of cancer, especially in the EMT process. While few miRNAs positively regulate EMT, others are yet to be explored. On the basis of their oncogenic activities, miRNAs are categorized into oncogenic miRNAs (oncomirs) or tumor-suppressor miRNAs. Table 1 lists some of the miRNAs that are altered during EMT and tumorigenesis.

miR-21 is a well-characterized oncomir known to target a major tumor-suppressor protein, PTEN (phosphatase and tensin homolog), to induce EMT [73]. However, miR-21 inhibition causes the restoration of the PTEN levels via inactivation of one of the indispensable arms of AKT/ERK1/2 signaling, which ultimately reverses EMT [74]. Alternatively, a more detailed study of molecular signatures of miR-21 also targets another tumor-suppressor, Leucine zipper transcription factor-like 1 (LZTFL1), for the restoration of EMT [75]. In a concerted effort to gear up advanced carcinogenesis, miR-10b, an oncogenic miRNA, is largely concerned

Table 1 EMT-regulating microRNAs

miRNA	Effect on invasion/metastasis	Target	Reference
miR-1	Suppression	Twist	[63]
Let-7 family	Suppression	NANOG, BCL2L1, Twist-1, Twist-2, c-MYC	[63, 64]
miR-10b	Promotion	HOXD10, Vimentin, KLF4, Apaf-1	[65]
miR-16	Suppression	Twist-2, CDK1, CDK2	[63, 66]
miR-22	Suppression	CDK6, SIRT1, SP1	[67]
miR-23a	Suppression	Twist-2	[63]
miR-30a/b	Suppression	Snail1	[68]
miR-33b	Suppression	Twist-2, Zeb-2	[63]
miR-34a	Suppression	Slug, Twist-1, Zeb-1, Snail1, and Notch	[69]
miR-141	Suppression	Zeb-1/Zeb-2	[63]
miR-200	Suppression	Zeb-1/Zeb-2	[70]
miR-300	Suppression	Twist-2	[63]
miR-337	Suppression	Twist-2	[71]
miR-373	Promotion	CD44	[72]
miR-506	Suppression	Snail2	[63]

because its regulation is controlled by Twist-1, and down the way, hyperactivated miR-10b targets the homeobox D10 (HOXD 10). On the other hand, recent research unveils that Twist-1 instigates the expression of miR-10b-mediated HOXD 10 suppression convincingly confers activation of pro-metastatic protein Ras homolog family member C (RHOC) [76]. In order to promote migration, another oncomir miR-9 directly targets CDH1 causing cell motility and invasion [77]. Downregulation of miR-9-mediated E-cadherin expression induces β-catenin signaling, contributing to the upregulation of VEGF tendering tumor angiogenesis. Clinically, miR-9 overexpression found in tumors is correlated with aggressive phenotypes and poor prognosis [78]. High levels of miR-103/107 are also associated with metastasis and poor outcome [79]. miR-103/107 functions to inhibit the expression of Dicer, causing global miRNA downregulation.

In contrast to the oncomirs, the tumor-suppressive miRNAs are attributed to stall malignant transformation. The cumulative miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) as prospective candidates to halt tumorigenesis is vividly characterized and known as an epithelial phenotype's guardians in breast cancer [80]. Predictably, loss of miRNA-200a is frequently observed in breast cancer, but this loss does not predict tumor recurrence or patient survival [81]. The miR-200 family activates the Sec23a-mediated tumor cell secretome, which leads to the secretion of metastasis-suppressive proteins [82]. miR-200 family members are encoded from two clusters and directly target the messenger RNAs of the E-cadherin transcriptional repressors Zeb-1 and Zeb-2. Notably, Burk et al. and other studies have shown that both promoter regions are repressed in mesenchymal cells by Zeb-1 and Zeb-2 through binding to the E-box elements [80, 83]. A double-negative feedback loop controlling Zeb-1-Zeb-2 and miR-200 family expression is vital for regulating the plasticity of the cancer cells. Another miRNA, miR-375, targets short stature homeobox 2 (SHOX2) to suppress EMT [84]. A novel miRNA, miR-506, significantly suppresses the expression of mesenchymal markers in the MDA-MB-231 human breast cancer cell line. In addition to restraining the transforming growth factor (TGF)-β-induced EMT, miR-506 also plays a vital role in the post-translational control of EMT-related genes [85]. miR-203 represses endogenous Snail, forming a double-negative miR-203/Snail feedback loop [86]. Additionally, miR-203 also targets Slug while TGF-β-induced Slug promotes EMT by repressing the miR-203 promoter to inhibit its transcription [87].miR-34 is one of the most studied tumor-suppressor miRNAs. It is implicated in the inhibition of EMT mediated by p53. It has been reported that activation of p53 downregulates the EMT induced by the transcription factor Snail via induction of the miR-34 gene. Suppression of miR-34 attributes the upregulation of Snail and endorses cell migration/invasion. Moreover, miR-34a prevents TGF-β-induced EMT, and the repression of the miR-34 gene by Snail is known to be a part of the EMT program [88].

1.3 EMT Markers

A variety of markers, including proteins as well as miRNAs, have been explored in pre-clinical settings to assess the extent of EMT. These markers have been categorized as (i) epithelial markers that are concerned with the maintenance of the epithelial state and (ii) mesenchymal markers that sustain the mesenchymal phenotype. Since EMT is characterized by the transition of an epithelial cell to a mesenchymal state, the attenuation of epithelial markers with simultaneous acquisition of mesenchymal markers lies at EMT's core. Table 2 highlights some of the EMT markers that are studied to assess EMT. Here, we summarize some of these well-accepted EMT markers that are analyzed to assess the EMT phenomenon.

Table 2 Markers of EMT and their role in tumorigenesis

Category	Marker protein	Role in EMT/ tumorigenesis	Reference
Epithelial markers	E-cadherin	Functional and expressional loss of E-cadherin during EMT and cancer. Downregulation increases cellular motility	[89]
	Claudins	Integral membrane proteins localized at tight junctions and maintain the epithelial cell polarity. Repressed during EMT to promote cancer cells invasion and migration	[90]
	Zonula occludins	Component of tight junctions and adherens junctions; controls cell migration; downregulated during EMT	[91]
Mesenchymal markers	N-cadherin	E-cadherin to N-cadherin switching during EMT. High expression in mesenchymal cells. Promotes cancer cell survival, invasion, and migration. High levels depict poor prognosis	[92]
	Vimentin	Established mesenchymal marker. Regulates cell shape as well as cell motility	[93]
	Fibronectin	Component of the tumor matrisome. Regulates the integrin signaling to facilitate EMT, invasion, and metastasis	[94]
	Snail1/2	Key repressor of E-cadherin and highly expressed in cancers. Promotes EMT and metastasis; predicts poor prognosis	[95]
	Twist-1	E-cadherin repprossor, promotes EMT, metastasis, and formation of cancer stem cells	[96]
	Zeb-1/2	Strong repressor of E-cadherin and aids EMT	[97]
	EPCAM	Highly expressed in circulating tumor cells	[98]

1.3.1 Epithelial Markers

Intact adheren junctions are a hallmark of epithelial morphology, which keep the cells tethered to each other. For maintaining cellular integrity, E-cadherin, encoded by the *CDH1* gene, is an important component protein identified at the adheren junctions, known to regulate the epithelial phenotype [99]. However, during malignant transformation, switching of E-cadherin to N-cadherin is prevalent and regulated by diverse signaling pathways [92]. Notably, loss of E-cadherin and subsequent EMT activation imparts overwhelming migration capability leading to metastatic dissemination. By analyzing clinical data, we can clarify how functional loss of E-cadherin, via chromosomal deletions, mutations, epigenetic silencing, or proteolytic cleavage, has been implicated in the development of pancreatic, breast, gastric, and skin cancers [100, 101]. On the other hand, the CDH1(E-cadherin) gene promoter's hypermethylation is extensively observed in malignant cells associated with EMT initiation [102].

Apart from E-cadherin, few other epithelial markers, including claudin family members, are involved in maintaining cell polarity and permeability. Claudins serve as a vital component of the tight junctions (TJs) [103]. Claudins comprise a large family of tetraspan membrane proteins, which are expressed in a tissue-specific manner. Similar to E-cadherin expression, a wide range of clinical samples display an altered expression of claudins, with claudin-1, -3, -4, and -7 being the most recurrently affected among the claudin family [104]. Strikingly, loss of claudin-3 (CLDN3) and claudin-4 (CLDN4) not only triggers robust morphological changes but adequately enhances growth, migration, and invasion processes. A deficit of CLDN3 and CLDN4 significantly boosts the E-cadherin protein levels with simultaneous N-cadherin downregulation [103]. Notwithstanding their EMT modulatory functions, some members of the claudin family are consistently downregulated during tumorigenesis, which is fairly constant with their function as a tight junction protein; however, claudin overexpression has also been reported in some cancers [104].

1.3.2 Mesenchymal Markers

In cancer, the role of EMT has been grossly corroborated into the severity of the disease and thus providing a mechanism for cancer cells to dislodge from their primary site and colonize at distant secondary sites. Rationally, therefore, a successful accomplishment of EMT warrants the activation of mesenchymal markers. In that context, major extensively studied mesenchymal markers include N-cadherin, Vimentin, and Epithelial cellular adhesion molecule (EpCAM) [105]. During malignant transformation, the induction of N-cadherin protein levels is a well-studied event. This E-cadherin to N-cadherin switch, also known as the cadherin switch, is a hallmark of EMT and is a designated biomarker for the evaluation of circulating tumor cells (CTCs). Based on substantial evidence, it can be assumed that elevated N-cadherin levels are significantly associated with increased tumor invasion, metastatic dissemination, and poor patient prognosis [106]. Surprisingly, N-cadherin also modulates the Wnt signaling because forced N-cadherin expression leads

to the elevated localization of β-catenin at the plasma membrane [107]. In order to promote β-catenin translocation, studies unveil that N-cadherin modulates the TCF/LEF-mediated gene transcription, which could be the causal root of excessive cell motility [108]. Additionally, the highly integrated cooperation between the FGFR-Akt with N-cadherin signaling in the perspective of EMT/ stemness induction has been extensively examined as well [109]. Sequentially, the next vital metastatic marker-Vimentin, which is a type III IF (intermediate filament) expressed during embryonic development as well as tumorigenesis. Out of the six major IFs, Vimentin is considered the most important facilitator for mesenchymal cellular stiffness. Vimentin is stimulated in epithelial cells as soon as EMT is activated; otherwise, these cells express keratin solely as a major IF. In order to analyze the coherent connection between Vimentin and Keratin, Polioudaki et al. [110] confirmed a significantly low Vimentin to Keratin ratio (Vim/K) in an epithelial phenotype, whereas a mesenchymal phenotype is associated with a high Vim/K ratio in CTCs in breast cancer patients. Although Vimentin overexpression is often ubiquitous in a diverse range of cancers, its aberrantly high expression is positively correlated with tumor progression, metastatic dissemination, invasiveness, and chemoresistance [111, 112].

Epithelial-cellular adhesion molecule (EpCAM), also known as CD326, is a transmembrane glycoprotein known to be associated as a vital cell adhesion protein in epithelial cells; however, its role in epithelial malignancies has been consistently emerging [113]. Contextually, a recent study unveils a high expression of EpCAM in triple-negative breast cancer (TNBC) cells [114]. Moreover, the metastasis incidence in TNBC is also directly correlated with EpCAM expression [115]. Importantly, EpCAM possesses a critical role in maintaining the pluripotency of cancer stem cells (CSCs), conferring it as a classical CSC marker [116].

1.4 EMT Paves the Way for Tumor Metastasis

Metastasis is a scientific terminology referring to the spreading and colonization of the primary tumor cells to distant secondary organs. It is responsible for the majority of cancer-related deaths. Although the lion's share of primary tumors can be treated with surgery and adjuvant therapy, the systemic nature of the metastatic disease renders it mostly incurable. Furthermore, the disseminated tumor cells are highly resistant to the existing anti-cancer therapeutic agents and often cause recurring disease [117]. The lethality due to metastasis is now well-recognized and remarkable efforts have been made to uncover the cellular and molecular basis of this systemic phenomenon. A series of cell-biological events, collectively termed as the invasion-metastasis cascade, are executed on the onset of the successful establishment of secondary metastases at an anatomically distant organ site. The invasion-metastasis cascade involves (1) local invasion of the cancer cells into the basement membrane and the surrounding extracellular matrix (ECM) and stromal cell layers, (2) intravasation into the endothelial lamina of blood vessels and entry into the systemic circulation, (3) surviving through the rigors of systemic transport (4) arrest at distant organ sites and extravasation into the parenchyma of distant tissues, (5) enduring the foreign microenvironments to form micrometastases and re-initiate their proliferative programs at metastatic sites, thereby generating macroscopic, clinically detectable neoplastic growths [12]. We have illustrated the various steps involved in the metastatic cascade in Fig. 2. While the majority of these events are controlled by the molecular mechanisms (genetic and epigenetic) functioning within the cancer cells, the non-neoplastic stromal cells also exert overwhelming resistance influencing the invasion-metastasis cascade [118]. In the next sections, we will critically analyze the inherent *modus operandi* of tumor metastasis.

1.4.1 EMT and Malignant Transformation

Post-EMT malignant transformation of TME is an intricately synchronized process driven by intrinsic genetic changes, alterations in the local microenvironment, or environmental factors. The initial escape from the primary site is an essential prerequisite for tumor cells to adopt motile phenotype and thus degrade the underlying basement membrane/ECM to initiate an invasion, which can only be accomplished upon EMT induction. Although EMT is typically considered a delayed event during overall tumorigenesis, metastasis commences with EMT induction in a tumor cell subset. The crucial role that EMT- associated transcription factors dictate the initial malignant conversion cannot be undermined. From the repository of emerging recent literatures, aberrant Twist-1 mRNA levels have been detected in the early stages of primary tumor development [119, 120]. Such information supports the critical role of Twist-1-mediated E-cadherin suppression during malignant conversion. One of the other mechanisms by which Twist-1 aids malignant transformation is binding to the tumor-suppressor protein, p53 leading to its degradation. Twist-1-mediated p53 degradation nullifies the oncogene-induced senescence and apoptosis executed by p53 [121]. Of note, the Twist-1-mediated p53 degradation, on the other hand, contributes to the Her-2 and H-Ras-driven malignant transformation [122]. Similarly, Wnt, Notch, and other signaling pathways that regulate EMT are also implicated in malignant transformation of TME, primarily through the activation of EMT-TFs [123, 124].

Post-activation of EMT-TFs, degradation of the underlying basement membrane is indispensable for invasion and is executed through the upregulation of various matrix degrad-

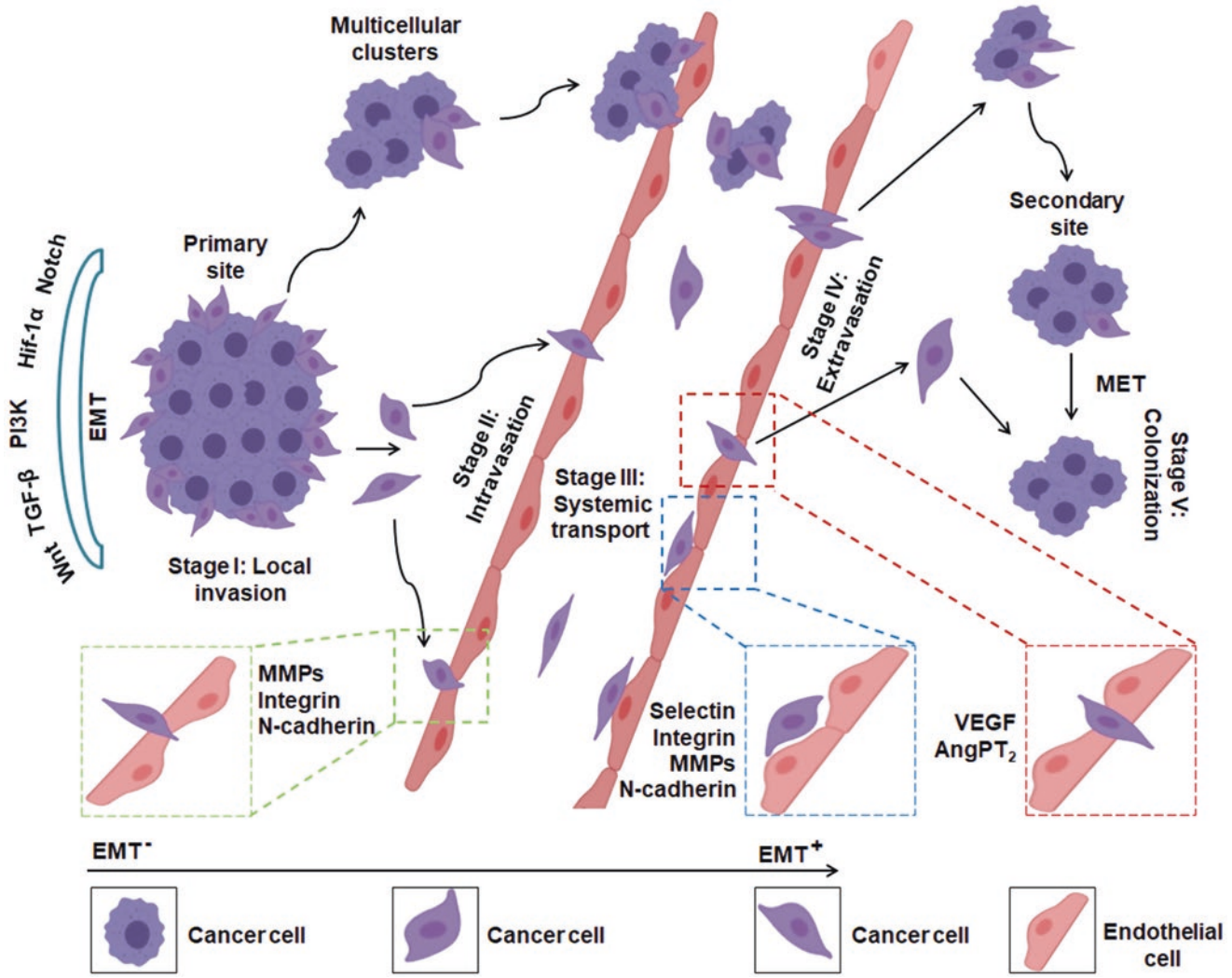

Fig. 2 EMT and tumor metastasis. The figure depicts the various stages of tumor metastasis. The tumor cells undergo EMT at the primary tumor site, travel to distant locations, and finally undergo MET to establish successful secondary metastases

ing enzymes. Interestingly, EMT-TFs orchestrate the formation of invadopodia to degrade ECM [125]. Notably, invadopodia facilitates the ECM degradation by means of involving diverse proteases, including matrix metalloproteases (MMPs), membrane-tethered proteases (MT-MMPs), and ADAMs (a disintegrin and metalloproteases) to the cell-matrix contact points [126]. Remarkably, EMT TF Twist-1 promotes invadopodia formation through the activation of PDGFRa/Src signaling. On the other hand, TGF-β equally contributes towards invadopodia formation by augmenting the expression of Twist-1 and the focal adhesion protein Hic-5 [127]. In a robust integrative approach, Zeppo1, another metastatic promoter found to impede E-cadherin expression along with the stimulation of invadopodia-like structures [128]. In a similar manner, Snail1 is known to assist the expression of MT1-MMP, MT2-MMP, and MMP9 as well as promotes the basement membrane's degradation [129]. Furthermore, EMT-TF Snail2 as well regulates tumor metastasis through induction of MT4-MMP and MMP2 [3]. Hence, cumulatively, all the above evidences imply that post-EMT preparatory phase is indispensable for tumor cells to ensure dissociate from tight gap junctions, attain migratory phenotype, and as a consequence, degrade the ECM to initiate the metastatic cascade.

1.4.2 Intravasation

Intravasation, the second step of metastasis, ensures the tumor cells invade the endothelial lamina, infiltrating into the lymphatic or blood vessels, and shelter into the vasculature accordingly. Following entering into the circulation, tumor cells either migrate directionally in response to chemokine or growth factor gradients, else get carried away passively by the stream of blood flow. Nevertheless, growing evidences suggest that cells in transition desperately need various

ligand-receptor molecules for stable adhesion. Besides, cytokines and growth factors that augment vascular permeability to allow transmigration through the vascular wall also adequately serve as an impediment [126]. Strikingly, during this initial phase of the journey, expression of N-cadherin spikes robustly; although present at the adherens junctions, N-cadherin is either negligibly expressed or absent in the epithelial cells. Notably, this newly synthesized pool of N-cadherin-mediated adhesion between the cancer cells and the endothelial cells governs the entire intravasation process. Eventually, following N-cadherin-mediated adhesion, downstream activation of Src kinase / β-catenin further potentiates the trans-endothelial migration [127].

What is the role of integrins in the intravasation cascade? Integrins are vividly implied in the metastatic intravasation process [128]. For example, melanoma cells expressing the integrin VLA-4 are known to stimulate adhesion and trans-endothelial migration via binding to VCA-1 localize on endothelial cells. Despite strong host-immunogenic resistance, aberrant expression of several selectins, viz. E-selectin (CD62E), P-selectin (CD62P), and L-selectin (CD62L) are known to facilitate binding and rolling of cancer cells on endothelium [129]. Contextually, EMT-transcription factors, for example, Zeb-1 and Snail1, also regulate the migration of the cancer cells through the endothelial barrier [130]. Of note, Snail1 overexpression specifically activates the membrane-bound MMPs (like MT1-MMP and MT2-MMP) but not secreted MMPs, suggesting physical contact of MMPs with the endothelium is a prerequisite for intravasation [131]. In the next phase, the EMT cells gradually degrade the surrounding matrix in order to pave the way for invasion and intravasation, while the non-EMT cells follow the course to infiltrate into the vasculature [132]. Paradoxically, non-EMT cells are believed to be more competent than the EMT cells in reestablishing colonies in the secondary sites due to their superior adhesive properties that allow them successful extravasation into the secondary site [133].

1.4.3 Systemic Transport

Once the tumor cells detach from each other and enter the vasculature, they must override immunological resistances, shear forces, and anoikis. Anoikis is a form of programmed cell death that is instigated when anchorage-dependent cells detach from the surrounding ECM [134]. Consequently, one such survival mechanism is initiated when the integrins on tumor cells interact with ECM, activating focal adhesion kinase (FAK), which phosphorylates its downstream effector molecules leading to Akt activation. Paradoxically, loss of contact between integrins and ECM impedes the survival signals and initiates cell death by triggering the expression of pro-apoptotic proteins [135]. EMT, on the contrary, supports the cancer cells to overcome anoikis by E-cadherin to N-cadherin switching, which is a decisive factor in promoting invasion. The importance of EMT can be evaluated from the landmark studies underscoring the presence of EMT markers in CTCs [136]. A handful of these relevant studies have demonstrated that the mesenchymal phenotype is adequately prevalent among the CTCs and solely accompanied by Zeb-2 overexpression [137]. Furthermore, in the squamous cell carcinoma-mouse tumor model, Twist-1 induction triggers a dramatic boost in the mesenchymal CTCs as indicated by low E-cadherin and high Vimentin levels [136]. Notably, the CTCs trigger tumor cell-induced platelet aggregation (TCIPA), which tether to the surface of CTCs via GPIIb-IIIa-fibrinogen bridge [138]. Platelets also secrete TGF-β that aids CTCs in maintaining the EMT state [139]. Moreover, platelet-derived TGF-β efficiently reduces the expression of the immunoreceptor-NKG2D, thus inhibiting Natural Killer (NK) cell activity [140]. On the other hand, mushrooming evidences elicit that platelets may shield the CTCs against immune assault by NK cells [141]. Furthermore, the transfer of the major histocompatibility complex (MHC) from activated platelets to CTCs favors the escaping of the immune surveillance [142]. The platelet-derived VEGF, at the same time, provides synergic impetus to CTCs by stalling the maturation of primary antigen-presenting cells/dendritic cells [143]. Further down the course, CTCs also take part in the construction of micro tentacles, which are believed to be microtubule-based membrane protrusions, probably aiding in CTC aggregation and tethering [144]. In that direction, burgeoning evidence implies that major EMT TFs, Twist-1/Snail1 play a pivotal role in promoting micro-tentacle formation, suggesting that CTC survival via micro-tentacle-based attachment of CTCs to platelets and endothelium could be potentiated by EMT [145].

1.4.4 Tumor Cell Extravasation and EMT

Most of the tumor cells trespassing into the bloodstream hardly confront the rigors of the circulation, including the hemodynamic shear forces as well as attacks of the immune system and anoikis due to the loss of adhesion to the ECM. Only a few surviving cells may arrest in the vascular lumen and manage to extravasate through the capillary endothelium into the parenchyma of distant organs and thus orchestrate micrometastasis [146]. However, the evidence for the involvement of extravasation in various pathogenic processes is mounting. For example, a multifunctional non-kinase receptor for semaphorins family, neuropilin-2 (NRP-2), is identified on the surface of renal carcinoma and pancreatic cancer cells. This receptor aids vascular adhesion and extravasation by interacting with endothelial α5 integrin [147]. Notably, in prostate cancer metastasis to bones, adhesion of E-selectin ligands as well as β1 and αVβ3 integrins, are sequentially required for extravasation. Furthermore,

while validating the role of integrins in adhesion and extravasation, a handful of emerging evidences uncover that integrins αvβ3, αvβ5, α5β1, α6β4 expressed on tumor cells correlating with metastatic progression in melanoma, breast carcinoma, pancreatic, lung, and prostate cancer [61]. Consequently, extravasation is orchestrated via active collaboration with prometastatic genes, Twist-1, Integrin beta-1 (ITGB1), and VEGFA [148]. In order to continue steady migration in this phase of metastasis, cancer cells deliberately recruit versatile motile structures. For example, filopodium-like protrusions (FLPs) by the tumor cells containing integrin-β1 constitutively interact with the ECM of the distant tissue parenchyma to alter the TME [148]. Similarly, Snail1 can induce the formation of FLPs, and most strikingly, the mesenchymal states of some breast cancer cells are closely associated with their ability to generate FLPs. Together, the above studies underscore the EMT program confers a significant role in promoting extravasation and dissemination of tumor cells to distant organs.

1.4.5 Metastatic Colonization and MET

Of the total fraction of tumor cells that metastasize from the primary site, only a minuscule subset of cells proceed to establish micrometastases under the catastrophic resistances by the unmet stromal environment [12]. As discussed above, the metastatic cascade till the extravasation stage is majorly driven by the EMT, as is evident by the EMT signatures noted in the primary carcinomas and CTCs. However, it is surprising that the macrometastases are largely epithelial, in contrast to the proposed mesenchymal nature, suggesting that EMT involvement during metastasis is likely to be functionally dynamic. In that context, Bonnomet et al. noted a heterogeneous expression pattern of Vimentin in the primary MDA-MB-468 tumor xenografts and the resulting lung metastases, while high levels of Snail1, Snail2, and Vimentin prevail in CTCs. This finding implies that the Vimentin-negative macrometastases might originate following MET in the Vimentin-positive CTCs, highlighting the epithelial-mesenchymal plasticity [149]. Similarly, another study rationally pointed out that EMT activation aids the metastasis's initial phases, including local invasion, intravasation, and extravasation. However, EMT inhibition is equally essential for tumor cell proliferation and macrometastasis formation at the distant site [136]. A novel EMT inducer, Prrx1, cooperates with Twist-1 to promote a more invasive phenotype in human breast cancer cells. On the basis of the evidence, downregulation of Prrx1 is an essential prerequisite to revert EMT and for lung metastasis colonization [150]. In an exactly similar fashion, EMT activation by Zeb-2/Snail1 leads to the inhibition of Cyclin D activity, thereby suppressing cell division [151]. All these studies together assert that EMT reversal could be essential to restart proliferation at the secondary site for metastasis colonization although these highly coordinated mechanisms warrant detailed investigation.

Another new perspective in this context is the emerging players of miRNA families, such as the miR-200 family (including miR-200a, miR-200b, and miR-200c). These miRs maintain the cells' epithelial nature by negatively regulating the EMT inducer Zeb-1 and vice versa (as we have discussed earlier). Interestingly, the Sec23a-mediated secretion of metastasis-suppressive proteins are by and large prevented upon the re-expression of miR-200 family members, which ultimately trigger colonization, possibly by repressing Igfbp4 and Tinagl1 [152]. These studies indicate that both the loss of EMT-inducing signals and the induction of MET-promoting cues may be required simultaneously to actively promote micrometastases. Given that micrometastasis outgrowth is a critical stage in the invasion-metastasis cascade, more studies on MET's molecular regulators could shed light on therapeutic approaches to inhibit tumor colonization.

1.5 EMT Acquisition by Mesenchymal Cells—A Real Challenge in the Development of Cancer Therapeutics

The association between major EMT-associated transcriptions factors and the development of novel therapeutic strategies on that basis is of great interest to the scientific community. The underlying molecular mechanisms involved in EMT acquisition are primarily governed by the EMT-associated transcription factors (EMT-TFs). EMT-TFs include transcription factors belonging to the basic helix loop helix family (Twist-1 and 2), zinc finger family proteins (Zeb-1/2, Snail, and Slug) and β-catenin. Acting in isolation or conjunction, EMT-TFs regulate certain EMT-associated marker proteins. Epithelial markers such as E-cadherin, Claudins, Occludins, and Cytokeratin are transcriptionally repressed by EMT-TFs with concomitant upregulation of mesenchymal markers like Vimentin, N-cadherin, Fibronectin, and matrix-metalloproteases (MMPs). Activation of EMT-TFs and subsequent EMT induction in cancer cells is invariably considered as the building blocks of acquired chemoresistance, leading to enhanced stemness/plasticity of the malignant cells. Stemness refers to the core properties exhibited by stem cells, for example, self-renewal and production of differentiated progeny. These properties related to stemness are physiologically displayed by embryonic stem cells and adult stem cells during development and tissue homeostasis as well as regeneration. Extensive studies of the tumor tissue have pointed towards the presence of stem-like cells, termed as the cancer stem cells (CSCs), within tumors [153]. The CSCs behave in an equivalent malignant manner to normal stem cells in terms of stemness

[154]. A mesenchymal phenotype is a commonality between the CSCs and the normal stem cells that allows them to retain the stemness as well as the migratory properties [155–157]. CSCs have been linked to EMT phenotypes by epigenetic programming in many types of cancer. The EMT process enables cancer cells to disseminate and to self-renew during tumor metastasis. For example, non-transformed immortalized human mammary epithelial cells undergo an EMT process upon Snail1, Twist-1 expression, or the presence of TGF-β1. The subpopulation of $CD44_{high}/CD24_{low}$ immortalized human mammary epithelial cells that possess stem-like properties increases with concomitant induction of EMT phenotype [158]. Contrariwise, CSCs confer prodigious carcinogenic potential and plasticity in comparison to the non-CSCs subset of cancer cells. This finding indicates that an EMT process generates cells with similar properties commonly observed in self-renewing stem cells. In this regard, it appears that the EMT process that enables cancer cells to disseminate from a primary tumor (i.e., metastasis) also promotes cancer cell self-renewal.

The potential application of the identification of interplay between CSCs and EMT has just begun to unveil. For instance, loss of the tumor suppressor p53 in mammary epithelial cells has been shown to induce EMT and enrich CSCs through repression of miR200c, suggesting that the p53–miR200c pathway can be activated to suppress EMT-associated CSCs to treat cancer [159]. Furthermore, EMT harboring CSCs are resistant to platinum-based conventional chemotherapies (oxaliplatin, cisplatin) (Fig. 3) due to the modulation of genes involved in cell survival or evasion of apoptosis. CSCs and EMT seem to be an axis of evil in cancer, a better understanding of which may contribute to establishing novel therapeutic platforms. Rationally, EMT acquisition in cancer cells puts forth a two-fold challenge, i.e., drug resistance and stemness, both of which are implicated in the progression of the metastatic cascade. Hence, the true challenges in anti-cancer therapy development must confront the EMT accretion of cancer cells.

2 Par-4 Emerges out as a Prospective EMT Modulatory Protein

Cellular fate between apoptosis or survival depends upon the balance between both survival (EMT) and pro-apoptotic cascades (Program cell death); this equilibrium stage is adequately explained in pre-clinical settings where therapeutic administration could modulate tumor suppressor's function to eradicate tumor burdens. Until recently, Par-4 as a tumor suppressor protein is well-established owing to its cancer-specific expression and apoptosis-inducing ability, but Par-4 research has attained a new height by illustrating EMT stalling properties of Par-4. Several research groups have indeed dissected the signaling mechanisms involved in Par-4 activation to augment apoptotic cascades [160–163]. However, new developments in Par-4 research have not only widened its therapeutic potential but dominantly proclaim its imperative role in modulating autophagy, senescence, and other therapeutically relevant avenues. One of such daunting task is the halt in EMT induction and prevention of metastasis by Par-4. In this section, we have extensively envisaged the prospective role of Par-4 with reference to EMT, the molecular signaling involved, and therapeutic implications. This section has also been summed up in Fig. 4.

2.1 Structural Aspects of Par-4(SAC Domain) that Link it with EMT

Par-4 is a leucine-zipper protein that has distinct nuclear localization and entry sequences. It comprises of two nuclear localization sequences (NLS1 and NLS2) at the N-terminal region, a nuclear export sequence (NES), a "selective for apoptosis of cancer cells" domain (SAC) unique to the Par-4 protein, and a leucine zipper domain (LZ) at the carboxyl-terminal region (Fig. 5) [164].

In an elegant study by Zhao et al., bone marrow from SAC transgenic mice transplanted into SAC-non-transgenic irradiated littermates serves as a pool for SAC-expressing cells that are resistant to tumor growth. In the tail vein mice metastatic model, recombinant Par-4 (TRX-Par-4) and SAC (TRX-SAC) proteins are competent in inhibiting the formation of metastatic lung nodules [165]. One of the principal mechanisms for Par-4 functionality is its inhibition of NF-κB. Par-4 that has a defective or lacking NLS2 (amino acid residues 137–153) domain is retained in the cytoplasm and is unable to block NF-κB activity [166], downstream targets of which include major EMT-related genes, for example, Twist-1, Snail1, and β-catenin. Of note, the NLS2 domain is encompassed by the larger SAC domain in totality. Rationally, the relevance of the SAC domain to the anti-EMT potential of Par-4 is beyond question. The question that begs to be asked is whether or not additional structural domains in Par-4 are unequivocally responsible for its anti-EMT activity. The leucine zipper (LZ) motif of Par-4 is essential for its interaction with other proteins and binding to DNA sequences to carry out its co-transcriptional activity. The interactions mediated by the LZ motif can be perceived as an alternate mechanism by which Par-4 can either interact with EMT markers (Vimentin) or bind to regulatory DNA sequences of EMT-TFs (Twist-1, Snail1, Zeb-1). However, unlike the SAC domain-mediated inhibition of NF-κB, the LZ motif's role in the abrogation of EMT needs in-depth exploration as there is a substantial dearth of evidence.

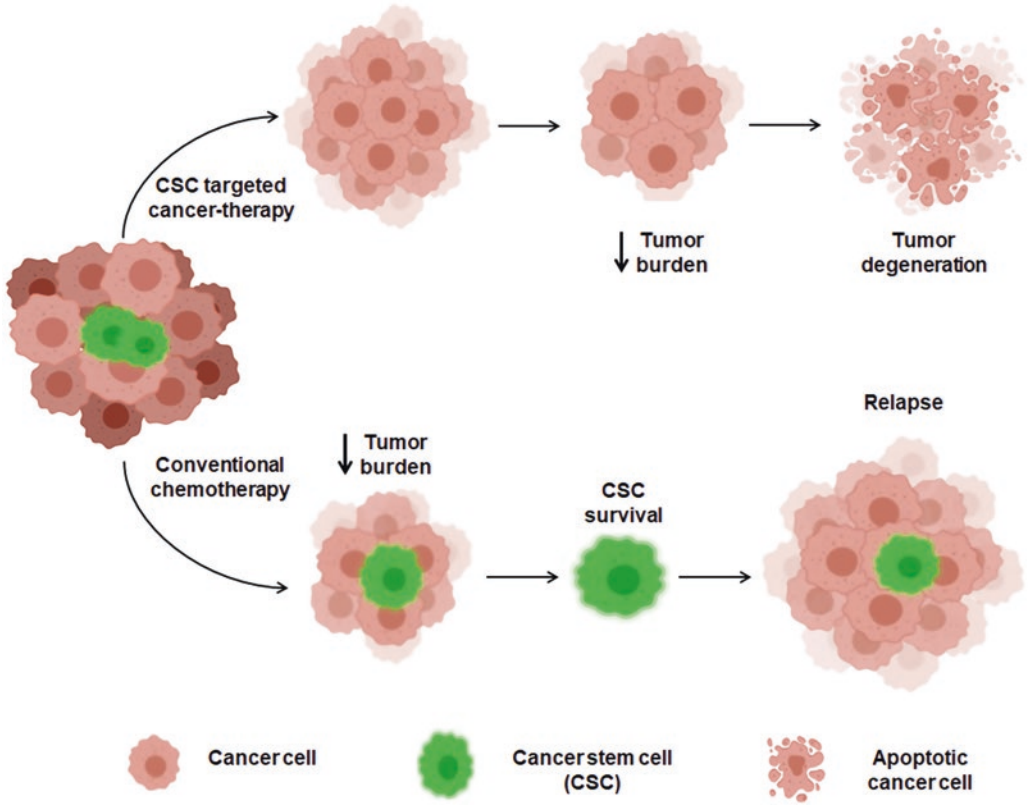

Fig. 3 Cancer stem cells (CSCs) and chemoresistance. The figure depicts the outcomes of conventional cancer therapies versus stem cell-specific therapies. The CSCs constitute a small subset within the tumor cells that drive chemoresistance as well as tumor recurrence following conventional chemotherapy

2.2 Regulation of NF-κB Activity by Par-4

EMT has been perceived as a deliberate ploy employed by cancer cells to evade cytotoxic threats and accomplish survival. Nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB, is a master transcription regulator that is essential for cell survival and is critically relied upon by cancer cells to ensure their survival. Notably, translocation of the NF-κB into the nucleus drives the expression of genes regulating diverse biological processes [167]. However, the majority of the cancer types are prone to altered levels of NF-κB that are positively correlated with tumor growth, invasion, metastasis, and chemoresistance. Interestingly, the NF-κB and Par-4 proteins are antagonistic to each other [168]. The NLS2 sequence found within Par-4 is essential for its nuclear translocation (as detailed earlier) and subsequent suppression of the NF-κB-dependent transcription activity, binding to Par-4 partner proteins WT1, ZIPK/DAXX, and THAP; and thus induction of apoptosis [169]. Apart from the direct inhibition of the NF-κB activity, Par-4 may also indirectly stall NF-κB via stabilizing AKT. AKT is a serine/threonine-protein kinase that regulates a variety of cellular processes, including proliferation, survival, and protein translation. However, AKT overexpression is a catastrophic event reported in almost all cancers, rendering it a very important therapeutic target. Of note, AKT activation not only stimulates NF-κB activity to instigate survival of cancer cells but at the same time, AKT blocks the pro-apoptotic transcription factor, FOXO3a [170]. However, an elaborate study by Joshi et al. has demonstrated that Par-4 autonomously inhibits AKT via PKCζ that phosphorylates AKT at Ser124 [171]. AKT phosphorylation at Ser124 impacts the phosphorylation status of the two most important residues, Ser473 and Thr308, that are critical for AKT activity [172]. Another detailed contextual study by Choudhry et al. reveals Par-4 to be one of the downstream targets of TGF-β signaling involved in the EMT induction. The TGF-β-mediated induction of Par-4 expression, as well as its nuclear localization, was revealed to be executed via the Smad4 and NF-κB pathways. Moreover, the study also reveals that the interaction of Par-4 with Smad4 results in the abrogation of the NF-κB and XIAP protein levels, culminating in an EMT halt [173]. Further, NF-κB also hinders the apoptosis process by enhancing the transcription of the anti-apoptotic protein, $Bcl_{xl,}$ and X-linked IAPs (XIAP) [174]. Par-4 counteracts the pro-survival effects of NF-κB by initiating the assembly of the death-inducing signaling complex (DISC) by augmenting the interaction of FAS receptor and

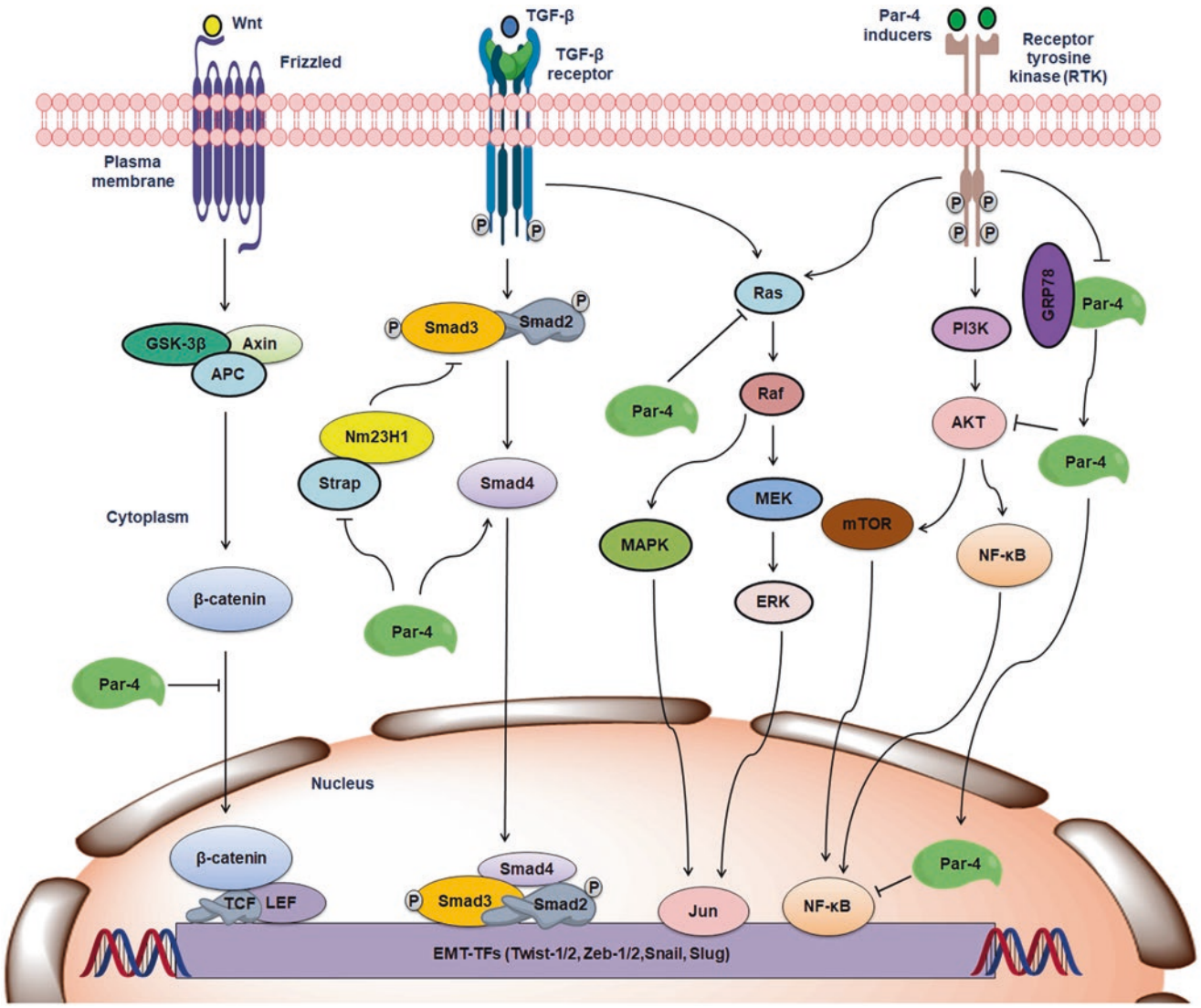

Fig. 4 Signaling involved in Par-4-mediated abrogation of EMT. The figure depicts various signaling pathways that initiate and/or facilitate EMT as well as the axes that are targeted by Par-4 to execute its anti-EMT and anti-metastatic function

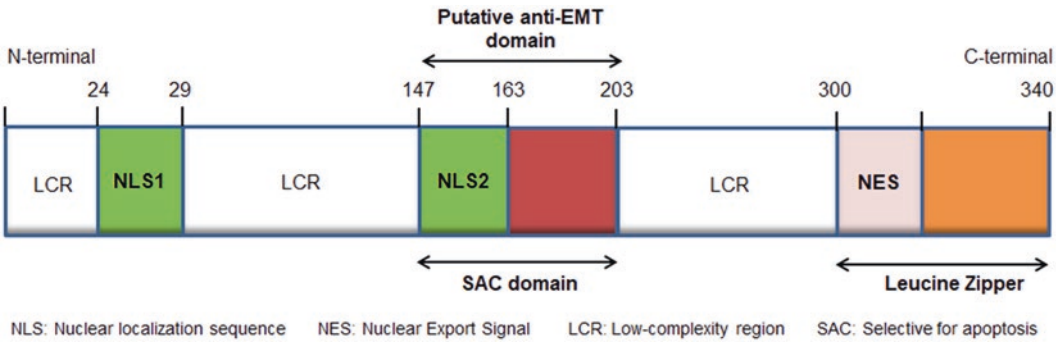

Fig. 5 Structural aspects that link Par-4 and EMT. Functional domains of Par-4 include two nuclear localization signals (NLS1 and NLS2) at the N-terminal, an SAC (selective for apoptosis in cancer cells) domain in the middle, and a leucine zipper (LZ) domain at the C-terminal

FAS Ligand with FADD and inducing apoptosis in a hormone-independent manner [175]. MMPs, the active players in ECM components degradation, are well-accepted for their involvement in cancer progression and metastasis. Importantly, MMPs can also confer apoptosis resistance to the cancer cells by negatively regulating the Fas-FADD-mediated death signaling [176]. However, the extracellular Par-4 can rescue the anti-apoptotic signaling associated with cancer cells by diminishing MMP-2 [177], activating downstream caspase-3 as well as nullifying the pro-metastatic effects of c-FLIP to exert an extrinsic apoptotic effect [178, 179]. Thus, the apoptotic induction, as well as abrogation of invasion, could be controlled independently by secretory Par-4 in diverse cellular background. Since Par-4 negatively regulates NF-κB protein which double-edged function is grossly equipped with the regulation of cancer cell survival through modulation of EMT-TFs. By and large, NF-κB induces the transcription of EMT-TF genes Twist-1, Slug, and SIP1 by directly binding to their promoter regions, ultimately attributing the EMT process to promote an aggressive phenotype [180]. Hence, all these above studies in this subsection authenticate the direct contribution of NF-κB in EMT promotion and as well provide compelling evidences that Par-4-mediated anti-EMT effects could majorly be attributed to the inhibition of NF-κB. Although Par-4 presents a foolproof theoretical approach to tackle the NF-κB-mediated tumorigenesis and chemoresistance, the feasibility of NF-κB-targeting therapies has to be carefully evaluated.

2.3 Regulation of EMT-Associated Transcription Factors by Par-4

In the horizon of Par-4 research, we have witnessed emerging evidences unleashing its novel functions. One of such fascinating functions emphasizes the anti-EMT role of Par-4. Multiple studies have recently revealed the anti-EMT role of Par-4 [177, 181]. Importantly, exogenous Par-4 is well-documented to positively correlate with E-cadherin expression and down-modulation of various EMT-TFs, including Twist-1, Snail, Slug, Zeb-1, and Zeb-2. As a consequence of diminished EMT-TF transcriptional activity, mesenchymal markers, viz. Vimentin, N-cadherin, MMPs, and fibronectin are consistently found to be repressed. However, whether or not EMT-TFs are directly regulated by Par-4 remains to be thoroughly examined. Although evolutionarily, Twist-1 transcription factors are attributed to embryonic development, their expression is limited post-embryogenesis in most of the cell types [182]. Elevated expression of Twist-1 is often associated with tumor progression, metastasis, and poor patient prognosis [183]. Twist-1-mediated E-cadherin suppression is critical for the induction of EMT that ultimately converges into metastatic dissemination [184]. Moreover, Twist-1 also positively modulates the expression of the mesenchymal markers Vimentin, Fibronectin, and N-cadherin to promote cellular motility. Recent studies elegantly postulate the relevance of Par-4-mediated Twist-1 inhibition in cancer cells concomitant with E-cadherin upregulation although the exact mechanism remains obscure [181, 185, 186]. One of the plausible mechanisms by which Par-4 may impede Twist-1 could be via the regulation of AKT1. AKT1, on the contrary, phosphorylates Twist-1 at the Serine-42 residue resulting in incremental Twist-1 transcriptional activity to mediate E-cadherin suppression [187]. Besides, we have discussed in the above section, Par-4, via the recruitment of PKCζ inhibits AKT activation to exert anti-tumorigenic effects [171]. These studies subtly point out the possibility of Twist-1 inhibition by Par-4, which requires more scientific validation.

Alternatively, Twist-1 is also an evolutionarily conserved target of NF-κB [188]. Since Par-4 is a well-known repressor of NF-κB, it may also possibly abrogate the NF-κB-Twist-1 upregulation. TNF-α is a pro-inflammatory cytokine that is deeply corroborated into EMT activation, cancer stemness as well as angiogenesis [189]. Both IKK-b and NF-κB p65 are required for TNF-α-induced expression of Twist-1, suggesting the involvement of canonical NF-κB signaling. Moreover, activation of NF-κB, as well as Twist-1, blocks programmed cell death (PCD). The protective activity of NF-κB is also crucial for oncogenesis as well as aids cancer chemoresistance. Together, these findings indirectly suggest that Par-4-mediated NF-κB inhibition may contribute to the Twist-1 suppression observed upon the ectopic expression of Par-4 in cancer cells. Although these could be the proposed mechanism of Twist-1 inhibition via the Par-4, more studies are warranted to validate the Twist-1-suppressing effects of Par-4 as well as its consequences on tumor progression and metastasis.

2.4 Role of Par-4 in Regulation of Cytoskeletal and ECM Remodeling

Basal levels of Par-4 secreted by cancer cells are generally inadequate to cause substantial apoptosis; secretagogues that augment the release of Par-4 represent an alternate approach to repurpose our objectives in Par-4-dependent therapeutic development. Notwithstanding, the implications of apoptosis-instigating mechanisms in relation to radiation or chemotherapy may provide clues to better explain the selection of proper targets in cancer. In a classical approach, Burikhanov et al. have utilized a unique chemical-genetic entity to underscore the ability of secretagogue-Arylquin to enhance Par-4 function. This secretagogue-Arylquin -mediated functional enhancement of Par-4 was executed by facilitating Par-4 secretion via the classical secretory pathway as

well as by aborting the interaction of Par-4 with Vimentin [190]. While the sequestration of Par-4 by Vimentin in cancer cells not only attributes an important role in the induction of EMT and maintenance of mesenchymal state, but it may also corroborate to drug resistance mechanisms and EMT, particularly in the advanced stage of cancer. Notably, in order to achieve a robust anti-tumor efficacy, such disruption of the Par-4–Vimentin interaction leads to the release of Par-4 to execute its pro-apoptotic function. Therefore, Par-4 rescue may not only sensitize the cancer cell to apoptosis but may abrogate the induction of EMT. This axis of Vimentin-mediated Par-4 regulation in cancer cells portraits a distinct post-translational therapeutic window to target Par-4, Vimentin, or both. Since our goal is to discover the novel function of Par-4, interestingly, our group has unfolded a potential MMP-2 inhibition by extracellular Par-4 [177]. Although secreted by a classical BFA-sensitive pathway, conditional media (CM) containing Par-4, in this research, found to abrogate ex vivo tumor growth in matrigel plug assay. Of note, MMP-2 is a highly proficient metalloproteinase that degrades the extracellular matrix and facilitates the invasion and migration capabilities of cancer cells. In this study, Rah et al. demonstrate that the MMP-2 expression and activity were simultaneously abolished by the secretory Par-4. These results were confirmed by the Par-4 knockdown studies where MMP-2 expression was restored along with a steady increase in invasion potential of cancer cells upon silencing of Par-4. Thus, the strategic use of small molecule inducers of Par-4 for the regulation of intracellular Par-4 could be an effective tool to control the cancer cell metastasis. These reports together put forward a novel paradigm of controlling deregulated malignant signaling by regulating Par-4 (Fig. 6), hence, revealing a new dimension of Par-4 extrapolation for advancement in metastatic cancer therapeutics.

2.5 Par-4 and Destabilization of β-Catenin Pathway

β-catenin signaling pathway is considered one of the critical axes concerning cancer metastasis and drug resistance issues. Deregulation of this pathway by activating mutations in the upstream components converges upon the nuclear accumulation of β-catenin, thereby driving the expression of genes implicated in cancer cell survival, proliferation, and EMT-TFs [191]. While intact cadherin–catenin complex is a critical prerequisite for the maintenance of the cellular homeostasis, however, the lack of cadherins regulating cell adhesion (primarily the E-cadherin) and/or altered subcellular distribution of β-catenin disrupts the cadherin–catenin complex, leading to increased invasiveness, migration, and poor clinical outcome.

Notably, constitutive activation of the phosphatidylinositol 3-kinase (PI3K) signaling triggers the dephosphorylation of β-catenin and finally its accumulation and translocation into the nucleus that culminates in the inactivation of glycogen synthase kinase 3-beta (GSK-3β) [192]. Contextually, in a breakthrough research, Amin et al. have elucidated that small-molecule inducer of Par-4 abrogates EMT and invasion by modulating β-catenin localization and its transcriptional activity in aggressive prostate and breast cancer cells [181]. This study revealed that 3-AWA (a withaferin-based potent Par-4 inducer) sequestered nuclear β-catenin and augmented its cytoplasmic pool as evidenced by diminished β-catenin transcriptional activity. Moreover, exogenous Par-4 attenuated AKT activity and rescue phospho-GSK-3β to promote β-catenin destabilization. Furthermore, Par-4-induced E-cadherin expression along with sharp downregulation of c-Myc and cyclin D1 proteins. The results from the Par-4 knockdown studies, as is performed using siRNA, validates that the 3-AWA-mediated inhibition of nuclear β-catenin is Par-4 dependent. Therefore, Par-4 and β-catenin proteins are mutually regulated and inversely correlated in normal as well as cancer contexts, and strategic modulation of intracellular Par-4 could be an effective tool to control and EMT and cancer cell metastasis.

3 New Insights Linking Par-4 and EMT

3.1 Lethal EMT: TGF-β Signaling and Par-4

Deregulation of transforming growth factor-beta (TGF-β) signaling is well-accepted to be one of the major deregulations observed in the pathophysiology of diverse cancer types. Through different stages of cancer initiation and progression, TGF-β plays a multifaceted and paradoxical role. TGF-β signaling can be pro-tumorigenic or tumor-suppressive. The particular cases where the duality of TGF-β role is observed are well-studied in pancreatic ductal adenocarcinomas (PDACs). TGF-β mediator-Smad4 is frequently found inactivated in PDACs, along with other gastrointestinal cancers. Typically, TGF-β-induced EMT program is considered to be a pro-tumorigenic phenomenon. But in TGF-β-sensitive PDAC cells, on the conversion of TGF-β-induced Sox4, from an enforcer of tumorigenesis into a promoter of apoptosis, the tumor-promoting EMT switches to lethal EMT [193].

Along with the already available therapeutic approaches to mitigate pro-survival/anti-apoptotic factors, the novel lethal EMT approach is a robust example of EMT-linked cellular transcription factor landscape remodeling, including the repression of Klf5, the gastrointestinal lineage master regulator. For the successful progression of cancer, vivid cooperation between Klf5 and Sox4 is crucial, and this asso-

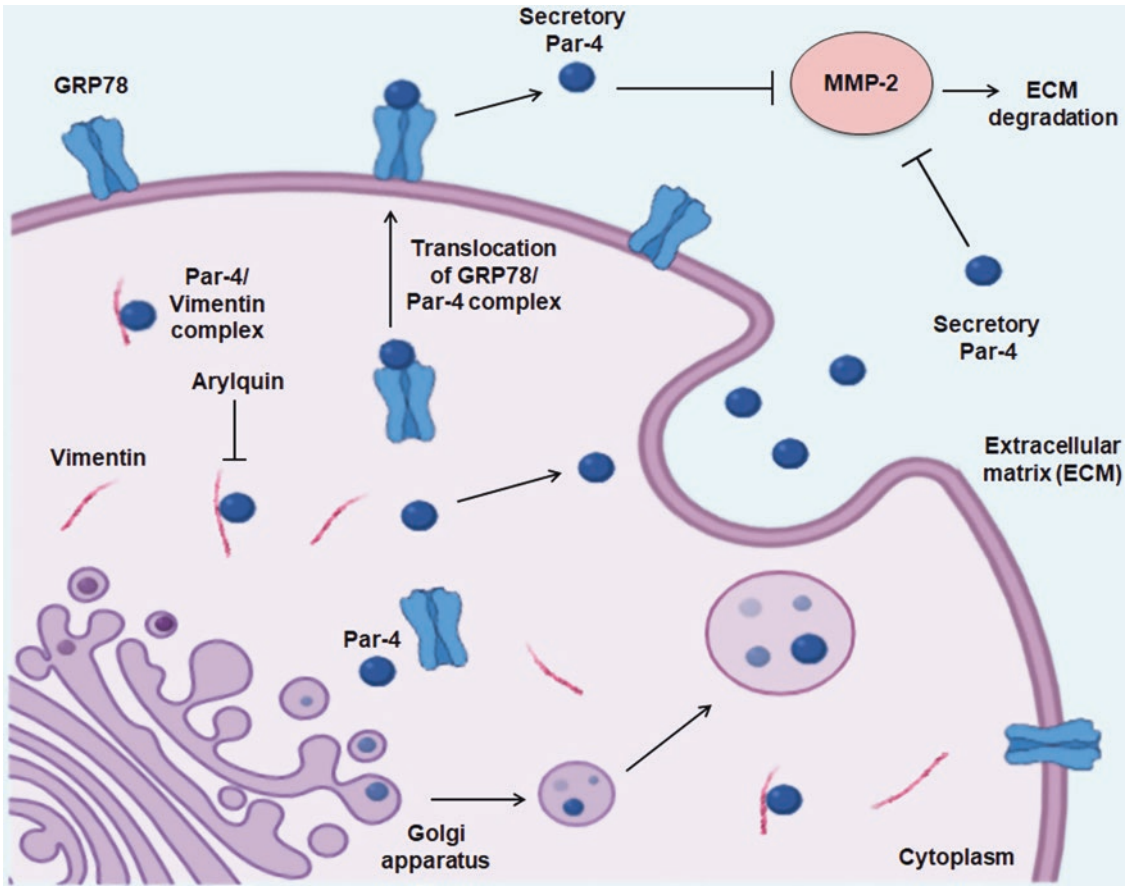

Fig. 6 Secretory Par-4 and ECM degradation**.** During ER stress conditions, Par-4 and GRP78 bind to each other, and the paired proteins relocate to the plasma membrane. Par-4 is then released as secretory Par-4, leaving GRP78 at the plasma membrane. Secretagogues like Arylquins disrupt the Par-4/Vimentin complex; as a result Par-4 is free to be secreted out. The secretory Par-4 then abrogates the ECM degradation, mainly through inhibition of MMP-2 activity

ciation impedes Sox4-induced apoptosis. Smad4 (also called DPC4) is a component of paramount importance in this axis. It is an established tumor repressor that is frequently lost/mutated in pancreatic cancer. However, it is noteworthy to mention that tumor growth of colon carcinoma cells is obstructed by the presence of Smad4 protein, which constitutively reactivates E-cadherin and therefore abrogates neo-angiogenesis. While Smad4 is indispensable for EMT, it is not an absolute prerequisite for Sox4 induction by TGF-β. On the one hand, TGF-β-induced Sox4 is spontaneously available to support progenitor identity. Simultaneously, an essential partner of Sox4 in oncogenesis is stripped away by Smad-dependent EMT. For achieving a viable therapeutic intervention, the Smad4-dependent EMT is grossly activated by induction of TGF-β in the PDAC cells. Intriguingly, the activation of Smad-dependent EMT successively acts as a whistleblower for apoptosis. However, to achieve the desired result, the pro-tumorigenic function of Sox4 needs to be switched to pro-apoptotic mode. This transition is obtained by Snail-mediated suppression of Klf5, a crucial master regulator of endodermal progenitors. These results, collectively, illustrate a paradigm shift in which TGF-β tumor-suppressive action revolves around an EMT-associated disruption of a pro-tumorigenic transcriptional network.

As mentioned above, a dual role is perceived by TGF-β in the successful accomplishment of tumor growth and invasion. The apoptosis promoting potential of TGF-β along with the termination of epithelial cell cycle progression leads to tumor suppression in the early stages of cancer. Contrariwise, in the later stages, it promotes tumor growth owing to interference with a chain of factors such as modulating genomic instability, cell motility, immune evasion, neo-angiogenesis, and metastasis. In recent studies, Par-4 has been emerging as a vital constituent to influence TGF-β-induced EMT. Most strikingly, the anti-metastatic function of Par-4 has been implicated as a crucial downstream target of the TGF-β signaling pathway [173]. Echoing this, Faheem et al. also demonstrate that Par-4 plays an essential role in regulating the TGF-β/Smad4 pathway in pancreatic ductal adenocarcinoma (PDAC) models [194]. In a breakthrough finding, authors

proclaim that overexpression/induction of Par-4 convincingly results in apoptosis in conjunction with TGF-β by positively regulating Smad4. Interestingly, Par-4$^{+/+}$ cells show far more significant Smad4 induction in comparison to Par-4$^{-/-}$ cells in the presence of TGF-β. Faheem et al. have diligently found that Smad4 expression is robustly spiked by ectopic Par-4 through the restoration of the TGF-β/Smad4 axis. Furthermore, Par-4 drags the PDAC cells to G1 arrest in the presence of TGF-β by boosting the p21 and p27 levels and attenuating Cyclin A and E to trigger lethal EMT via caspase 3 cleavage augmentation. Interestingly, in this report, the authors hypothesize that Par-4 dependent and TGF-β-mediated lethal EMT is embarked in these cells following restoration of Smad4 in the Smad4 null BxPC3 cell line. However, mechanistically this research work underscores that disruption of Nm23H1-Strap interaction is the cornerstone of Par-4-mediated Smad4 activation. Nm23H1 is a nucleoside diphosphate (NDP) kinase and a putative metastatic suppressor. Nm23H1–Strap interaction is not only essential for simultaneous p53-mediated apoptotic functions as well as regulating TGF-β-mediated biological activity. In addition to this, this interaction controls intrinsic Nm23H1 activity [195, 196]. Nm23H1/Strap interaction obstructs the downstream signaling of TGF-β as an intact Nm23H1/Strap complex acts in tandem with the inhibitory Smads (Smad7 particularly), which results in a lowered capacity of receptor Smads (Smad2 and 3) to couple with Smad4 [197]. Given that Par-4 positively modulates the TGF-β/Smad4 pathway in PDAC cells and favors the tumor-suppressive role of TGF-β. Hence, Par-4 is a crucial element that helps to restore the apoptotic functions of the TGF-β pathway.

3.2 BMP and ALK Signaling

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and constitute a diverse, evolutionarily conserved family of secreted signaling molecules critical for various developmental processes [198]. BMP7 is known to counteract TGF-β-induced EMT in developmental stages [199]. ALK2, on the other hand, also termed ACTRI, is an activin type I receptor that mediates responses for BMP7 [200]. ALk2 phosphorylates Smad1/5/8 and, as a result, triggers its association with Smad4 incurring MET phenotypes. Apart from its EMT alleviating role, Par-4 has been reported to induce MET in highly aggressive cancers [201]. Recently, Katoch et al. have conceived a dual mechanism of Par-4-mediated inhibition of EMT and concomitant alleviation of MET in metastatic pancreatic cancer cells [186]. Authors demonstrate that induction of Par-4, either ectopically or by NGD16 (a small molecule derivative of diindolylmethane), strongly impede invasion, migration, and metastatic index of these cells. In the same experimental setup, authors have found a robust amplification of epithelial marker E-cadherin concomitant with downregulation of canonical mesenchymal marker Vimentin. However, siRNA-mediated silencing of either endogenous Par-4 or Smad4 resulted in the reversal of MET phenotypes with diminished E-cadherin levels underscoring the appearance of MET phenotypes were due to the augmentation of ALK2/ Smad4 signaling in a Par-4-dependent manner. These findings are in concordance with the emerging role of BMP7 in MET induction, possibly by ALK2, phosphorylation of Smad 1, 5, and 8; and inhibition of EMT-TFs, viz. Slug, Twist-1, and Snail. Therefore, ALK2 induction can be perceived as a plausible mechanism of Par-4-mediated abrogation of EMT and induction of MET in PDAC cells.

3.3 Anti-Metastatic miRNAs and Par-4

microRNAs (miRNAs) are non-coding single-stranded RNAs that negatively control post-transcriptional gene expression to degrade multiple target mRNAs and execute translational suppression [202]. miRNA dysregulation has been implicated in the etiology, pathogenesis, diagnosis, and treatment of cancer [203]. In the myocardium, miR-17-3p-mediated Par-4 abrogation was demonstrated to attenuate cardiac aging [204]. This event leads to the upregulation of its downstream proteins, including CEBPB, FAK, N-cadherin, Vimentin, Oct4, and Sca-1 (stem cell antigen-1), and downregulates E-cadherin. Thus, repression of Par-4 by miR-17-3p augments the transcription of CEBPB and FAK. This, in turn, results in EMT acquisition and self-renewal, culminating in cellular senescence and apoptosis resistance. A growing number of studies have demonstrated altered levels of the miRNA-200 family members in the cells undergoing EMT [205, 206]. miR-200c is a positive regulator of E-cadherin and represses the expression of E-cadherin repressor, Zeb-1, to maintain the epithelial phenotype in the cells, thus attenuating EMT [207]. Consequently, extensive investigation has unveiled the role of miR-200c in cell proliferation, apoptosis, EMT, invasion, therapy-induced resistance, and metastasis in diverse cancer types [208]. However, miR-200c and Zeb-1 possess an inverse relationship in the context of the EMT phenomenon as well as their regulation *vis a vis;* miR-200c directly targets and impedes Zeb-1 and Zeb-2 expression. Albeit, the aberrant miR-200c loss with a simultaneous increase in Zeb-1 expression has been correlated to orchestrate EMT by downregulating E-cadherin [209]. From that standpoint, our group recently demonstrated that the consequences of Par-4 upregulation in the amelioration of Zeb-1-mediated EMT by enhancing the miR-200c levels [185]. Of note, the global proteome changes in Panc-1

cells upon ectopic restoration of miR-200c / Par-4 identify overlapping protein targets in the miR-200c and Par-4 axis. Intriguingly, reverse phase protein (RPPA) analysis for the whole proteome of miR-200c and GFP-Par-4-transfected Panc-1 cells identify 82 proteins which consistently overlap in both the sample sets. Cumulatively, these proteins include phospho-p44/42 MAPK; Bcl-xl; Bim; phospho-Rb (Ser807, Ser811); phospho-Akt (Ser473); phosphor-Smad1/5 (Ser463/Ser465); and Zyxin. The expressional changes in these distinct proteins might be exerted independently by different arms of the miR-200c and Par-4 signaling pathways. This work by Katoch et al. has unveiled a novel role of Par-4 as a positive regulator of miR-200c expression that results in halt in EMT progression.

4 Conclusion, Limitations, and Future Perspectives

Over the years, Par-4 research has been largely focused on unveiling its pro-apoptotic role. Mounting evidences, however, suggest towards the beneficial role of Par-4 in the abrogation of EMT and subsequent metastasis in various cancers. Therefore, exploration of Par-4 in EMT progression warrants detailed investigation. Small-molecule inducers of Par-4 or recombinant Par-4 are ideal for examining the effects of Par-4 on EMT associated markers (epithelial/mesenchymal) both in in vitro and in in vivo contexts. Since Par-4 modulates major metastasis-related proteins like Vimentin and MMPs, targeting bystander effects of Par-4 would be an attractive strategy to control EMT in aggressive cancers. Furthermore, induction of programmed cell death by Par-4 is independent of its novel β-catenin signaling modulatory role; however, future studies need to divulge into the mechanisms by which Par-4 deters Wnt/ β-catenin signaling. Whether the pro-apoptotic role of Par-4 is mutually exclusive to its anti-metastatic role or these roles are concomitantly intertwined with each other needs deciphering. In this context, the integration of the SAC domain with respect to the anti-metastatic potential of Par-4 is of significant relevance and yet to be comprehended. Pertinently, a dearth of evidence so far links the effects of Par-4 on EMT-associated markers (epithelial/mesenchymal) and *vis-à-vis* subsidiary signaling nodes like NF-κB, β-catenin, etc. There is a shortfall of evidences underscoring the regulation of EMT-TFs by Par-4. The need of the hour is to decipher whether or not Par-4 directly interacts with any of the EMT-TFs. Albeit, with the identification of novel signaling intersections between Par-4 and EMT programs, the opportunity to examine this axis holds a promising field in future study. Further, identification, development, and exploration of novel Par-4 inducing small molecules that impede EMT cascades represent significant progress in the right direction. All this relevant information should facilitate the development of Par-4 targeted novel anti-metastatic therapeutic regimens in the future.

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Prostate Apoptosis Response-4 in Inflammation

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Abstract

Chronic inflammation is the underlying cause of about a quarter of the world's cancer. Regular use of inhibitors of inflammation such as aspirin has a significant chemopreventive effect, protecting against the development of colorectal cancer and some other cancers.

The tumor suppressor Par-4 has been associated with the inhibition of tumorigenesis of different cancers. Recently, it has been shown that inflammatory signals downregulate Par-4 in breast cancer cell lines, suggesting a possible role for Par-4 in inhibiting cancer initiation in response to inflammatory signals. More importantly, inhibition of inflammation by a number of different NSAIDs results in upregulation of Par-4 and induction of apoptosis in different cancer cell lines. It is interesting to note that the mechanisms of apoptosis induction by Par-4 share remarkable similarities with those of different NSAIDs. Both Par-4 and NSAIDs affect Ras signaling, NF-κB activation, and Bcl-2 levels. These similarities suggest that Par-4 may be mediating apoptosis induction by NSAIDs.

Characterization of the exact involvement of Par-4 downstream of NSAIDs is essential for understanding their mechanism of function and tailoring better drugs for chemoprevention and the development of better cancer treatment protocols.

In this chapter, we will discuss the role of inflammation in the initiation and progression of tumorigenesis, the mechanism of inflammation inhibition and apoptosis induction by NSAIDs, and finally the possible role of Par-4 as a mediator of NSAIDs effects on carcinogenesis.

Keywords

Par-4 · Chronic inflammation · Cancer · NSAID · Macrophage · TAM · TME · COX-2 · Ras · NF-κB · Bcl-2

1 Introduction

Immunity is a complex system adapted to protect the body from a different array of invaders while able to differentiate between self and non-self. It can also identify host cells that are altered to form cancer. The immune system generates a wide variety of cells and molecules that perform different functions to achieve these goals.

The immune response is traditionally divided into two main arms: the innate immunity and the cellular or adaptive one. The innate immunity causes a wide range of reactivity while the cellular one requires antigen specificity and confers "memory immunity" (Fig. 1). Innate immunity essentially works through pattern recognition receptors (PRR) on the immune cells that recognize molecules (pathogen-associated molecular patterns or PAMP) shared by many pathogens but are not present in the host. This is followed by recruiting phagocytes and other leukocytes that destroy the invading microbes, in a process called inflammation.

Inflammation is one of the most important features of the innate immune system, and it is involved in the induction of adaptive immunity. In response to invaders or injury, the innate immune cells, which include macrophages, dendritic cells (DC), neutrophils, mast cells, and natural killer (NK) cells, initiate the inflammatory response. Activation of the immune cells results in the activation of several pathways and transcription factors such as NF-κB (nuclear factor-κB), activation protein 1, interferon response factors three and seven. These transcription factors induce the expression of genes encoding inflammatory cytokines, chemokines, and adhesion molecules. Cytokines, through cell receptors, induce specific activity in different cells. The activated immune cells will also release matrix-remodeling proteases,

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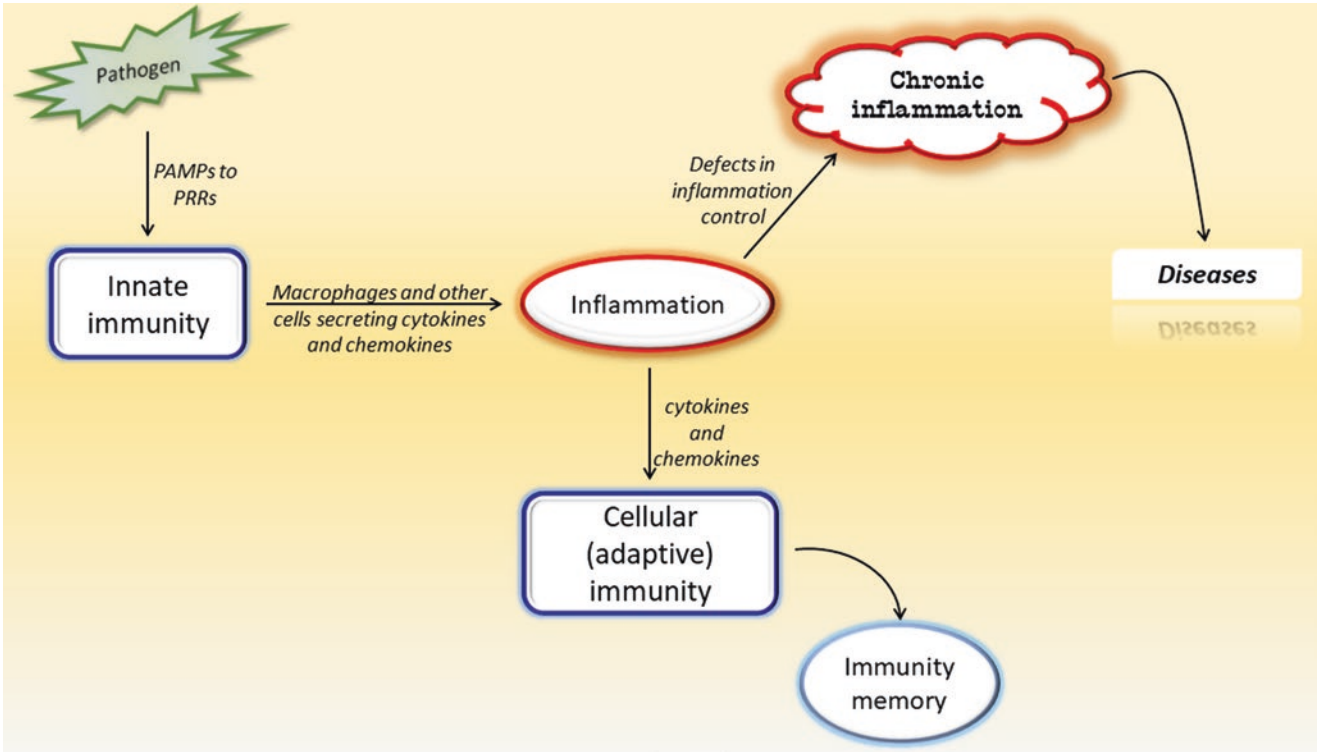

Fig. 1 Overview of adaptive and innate immunity: Pathogen invasion activates elements of the innate immunity through PAMP interaction with PRR on immune cells. This will induce the release of cytokines and chemokines recruiting more macrophages and other immune cells thus initiating inflammation. Antigen presentation by APCs (e.g., dendritic cells) together with the presence of cytokines, activate the cellular (adaptive) immune response (T cells and B cells). Later, some persisting B and T cells will form immunity memory. After the infection resolution, failure to terminate the inflammation, as a result of the persistence of the pathogen or irritant, or because of a misfunction in the termination machinery, leads to chronic inflammation.
PAMP Pathogen-associated molecular patterns, *PRR* Pattern recognition receptor, *APC* antigen-presenting cell

and reactive oxygen and nitrogen species, leading to the recruitment of leukocytes and plasma proteins to the affected site resulting in the elimination of pathogens and repair of tissue damage.

Three of the most important pro-inflammatory cytokines of the innate immune system are TNF-α (Tumor necrosis factor α), IL-1, and IL-6 (Interleukin 1-6). The production of these cytokines results in the characteristic tissue changes that accompany inflammation namely redness, heat, pain, and swelling: rubor, calor, dolor, and tumor, respectively.

Even though the immune system is of utmost importance as it protects the body from pathogens and uncontrolled replication of cells causing cancers, sometimes it fails by under or over-reacting causing a wide array of diseases. Immunodeficiencies occur when one or more components of the immune system are defective, and it can be inherited or acquired. Allergy, asthma, and autoimmune diseases result from over-activation towards innocuous antigens. Recently, a form of over-reaction of the immune system has been wildly mentioned in the context of the COVID-19 pandemic; the "cytokine storm" which results from a sudden acute increase in circulating cytokines including IL-6, IL-1, TNF-α, and interferon leading in some cases to mortality [1–4].

2 Diseases Caused by Inflammation

Some inflammation is good. Too much inflammation is bad. When inflammation is mild and controlled, normal tissue architecture and function can be restored after the inflammation is resolved. Uncontrolled persistence of inflammation can be very harmful to the host resulting in lasting tissue damage, especially if the microbes resist being killed and continue to stimulate the innate immune responses. The proteolytic enzymes and reactive oxygen species produced by immune cells to kill microbes during acute inflammation can injure host cells and degrade the extracellular matrix. For this reason, there is an elaborate mechanism to control the immune response, and any failure in the precise control of immune components can lead to chronic inflammation [4, 5].

Chronic, persistent inflammation is behind a host of health problems that result in the death of 3 out of 5 people in the world. Diseases associated with chronic inflammation

include cardiovascular disease, diabetes, chronic obstructive pulmonary disease, arthritis and joint diseases, neurological diseases, psoriasis, allergies, bowel diseases and cancers [2, 4, 6] (Fig. 2).

Cardiovascular diseases (stroke, heart disorders): Inflammation is an important factor in the progression of atherosclerosis, which is the main underlying cause of cardiovascular diseases. Atherosclerosis starts with the adherence then migration of monocyte through the arterial walls. The monocytes then differentiate into macrophages that will secret inflammatory signals to recruit more immune cells into the forming plaque, leaving the artery narrowed and more susceptible to blockage. This block chokes off blood flow to regions of the heart causing a heart attack. Studies show that lowering levels of CRP (C-reactive proteins), which is an important marker of inflammation, is correlated with reduced rates of heart attacks [2, 7].

Diabetes: Involvement of inflammation in diabetes was first suspected over a hundred years ago when high doses of salicylates were shown to lower glucose levels in diabetic patients. Type 2 diabetes is caused by deficiency in insulin production by the pancreatic beta cells and by insulin resistance in other cells. Inflammatory factors are implicated in both beta cell failure and insulin resistance. All major risk factors for type 2 diabetes (such as over nutrition, sleep deprivation, and depression) have been found to induce low-grade inflammation in susceptible individuals. Immune cells like macrophages infiltrate pancreatic tissues, releasing pro-inflammatory molecules participating the killing of beta cells. In addition, pro-inflammatory cytokine such as TNF-α and IL-6 produced by adipocyte and associated adipose tissue macrophages can directly interfere with insulin signaling thus inducing insulin resistance [8–10].

Chronic Obstructive Pulmonary Disease (COPD) is an obstructive lung disease that develops as a chronic inflammatory response to inspired irritants (mainly through smoking) that results in progressive and irreversible airflow blockage [11].

Arthritis and Joint Diseases such as rheumatoid arthritis, which is a systemic autoimmune disease that develops in genetically susceptible hosts. It is induced by several environmental factors such as smoking, infections, and other unknown factors that lead to infiltration of immune cells and release of cytokines causing local inflammatory response in joints and potentially other parts of the body [6, 12].

Neurological diseases such as Parkinson's and Alzheimer's diseases: In older adults, chronic low-level inflammation is linked to cognitive decline and dementia. Increasing evidence suggests that Parkinson's and Alzheimer's diseases strongly interact with immunological mechanisms in the brain. In Alzheimer's disease, misfolded and aggregated proteins bind to PRRs on micro- and astroglia and induce the release of inflammatory signals and recruitment of leukocytes, which contribute to disease progression and severity [13, 14].

Psoriasis is a systemic, immune-mediated disorder with a strong skin manifestation. Dermal infiltration by T cells and macrophages appear in early skin lesions before epidermal changes [15, 16].

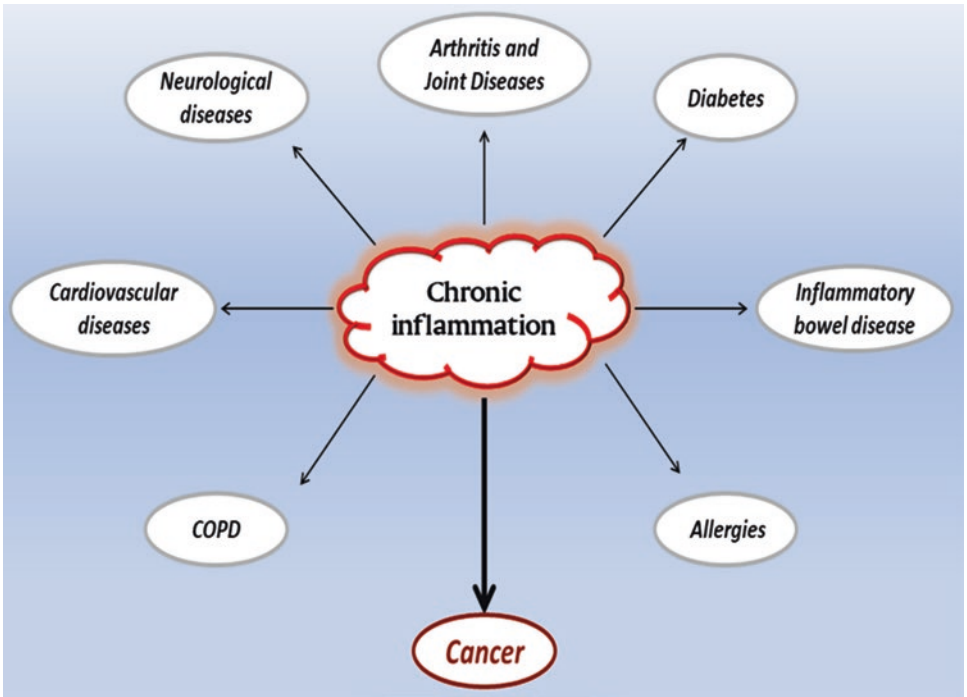

Fig. 2 Diseases caused by chronic inflammation: Chronic inflammation has been implicated in the development of a myriad of diseases responsible for three out of five deaths worldwide

Allergies are caused by inappropriate immune response and inflammation (hypersensitivity) to certain environmental substances (allergens) that do not usually affect most people. Allergy types include hay fever, food allergies, atopic dermatitis, allergic asthma, and anaphylaxis [17].

Bowel diseases such as Crohn's disease, ulcerative colitis, inflammatory bowel disease, and celiac disease: Inflammatory bowel disease is a group of chronic inflammatory disorders of the digestive tract. Ulcerative colitis causes ulcers and chronic inflammation in the lining of the large intestine and rectum, whereas Crohn's disease is characterized by a more spread inflammation of the lining of the digestive tract in the mouth, esophagus, stomach, and anus [6, 18].

3 Inflammation and Cancer

Although the first mention of a link between inflammation and cancer was back in 1863 by Rudolf Virchow, evidence of the involvement of inflammation in cancer did not become available for more than a century [19]. According to Colotta et al., cancer-related inflammation is now considered the seventh hallmark of cancer after self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, unlimited replication potential, sustained angiogenesis, tumor invasion, and metastasis [20, 21].

It is estimated that inflammation, caused by infection or chronic inflammation, is the underlying cause of about 25% of cancers [3]. For example, chronic inflammatory diseases, such as Crohn's disease are associated with colon cancer [22] and Barrett's esophagus with esophageal cancer [23]. Many infectious agents, characterized by the development of chronic inflammation are also associated with cancer. Some such examples are schistosomiasis causing bladder and liver cancers [24], *Helicobacter pylori* stomach infection and gastric cancer [25], human papillomavirus relation to cervical cancer [26] and others [27].

Inflammation contributes to both the initiation and the survival and proliferation of malignant cells which we will discuss in the following subsections.

3.1 Inflammation as an Initiation Factor

So how does inflammation lead to cancer? Here's the current thinking;

In response to pathogen invasion, internal or external injury, inflammatory cells are recruited to the injury site. Failure in the precise control of immune response, in a timely manner, leads to chronic inflammation which is predominated by macrophages. These cells, together with other leukocytes as well as epithelial cells in the site of inflammation, produce inflammatory mediators such as prostaglandins, pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-15, and chemokines IL-8 and growth-regulated oncogene 1 (GRO-1/CXCL1), in addition to inducible nitric oxide synthase (iNOS or NOS2) and cyclooxygenase-2 (COX-2). Transcription factors such as NF-κB, signal transducers, and activators of transcription 3 (STAT3), nuclear factor erythroid 2-related factor 2 (Nrf2), and nuclear factor of activated T cells (NFAT) are also activated and create a positive feedback loop activating more inflammatory mediators. All of these are implicated in carcinogenesis as they are enhancers of proliferation and inducers of tumorigenesis [3, 28].

iNOS expression, which is regulated by transcriptional factors such as NF-κB and STAT3, is responsible for the production of high levels of reactive oxygen (ROS) and nitrogen species (RNS), which are usually involved in fighting infection. The continuous presence of ROS/RNS causes damage to various cellular components such as nucleic acids, proteins, and lipids [28]. In DNA, oxidative and nitrative damage caused by ROS/RNS, have been shown to induce G:C to T:A transversions. These mutations have been observed in several oncogenes and tumor suppressor genes such as *k-ras* and *p53* in cancers like lung and liver [3]. ROS/RNS, pro-inflammatory cytokines such as IL-6 and its downstream STAT3, have been shown to cause epigenetic changes in DNA in different cancer settings. They affect the DNA methyltransferase 1 (DNMT1) resulting in enhanced DNA methylation of tumor suppressor genes and microRNAs [29]. ROS/RNS also induce global DNA hypomethylation, resulting in genomic instability. Changes in DNA methylation patterns, such as global hypomethylation of the genome and the hypermethylation of specific gene promoters have been associated with different human malignancy [30]. In addition to the damage caused to the DNA, inflammation-derived ROS/RNS can induce protein carbonylation which is an irreversible and irreparable protein modification induced by oxidative stress [27] (Fig. 3).

From the above, we can see that chronic inflammation can lead to point mutations and changes in gene expression in both oncogenes and tumor suppressor genes, and to post-translational modifications of proteins, which could lead to genetic and physiological instability and cancer [3].

3.2 Inflammation as a Factor in Cancer Progression and Metastasis

Not only can chronic inflammation lead to the initiation of cancer, but it is now well known that inflammation contributes to tumor progression and invasion. As a tumor grows and accumulates mutations, the cancer cells release chemical

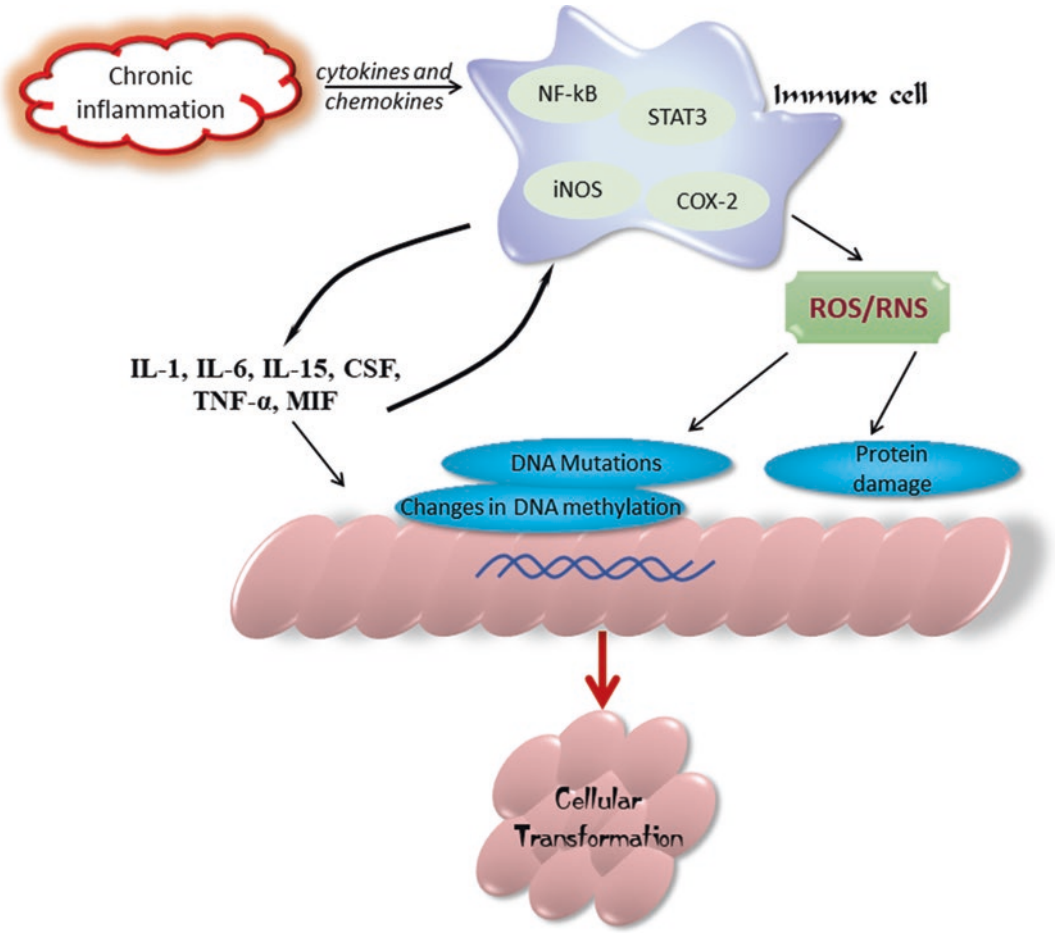

Fig. 3 Chronic inflammation and initiation of tumorigenesis: Secretion of cytokines and chemokines by immune cells activates a number of signaling pathways which persistence could lead to cellular transformation. During inflammation, activation iNOS, NF-κB, and COX-2 will induce the production of ROS and RNS and more cytokines, chemokines, and other elements that participate in the induction of transformation. ROS/RNS can cause mutations and epigenetic changes in DNA, and permanent damages in proteins. Activation of transcription factors such as NF-κB and STAT3 gives a survival advantage to cells with damaged DNA, increasing the chance of initiation of tumorigenesis.
ROS Reactive oxygen species, *RNS* Reactive nitrogen species

signals that recruit immune cells to infiltrate the tumor, forming the "Tumor microenvironment" (TME).

TME consists of tumor cells, tumor stromal cells including stromal fibroblasts, endothelial cells, and infiltrating immune cells (mainly macrophages), and the non-cellular components of the extracellular matrix such as collagen, fibronectin, hyaluronan, and laminin. TME is now considered a main component of almost all types of cancer. Tumor cells control the function of cellular and non-cellular components of the TME through complex signaling networks to support tumor growth and invasion by inducing angiogenesis, inflammation, and of course, immunosuppression.

In TME, the infiltrating immune cells such as macrophages, microglia, and lymphocytes release cytokines and chemokines that, in addition to their function to attract more cells to the TME, affect the tumor cell signaling and induce the expression of a large number of proteins. These proteins have been found to increase angiogenesis, replication, EMT (epithelial mesenchymal transition), thus metastasis, and other protein involved in transforming cancer cells into more aggressive invasive cells [31–33].

Macrophages are one of the main populations of inflammatory cells in the stroma of many tumors. In breast cancer TME, macrophages constitute about 50% of the cancer mass [34]. They are generally recruited from blood monocytes by CC chemokines such as CCL2 (MCP-1) [35, 36]. Normally, macrophages play a critical role in the emergence and resolution of inflammation. In addition to their ability to kill and clear pathogens, they also play a role in the coordination of other cells and tissues of the immune and other supporting systems mainly by secreting cytokines and complement proteins. Once recruited to the tumor site, they differentiate into tumor-associated macrophages (TAMs). The resulting TAMs secrete multiple cytokines and growth factors including

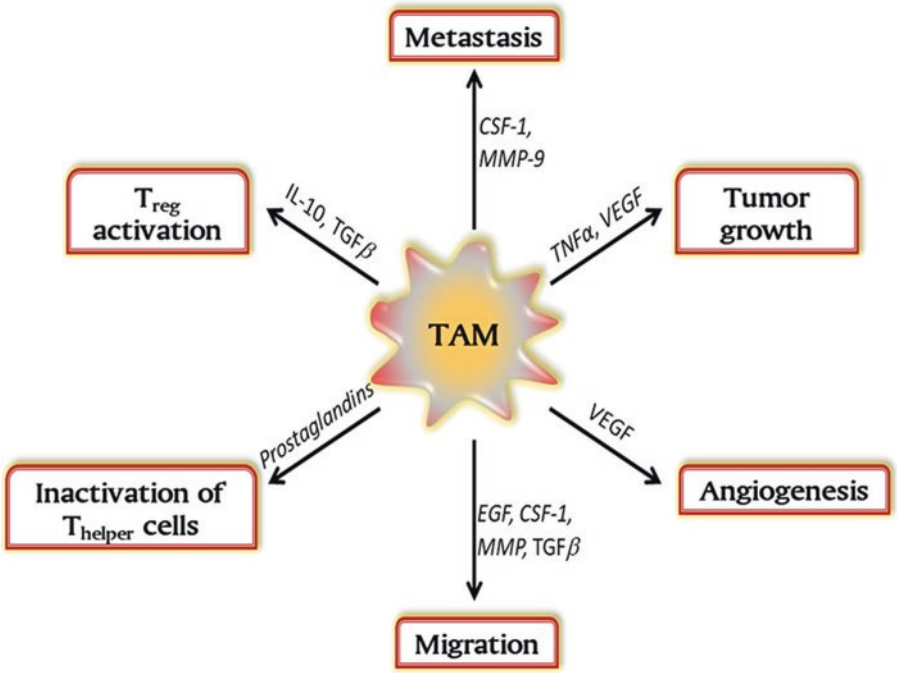

Fig. 4 Chronic inflammation and progression of tumorigenesis; Role of TAM in TME: Tumor-associated macrophage (TAM) is a main component of the tumor microenvironment (TME). TAM presence contributes to the growth, progression, and metastasis of tumors through multiple mechanisms: Inhibition of anti-tumor immunity through inhibition of T cytotoxic cells recruitment while enhancing recruitment of T_{reg} cells; Supporting tumor growth through TNF-α and VEGF secretion; Enhancement of angiogenesis through VEGF secretion; and finally, enhancement of migration and metastasis through CSF-1 and MMPs production.
T_{reg} regulatory T cells, *VEGF* vascular endothelial growth factor, *CFS-1* colony-stimulating factor 1, *MMPs* Matrix metalloproteinases

TNF-α, IL-1 and IL-6, CSF-1, and activate COX-2, NF-κB, and enzymes involved in tissue remodeling, which are all known factors that assist cell malignant behavior [37].

The association between the high macrophage content of tumors and poor patient prognosis supports the notion of the protumoral role of TAM. Moreover, the reduction of the risk of several cancers, which is provided by long-term use of anti-inflammatory drugs, has further supported this notion.

TAM plays multiple roles in cancer development and progression:

- One of the important functions of TAM in assisting tumor growth is their ability to dampen host immune responses against the tumor by preventing the accumulation of anti-tumor cells (T cytotoxic) and by help recruiting cells that inhibit immune response such as regulatory T cells (T_{reg}) cells. Thus, TAMs act as a double-edged knife in enhancing tumor growth [38].
- They produce factors that facilitate **angiogenesis** (blood vessel development) and **lymphangiogenesis** (lymphatic vessel development) [39].
- TAMs produce a variety of proteases and other factors to break down the basement membrane and mobilize tumor cells. Thus, enhancing tumor **metastasis**.

The overall effect of TAM in TME is protection from the immune system, increased growth, and enhancement of angiogenesis and metastasis [40, 41] (Fig. 4).

In the following section, we will discuss the role of non-steroidal anti-inflammatory drugs in inhibiting the effects of inflammation in cancer initiation and progression.

4 Non-Steroidal Anti-Inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs commonly used for the treatment of inflammation, pain, and fever. Their classical mode of action is attributed to the inhibition of cyclooxygenase-1 and -2 (COX-1 and -2). These enzymes are involved in the synthesis of the key biological mediators; eicosanoids (prostaglandins (PGs), prostacyclin, and thromboxane A_2) from arachidonic acid. PGs are responsible for inflammation, pain, and fever. Studies in experimental animals provided evidence that PGs participate in the transition to and maintenance of chronic inflammation by acting as a cytokine amplifier. PGs and cytokines synergistically activate NF-κB to induce the expression of inflammation-related genes, including COX-2 thus main-

taining a positive feedback loop amplifying chronic inflammation. PGs also contribute to angiogenesis and fibrosis [42]. The main mode of action of most NSAIDs is to inhibit COX-1 and -2, preventing them from the generation of eicosanoids thus reducing pain, fever, and inflammation. Eicosanoids are also critically important for the homeostatic maintenance of the gastrointestinal mucosa, blood clotting, regulation of blood flow, and kidney function which explains some of the side effects generated by their inhibition [43].

COX-1 is expressed constitutively in most tissues, including gastrointestinal (GI) mucosa, whereas COX-2 expression is usually transiently induced by cytokines, inflammatory stimuli, growth factors, mitogens, and is generally associated with pathological processes. Conventional NSAIDs act on both COX-1 and -2, but in addition to their anti-inflammatory, analgesic, and antipyretic action, they may cause serious gastrointestinal, cardiovascular, and renal adverse effects. The inhibition of COX-1-induced PG's production in GI is regarded as the cause of the most frequent and potentially most dangerous side effects of NSAIDs, namely gastric/duodenal ulceration and bleeding [44]. As COX-2 is the main mediator of inflammation, inhibitors with the highest effect on COX-2 and less effect on COX-1 should have potent anti-inflammatory activity with fewer side effects. Unfortunately, COX-2 inhibition is associated with cardiovascular adverse effects, which resulted in the restricted use of this category of NSAIDS [45, 46].

Other non-COX mechanisms have also been suggested as a mode of action for NSAIDs. They have been shown to affect different transcription factors such as NF-κB, which is as we mentioned before, an essential transcription factor for pro-inflammatory cytokines, chemokines, and adhesion molecules [47]. In addition, NSAIDs inhibit activator protein 1, membrane stabilizing, Ras, and ROS production. It is unclear how these other mechanisms contribute to the clinical benefits of NSAIDs [48–50].

For many years, NSAIDs have also been studied for their chemopreventive and anti-tumor effects. Their role as chemopreventive agents was first noticed in colorectal cancer. Population studies have shown a 40–50% decrease in the relative risk of developing colorectal cancer in persons who are continuous users of aspirin [51]. Other NSAIDs have also been found to protect against colon cancer. The NSAID sulindac has a unique ability to partially compensate for the loss of adenomatous polyposis coli (APC), the tumor suppressor affected in patients with familial adenomatous polyposis (FAP), and sporadic polyps in colon cancers [52]. The anti-tumorigenic activity of sulindac against colon cancer may involve both COX-dependent and independent inhibition activities. It was found that sulindac enhances the killing of tumor cells by oxidative stress, with no COX inhibition involved [53].

Although most epidemiologic studies have been conducted in colorectal cancer, the use of NSAIDs had similar anti-neoplastic effects with other tumors such as breast [54], ovary [55], lung [56], esophagus, rectum, and stomach [43, 57]. Sulindac has also been reported as a chemopreventive agent for mouse urinary bladder cancer [58].

Anti-tumor mechanisms of NSAIDs are not fully understood. Some evidence points to the involvement of COX-2 inhibition. COX-2 is upregulated from 2- to 50-fold in 85-90% of colorectal adenocarcinomas, which makes the COX-2 enzyme a possible target. On the other hand, different studies point to COX-independent mechanisms [59, 60]. The potential COX-2-independent anti-neoplastic mechanisms of NSAIDs may include downregulation of proto-oncogenes, such as *c-myc*, *ras,* and *bcl-2,* and transcriptional factors such as PPARδ and NF-κB and upregulation of apoptotic genes such as Par-4 as will be discussed in more details later [50].

Furthermore, different NSAIDs may have different anti-cancer mechanisms of action. Aspirin has a unique property of acetylating COX-2, which is not seen in other NSAIDs. Acetylated COX-2 has anti-inflammatory and anti-tumorigenic effects. This unique ability of aspirin may be the cause of its anticancer potential [50]. Aspirin and sodium salicylate can inhibit NF-κB activity through inhibition of IκB degradation in a different range of cell types and conditions which would contribute to the anticancer properties of these drugs [49]. Sulindac sulfide, but not other NSAIDs, was found to significantly increase NSAID-activated gene (*nag-1*), an anti-tumorigenic and pro-apoptotic gene, in COX-2-deficient gastric cancer cell lines, resulting in increasing apoptosis [61].

5 Par-4 and Cancer

Rat Par-4 was first discovered in a differential screening in androgen-independent prostate cancer cells forced to undergo apoptosis. Although it was later found to be ubiquitously expressed in all tissue (of both human, rat, and mouse), Par-4 was found to be inhibited or inactivated in several cancers.

Par-4 is lost or inactivated in over 70% of renal cancers [62]. In breast cancer, downregulation of Par-4 is associated with resistance to therapy and recurrence and progression of more aggressive breast cancers [63, 64]. Par-4 down-expression was observed in about 40% of endometrial carcinomas which was mainly due to promoter hypermethylation [65]. Similar observations have been made in neuroblastomas [66].

Moreover, the human *par-4* gene (*pawr*) is located on chromosomal 12q21, a region that is unstable in several can-

cers [67]. Pancreatic and gastric cancers and acute lymphoblastic leukemia (ALL), frequently show a deletion or instability in chromosome 12q21. Duplication of this region has also been shown to occur in about 10% of Wilm's tumor cases [68–71].

In prostate cancer, Par-4 phosphorylation by AKT (PKB) prevents its nuclear translocation which is essential for inhibition of NF-κB and induction of apoptosis by Par-4. Retaining Par-4 in the cytoplasm, by AKT, abrogates its ability to kill cancer cells [72].

In ovarian cancer cell lines, Par-4 is required for apoptosis-sensitizing to Taxol treatment. Furthermore, Par-4 co-localizes with GRP78 and enhances its translocation to the membrane in these cells [73].

In hematopoietic stem cells, Par-4 inhibits IL-3-independent proliferation and transformation induced by p185BCR-ABL, the protein produced by BCR-ABL translocation [74].

Par-4 knockout mice are more susceptible to chemical- or hormone-induced lesions and to spontaneous tumor development in various tissues. The endometrium and the prostate gland appear to be relatively sensitive to Par-4 loss as Par4-null mice were more prone to the development of proliferative lesions. This further confirms the role of Par-4 in the development of hormone-dependent tissues [75]. In mouse TCL1 leukemia system, overexpression of human Par-4 has an anti-leukemic effect through downregulation of NF-κB signaling [76].

The tumor suppressor activity of Par-4 is achieved through several pathways that involve suppression of cell survival mechanisms and activation of the pro-apoptotic machinery. Par-4 inhibits oncogenic Ras, Bcl-2, atypical protein kinase C (aPKC), and NF-κB and sequesters Topoisomerase I, thus stopping crucial survival pathways. On the other hand, it increases translocation of Fas and Fas ligand and activates TRAIL apoptosis induction through GPR78-dependent mechanism [77].

In addition to its confirmed intracellular tumor suppressor function, it was found that Par-4 is excreted from cells and extracellular Par-4 is as potent in inducing apoptosis in neighboring cells. The ability of extracellular Par-4 to induce apoptosis is dependent on its binding to the stress response protein, glucose-regulated protein-78 (GRP78), expressed at the surface of cancer cells. The interaction of extracellular Par-4 and cell surface GRP78 leads to apoptosis via ER stress and activation of the FADD/caspase-8/caspase-3 pathway through TRAIL receptor [78].

These and several other studies proved without a doubt the importance of Par-4 in preventing tumor initiation and progression. In the following section, we are going to explore different aspects of Par-4 involvement in tumor-associated inflammation and possible mechanisms of this involvement.

6 Par-4 and Inflammation

Par-4 role in cancer inflammation has been largely overlooked. Only a couple of studies have shown that Par-4 is involved in modulating cancer-associated inflammation. In both studies, the expression of Par-4 has been upregulated by anti-inflammatory drugs and mediated their induction of apoptosis in cancerous cells.

In the first study, Zhang and DuBois, have shown that Par-4 mediates apoptosis induction by different NSAIDs. They demonstrated that Par-4 expression levels are strongly increased in colon cancer cells, HCA-7, treated with high levels of several NSAIDs, namely NS-398, a selective COX-2 inhibitor, and sulindac sulfide, SC-58125 and, nimesulide which are COX-1 and-2 inhibitors [79].

A recent study by the Shouman group showed that breast cancer cell lines treated by inflammatory factors generated from activated macrophages will undergo apoptosis in response to the NSAIDs sulindac sulfide and piroxicam. In the study, induction of apoptosis by sulindac sulfide and piroxicam was associated with increased expression of Par-4.

This study went a step further as they showed that the conditioned media generated from activated macrophages, was able to reduce levels of Par-4 by 50% in breast cancer cell lines compared to control cells. The conditioned media also increased the expression of hallmarks of inflammation: cytokines IL-1β and IL-6, Ras, and COX-2, confirming the effect of TAM on cancer cells [80].

These two studies suggested that Par-4 contributes to the apoptosis induction by NSAIDs whether they are selective COX-2 inhibitors or not. As we discussed before, different NSAIDs can have different mechanisms to achieve COX inhibition and to induce apoptosis. This suggests that Par-4 may be acting through different mechanisms with different COX inhibitors.

In the following section, we will discuss the possible mechanisms through which Par-4 may be involved in apoptosis induction by NSAIDs, mainly through presenting the molecules that are downstream of both Par-4 and different NSAIDs.

6.1 Role of Ras in Cancer and Inflammation

Ras is the most commonly mutated oncogene in cancer. Mutations in *k-ras* are the known drivers of three of the most lethal cancers: lung cancer, colorectal cancer, and pancreatic cancer. Mutations in *k-ras* reach 60% in pancreatic cancer [81]. In normal cells, the Ras family of GTPases are small membrane-associated proteins that play essential roles in development, survival, growth, and inflammation. They are

activated at the membrane in response to growth factor receptors binding to their ligands. Ras is responsible for the activation of a constellation of pathways including the famous Raf-MEK-ERK, PI3K-Akt, and the MEKK-SEK-JNK pro-survival pathways [82].

The Ras family includes H-Ras, K-Ras, R-Ras, and N-Ras. Their activity is controlled by a regulated GDP/GTP cycle. Mutations in Ras render them insensitive to external signaling and they become constitutively active, activating downstream signaling pathways. In the oncogenic state, Ras activates diverse pathways that are known to promote tumorigenesis [83, 84].

The Ras family plays a vital role in the regulation of immunity and inflammation. In the oncogenic state, one consequence of Ras signaling in cancer cells is the upregulation of an array of cytokines and chemokines. These include CCL2 (MCL-1), CCL5, IL-8, IL-6, and GRO-1/CXCL1, which, among other chemokines, are known to recruit monocytes to tumors where they differentiate to TAMs. Oncogenic Ras has also been shown to upregulate and stabilize COX-2 expression [85].

In addition to the role of activated Ras in recruiting TAM, Thabet et al. study showed that Ras is upregulated in breast cancer cell lines in response to stimulation by macrophage conditioned medium suggesting the formation of a positive feedback loop in maintaining the inflammation in the TME [80]. Li and colleagues found a strong correlation between the presence of oncogenic Ras, infiltration of TAM, and formation of neovascularization. These studies and others suggest an essential role of Ras in the TME and cancer progression [41, 86, 87].

Oncogenic Ras has also been linked to the induction of tumor angiogenesis and metastasis by upregulating vascular endothelial growth factor (VEGF) and by inducing matrix metalloproteinases (MMPs) that degrade the extracellular matrix allowing metastasis [88] (see Fig. 5).

In the early years of Par-4 research, a strong relation between Par-4 and Ras was established. It was found that Par-4 induces apoptosis in a Ras-dependent matter. Cells overexpressing oncogenic Ras will undergo apoptosis when Par-4 is introduced. This apoptosis is dependent on the inhibition of NF-κB transcription activity. In addition, Ras-induced cellular transformation is abrogated when Par-4 levels are restored through MEK inhibition or overexpression of ectopic Par-4. This anti-transformation action of Par-4 appears to be distinct from its apoptotic function. On the other hand, expression of oncogenic Ras has been shown to strongly downregulate Par-4 in a variety of cells via the Raf-MEK-ERK pathway, and this inhibition is essential for cellular transformation by Ras. Par-4 inhibition by Ras is most probably achieved by inducing methylation of the Par-4 promoter through Raf-MEK-ERK-dependent and independent pathways [89–92].

In pancreatic cancer tissues and cell lines, Par-4 is significantly downregulated in the presence of *k-ras* point mutations. In addition, higher levels of Par-4 in pancreatic cancer tissue samples were correlated with prolonged survival. Moreover, transient overexpression of oncogenic Ras in a pancreatic cancer cell line with WT-k-ras, significantly downregulated endogenous Par-4 and conferred accelerated growth [93].

The translocation generating BCR-ABL fusion gene is found in about 25% of adult ALL. This gene encodes p185BCR-ABL oncoprotein which is able to transform immature hematopoietic cells. Par-4 was found to antagonize the transformation induced by p185BCR-ABL in hematopoietic stem cells by interfering with Ras activation and disrupting signaling downstream of p185BCR/ABL [74].

Targeting Ras as a therapeutic option for cancer treatment has been attempted repeatedly. Unfortunately, clinically, direct inhibition of Ras has been very difficult and associated with toxicity. On the other hand, inhibition of downstream signals, such as Raf, MEK, mTOR, and Ras-induced cytokines has shown better results [86]. Like Par-4, several NSAIDs have been shown to act on the Ras signaling pathway with favorable results suggesting the need for more studies to evaluate the possibility of using NSAIDs as Ras pathway inhibitors.

6.2 Role of NF-κB

NF-κB transcription factors are involved in the control of a large number of processes, such as immunity and inflammatory responses, development, cellular growth, and apoptosis. They are dimers formed from a related group of proteins; RelA (p65), RelB, c-Rel, p50, and p52. The p50 and p52 proteins are unable to activate transcription independently since they lack transactivation domains. p50-RelA heterodimer represents the major and best studied NF-κB dimer. NF-κB transcription factors bind a DNA sequence motif, named κB site, in its target genes that are mainly involved in immunity and inflammation, in addition to a number of antiapoptotic genes [94].

Under normal conditions, NF-κB is kept inactive in the cytoplasm, bound to IκB. Stimulation by pro-inflammatory cytokines (e.g., TNF-α, IL-1), microbial and viral infections, stress factors and carcinogens, results in phosphorylation of the inhibitory IκB by the IKK complex and the subsequent proteasomal-degradation of IκB. Once IκB is degraded, NF-κB is free to translocate to the nucleus where it activates the transcription of its target genes. Normally, the activity of NF-κB is highly controlled; however, during oncogenesis, it becomes constitutively activated mostly due to the continuous presence of external signals and not because of activating mutations [95–98].

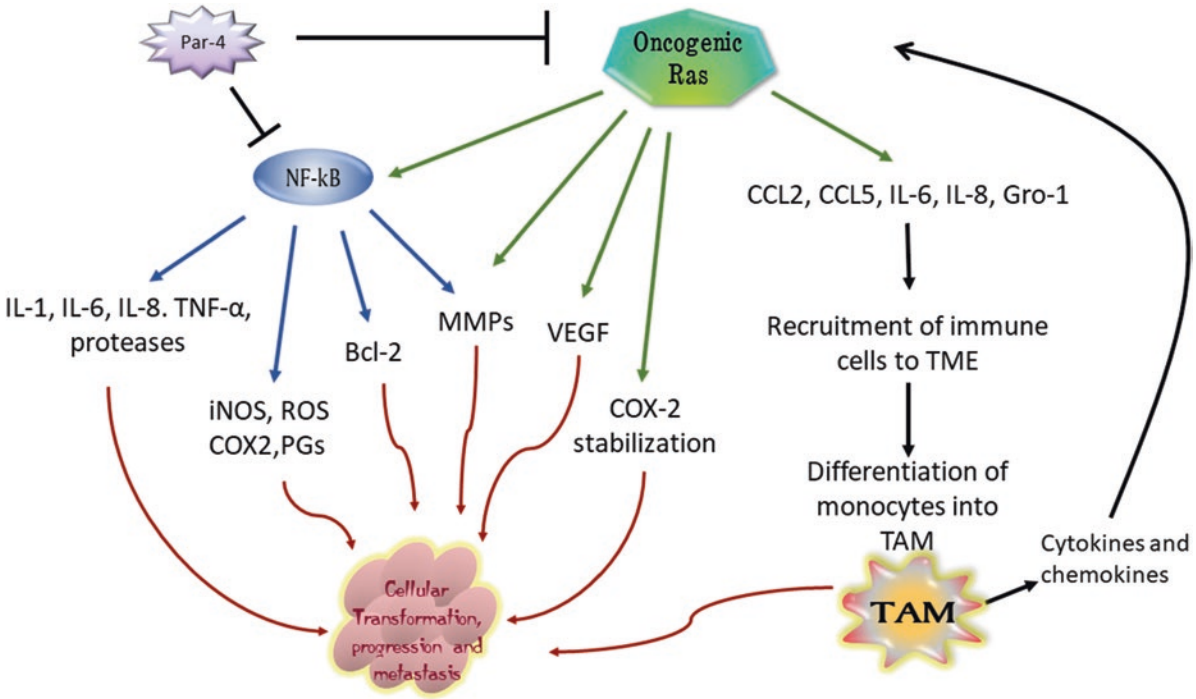

Fig. 5 Ras and NF-κB, role in inflammation and tumorigenesis: Ras and NF-κB play an essential role in tumor survival, growth, and invasion by inducing protein such as Bcl-2, MMPs, and VEGF, and by increasing the expression of a number of cytokines and chemokines which participate in maintaining the tumor-associated inflammation.
MMPs Matrix metalloproteinases, *VEGF* vascular endothelial growth factor

NF-κB is one of the main regulators controlling inflammation in general and cancer-associated inflammation in particular. NF-κB controls the expression of many inflammatory cytokines and chemokines such as IL-1, IL-6, IL-8, IL-23, and TNF-α. NF-κB regulates the expression of MMPs which are crucial mediators of local inflammation and leukocyte chemotaxis, and the expression of adhesion molecules on leukocytes and endothelial cells, which allow the recruitment of leukocytes to the sites of infections [99, 100]. In addition, iNOS and COX-2 are both NF-κB target genes, thus NF-κB is involved in the production of prostaglandins and ROS. Like Ras, some of the targets of NF-κB participate in its activation, making it a crucial element in the propagation and elaboration of cytokine responses. It is important to note that NF-κB also encodes its negative regulators genes [101–103] (Fig. 5).

In addition to the essential role in immunity and inflammation, NF-κB controls the transcription of genes that regulate cellular differentiation, survival, and proliferation. It activates genes encoding antiapoptotic proteins from the Bcl-2 family, e.g., Bcl-2 and Bcl-x_L, and other antiapoptotic genes like X-linked inhibitor of apoptosis (XIAP) which can protect the cell from TNF-α-induced apoptosis thus preventing apoptosis of epithelial and cancer cells [104]. To sum it up, NF-κB targets genes that are involved in inflammation, cancer development, cell cycle progression, angiogenesis, and metastasis making it an essential contributor in tumorigenesis [32, 102].

Many studies showed that one of the main mechanisms of apoptosis induction by Par-4 is through inhibition of NF-κB-mediated cell survival mechanisms [89, 105].

Par-4 was found to inhibit TNF-α-induced nuclear translocation of the RelA subunit of NF-κB, by blocking the ability of aPKC to activate the IKK complex. Although Par-4 translocation to the nucleus is known to be required for apoptosis induction [106], in this study, Par-4 was found to bind and inhibit aPKC in the cytoplasm. Expression of oncogenic Ras restored TNF-α ability to translocate RelA to the nucleus, by decreasing levels of endogenous Par-4 [107].

In PC12 neuronal cells, overexpression of Par-4 causes suppression of NF-κB activation and cause Bcl-2 levels reduction, which correlates with enhanced apoptosis [108].

In prostate cell lines overexpressing Par-4 (PC3/Par-4), radiation-induced NF-κB activation was inhibited as Par-4 directly inhibits the phosphorylation and degradation of IκB-α, causing repression of radiation-induced Bcl-2 protein [109].

In the mouse TCL1 leukemia system, overexpression of Par-4 has an anti-leukemic effect. It delayed the development of leukemia in these mice through downregulation of NF-κB signaling which was mainly accomplished by reducing RelA translocation to the nucleus [76].

The activity of NF-κB can be inhibited by the NSAIDs sodium salicylate and aspirin through inhibition of IκB degradation in a different range of cell types and conditions. This results in inhibition of NF-κB translocation to the nucleus which blocks IL-1, IL-6, IL-8, and TNF-α-induced NF-κB activation [49, 110].

In summary, NF-κB is an essential player in the initiation of inflammation and is required for sustaining the inflammation and providing a positive feedback loop for its own production (e.g., TNF-α). It induces the expression of a large number of genes with known involvement in sustaining inflammation and in the initiation of carcinogenesis. Par-4 has been shown to induce apoptosis through inhibition of NF-κB activation and inhibition of nuclear translocation of p65 subunit of NF-κB.

6.3 Role of Bcl-2

The Bcl-2 family contains both antiapoptotic (e.g., Bcl-2, Bcl-x_L, Bcl-w, Mcl-1,) and pro-apoptotic (e.g., Bax, Bak, Bad, Bid) members. The first group members protect cells from apoptotic stimuli by binding and inactivating their pro-apoptotic antagonists [111]. Bcl-2 helps oncogenesis through cell death resistance. High levels of Bcl-2 are found in many types of cancers. Overexpression of Bcl-2, following chromosomal translocation, is linked to the development of B cell lymphoma in humans. Drugs specifically targeting the pro-apoptotic members of the Bcl-2 family have shown promising results in treating several types of cancer such as relapsed chronic lymphocytic leukemia (CLL) [112] confirming the importance of Bcl-2 in supporting the survival of tumors.

Bcl-2 essentially acts as an anti-oxidant thus protecting cells from the effects of inflammation, but it also protects the cells from undergoing apoptosis, which allows the accumulation of transforming mutations [113]. Bcl-2 levels are regulated by various cytokines, including IL-1β and IL-6. In TAM, high levels of secreted IL-10 induce drug resistance through IL-10/STAT3/Bcl-2 signaling pathway [114].

Early on, it was found that overexpression of Par-4 in mouse fibroblast cells (NIH-3T3) or in human prostate cancer cell lines (PC-3) resulted in a reduction of endogenous Bcl-2 levels. In addition, androgen-independent CWR22R tumors derived from the CWR22 xenografts showed mutually exclusive expression patterns of Par-4 and Bcl-2 [115].

In lymphatic cell lines and in blast cells of acute lymphocytic leukemia (ALL) patients, there is an inverse pattern of expression between Par-4 and Bcl-2. In addition, overexpression of Par-4 in lymphocytic cell lines (Jurkat T cells) decreases levels of Bcl-2 with no effect on Bax. In these cells, overexpression of Par-4 was sufficient to cause PARP cleavage. The addition of a chemotherapeutic agent was required to activate caspase-3 and cause apoptosis in the cells overexpressing Par-4 [116, 117].

In PC12 neuronal cells and PC3 cells overexpressing Par-4 and suppression of NF-κB activation was accompanied by reduction in Bcl-2 levels followed by apoptosis [108, 109].

In androgen-independent prostate cancer cell lines (LNCaP), Par-4 was found to downregulate Bcl-2 through a WT1-binding site on the *bcl-2* promoter, suggesting that Par-4 nuclear translocation is essential for the downregulation of Bcl-2 [118].

6.4 Role of Fas

CD95 (Fas) and CD95L (FasL) belong to the TNF receptor superfamily of death receptors. Trimerization of Fas after binding to its ligand recruits the adaptor protein Fas-dependent death domain (FADD) which induces the formation of the death-inducing signaling complex (DISC), activating caspase-8 and thus inducing apoptosis [119, 120]. FasL is implicated in immune homeostasis and immune surveillance. It mediates lymphocyte-dependent cytotoxicity and clonal deletion of alloreactive T cells. The soluble form (sFasL) results from cleavage of membrane FasL by metalloproteinases and induces apoptosis in susceptible cells [121].

One of the mechanisms by which Par-4 induces apoptosis in androgen-independent prostate cancer cell lines is by increasing the translocation of Fas and Fas ligand (Fas/FasL) to the plasma membrane alongside the inhibition of NF-κB activity [105].

Par-4 was also found to increase Fas-induced apoptosis by protecting FADD from PKC zeta phosphorylation in KG1a acute myeloid leukemia (AML) cell lines, thus overcoming drug resistance and increasing apoptosis in these cells [122].

Surprisingly, in addition to the apoptotic function of Fas/FasL, it also has a non-apoptotic function as it was found that sFasL can promote cancer cell migration in some systems [123]. It is not yet clear what are the conditions that contribute to the pathway that Fas/FasL chooses, but it is evident that Par-4 is part of the apoptotic induction mechanism of Fas/FasL.

6.5 Inhibition of Par-4 in Response to Inflammatory Stimuli

In the study conducted by Thabet and colleagues, they showed that treating breast cancer cell lines (estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231) with a conditioned medium of activated macrophages,

increased inflammatory signals in the cells. These breast cell lines showed increased expression of IL-1β, IL-6, COX-2, and PGE2 (Prostaglandin E2). Together with these inflammatory signals, Ras expression was increased in MCF-7. In MDA-MB-231, Ras expression was only slightly affected since it is already high in these cells. Interestingly, Par-4 expression was decreased in response to inflammatory stimulation by the conditioned medium. The decrease of Par-4 level was well pronounced in the hormone-dependent MCF7 cells while the hormone-independent cells showed a slight decrease consistent with the changes seen in Ras levels, suggesting Ras may be responsible for the change in Par-4 expression in these cells [80].

It would be interesting to know if other inflammatory signals are involved in the inhibition of Par-4 expression and whether this is restricted to tumor-associated inflammation or is a general inflammatory response.

6.6 Par-4 as a Mediator of Anti-Inflammatory Drug Action

Although the role of NSAIDs as chemopreventive and anticancer agents is quite well established, their mechanism of action is still not yet fully elucidated.

The effect of NSAIDs on Par-4 expression became known for the first time through the work of Zhang and Dubois in 2000. They found that several NSAIDs are able to upregulate Par-4 expression in human colon carcinoma cell lines. These NSAIDs were either COX-1 and -2 (sulindac sulfite) or COX-2 specific inhibitors (NS-398, nimesulide, and SC-58125). Both categories of NSAIDs caused an increase in Par-4 expression at the RNA and the protein levels [79]. Almost two decades later, a second study showed that the expression of Par-4 is increased in stimulated breast cancer cell lines after treatment with sulindac sulfite or piroxicam COX inhibitors. Par-4 increase in the breast cells was accompanied by an increase in caspase-3 expression and a decrease in Ras, Bcl-2, and other inflammatory signals; IL-1β, IL-6, and COX-2 [80].

The mechanism of Par-4 increased expression by NSAIDs is unknown. The observation was made in different cell types, under different conditions. The breast cancer cells were stimulated with inflammatory signals, but not the colon cancer cells. The NSAIDs involved in Par-4 regulation are also known to have different mechanisms of action since some are selective COX-2 inhibitors and others act on both COX-1 and COX-2.

In both studies, the increase of Par-4 in response to NSAIDs treatment was associated with increased apoptosis in the cancer cell lines under investigation. Inhibition of the Ras pathway by Par-4 may be essential in this regard since Ras is increased in inflammation and it has been suggested to be a common target of several NSAID inhibitors. NS-398 and sulindac sulfide have been shown to inhibit ERK activity through attenuation of Ras/c-Ras interaction [124, 125]. Piroxicam downregulates Ras in colon tumors in rats [126]. Other studies have shown that COX-2-specific and nonspecific inhibitors inhibit NF-κB and the Raf-MAPK (ERK2) pathway, which is very similar to the effect of Par-4 on these pathways [127]. This suggests that the effect of different NSAIDs on Ras levels and their apoptosis induction may be mediated by Par-4.

Of course, there is the possibility that Ras is inhibited by a different mechanism and this inhibition leads to an upregulation of Par-4, and subsequent induction of apoptosis since it has been previously shown that inhibition of RAS\RAF\MAPK signaling restores Par-4 in cancer cells [92]. Par-4 would then be able to induce apoptosis through other mechanisms such as inhibition of Bcl-2 and NF-κB and activation of Fas/FasL and TRAIL apoptotic pathways (Fig. 6).

7 Future Research Directions

Discovering that Par-4 is involved in tumor-associated inflammation raises so many new questions.

- Since Par-4 was found to be downregulated by inflammatory conditions, it is important to characterize the inflammatory mediators involved. Ras represents a good candidate for mediating downregulation of Par-4, but this has to be confirmed experimentally. The involvement of other factors has to be considered as well.
- Determination of the possible involvement of Par-4 in other types of inflammation is of great interest. Par-4 has been previously implicated in neurodegenerative disorders [128], and it is interesting to know if it has a role in the inflammation associated with the development of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.
- Par-4 knockout mice can be used for the elucidation of the role played by Par-4 in the apoptosis induction by NSAIDs. They can be used to identify the place of Ras compared to Par-4 in response to NSAIDs signaling.
- Is Par-4 required for apoptosis induction by NSAIDs? Downregulation of Par-4 using siRNA can be used in such a study.
- Characterization of the role Fas/FasL downstream of Par-4 in response to NSAIDs treatment should shed more light on the mechanism of action of Fas/FasL in inflammation and cancer.
- Although several NSAIDs were shown to upregulate Par-4, it is still interesting to know if other NSAIDs, with different mechanisms of action, have similar effects on Par-4 levels.

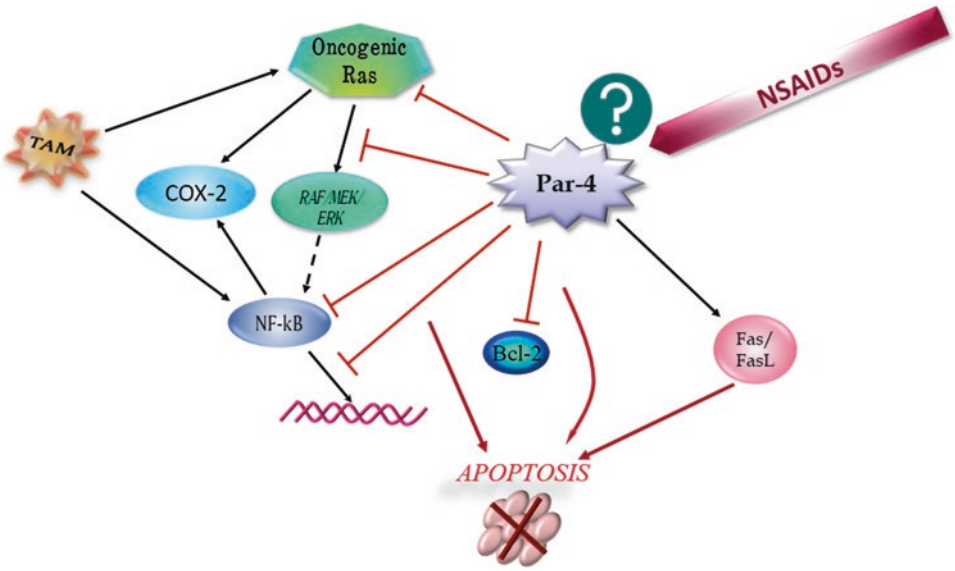

Fig. 6 Possible role of Par-4 downstream of NSAIDs: NSAIDs mechanism of chemoprevention and anti-tumor action is not yet fully elucidated. Inhibition of NF-κB, and downregulation of Ras and Bcl-2 have been shown to be part of the mechanisms of apoptosis induction in cancer cells following treatment with NSAIDs. The recent findings, showing that several NSAIDs upregulate Par-4, suggest that Par-4 can act as a mediator of apoptosis induction by NSAIDs in cancer cells as they share same mechanisms of apoptosis induction. Upregulation of Par-4 by NSAIDs could be responsible for Ras, Bcl-2, and NF-κB inhibition leading to inhibition of COX-2, interruption of the positive feedback loop activating TAMs and induction of apoptosis.
TAM Tumor-associated macrophage, *NSAIDs* Non-steroidal anti-inflammatory drugs

- So far, the relation between Par-4 and inflammation has been studied in cancer cell lines. It is essential to confirm this relation in vivo. Determination of Par-4 levels in different inflammatory diseases and TME should be very informative. Of equal importance is the determination of the effect of NSAIDs on Par-4 levels in patients.

8 Conclusions

The study of cancer-related inflammation has greatly evolved in recent years to provide new prophylactic and therapeutic strategies in the form of inhibitors of inflammation. A better characterization of the mechanism of action of these inhibitors and the identification of their downstream signaling molecules is essential to tailor more targeted therapies that would avoid the side effects of the currently used NSAIDs.

As we have seen presented in this chapter, accumulating evidence suggest that Par-4 could be an essential player in inhibiting inflammation associated with tumors. As we discussed above, Par-4 affects different components of the immune system such as Ras, NF-κB, and Bcl-2. These interactions have been studied in the context of transformation inhibition and apoptosis induction but not in relation to the immune response and inflammation. We have seen similarities between the mechanism of apoptosis induction by Par-4 and by different NSAIDs such as the inhibition of the Raf-MEK-ERK pathway, NF-κB activation, and Bcl-2 expression suggesting a mediatory role for Par-4 in NSAIDs function.

The identification of the role played by Par-4 in tumor-associated inflammation adds a new piece to the repertoire of actions of Par-4 as a tumor suppressor protein and sheds new light on a possible use in managing chronic inflammatory diseases and in cancer prevention.

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Par-4 in Chemoresistant Ovarian and Endometrial Cancers

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Abstract

Ovarian cancer is the most lethal gynecological cancer; on the other hand, endometrial cancer is the most prevalent, with exceedingly poor prognostic when found to be recurring. In both cases, robust resistance to chemotherapeutic compounds, may they be genotoxic in nature or specifically targeting dysregulated pathways, drastically reduce our ability to treat these diseases. In order to overcome the chemoresistant nature of these neoplastic entities, novel therapeutic avenues must be explored. In that context, Par-4, a pro-apoptotic protein that has been reported to selectively induce cell suicide in cancer cells, appears to be of particular interest. This review aims to objectively assemble the available evidence, which predominantly emanates from non-gynecological tissues, and underline the convergent findings of past investigations, highlight divergences, and help direct future research endeavors. Considering that both of these tumors are characterized by prevalent mutations in the PI3K/Akt/PTEN axis as well as alterations in p53, our work provides a sharp focus on the regulatory role of these molecular pathways on Par-4 function. We hope this chapter will allow the potent abilities of Par-4 to be fully leveraged in these tissular contexts and augment the available armamentarium, so that we may eventually improve the prognostic of women afflicted with these diseases.

Keywords

Par-4 · Chemoresistant tumors · Ovarian cancer · Endometrial cancer · Pathogenesis · Molecular mechanisms · Gynecological tissues · PI3K/Akt/mTOR axis · NF-kappaB · p53 · Autophagy

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1 Introduction

1.1 Pathogenesis and Molecular Mechanisms of Chemoresistance

One of the main causes of treatment failure in the context of cancer therapeutics, and implicitly the main driver of mortality, is the ability of tumors to resist to the various pharmacological and biological tools we have at our disposal. The capacity of these transformed cells to withstand chemotherapeutic assaults is the main determinant of chemoresistance; however, the underlying mechanisms resulting in the emergence of such characteristics among a cell population are multifaceted and their plurality is one of the most potent hurdles in our ability to improve patient's prognostic, especially in gynecological cancers. Indeed, ovarian cancer (OC) and recurrent endometrial cancer (EC) present with exceedingly unfavorable outcomes mainly due to late-stage disease discovery, which is consistently associated with intratumoral heterogeneity and robust resistance to chemotherapeutic agents, both in the context of adjuvant or neoadjuvant to cytoreductive therapy.

Multiple aspects of intrinsic cellular homeostatic components can be disrupted by the oncogenic progression, which will impart a varied number of features enabling proliferation, dissemination, and resistance. Through mutation in key regulatory pathways, which we will explore in more details below, cells will acquire hallmarks of cancer, which will allow the neoplastic entity to become highly genetically unstable as well as disjoined itself from signaling constraints that should regulate its proliferative program. This gives rise to cells that can acquire a massive mutational burden through successive round of replication and selection. Such events will often result in an initial remission of tumors following cytoreductive therapy and adjuvant chemotherapy, which will be followed by a re-emergence of resistant, occult metastases, a process reminiscent of bacterial selection in the context of antibiotic use. Additionally, the genomic instability that is a hallmark of many of these tumors, and which

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induces the development of highly heterogeneous primary tumors, makes the selection of an effective chemotherapeutic regimen all the more challenging. Various cells will exhibit diverse levels of sensitivity, which will only increase the likelihood of resistant subpopulations' appearance. As previously stated, while eminently complex and varied, cancer can be primarily construed as a disease of dysregulated proliferation and survival that is frequently driven by failure of the genomic integrity-maintenance apparatus. Multiple pathways can lead to such outcomes, many of which are directly associated with cell death and proliferation. At the epicenter of those dysregulations is the apoptotic machinery, which is often compromised, either intrinsically or through inhibition of some of its crucial signaletic components. This deeply imbedded, cardinal cell suicide program, can be activated either through intrinsic mechanisms or provoked by the action of extrinsic effectors; as such, apoptosis is generally thought as the primary bulwark against neoplastic transformation, enabling cell death in response to genotoxic stressors, immunological inducers or simply cessation of pro-proliferative or survival signaling. Consequently, and considering Par-4 primarily suggested function as an intrinsic regulator of the apoptotic programming, a significant portion of this chapter will focus on the dynamic and integrative molecular control of this cellular process of central importance in chemosensitivity.

1.2 Par-4 in Gynecological Tissues

Par-4 has been proposed as a pivotal inducer of cell suicide, through a multiplicity of pathways that will be expounded on throughout this chapter. While Par-4 role in EC and OC hasn't been as well described as other tissular contexts, initial reports showing Par-4 ability to induce apoptotic cell death in hormone-independent models arouse interest regarding the protein's role in gynecological tissues. Mice supplemented with estrogen have been shown to display reduced Par-4 levels in the uterus, suggesting a negative regulatory loop enacted by steroid hormones in that tissue [1]. Estradiol has also been shown to negatively regulate Par-4 mRNA in breast cancer cell lines [2, 3]. We have also recently demonstrated, using ChIP-seq assay, that ERα directly binds to *PAWR* promoter, reducing Par-4 mRNA transcription [4]. The exact role of hormone receptors and their associated pathways in regard to Par-4 remain, however, ill-defined. Nevertheless, in a variety of models, low levels of Par-4 have been associated to poor prognostic, with tumors presenting decreased or absent Par-4 being generally more resistant to therapies as well as displaying increased aggressiveness [5–10]. More specifically, the first report underlining the role of Par-4 in the endometrium arose following the demonstration that 80% of Par-4 knockout mice developed endometrial hyperplasia; even more interestingly, the same study reported that 36% of these mice would go on to present endometrial adenocarcinomas within their first year of life [1]. These intriguing data highlighted the role of Par-4 in endometrial tumorigenesis and correlated the loss of Par-4 with increased level of XIAP, a well-characterized driver of chemoresistance. Some reports have also highlighted a pro-apoptotic, chemosensitizing activity of Par-4 in OC [11]; however, very little data is available at the present time. Par-4 mRNA is widely expressed throughout gynecological tissues, is seldomly mutated in all cancer types, an observation that holds true in OC and EC (Fig. 1). As will be expounded further in this chapter, Par-4 appears to be functionally silenced rather than mutated, allowing us to think it could be effectively instrumentalized in these tumors.

Altogether, we are allowed to believe that Par-4 could act in these tissues in a similar fashion as other reported models, suggesting that Par-4 regulation could be instrumentalized in order to overcome chemoresistance through reinstatement of apoptotic programming. The present chapter will thus mainly focus on the regulatory pathways impeding, or stimulating, programmed cell death, and the intricate position Par-4 occupy within these networks. We will also make a brief foray into the role of autophagy in chemoresistance considering the emerging and dualistic role of this process in cancer, and the involvement of Par-4 in its regulation. However, it appears necessary to first describe these diseases more fully so that their clinical, histological, molecular, and therapeutic specificities can be used to contextualize the molecular mechanisms that will be expounded on later; we also hope this overview will also help direct future work aimed at instrumentalizing Par-4 to reverse chemoresistance in these neoplastic entities.

2 Ovarian Cancer

2.1 Statistics

Ovarian cancer (OC) is a complex disease that presents challenging problems mainly caused by late diagnosis, tumoral heterogeneity, and widespread chemoresistance. Annually, it was estimated in 2018 that 295,414 new cases of OC would be diagnosed worldwide with 184,799 related deaths [12]. In the USA alone, OC ranks 11th in new cases, fifth in related deaths for female cancer and was estimated to read 21,750 cases and 13,940 deaths in 2020 [13]. Despite a reduction of 29% in overall cancer deaths in the last 30 years, OC prognostic has nearly stayed the same, remaining the most fatal gynecologic cancer. Indeed, based on the NIH statistics, the 5-year relative survival rate is at an average of 48.6% but is highly variable depending on the cancer stage at diagnosis; however, almost 60% of the patients are initially diagnosed

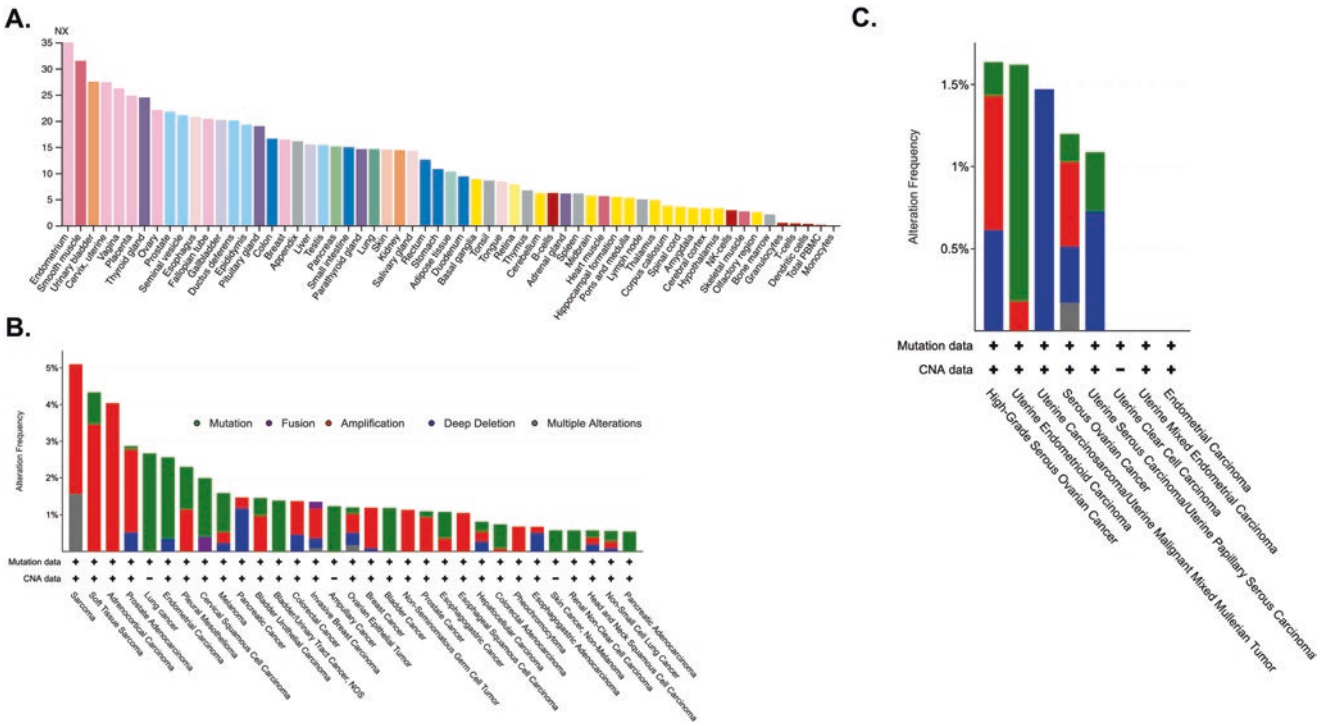

Fig. 1 Salient information regarding Par-4 gene (PAWR) expression and mutations. (**a**) Data taken from https://www.proteinatlas.org/ENSG00000177425-PAWR/tissue showing PAWR mRNA expression profiles across human tissues (**b**) Overall alteration profile of PAWR. Data extracted from TCGA using the curated set of non-redundant studies and excluding all cancer type presenting less than 0.5% alteration rate. Represents 45,142 patients in 47,571 samples, from 181 studies. (**c**) PAWR alteration profile in ovarian and endometrial cancers. Graphical representation of Par-4 alterations in endometrial and ovarian cancers. Data extracted from all available non-overlapping uterine cancer datasets (which include the following studies: MSK, 2018; TCGA PanCancer Atlas; John Hopkins, Nat Commun 2014; NIH, Cancer 2017; MSK, Clin Cancer Res 2020) and ovarian cancer datasets (which include the following studies: MSKCC, Nat Genetics 2014; TCGA, Nature 2011; TCGA PanCancer Atlas). Graphs **b** and **c** were produced using cBioPortal.org interface and use the same coloring legend, as found in **b**

with distant metastasis, with an associated survival of 30.2% [14]. The lack of symptoms at early stages of the disease combined with inadequate screening tools delays detection and explains the extensive dissemination often observed at diagnosis. Incidentally, metastatic and recurrent diseases are associated with widespread, robust resistance to classic chemotherapeutic compounds, owing to various molecular alterations, intratumoral heterogeneity and resistant subpopulations [15, 16].

2.2 Histological, Morphological Subtyping, and Pathogenesis

Morphologically, OC is classified into two broad categories: epithelial OC (surface epithelial-stromal) and non-epithelial OC, which comprise sex cord-stroma and germ cells. Surface epithelial-stromal type accounts for approximately 60% of all OC followed by germ cells (25%) and sex cord-stromal (10%). Each type can be further subdivided into different histotypes, then classified as benign, borderline, or malignant tumor. Surface epithelial-stromal constitutes 90% of malignant ovarian lesions [17] and can be further subdivided in five main different histologic subtypes: high-grade serous (HGSOC)(71%), which will be the focus of this chapter, low-grade serous (LGSOC)(4%), endometrioid (8%), clear-cell (10%), and mucinous (3%) [18, 19]. Table 1 highlights the cellular origin, as well as the salient molecular characteristics, of each OC subtypes.

In 2004, a new model based on morphological and molecular genetic was proposed where epithelial carcinomas were divided into two groups designated type I and type II tumors [20]. Type I tumors include LGSOC, endometrioid, clear-cell, and mucinous which are associated with low proliferation, low-grade neoplasms, chromosomal stability and arise from a borderline tumor in a stepwise manner. Type II tumors include HGSOC, carcinosarcoma, and undifferentiated carcinoma. They are characterized by rapid evolution, early metastasis, high-grade neoplasms, chromosomal instability, and uncertain precursor lesions. However, the typical model somewhat lacks granularity have been challenged [21–23]. Indeed, the five major OC subtypes could be considered dis-

Table 1 Ovarian cancer histologic and molecular classification

Histologic subtype	HGSOC	LGSOC	Endometrioid	Clear cell	Mucinous
Epithelial OC %	71%	4%	8%	10%	3%
Tissue origin	Fallopian tube	Fallopian tube	Atypical endometriosis	Atypical endometriosis	Unknown
Precursor lesion	STIC	Fallopian epithelial cells Borderline tumor	Borderline tumor	Borderline tumor	Cystadenoma Borderline tumor
Mutation profile	*TP53*	*BRAF*	*PTEN*	*ARID1A*	*KRAS*
	BRCA1/2	*KRAS*	*CTNNB1*	*PI3KCA*	
	NF1	*ERBB2*	*PI3KCA*	*PTEN*	
	CDK12		*ARID1A*	*CTNNB1*	
			PPP2R1A	*PPP2R1A*	
Characteristics	Chromosomal instability PI3K/Ras, Rb, Notch, FoxM1 alterations		MMR deficiency	*ERBB2* amplification	*ERBB2* amplification
Prognosis	Poor	Intermediate	Favorable	Intermediate	Favorable
Chemosensitivity	High low at recurrence	Intermediate	High	Low	Low

General information used to elaborate this table was extracted from [181]; percentages were obtained from Prat, 2012 review [182], tissular origins were obtained from Kurman et al., 2010 review [183]. Finally, molecular profiles were identified using Jayson et al., 2014 review [184] and the TCGA genomic analyses effort of 2011 [35]

tinct diseases since they are associated with vastly different pathogenesis, molecular abnormalities, response to chemotherapy, and prognosis [24].

OC pathogenesis has been an actively debated topic in the scientific community. Initially, it was believed that OC arose from the ovarian surface epithelium, with the surface epithelium, ruptured following ovulation, would invaginate during the repair process and form cortical inclusion cysts [25, 26]. However, data suggested that women presenting genetic predisposition to OC had increased tubal dysplasia and an associated heightened tubal cancer risk [27]. Further investigations also demonstrated that 70% of sporadic HGSOC patients presented serous tubal intraepithelial carcinoma (STIC) with similar molecular alterations [28]; compelling molecular evidence have since shown that invading cells from STIC lesion act as precursors to HGSOC [29, 30], with most surface epithelial-stromal carcinoma subtypes sharing this external origin [21]. Alternatively, molecular evidence have also shown that endometriosis and adjacent endometrioid OC share a common lineage, underlining the profound pathophysiological difference between serous and non-serous OC [31, 32]. Altogether, it appears clear that the pathogenesis of most surface epithelial-stromal OC originate from outside the ovary and where the ovary is involved secondarily. Unsurprisingly, the divergent tissular origins and the distinct pathogenesis of each subtype are associated with discrete molecular signatures, which will be discussed in the next section.

2.3 Molecular Subtyping

With the advancement of molecular genetic analysis (omics), characterization and better classification of different subtypes have improved our understanding of the molecular abnormalities found in epithelial OC. First and foremost, type I tumors present gene mutations in *BRAF*, *KRAS*, *PTEN,* and *ARID1A* while type II tumors are associated with *TP53*, *BRCA1, and/*or *BRCA2* gene abnormalities [20]. HGSOC is prototypically characterized by mutations in *TP53*, present in 96% of cases [33], as well as *BRCA1/2,* found in 33% of cases [34, 35]. A molecular pathogenetic model, in which p53 loss is considered as the initiation event which is then followed by BRCA1/2 loss and subsequent chromosomal instability, has been suggested for HGSOC [36]. Interestingly, *BRCA1* germline mutation was shown to lead to decreased PTEN mRNA levels whereas epigenetic silencing leads to an increased copy-number of *PIK3CA* [37]. Additionally, in 2011, The Cancer Genome Atlas (TCGA) analyzed 489 HGSOC tumors to evaluate mRNA and miRNA expression, promoter methylation, and DNA copy-number [35]. The study confirmed the importance of *TP53* and *BRCA1/2* genes and shed light on other involved pathways such as FOXM1(87%), Rb(67%), RAS/PI3K(45%), and also NOTCH(22%). Finally, a recent large-scale study that aimed to use gene expression to predict response to platinum-based chemotherapy showed that alteration of the homologous repair system and promotion of EMT were central to OC ability to resist chemotherapy [38]. Table 1 summarizes the various molecular abnormalities that characterize each OC histological subtypes; these alterations are the bedrock upon which rest the tumoral cell's ability to resist therapies.

2.4 Current Therapeutic Strategies

Standard treatment strategy for OC has remained generally similar in the last 20 years, with recent improvements in the sphere of maintenance therapy. The archetypal protocol relies mainly on surgical debulking, also called cytoreductive surgery [39]. This is then followed by platinum-taxane-based chemotherapy in order to eliminate the remaining mass in cases of advanced OC [40]. After surgery, chemotherapy will begin using carboplatin (cisplatin replacement [41]) and paclitaxel doublet administered every 3 weeks although dosage, choice of platinum and/or taxane, schedule, and mode of administration have been the subject of debate [42]. This regiment presents a high response rate of 60 to 75%, but many women will experience disease recurrence displaying chemoresistant lesions, with advanced OC exhibiting 5-year relative survival rate of 30.2% [43]. Table 1 summarizes chemosensitivity profiles in the context of OC subtypes.

Different mechanisms can lead to OC chemoresistance including efflux pumps, altered DNA repair mechanisms such as homologous recombination, nucleotide excision and mismatch repair, and signaling pathways deregulations [4]. To interact with these alterations and overcome chemoresistance, targeted molecular therapies were developed and investigated. To date, two have been integrated to the treatment strategy as maintenance therapy; bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), and poly (ADP-ribose) polymerase (PARP) inhibitors [44–47]. Other agents, targeting p53, PI3K/Akt, mTOR, and estrogen signaling, are being tested in clinical trials either as single agents or in the context of combination treatments [4]. Unfortunately, as of now, no targeted inhibitor that has undergone clinical trials displayed satisfying response rates against chemoresistant tumors when used as monotherapy or in combination with current therapeutic strategies.

3 Endometrial Cancer

3.1 Statistics

Endometrial cancer (EC) incidence varies among different regions of the world, with the highest incidence found in North America and Europe. Worldwide, EC is the sixth most common female malignant disorder with 382,069 cases estimated in 2018 and 89,929 related deaths [48]. In the USA, EC ranks fourth in female cancers incidence with 65,620 cases and 12,590 deaths making it the most common gynecologic malignancy [13]. This variation throughout regions is due to the fact that obesity and conditions associated with metabolic syndrome such as diabetes are risk factors for EC [49, 50]. As obesity is rising in the USA, the incidence of EC is increasing, as 57% of all cases were attributable to obesity in 2004 [51]. EC has a fairly good prognosis, 67% of tumors diagnosed at a localized stage, which is associated with a 5-year relative survival of 81.2%. Unfortunately, 9% of patients are diagnosed with metastasized cancer which has a poorer prognosis, with a 5-year survival rate of 17% [52]. Furthermore, 10–15% of patients will experience recurrence after initial surgery, an event associated with poor survival outcome [53, 54].

3.2 Histological, Morphological Subtyping, and Pathogenesis

Histologically, EC begins in the endometrium, mainly from the inner single layer of epithelial cells from the uterus (carcinoma). Cancer cells emanating from the stroma, known as sarcoma, are possible although rare. Depending on the degree of invasion and metastasis, tumors will be staged from I to IV based on the FIGO classification [55]. There are three distinct but overlapping ways to categorize EC based on their histomorphology, pathogenetic, or molecular profile. The World Health Organization (WHO) developed a classification in 2014 using the morphological characteristic of the tumors [56]. The four main histotypes are endometrioid carcinoma (85%), followed by serous carcinoma (3–10%), clear-cell carcinoma (2–3%), and carcinosarcoma (<2%) [57]. Other subtypes include mucinous, neuroendocrine, mixed, undifferentiated, and dedifferentiated carcinomas, but will not be discussed in this section of the present chapter. Table 2 summarizes the various histological and molecular characteristics of each subtype.

Another categorization used for EC was established around the pathogenesis of the disease and proposed by Bokhman in 1983 which separates endometrial carcinomas into two groups defined as type I (80–90%) and type II (10–20%) [58]. The prototypical type I tumor histotype is the endometrioid carcinoma whereas type II is the serous carcinoma, but also includes clear-cell [57]. Type I tumors are linked to obesity, hyperlipidemia, and diabetes and is predominantly estrogen-driven, generally because of estrogen-producing tumors, endogenous estrogen from adipose tissue, or estrogen hormone replacement therapy [59]. Usually, at the moment of diagnosis, patients are peri- or postmenopausal, presenting carcinomas of low stage, with high expression of estrogen and progesterone receptors; this portrait ultimately coalesce into a favorable prognosis [59]. However, high-grade endometrioid carcinomas have a poor prognosis. On the other hand, type II tumors are not typically related to estrogens and are considered hormonally independent, with low expression of estrogen and progesterone receptors. Patients also present low serum estrogens level, which is reflected by an atrophic uterus and inactive endometrium [59]. This type of carcinoma, which is responsible for 47% of EC-associated death [52], is diagnosed in older, postmenopausal women with invasive, high-grade tumors, which leads to poor prognosis [60].

3.3 Molecular Subtyping

Briefly, endometrioid carcinomas are characterized by alterations in the PI3K/PTEN/Akt pathway, *KRAS*, *CTNNB1*, and *ARID1A* gene mutations [61], with molecular aberration in the PI3K/PTEN/Akt pathway occur in 80–95% of endometrioid carcinomas [62]. *PTEN* gene mutation, which occurs at a frequency of 67–90%, is considered as an early event in the pathogenesis of endometrioid carcinoma, and is found in endometrial hyperplasia [63, 64]. Furthermore, somatic mutations in *PI3KCA* and *PIK3RI* genes often co-occur with *PTEN* which emphasize the importance of the pathway as a tumorigenesis driver [62, 65]. Regarding serous endometrial carcinomas, studies have shown somatic mutations in *TP53*, *PPP2R1A*, *FBXW7*, and *PI3KCA* with amplification of *ERBB2*, *MYC* and *CCNE1* [61, 66]. The most prominent mutation occurs in the *TP53* gene with a 85% frequency [67], an event which is also considered pathogenetic for this subtype [68]. Interestingly, 50% of high-grade endometrioid carcinomas have *TP53* mutation, a rare occurrence at low grade, and could potentially explain their poorer prognosis [67].

Although used clinically for decades, Bokhman's binary classification is now being challenged by high-throughput molecular data and large epidemiologic studies, which revealed the molecular diversity found within each histotypes [69]. In 2013, The Cancer Genome Atlas (TCGA) analyzed 373 endometrioid and serous endometrial carcinomas leading to the description of four distinct molecular subgroups [70]: *POLE* ultra-mutated, micro-

Table 2 Endometrial cancer histologic and molecular classification

Histologic subtype	Endometrioid	Grade 3 endometrioid	Serous carcinoma	Clear-cell
% of EC	85%	Unspecified	3–10%	2–3%
Bokhman's classification	Type I	Type II	Type II	Type II
Precursor lesion	Atypical endometrial hyperplasia (AEH)		Serous endometrial intraepithelial carcinoma (SEIC)	Unknown
	Endometrioid intraepithelial neoplasia (EIN)			
Mutation profile	*PTEN*	***TP53***	*TP53*	*TP53*
	PIK3CA/PIK3R1	*PTEN*	*PPP2R1A*	*PIK3CA/PIK3R1*
	KRAS	*PIK3CA/PIK3R1*	*FBXW7*	*PPP2R1A*
	CTNNB1	*KRAS*	*PI3KCA*	*KRAS*
	ARID1A	*CTNNB1*	*PTEN*	*ARID1A*
	POLE	*ARID1A*	*ERBB2* (amplification)	*CCNE1* (amplification)
		POLE	*CCNE1* (amplification)	*ERBB2* (amplification)
Characteristics	Usually hormone sensitive		Usually hormone insensitive	Usually hormone insensitive
	MSI/MMR alterations			
Prognosis	Good	Poor	Poor	Intermediate
TCGA classification	**CN-low** (60%)	**MSI** (54.3%)	**CN-high** (100%)	Unknown
	MSI (28.6%)	CN-high (19.6%)		
	POLE (6.4%)	*POLE* (17.4%)		
	CN-high (5%)	CN-low (8.7%)		

Data used to elaborate this table was extracted and interpreted from Daphne et al., 2019 review [185]

satellite instability (MSI) hypermutated, copy-number low, and copy-number high. Those subgroups were based on consideration of somatic mutation rates, microsatellite instability (MSI) status, and somatic copy-number alterations (SNCA). *POLE* ultra-mutated has a high mutation rate with somatic mutation in the exonuclease domain of *POLE* whereas MSI hypermutated has high mutation rate, MSI, and frequent MLH1 promoter hypermethylation. Copy-number low and high subgroups both present low mutation rate and copy-number alterations, with oncogenes *MYC*, *ERBB2,* and *CCNE1* being often amplified. A summary of the histotype's distribution within the TCGA molecular classification can be found in Table 2. Interestingly, while almost all serous endometrial carcinomas are classified in the copy-number high subgroup, endometrioid carcinomas are present in all four subgroups. Low-grade endometrioid carcinomas are mostly in copy-number low (60%) while high-grade endometrioid carcinomas are in MSI (54.3%) [61]. Considering that endometrioid and serous histotypes does not share the same therapeutic strategy, this new molecular classification could have relevant clinical application in the future.

3.4 Current Therapeutic Strategies

Optimal treatment strategy for EC is still the subject of deliberation. Total hysterectomy with bilateral salpingo-oophorectomy is the preferred surgery for women with stage I endometrial carcinomas and low risk of recurrence, in which case surgery alone is often sufficient [60]. However, in cases harboring high risk for recurrence, combination with adjuvant radiotherapy or chemotherapy is necessary [71]. Radiation therapy has been found effective for local control but has been reported to offer no benefit to overall survival (OS) [72]. Regarding chemotherapy, the use of doxorubicin and cisplatin improves OS in advanced-stage disease but not in high risk, early-stage patients [73]. Trials have also suggested that the addition of chemotherapy to adjuvant radiotherapy did not benefit patients with low stage cancer and high risk of recurrence, but only those presenting with advanced EC [74–76]. In case of persistent recurrence, first- and second-line chemotherapy regimen, which are comprised of platinum and taxane, with limited effectiveness regardless of platinum-sensitivity [77]. Alternatively, considering the lack of effective treatment options for systemic recurrence and its associated poor prognosis, studies exploring the effectiveness of targeted therapeutic strategies are underway for various canonically altered pathways such as HER2, p53, PI3K/Akt, PTEN, and RAS [71].

While some monotherapies show encouraging but not satisfying response rate [78–80], other combination therapies seem promising such as everolimus and letrozole [81] or the combination of trastuzumab with paclitaxel-carboplatin in HER2+ serous endometrial carcinomas [82]. Recently, using the TCGA classification, personalized targeted therapies selected on the basis of tumor's molecular subgrouping were used to treat recurrent EC, an effort that was met with a clinical benefit rate of 62.5% [83]. The routine use of molecular fingerprinting and the integration of the TCGA molecular classification show great potential for the development of targeted therapies. In order to facilitate this, ProMisE, a pragmatic molecular classification tool, was developed to facilitate the categorization of tumors based on TCGA classification system [84]; this research methodology should be considered by investigators in order to better dissect the molecular mechanisms responsible for treatment failure in these tumors.

4 Molecular Pathways Responsible for Chemoresistance in Ovarian and Endometrial Cancers and the Place of Par-4 in those Signaling Networks

4.1 The PI3K/Akt/mTOR Axis

PI3K/Akt has been shown to be a potent inducer of multidrug resistance and is widely considered as one of the most eminent nexus of cell fate decisions [85, 86]. Mutations or overactivation of this pathway is almost sine qua non of chemoresistance development within neoplastic ovarian and endometrial tissues; it is also well accepted that cancer cells can respond to chemotherapeutic insults by increasing the PI3K/Akt activity [87–92]. Briefly, PI3K phosphorylates PIP2 to PIP3, which allows the docking of Akt and PDK1 to the plasma membrane. PDK1 will subsequently phosphorylate Akt on thr308 and complete activation of Akt will be achieved upon phosphorylation of its ser473 by mTORC2. Akt will then phosphorylate its multiple downstream targets, which are almost inescapably involved in the balance of proliferative and apoptotic stimuli and have been associated with chemoresistance, such as TSC1/2, NF-κB, GSK3-β, BAD, and potentially Par-4 [85, 90, 93]. Akt, through TSC1/2 and PRAS40 phosphorylation, also regulates mTORC1 activity, which operate as the cellular linchpin of protein synthesis, growth signaling and metabolic decision-making. On the other hand, PTEN acts as the main antagonist to this signaling cascade through his phosphatase activity which dephosphorylates PIP3 to PIP2, abrogating PI3K stimulation of its downstream effectors. A more complete, in-depth review of these intricate and complex regulatory events can be found in Manning and Toker 2017 review [86]. It should also be noted that Akt presents three isoforms that display structural homology but also distinct cellular functions; while pivotal to Akt role, this will not be discussed in the chapter (see Reproduction 2014 for a review [94]).

In addition to cellular survival and proliferation, Akt is also involved in cell motility and systemic dissemination. While not directly responsible for resistance to chemotherapeutic compounds, metastatic relocalization is a fundamental driver of treatment failure and thus chemoresistance. Even though most patients diagnosed with disseminated OC will present metastasis in the peritoneal cavity, with a clear predilection for the omentum as the primary target for novel tumor foci, it has been suggested that OC is also capable of hematogenous spread [95]. Interestingly, a research team used a parabiosis model that unveiled the ability of OC cell to localize to the omentum and establish distant metastasis solely through systemic circulation [96]. The team also showed that ErbB3, an EGFR-family receptor which signals through PI3K, was hyperphosphorylated following NRG1 stimulation. This culminated in the overactivation of Akt, which was correlated to the expression of canonical EMT markers and accompanied by changes in cell polarity; these results suggest that, in that context, the PI3K/Akt axis could potentially act as the main inducer of cell motility, EMT phenotype instigator, and hematogenous dissemination vector. Strikingly, it has been shown that Par-4 is partly responsible for the ability of TGF-β to promote EMT in endometrial and cervical cancer cell lines [97]. Additionally, it has been shown that ErbB3 expression, widespread in OC, is a potent mediator of chemoresistance [98–100] and that inhibition of the ErbB3/PI3K/Akt axis sensitized OC cells to doxorubicin-mediated induction of apoptosis [101]. Very interestingly, adipocytes, which are a dominant cell type in the microenvironment of the omentum, have been shown to be potent inducers of chemoresistance through the secretion of arachidonic acid, which results in the activation of Akt and resistance to cisplatin, doxorubicin, and taxol [102].

One of the most compelling and straightforward role of Akt in chemoresistance in regard to Par-4 dynamic is that of its ability to phosphorylate Par-4 on ser249 in the rat, which induces its interaction with 14-3-3 [93]. This post-translational modification could, potentially, limit the ability of Par-4 to enter the nucleus, which would in turn hinders its apoptosis-inducing function; however, the presence and role of similar regulation mechanisms in human tissues remains to be fully investigated. Considering the overactivity of PI3K/Akt/PTEN axis in cancers, either through signaletic dysregulations or direct mutations of the pathway's components, it appears plausible that this signaling network could abrogate the ability of Par-4 to enter the nuclei; manipulation of these regulatory pathways would be an interesting direction for future investigations to take, in order to clarify the robustness of a PI3K/Akt/Par-4 axis of chemoresistance.

This posited axis is also reminiscent of the PKA/Par-4 proposed relationship, in which PKA, through its suggested enhanced activity in cancer cells, would phosphorylate Par-4 on threonine 163, which would enhance its pro-apoptotic effect once nuclear entry was completed [103]. However, the authors also underlined that Par-4 nuclear localization did not seem to depend on PKA-dependent phosphorylation of Par-4 but that the post-translational modification was necessary for successful apoptosis induction following nuclear localization. Interestingly, this effect was specific for the selected cancer cells used in the course of the experiments, an effect surmised to be dependent upon elevated levels of PKA activity. We believe that the regulated compartmentalization of Par-4 is central to its effect and the role of this dynamic nuclear translocalization will be further discussed in the following sections.

4.2 NF-kB

Nuclear factor kappa beta (NF-κB) is a family of five DNA-binding proteins (p50(NF-kB1), p52(NF-kB2), p65(RelA), RelB, and c-Rel) acting as transcription factors that act as a central stress-response mechanism, capable of regulating a multiplicity of immunogenic pathways as well as pro-survival functions, anti-apoptotic mechanisms and enhancers of cell motility, among others [104]. Briefly, NF-κB is normally sequestered in the cytoplasm by the inhibitor of NF-κB (IκB) in an inactive form, incapable of localizing to the nuclei in order to exert its expressional regulation. Phosphorylation of IκB by IκB kinase (IKK), a complex constituted of two catalytic subunits IKK-α, IKK-β, and a regulatory subunit IKK-γ, relieves NF-κB from this sequestered state and will reveal its nuclear localization signal (NLS) motif. IκB phosphorylation will induce its proteasomal degradation [105] and will allow the nuclear importation of either NF-κB hetero or homodimers, canonically RelA and p50 [106], followed by subsequent DNA binding and transcription of various target genes. Various stimuli have been described as activating IKK, with the canonical pathway being through the activation of Toll-like receptors (TLR) which culminates in the activation of immune-mediated host defense against a pathogen. It should be noted that the role of NF-κB in carcinogenesis and therapy resistance is, while uncontested [104, 107], still under intense scrutiny. Crosstalk with a variety of oncogenic pathways have been investigated [108], with evidence showing the involvement proteins such as EGFR [109], p53 [110, 111], and the PI3K/Akt/mTOR axis [112–114]; in the latter case, the possibility of a direct activation of the NF-κB pathway by PI3K/Akt has been underlined [115, 116]. Activation of the NF-κB pathway has been strongly associated with chemoresistance in a variety of cancer types [85, 117–120], especially in regard to resistance to platinum compounds [121]. While complex, this relationship is a corollary of the canonical targets of NF-κB, which include anti-apoptotic proteins such as XIAP, Bcl-2, cIAP1/2, and c-FLIP, which counteract both the intrinsic and the extrinsic pathways of apoptosis [104, 122].

One of the main manners by which Par-4 has been thought to act as a pro-apoptotic stimuli is by antagonizing the NF-κB pathway, which in turn could potentially reduce Bcl-2 expression, either through WT1 or atypical PKC [8, 123, 124]. However, an alternative inhibitory mechanism involving direct binding of Par-4 to a WT1 consensus site on Bcl-2 promoter has been put forward in endometrial cell lines [125]. On the other hand, reports from other investigators suggested that NF-κB could potentially act as an inducer of Par-4 expression in endometrial adenocarcinomas [126] and HeLa cells [97], again underlining how complex is the regulatory network surrounding Par-4. It should be noted that this article also highlighted an inverse relationship between pAkt and Par-4 levels, a negative feedback loop that we will discuss in the following sections of the chapter. Overall, we believe that further investigations are required to fully dissect the relationship between Par-4 and NF-κB in order to determine their impact on the emergence of chemoresistance in OC and EC. Alternatively, pro-apoptotic roles of NF-κB have also been described [127], such as its capacity to induce the expression death receptor protein 4 and 5 (DR4/5) promoters [128]. The same article also showed the parallel ability to upregulate Bcl-xL expression, showcasing the dualistic nature of NF-κB effect on cells, which could be explained partially by the intricate interplay of its diverse subunits, their dimerization dynamic and the resultant induced gene expression. The interplay between Par-4 and this sophisticated functionality spectrum is still unknown.

4.3 p53

Classic chemotherapeutic compounds such as cisplatin and doxorubicin exert their cytocidal activity through DNA damage and genotoxic stress. p53 acts as the sensing organ of this type of damage and is the most comprehensively described tumor suppressor to date. Canonically, p53 acts as a central node of the intrinsic apoptotic pathway [129] and is considered to be one of the most profound determinant of chemoresistance [130, 131]. Its role, function, and regulation mechanisms will not be expounded on considering the vast amount of literature that have abundantly described p53 central place in cellular homeostasis.

Interestingly, the induction of secreted Par-4, in the context of chloroquine (CQ) use, appears to be dependent on p53 [132]. However, while normal cells retain wild type p53, numerous cancer cells will present with mutated or absent p53, especially in the case of OC and recurrent EC. This, in

turn, would allow CQ to act as a secretagogue of Par-4 in normal, non-neoplastic surrounding tissues; this extracellular Par-4 could potentially induce apoptosis in cancer cells through a suggested GRP78-dependent mechanism that is entirely independent of p53 [132]. Additionally, epithelial OC generally overexpress GRP78, which is a prognostic biomarker of poor survival [133]; EC has also been shown to display GRP78 enrichment [134]. This overexpression, which is an expected response to heightened levels of protein misfolding, allows the cells to more effectively manage the large amount of aberrantly folded proteins [135], actively enabling the cell to evade apoptotic stimuli that would normally be induced by the UPR; this process has also been linked to chemoresistance in various models, with membrane localization of GRP78 being associated with therapy resistance [136]. Therefore, it is possible that GRP78, through the use of Par-4 secretagogues, could be instrumentalized in order to induce apoptosis specifically in OC and EC, thus revectoring the overexpressed GRP78 pro-survival role, which support the chemoresistant nature of the tumors, to that of a Par-4-dependent inducer of apoptosis.

4.4 Autophagy in Chemoresistance

Autophagy is a physiological process that allows the degradation of dysfunctional or damaged organelles as well as the management of misfolded proteins. Considering that some of the most potent regulators of autophagy are mTORC1, as well as the PI3K pathway, and the importance of those pathways in malignancies, it appears necessary to delve slightly deeper into that axis of cell fate regulation [137]. This recycling process is thought to have mainly evolved as a response to starvation in order to extract necessary nutrients from damaged components of the cell; demonstrations using ATG7 deletion in mice models have shown that the autophagotic apparatus is indeed necessary for glucose homeostasis under fasting conditions [138]. However, autophagy is also involved in the homeostatic regulation of cell components, either through non-selective degradation of cytoplasmic portions, or the targeted autophagy of disposable cellular waste products.

As such, autophagy has been widely considered to be an effective regulator of tumor suppression, through its ability to dispose of defective proteins and respond to cellular stressors. As such, basal level of homeostatic autophagy is considered a growth-promoting, tumor-suppressing process, first underlined by the tumorigenic effects of beclin-1 haploid suppression [139]. However, more recently, and in stark contrast to this role as a tumor suppressor, autophagy has been posited as a powerful promoter of survival, particularly in the context of the central microenvironment within solid tumors, which is characterized by hypoxia as well as nutrient and growth factors deprivation [138, 140, 141]. In that case, autophagy allows the cells to resist higher threshold of metabolic stress and the abrogation of autophagic pathways sensitize them to apoptotic insults [142]. Indeed, it has been suggested that autophagy profoundly increases the metabolic flexibility of tumors, providing whichever nutrients is necessary for tumor sustenance, especially when confronted with reduced vascular support and with increased ROS production [140]. In that sense, while the baseline level of autophagy might have fundamentally necessary homeostatic effects as well as tumor-suppressing activities, local activation of autophagic mechanisms are plausibly responsible for increased tumor plasticity, survival and enhanced resistance to chemotherapeutic compounds [143]. More specifically, some results suggest that inhibition of key autophagic pathway mediators could sensitize OC cells presenting stem-like characteristic to chemotherapeutic regimens, reducing the potential for later relapse and emergence of chemoresistant tumors [144]. Similar results were found in the context of EC, in which inhibition of autophagy promoted cisplatin sensitization [145] as well as taxane sensitivity [146]. Overall, autophagy is often construed as a driver of chemoresistance processes [147]. It should be noted that Par-4 has been linked with the induction of autophagy in glioma cell lines [148] and has been suggested to be a component of the p53-dependent induction of autophagy in various cancer cell lines [149]; alternatively, secreted Par-4 appears to induce apoptosis through a GRP78-dependent pathway [150], an effect that could potentially be autophagy-independent, or even dependent upon CQ ability to abrogate autophagy [132]. The exact role of Par-4 in the heterogenous functions of autophagy, and the translation of these functions to chemoresistance abrogation, remains to be elucidated.

5 Molecular Determinants of Par-4 Function

5.1 Role and Regulation of Nuclear Par-4

Par-4 is seldom mutated in human cancers; its function is generally considered to be inhibited by functional silencing, either through downregulation of its expression or various post-translational modifications that impedes its activity. In the context of EC, data obtained using tissue microarray (TMA) and cancer cell lines suggest that promoter hypermethylation could be responsible for Par-4 reduced expression [151]. Other studies, on the other hand, reported that hypermethylation was not responsible for Par-4 reduced expression in human EC, further confounding our understanding of the regulatory pathways at work in this tissue [126]. Observations in other tumor types, such as melanoma, failed to identify promoter hypermethylation, suggesting

that this event might be tissue dependent [152]. Alternatively, OC cells do not seem to display reduced levels of Par-4 when compared to normal tissues, also highlighting the heterogeneity of Par-4 epigenetic and transcriptional regulation [11].

Multiple studies have already suggested the ability of Par-4 to potentiate the effectiveness of various chemotherapeutic compounds and overcome chemoresistance in a variety of cellular contexts [6, 7, 153, 154]. As mentioned throughout this chapter, nuclear localization of Par-4 appears to be of paramount importance for its apoptotic activity in a large number of studied models. Primarily, phosphorylation by Akt appears to act as the primary modulator of Par-4 nuclear translocalization, which could potentially abrogate Par-4 antitumoral capabilities [93, 155] and plausibly increase its Fbxo45-dependent degradation [156]; Akt also appears to impede Par-4 activity, regardless of nucleocytoplasmic shuttling [157, 158].

The nuclear dependency of Par-4 regulation has been underlined since 2003, when El-Guendy et al. first described the core domain of Par-4 capable of inducing cell death specifically in cancer cells [159]. At the time, the authors reported a strong correlation between cells presenting nuclear Par-4 and their androgen independence; this relationship was also even more interesting considering that only cells presenting endogenous levels of Par-4 in their nuclear compartment were sensitive to Par-4 overexpression-induced apoptosis. Moreover, transfection of the core domain with an intact NLS2 nuclear-import sequence (137–195 construct) induced apoptosis in all tested cell lines irrespective of their hormonal requirements, with the notable exception of immortalized and normal cells which did not respond to the 137–195 protein construct. This core domain, since named SAC (selective for apoptosis in cancer cells), is still under intense scrutiny as its exact nature and mechanism of action is still incompletely elucidated. This experiment underlined two important findings that should be explicit, which are that Par-4 core domain must be in the nuclei in order to induce apoptosis and that the leucine zipper domain is not necessary for that to occur. The same study even suggested that leucine zipper removal, through ablation of the C-terminal component of the protein, stimulated nuclear entry of the protein constructs. Other reports, in prostate models, have also highlighted the importance of the central portion of the protein, potentially the SAC domain, for Par-4 mediation of AR-transcriptional regulation [160].

Limited amount of direct data, however, is available in the context of gynecological malignancies; nonetheless, some evidence does shed some light on Par-4 role in these tissues. Par-4 overexpression sensitized SKOV-3 cells, an OC cell line, to taxol, an effect that was correlated to nuclear entry of Par-4 but did not depend on Par-4 secretion and Par-4 binding to GRP78 [11]. Alternatively, reports from experiments performed in EC samples showed that almost 40% of the examined tumors were negative for Par-4, with more than 90% of the Par-4-positive tumors presenting a complete nuclear exclusion pattern of localization for the protein, underlining the potential importance of Par-4 expulsion from the nuclei in EC [151].

CRM1, also known as exportin-1 (XPO1), is a protein belonging to the RanGTP transporter superfamily. The primary function of this type of protein shuttle is to regulate RNA, proteins, and RNPs transit through the nuclear pore in order to extrude these molecules from the nuclear to the cytoplasmic compartment [161]. The presence of a nuclear export signal (NES) allows the interaction between CRM1 and its cargo; a $\Phi1\text{-}X_{2,3}\text{-}\Phi2\text{-}[\hat{}W]_3\text{-}\Phi3\text{-}[\hat{}W]\text{-}\Phi4$ motif has been identified as a strong predictor of protein–CRM1 interaction [162]. Such a sequence is found on Par-4 and has been validated experimentally and appears to be masked upon potential Par-4 homodimerization, which would impede NES recognition by CRM1 [163]. We suggest that unbalanced shuttling and nuclear export of Par-4, either through its inability to homodimerize in the nucleus or through upregulation of its potential nuclear exporter CRM1, could be a putative mechanism of resistance. Interestingly, data using small molecule inhibitor of nuclear export (SINE), which blockade CRM1 action, have been found to enrich pancreatic cancer cells nuclear compartment with Par-4 [164]; whether that translates to other models, however, remains unclear. Taken together, we believe that the literature supports the importance of Par-4 nuclear localization to exert its antitumoral effect; intervention capable of inducing nuclear localization of either full length or the 25 kDa C-terminal fragment generated by Par-4 cleavage, which will be discussed in the next section, should be investigated. However, it appears critical to first determine whether abnormally increased Par-4 nuclear export, failure to import, or a combination of both, operate as a resistance mechanism in OC and EC.

5.2 Post-Translational Regulation of Par-4

As mentioned earlier, the low incidence of mutation and deletion of the Par-4 gene makes it an attractive target for therapeutic intervention. Instrumentalizing the protein mechanism of action, either through manipulation of its nuclear localization or protein partners, is a potential avenue if Par-4 is to be leveraged in the context of chemoresistant gynecological cancers. Multiple mechanistic theories explain the capacity of Par-4 to induce apoptosis specifically in cancer cells, either through PKA-dependent potentiation of nuclear Par-4 functions, or through nuclear entry of the SAC domain, with reports also underlining the non-importance [159] as well as the concurrent importance [165] and antagonistic action of the leucine zipper [166]. Alternatively, Par-4

is suggested to function through extracellular pathways involving GRP78, acting both as a transporter complex, allowing plasma membrane localization of GRP78, and as a ligand, inducing caspase-8-mediated cell death [167]. As expounded on in previous sections, multiple post-translational events regulate Par-4 localization and activity. However, we believe that two regulatory pathways require further description as they potentially could define functional specificities of Par-4 in EC and OC and provide avenues of investigation in order to fully decipher Par-4 convoluted network of regulation.

First, evidence has suggested that Par-4 can be cleaved by caspase-3 at EEPD^{131}↓G in the context of apoptosis [168]. This 25 kDa truncated form of Par-4, which we will refer to as cleaved Par-4/25, represents the protein's C-terminal component and can be observed in a multiplicity of cell lines following cisplatin treatment. Moreover, cleaved Par-4/25 localized to the nucleus and appeared to be capable of sensitizing cells to chemotherapeutic agents, and potentially induce apoptosis directly, an effect that was abrogated upon mutation of the caspase cleavage site (Asp to Ala, D131A). Cleaved Par-4/25 is also observable in the context of TRAIL-induced apoptosis in a plethora of OC and EC cell lines [169], and the necessity of this cleavage event for the induction of Par-4-dependent apoptosis has since been evidenced in other cell lines [157, 170]. Nuclear importation of cleaved Par-4/25 has been suggested to be dependent on RASSF2 in prostatic cancers cells [171], an effect that has yet to be replicated in gynecological tissues. Interestingly, because of the location of the caspase cleavage site, cleaved Par-4/25 retains both of its crucial phosphorylation site, thr163 and ser249, respectively, targeted by PKA and Akt1, as seen in Fig. 2.

The role of Par-4 cleavage was also expounded on by the identification of an amino-terminal product of caspase-3-dependent cleavage capable of inducing apoptosis in a paracrine fashion. Par-4 amino-terminal fragment, or PAF, is produced by sensitive cells undergoing apoptosis; the fragment, which contains the VASA domain and is targeted by Fbxo45 for subsequent proteasomal degradation [156, 172], also act as a natural decoy, protecting Par-4 from degradation and potentiating resistant cells entry into the apoptotic program. The presence and function of PAF in the context of chemoresistant OC and EC is, however, uncharacterized. Alternatively, the function of the VASA/SPRY domain in these tissular contexts is also yet to be clarified. Further investigations are required to determine whether PAF is capable of stabilizing full-length Par-4 through its activity as a secreted decoy protein. If that is the case, the ways by which PAF could be leveraged to overcome chemoresistance would be diverse; potentially, increased cleavage of Par-4 would yield more PAF, which would in turn stabilize Par-4 and potentiate its activity. Alternatively, identification of PAF secretagogues, as well as a better understanding of PAF uptake mechanisms by resistant cells, would be an attractive therapeutic avenue. The C-terminal cleaved Par-4/25 produced by caspase-3 cleavage could conceivably be protected from Fbxo45-induced degradation, partially explaining the fragment enhanced death-inducing capabilities. However, it is our opinion that in order to fully mobilize Par-4 antitumoral potential in the context of chemoresistant OC and EC,

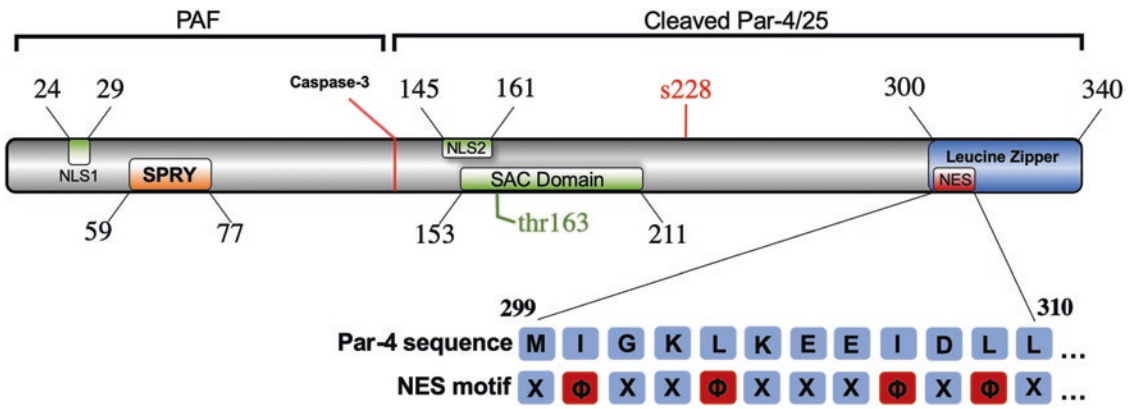

Fig. 2 Schematic representation of the major functional domains and post-translational modifications of Par-4. Briefly: the SPRY domain, potentially acts as a protein–protein interaction domain and the target for Fbxo45-dependent ubiquitination; the second nuclear localization signal (NLS) domain allows nuclear entry of the protein; the specific for apoptosis in cancer cells domain (SAC), necessary for Par-4 pro-apoptotic function; thr163, phosphorylated by PKA, potentiate Par-4 apoptosis-inducing capabilities once nuclear entry has been achieved; s228, which is potentially phosphorylated by Akt, could blockade nuclear entry of Par-4 in a similar fashion to s249 in the rat, although further experimental demonstrations are required; the putative nuclear export signal (NES), which could mediate nuclear export of Par-4 through CRM1-dependent shuttling; the leucine zipper, which could act as a mediator, regulator or inhibitor of Par-4 activity. Caspase-3 cleavage site at EEPD131↓G. The Par-4 amino-terminal fragment (PAF), which corresponds to a.a 1–131, which could act as a decoy to stabilize Par-4 accumulation and escape degradation; the cleaved Par-4/25 fragment, which corresponds to a.a 132–340, which could induce apoptosis following nuclear entry

another post-translational regulatory network must be considered.

As previously mentioned, PI3K/Akt is one of the most frequently altered signaling pathways in all cancers and is especially important in gynecological malignancies. If nuclear localization of Par-4 is necessary for the induction of apoptosis in these neoplastic entities, first and foremost, the inhibition of Akt-dependent cytoplasmic sequestration must be investigated. Akt has been shown to act as a potentiator of cleaved Par-4/25 destabilization, a negative regulation that appears to be dependent on proteasomal degradation. Additionally, cleaved Par-4/25 levels are stabilized by cisplatin, an effect that is robustly enhanced by the concomitant use of PI3K/Akt inhibitors [158]. We are allowed to think that stabilizing cleaved Par-4/25, which is inherently structurally immune to Fxbo45, through the modulation of the PI3K/Akt signaling pathway represents an attractive therapeutic strategy for chemoresistant OC and EC. Finally, XIAP has been shown to negatively influence Par-4 cleavage [97]. Its functions are well characterized, acting as a potent inducer of chemoresistance in OC and EC [173, 174] which directly inhibits caspases through its BIR domains [175] but also as an E3-ligase for various targets, namely PTEN, through its RING domain [176]. XIAP role in the regulation of cleaved Par-4/25 stability, however, remains unclear. Ultimately, because of its ability to induce resistance to TRAIL, cisplatin, and doxorubicin [4, 173, 174, 177], and the multilayered relationship it appears to have with Par-4, we believe that XIAP plays a central role in a potential Par-4-dependent chemosensitization therapeutic strategy.

6 Conclusion

6.1 The Functional Pleiotropy of Par-4

Sells et al. first investigated the role of the leucine zipper found at the C-terminal of Par-4 in AT-3 and PC-3 cells, both androgen-independent cell lines from rat and human origin, respectively; A375-C6 cells were also used, which are a human melanoma cell line. The experiments suggested that Par-4 was dependent upon its leucine zipper in order to induce apoptotic sensitization to thapsigargin. Moreover, and very interestingly, the authors demonstrated that co-expression of both full-length Par-4 as well as the leucine zipper domain in PC-3 cells abrogated the ability of Par-4 to induce apoptotic cell death [166]. The authors interpreted this observation through two possible mechanism, either competitive binding of the overexpressed leucine zipper with potential Par-4 partners, namely WT1, or through homodimerization between the intact Par-4 protein and the separately overexpressed leucine zipper domain. Six years later, El-Guendy et al. would demonstrate that Par-4 did not require its leucine zipper domain to induce apoptosis and that the core SAC domain alone was sufficient for induction of apoptosis in cancer cells [159]. That effect, as mentioned previously, was mediated by nuclear translocation of Par-4 and inhibition of NF-κB transcriptional activity. These observations, which are considered cornerstones of the Par-4 literature, allow us to think that Par-4 functional mechanisms still require investigations; this is especially true for models in which Par-4 regulation hasn't been as comprehensively studied as others, such as OC and EC cells.

Some avenues, however, are compelling if we are to decipher the seemingly conflicting conclusions that have emerged from the literature so far. Interestingly, reports have underlined the conformational heterogeneity and flexibility of the leucine zipper domain of Par-4 which could modulate its DNA-binding specificity as well as alter its intracellular binding partners; these findings suggest that some of the discordant findings regarding Par-4 function, as well as the effect of its localization and binding partners, could be dependent on cellular context and leucine zipper domain folding [178]. Binding of Par-4 with a potential isoform (Par-4/p33) has also been described as a negative regulator of nuclear import and apoptosis induction, highlighting the potential for Par-4 homodimerization and potential auto-regulation [179]. Direct demonstration of that homodimerization was also obtained in prostatic cell lines [160]; however, the role and effects of this multimerization remains unknown. Whether this isoform plays a role in the context of human pathologies is still unclear; the question regarding Par-4 homodimerization, and the subsequent effect of such an event on nuclear localization, remains also unanswered.

Influence of various pathways also seem to be tissue-dependent. The discovery that Par-4 is upregulated by TGF-β and acts as a direct inducer of EMT in HeLa cells [97] emphasize that factors that are still improperly understood allows Par-4 to act as a driver of cell transformation in some cell types, rather than an inducer of cell death, a result that echoes findings reported by other authors underlining atypical regulation and function of Par-4 [126, 180]. XIAP, an inhibitor of both caspases 3 and 8 [175], has been suggested as a regulator of Par-4 role, a regulatory relationship in which XIAP activity inhibits Par-4 cleavage and supports its pro-EMT functions [97]. However, no causal relationship between Par-4 cleavage and EMT induction has been established so far and the exact mechanism by which XIAP regulates Par-4 function remains to be elucidated.

7 Future Perspective

7.1 Deciphering the Role of Par-4 in Chemoresistance of OC and EC Cells

Altogether, it appears clear that Par-4 multifaceted, intricate molecular regulatory events, may it be post-translational modification such as phosphorylation events, nuclear localization as well as DNA and protein binding, could all be conceivably construed as potential mechanisms of functional silencing. Reports are still contrasting, with stark discrepancies, as well as similitudes, emerging between models and tissue types. We believe that further investigations should be centered on the role of nuclear Par-4, the identity of Par-4 proteic partners in both the cytoplasmic and nuclear compartment, the regulation mechanisms and cellular roles of cleaved Par-4/25, and the involvement of the PI3K/Akt axis in those events, especially in regard of Par-4 intracellular localization and stabilization. The information extracted from such research endeavors are of paramount importance if Par-4 is to be instrumentalized in the treatment of chemoresistant gynecological cancers.

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Regulation and Role of Par-4 in Gastrointestinal Tumors

Rosalyn B. Irby, Christina Leah B. Kline, and Arun K. Sharma

Abstract

Cancers of the gastrointestinal (GI) tract are the second leading cause of cancer deaths worldwide for both sexes. These include esophageal cancers, gastric cancers, cholangiocarcinomas, cancers of the small bowel, and colorectal cancer. Of these, colorectal cancers account for almost half of the cancers of the GI tract. In a number of these cancers, Par-4 has been shown to play a negative role in the progression of cancer. Higher Par-4 activity has been shown to indicate a better prognosis, as, when activated, it causes apoptosis in the cancer cells, both locally and distally. However, many factors affect both the expression and the activity of Par-4 in GI cancer cells. This chapter details what is known about the role and regulation of Par-4 in tumors of the GI tract.

Keywords

Gastrointestinal cancer · Colorectal cancer · Oral cancer · Cholangiocarcinoma · Par-4 · Therapy

1 Background

Cancers of the gastrointestinal (GI) tract are the second leading cause of cancer deaths worldwide for both sexes. These include esophageal cancers, gastric cancers, cholangiocarcinomas, cancers of the small bowel, and colorectal cancer. Colorectal cancers account for almost half of the cancers diagnosed in this category (Table 1) [1].

While cancers of the individual organs of the GI tract have unique risk factors, treatments, and molecular signatures, they have a number of features in common. As with other cancers, GI cancers often arise as a result of inflammation and the cellular responses to it. The GI tract is closely connected with the outside environment and, as such, is at risk for the invasion of pathogens directly into the lumen. Cancers of the GI tract have been noted often as being related to specific pathogenic organisms from the environment. For example, HPV is often associated with oropharyngeal cancer [69] and one of the risk factors of gastric cancer is *H. pylori* [70]. Colon cancers arising in the setting of inflammatory bowel disease are thought to be a combination of genetics and environmental factors.

GI cancers, as detailed below, often result from and/or are exacerbated by inflammatory response to the pathogens. In addition, there are a number of shared pathways involved in tumorigenesis between individual cancers that occur within, and outside of, the GI tract. These include, but are not limited to, MAPK, receptor tyrosine kinases, intracellular kinases, and the JAK/STAT pathway [2–6]. One of these pathways is the PI3K/Akt pathway. Prostate apoptosis response-4 (Par-4), the produce of the *PAWR* gene, has been shown to be regulated by Akt1 [7], TNFα and IFNγ [71], and Src [8], among others. These pathways are activated in many cancers, portending the importance of Par-4 in tumor development and progression.

The presence of *PAWR* mutations in GI cancers has been investigated to help in the understanding of the role of the Par-4 protein in these cancers. In the COSMIC database [72], 8% of stomach cancers analyzed showed an overexpression of Par-4. Focusing on *PAWR* mutations, esophageal and colon (large intestine) cancers are two of the top 10 cancers which have the highest preponderance of point mutations (1.57% and 1.19% of samples analyzed had point mutations) in the *PAWR* gene. Out of 14 major cancer types, cancers of the colon (along with lung) have the highest percentage of non-synonymous mutations (21%) [9]. The mutations that have been identified in different GI cancers are listed in Table 2. Out of the *PAWR* mutations in the COSMIC database, the R243Q mutation is one of the most common mutations that has been identified, based on the number of samples that contained a particular mutation. Out of the four samples

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Table 1 Specific Sites and Case Numbers of Gastrointestinal Tumors

Tumor site	Estimated cases	Estimated deaths	% GI Cancers	% Total cancers
Oral cavity and pharynx	53,260	10,750	16.17173741	2.948095583
Esophagus	18,440	16,170	5.599076942	1.020707521
Stomach	27,600	11,010	8.380397158	1.527740107
Small intestine	11,110	1700	3.373413494	0.614970746
Colon	104,610	52,200	31.76352705	5.790467123
Rectum and anus	51,930	1350	15.76789944	2.874476223
Liver, intrahepatic bile duct	42,810	30,160	12.99872472	2.369657753
Gallbladder and biliary	11,980	4090	3.637578187	0.663127771
Other digestive organs	7600	3060	2.307645594	0.420682058
Totals	**329,340**	**130,490**	**100**	**18**

Table 2 Mutations Found in Gastrointestinal Cancers

Mutation; Amino acid change	Cancer type	Mutation type	Par-4 protein domain affected
c.489C>T; p.T163T	Large intestine; colon	Substitution—coding silent	SAC
c.71A>G; p.K24R	Large intestine; colon		Substitution—missense
c.863G>A; p.R288K	Large intestine; rectum	Substitution—Missense	DD
c.809delA; p.K270fs*10	Large intestine; colon		Deletion—frameshift
c.937-2A>G; p.?;	Esophagus; lower third		Unknown
c.728G>A; p.R243Q	Large intestine; colon and rectum	Substitution—Missense	DD
c.909C>G; p.L303L	Esophagus; middle third	Substitution—coding silent	LZ
c.33C>T; p.G11G;	Large intestine		Substitution—coding silent
c.597T>A; p.I199I;	Large intestine; colon	Substitution—coding silent	SAC
c.403G>C; p.E135Q;	Esophagus; middle third		Substitution—Missense

where this mutation was identified, three were colorectal cancer samples. This mutation is predicted to be pathogenic using the FATHMM-MKL algorithm [73].

2 Colorectal Cancer

2.1 Background

Colorectal cancers are diagnosed primarily through direct viewing by colonoscopy or sigmoidoscopy, DNA assessment in stool samples, and observance of blood, either occult or frank, in stools. Periodic screening often identifies the presence of sporadic cancers or of precancerous polyps (See Fig. 1). Cancers arising secondary to inflammatory bowel disease are more difficult to diagnose, as they arise from dysplasias rather than observable polyps and can be missed on colonoscopic surveillance. Often these cancers can be prevented by removal of the entire colon, i.e., a complete colectomy.

Cancers of the colon and rectum remain the second leading cause of cancer-related death worldwide [1] despite the expanding number of targets for therapy [10]. The 5-year survival rate for patients with colorectal cancer is 64% although for patients who present with distant metastases at diagnosis, the 5-year survival rate is only 14% [1].

Colorectal cancer is more prevalent in developed countries, and the incidence of this cancer will likely increase as the population ages [1]. However, death from colorectal cancer has been decreasing in developed countries, likely as a result of increased screening resulting in early detection and intervention and from more advanced treatments. Developing countries, on the other hand, show rising rates of incidence and death as more people adopt the lifestyle and eating habits of the Western world, and they are challenged by the limitations of their healthcare systems [11].

In the United States, a distinct disparity exists in the incidence of colorectal cancer. While cancer at any age is a larger problem in rural and poorer communities, it was recently reported that the development of colorectal cancer in younger people, those under the age of 50, is also rising, particularly in certain areas of the country [12]. The hotspots were once in the Northeast states, but not the hotspots in states in the south and the Midwest, likely as a result of an increase in rural populations and those living in poverty. The inhabitants of these areas have a higher incidence of obesity and smoking, and a lower incidence of physical activity, access to proper diets, lower education status, and have a limited access to physicians for both surveillance and treatments, all risk factors for the development of colorectal cancer. In addition, the population in this demographic with the highest mortality rate is non-Hispanic Blacks in comparison with

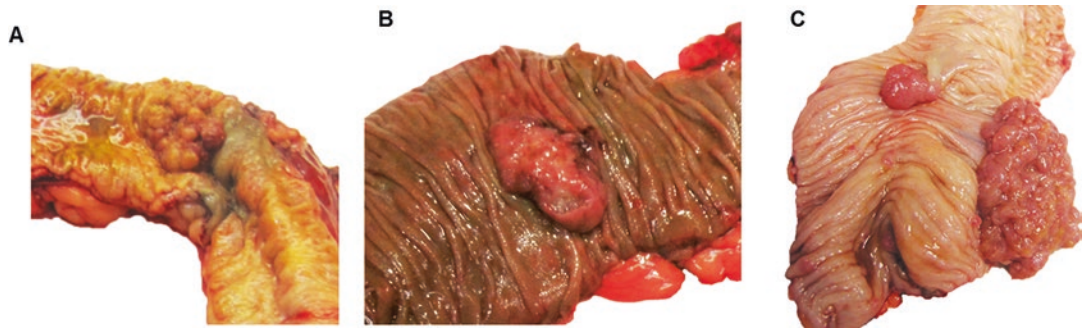

Fig. 1 Cancers of the colon. A. Right colon. B. Left colon. C. Rectal cancer with polyposis. Images are the kind gift of Dr. Walter Koltun

patients across all ethnic groups matched for age of onset and stage of cancer.

Typically, colorectal cancers develop from polyps, either adenomatous polyps which then progress to adenomas and carcinomas, or sessile polyps. While colorectal cancers can have a genetic component, only about 4–6% are hereditary and 30–40% are familial. The majority of these cancers, 50–60%, are sporadic.

2.2 Current Treatment of Colorectal Cancers

Once diagnosed, treatment is typically surgery with adjuvant drug and/or radiation therapy. Drug therapies, including chemotherapy, can be a single drug or a cocktail of drugs that treat cancer systemically and often have adverse effects on normal dividing cells. Common chemotherapeutic drugs used for colorectal cancer include 5-fluorouracil (and its oral prodrug form, capecitabine) irinotecan, and oxaliplatin. Although much research has been done in developing drugs against different colorectal cancer signaling pathways, the only FDA-approved targeted drugs primarily inhibit the epidermal growth factor receptor and the vascular endothelial growth factor receptor pathways [10]. Immunotherapy is an emerging therapeutic strategy where the body's immune system is mobilized to fight the cancer. Antibodies against the immune checkpoint protein programmed cell death-1 (PD-1) and its ligand (PDL-1), pembrolizumab and nivolumab, are FDA-approved for treatment of a subset of colorectal cancer patients, those with deficient mismatch repair. However, despite clinical benefits, the use of immune checkpoint blockade antibodies is associated with a unique spectrum of side effects termed immune-related adverse events (irAEs). IrAEs include dermatologic, gastrointestinal, hepatic, endocrine, and other inflammatory events [13]. Each of the therapies has drawbacks and failure rates, and new treatment strategies are constantly being explored.

The ability of colon cancer cells to resist apoptosis is correlated with chemoresistance to the chemotherapeutics 5-fluorouracil and oxaliplatin [14]. There is also evidence that as colon cancer cells metastasize, they acquire additional apoptosis resistance characteristics. In addition to a decrease in the Fas receptor of the extrinsic pathway [15–17], expression of the intrinsic apoptotic pathway protein Apaf-1 is also reduced. Moreover, expression of two members of the inhibitor of apoptosis family, survivin and XIAP is elevated [15, 17]. The downregulation of pro-apoptotic proteins and upregulation of anti-apoptotic proteins in colon cancer result in resistance to apoptosis.

Different strategies have been employed to induce apoptosis in colon cancer cells. One is to leverage pro-apoptotic proteins for therapy, an example of which is TNF-related apoptosis-inducing ligand (TRAIL/Apo2L). Because of its cancer-selectivity, much effort has been put forth to engage the TRAIL signaling pathway to induce cancer killing. Unfortunately, at this point, clinical benefits have not been realized using TRAIL or similar molecules called TRAIL receptor agonists [18].

Given the body of work that has shown that Par-4 is involved in apoptosis induction in different contexts, research efforts have pursued the long-term goal of developing treatment regimens for colon cancer that exploit the pro-apoptotic activity of Par-4. This chapter details what has been learned about how Par-4 functions in colorectal cancer and how this knowledge can be leveraged to modify the activity of Par-4 to treat this disease.

2.3 Regulation of Par-4 Expression in Colorectal Tumors

Par-4 expression was first reported in colon cancer, specifically, in HCA-7 colon carcinoma cells treated with cyclooxygenase (COX) inhibitors/nonsteroidal anti-inflammatory agents (NSAID's). Although the COX inhibitors induced apoptosis and Par-4 expression, the link between the observed

cell death and Par-4 expression was not established [19]. Par-4 is expressed in normal colon [20] and by colon cancer cells [21, 22]. However, *PAWR* gene expression has been shown to be downregulated in colon cancer tissue [22]. Moreover, the levels of Par-4 expression in colon tumors clearly indicated a prognosis, as the Par-4 levels showed a relationship to survival. Our ongoing unpublished studies have shown that patients having tumors with significantly reduced to undetectable levels of Par-4 succumbed to the disease within 5 years, while those with Par-4 levels close to those of matched normal tissue were living 10 years later.

Par-4 gene expression can be downregulated through different mechanisms. Par-4 downregulation can occur as a result of promoter methylation, as has been observed in endometrial and lung cancers [23, 24]. Oncogenic Ras can increase the methylation of the *Par-4* promoter [25]. Another oncogenic protein, v-Src, has also been shown to increase the methylation of the *Par*-4 promoter in fibroblasts. V-Src was shown to increase the expression of DNA methyltransferase 1, and consequently, *PAWR* promoter hypermethylation [8].

A second mechanism of Par-4 downregulation is through the E3 lyase, TRIM21. TRIM21 targets proteins for degradation, through the proteasome pathway. Although TRIM21 does not constitutively regulate Par-4 levels alone, cisplatin treatment induces TRIM21/Par-4 interaction through the SPRY domain of TRIM21, resulting in Par-4 degradation [26].

2.4 Regulation of Par-4 Activity in Colorectal Tumors

Par-4 is endogenously expressed in colon cancer [21], albeit at lower levels than normal tissue, with respect to mRNA expression [22]; however, it does not always cause apoptosis. This strongly suggests that it is endogenously inactivated in colon cancer. The activity of Par-4 is dependent on its cellular localization. Cells that have endogenous or ectopic Par-4 in the cytoplasm are not susceptible to Par-4-mediated apoptosis. Par-4 overexpression has to be combined with another apoptotic stimulus to increase cell death in these cells. On the other hand, other cell types have Par-4 in their nuclei. These undergo cell death when Par-4 is overexpressed [27].

Immunocytochemical and immunoprecipitation experiments show that Par-4 is detected in both the cytoplasm and nucleus of different colorectal cancer cells [21, 22, 28]. The phosphorylation of Par-4 by Akt at a serine 230 site (serine 249 in rat sequence) promotes an interaction of Par-4 with the scaffolding protein 14-3-3, and sequestration of Par-4 in the cytoplasm in other cancer types [7]. We confirmed that Par-4 interacts with Akt1 and 14-3-3 in colorectal cancer cells. In HT29 colon cancer cells, Par-4 endogenously associates with the sigma isoform of 14-3-3 [28]. This isoform is expressed in epithelial cells [29] and is mainly localized in the cytoplasm [30]. However, downregulation of the interaction of Par-4 with 14-3-3σ [28], or of Akt1 levels has not been sufficient to induce mobilization of Par-4 into the nucleus [22]. Instead, Akt1 downregulation has been shown to result in the interaction of Par-4 with the p65 and p50 subunits of NF-κB [22]. Figure 2 shows elements involved in the regulation of Par-4.

A significant relationship between Src and Par-4 may exist in colorectal cancer that expands beyond the promoter methylation activity. Src is upregulated early in colon cancer development, showing increased levels as early as the adenomatous polyp stage. Src levels increase progressively as carcinomas develop, and the activity of Src increases further with metastatic lesions [2, 31]. As mentioned above, Src activity can reduce Par-4 expression (Sung). However, Lee et al showed that Src increases translocation of the endoplasmic reticulum chaperone, GRP78, to the cell surface [32]. As this protein also moves Par-4 to the surface for export out of the cell and serves as a docking site for external Par-4, this has the potential to enhance the ability of Par-4 to bind the outer cell surface and initiate the TRAIL (Tumor necrosis factor (TNF)-related apoptosis-inducing ligand) pathway of apoptosis, the extrinsic apoptotic pathway**.** This conundrum may be explained by the finding that CD109, which also interacts with GRP78, may provide interference with the ability of Par-4 to bind; yet, in the colon cancer cell line tested, HCT116, GRP78 was Src independent, and, thus, Src may not affect the activity of Par-4 through its interaction with GRP78 in all colon cancers.

TNFα and IFN-γ may play a role in the activation of the extrinsic apoptotic pathway by Par-4. First, SW620 colorectal cancer cells are resistant to TRAIL-mediated apoptosis. Even treatment using TNFα and IFN-γ separately does not induce apoptosis, but the two together sensitize these cells to TRAIL-mediated apoptosis. It was found that one mechanism for this effect was the downregulation of pro-survival proteins, surviving and Bcl-XL [33]. Next, it was shown that TNFα and IFN-γ together upregulate Par-4, causing apoptosis in human neuroblastoma cells [33]. Together these findings suggest that the effects of TNFα and IFN-γ in colorectal cancer cells induce the upregulation of Par-4, which also is involved in downregulation of pro-survival genes.

Although transient expression of the protein in colorectal cancer cell lines reduces proliferation [21, 34, 35], stable overexpression of Par-4 has not been sufficient to induce apoptosis, as assessed by PARP cleavage [21, 26]. Nevertheless, Par-4 overexpression sensitizes cells to apoptosis in response to 5-fluorouracil [21], and an Akt inhibitor ISC-4 [16, 36]. The presence of substantial Par-4 expression levels in colorectal tumors has been shown to indicate a posi-

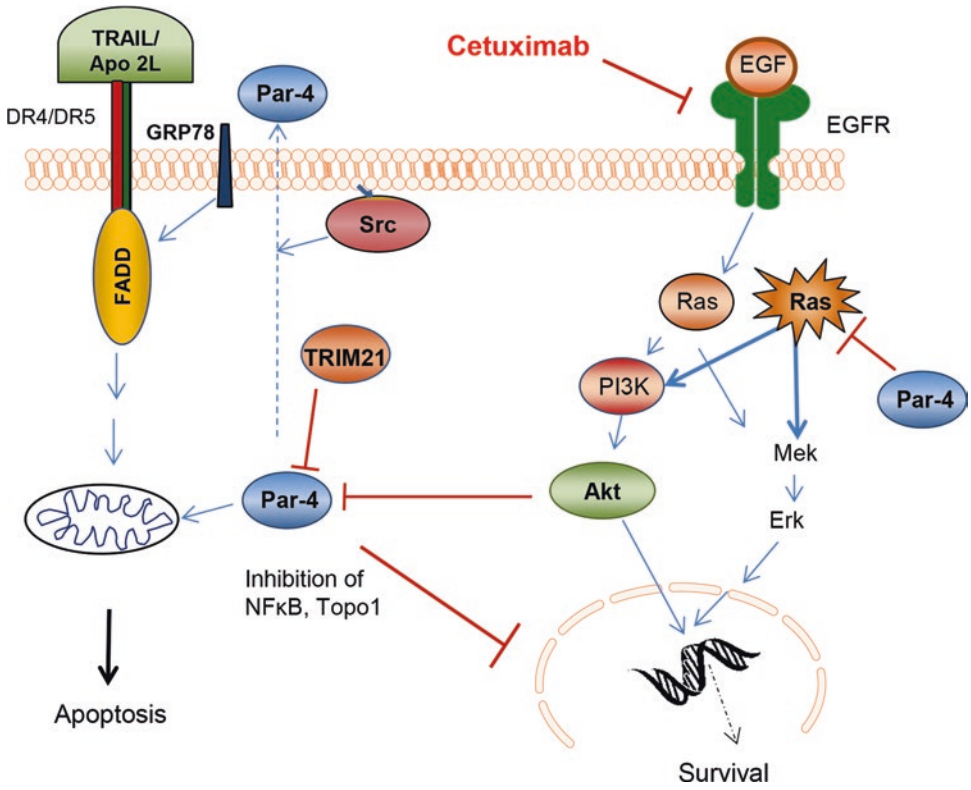

Fig. 2 Schematic of Par-4 regulation in colorectal cancers

Table 3 US FDA-approved drugs used in the treatment of colorectal cancer

Drug	Mechanism of action
5-fluorouracil (5-FU) and its oral prodrug form, capecitabine	Inhibition of DNA synthesis, induction of DNA strand breakage, disruption of RNA metabolism, as a result of thymidylate synthase (TS) inhibition
Leucovorin	Increases efficacy of 5-FU by stabilizing binding of 5-FU metabolite to TS
Oxaliplatin	Apoptosis resulting from DNA adduct formation
Irinotecan	Inhibition of DNA replication, and transcription, as a result of DNA topoisomerase I inhibition
Cetuximab and panitumumab	Inhibition of the epidermal growth factor receptor
Bevacizumab	Inhibition of the vascular endothelial growth factor
Ramucirumab	Inhibition of the vascular endothelial growth factor receptor (VEGFR) 2
Ziv-aflibercept	Sequestration of VEGF by functioning as a soluble decoy receptor
Regorafenib	Inhibition of VEGFRs 2 and 3, receptor tyrosine kinases RET, KIT, and PDGFR, and the serine/threonine-specific Raf kinase
Ipilimumab	Inhibition of the immune checkpoint protein CTLA-4
Pembrolizumab, nivolumab	Inhibition of the immune checkpoint protein PD-1

tive prognosis. Thus, the presence of Par-4 protein may well enhance the anti-tumor effects of commonly used FDA approved drugs used in the treatment of colorectal cancer. Data taken from the NCI website. (Table 3) [74].

2.5 Par-4 as a Target for Treatment of Colorectal Cancers

Par-4 has been shown to play a role in treatment response to chemotherapy [37] and radiation [38] suggesting that Par-4 may be an attractive target for treatment. One potential strategy involves the inhibition of proteins that downregulate the apoptotic activity of Par-4. Inhibition of Akt1 has shown promise as a therapeutic agent (Fig. 2) [22, 36]. Inhibition of Akt has resulted in slower tumor growth, tumor size regression, and complete resolution of tumors that overexpress Par-4 in a mouse model of colorectal cancer [22].

A second treatment modality involves increasing the expression of Par-4 in cancer cells. Increased expression of the protein in colorectal cancer cell lines has been shown to reduce proliferation [21, 34, 35]. Although stable expression of Par-4 has not been sufficient to induce apoptosis as assessed by PARP cleavage, [21, 26], Par-4 overexpression sensitizes cells to apoptosis in response to 5-fluorouracil [21], and an Akt inhibitor ISC-4 [36]. In the clinic, Par-4 overexpression can potentially be facilitated using gene therapy.

Although currently this therapy is not being performed in colorectal tumors, several methods of in vivo gene delivery exist, including viral vector gene delivery, electroporation of visualized tumors such as rectal tumors, and nanoparticle delivery of plasmid DNA carrying the Par-4 gene. Different strategies have been explored to take us closer to adopting Par-4-based therapy in the clinic to treat GI tumors. Given the lower levels of Par-4 in colorectal cancer cells, treatments that can increase expression of Par-4 may be effective. Par-4 cDNA can be encapsulated in nanoliposomes. These Par-4 containing nanoliposomes effectively reduce tumor cell growth in vitro and can be delivered intratumorally and reduce tumor growth in vivo [21].

3 Par-4 in Cholangiocarcinoma

3.1 Background

Cholangiocarcinomas are a diverse group of epithelial diseases arising from cells lining the biliary tract, both intrahepatic and extrahepatic [39–41] (See Fig. 3). These are typically considered to be sporadic although a number of risk factors have been determined. Most of the risk factors involve inflammatory issues leading to cell proliferation and, ultimately, to genetic mutations in both proto-oncogenes and tumor suppressor genes [39].

3.2 Expression and Activity of Par-4 in Cholangiocarcinoma

It has been shown that Par-4 protein and mRNA are downregulated in cholangiocarcinoma. While Par-4 is expressed in both hepatocytes and cholangiocytes, this expression is significantly decreased in cholangiocarcinoma cells and cell lines, down to as low as 5% of normal cholangiocyte expression [42]. The reduction in Par-4 expression is accompanied by a concomitant decrease in PTEN expression and an increase in the anti-apoptotic protein, BCL2, and in NF-κB expression. Furthermore, in vitro studies on cholangiocarcinoma cells showed that when apoptotic stimuli were applied to the cells, Par-4 increased, as has been shown in other cancers. Apoptosis induced by both mitochondrial and membrane pathways causes an increase in Par-4 expression, an increase in the pro-apoptotic protein Bax, and an increase in apoptosis. Conversely, a reduction in Par-4 expression results in an increase in proliferation and a concomitant decrease in caspase 3 and 8 activity. Finally, Par-4 is present in both the nucleus and the cytoplasm of normal cholangiocytes, so its regulation for maintaining a normal apoptotic/proliferative state occurs on multiple levels.

4 Par-4 in Stomach Cancer

4.1 Background

Gastric cancer, cancer of the stomach, is a significant cause of cancer-related deaths worldwide. First symptoms of gastric cancers are often weight loss and nausea, which may be ignored by patients, resulting in a late diagnosis of these tumors. While the incidence of gastric cancer is declining as a result of better surveillance and treatments, there remain areas of high incidence, including 25–39 year olds in the United States [43]. Inhabitants of Japan and Korea have approximately ten-fold higher rates of gastric cancer than the United States which cannot be explained fully by diet and genetics [44].

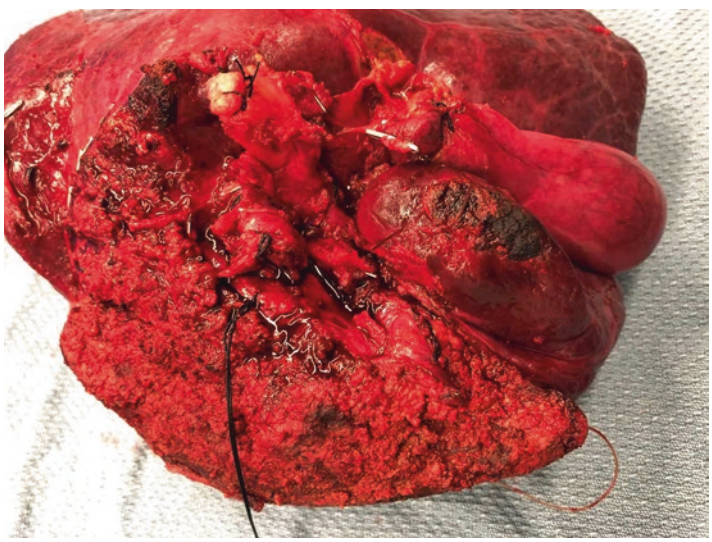

Fig. 3 Cholangiocarcinoma. Intrahepatic specimen from a trisectionectomy with extrahepatic removal of bile duct and resection of gall bladder en bloc. Image is a kind gift of Dr. Matthew Dixon

Gastric cancer rates have fallen since the discovery that *H. pylori* is a major cause of gastric ulcers which then develop into cancer. Antibiotics have resulted in a drop in *H. pylori* caused stomach cancer. In addition, improved diets that include more fruits and vegetables and fewer processed and high salt foods have lowered stomach cancer rates, as with other GI cancers [44].

Gastric cancers fall into three categories: gastroesophageal (GE) junction cancers, distal intestinal types, and diffuse, signet-ring cancers. Genetic studies have been conducted to further identify etiologies of gastric cancer. One of these was the Cancer Genome Atlas Research Network in 2014, from which gastric cancer was further divided into four molecular subtypes: Epstein–Barr virus-related tumors, microsatellite unstable tumors, genomically stable tumors, and tumors with chromosomal instability. The last subtype is often found in the aggressive tumors of the GE junction, but otherwise the four types do not closely correlate with the anatomical locations [45, 46]. Finally, Parry Guilford found a mutation in the E. cadherin, *CDH1,* gene, which was present in patients presenting with familial gastric cancers [47].

4.2 Putative Role of Par-4 in Gastric Cancer

While studies of Par-4 expression in gastric cancer have not been reported, other findings suggest that Par-4 can play a role in these cancers. The report that E. cadherin is involved in suppression of gastric cancer [47], coupled with the report that Par-4 upregulates E. cadherin is consistent with upregulation of Par-4 causing inhibition of gastric cancer through this pathway [34, 35, 48–50]. Furthermore, the expression of Akt and phospho-Akt are increased in gastric cancer as compared to normal adjacent tissue, and this high expression in gastric cancer is associated with a poor prognosis [51, 52]. Upon reduction of these proteins, cells become apoptotic [53]. The fact that Par-4 is downregulated by Akt, in particular phospho-Akt, is consistent with Par-4 becoming activated in this event and playing a role in apoptosis of gastric cancers.

Surgery remains a mainstay of gastric cancer treatment. Current chemotherapy treatments for gastric cancer are similar to many of the therapies used for colorectal cancer, including: Ramucirumab, docetaxel, doxorubicin, 5-FU with and without leucovorin, trastuzumab, pembrolizumab, and mitomycin [54]. Despite aggressive treatment, gastric cancer maintains a significant mortality rate, and new treatments are needed.

5 Par-4 in Oral Cancers

5.1 Background

Oral cancers, including those of the oral cavity and the pharynx, are one of the most commonly diagnosed cancers worldwide [1]. However, these cancers have a greater prevalence in east and southeast Asia [55]. The most common of these cancers is oral squamous cell carcinoma (OSCC) [56, 57]. Because there are few symptoms, it is often diagnosed at a late stage which can result in a poor prognosis [58]. Risk factors for OSCC include genetics and environmental factors such as smoking, alcohol use, and exposure to toxins, carcinogens (particularly in the workplace), and viral agents such as Epstein–Barr virus (EBV) [59], and, increasingly, human papilloma virus (HPV) [60].

5.2 Role of Par-4 in Oral Cancer

Par-4 expression and location have been studied and have been suggested to have minor significance in oral cancer. Cytoplasmic expression of Par-4 may be associated with more advanced disease and metastasis, while disease-free survival is better in patients with nuclear localization of Par-4, suggesting that it is the location of the protein rather than simply expression levels that predict disease outcome [61]. While the digestion of food does begin in the oral cavity, this area is covered in the chapter on head and neck cancers and is not discussed further in this chapter.

6 Par-4 in Other Tumors of the Gastrointestinal Tract

Although there is no published direct evidence of a role for Par-4 in esophageal, small bowel, or appendiceal cancers, the nature of these cancers suggests that it could play a role. Esophageal cancers are frequently aggressive and have a poor prognosis. They often arise as a result of Barrett's esophagus, potentially resulting from inflammation in the distal area of the esophagus [62]. Current treatments include radiation, and both neoadjuvant and adjuvant chemotherapies, including carboplatin, oxaliplatin, 5-FU, cisplatin, irinotecan, paclitaxel, and capecitabine. However, Par-4 has not been shown to play a role in these cancers.

Small bowel cancers are relatively rare. They consist of adenocarcinomas, carcinoids, stromal tumors, and lymphomas [63, 64]. A role of Par-4 in small bowel cancer has not been reported. As with esophageal cancer, Par-4 has been shown to exhibit mutations in cancers of the small bowel, but

they are silent mutations, and the significance of these mutations is not clear. It is possible that such mutations may actually have significance; for example, silent mutations can interfere with exonic splicing enhancers (ESEs), resulting in changes in mRNA processing of the genetic information.

Appendiceal cancer is rare, occurring in approximately 1–2 per million people. Because these cancers appear in a variety of types, they have no known growth etiologies. Data from molecular profiling performed by next-generation sequencing has not suggested a role for Par-4 in these cancers, including the presence of alterations in Akt or PKA [65].

7 Summary

Par-4 has been shown to play a role in the balance between apoptosis and proliferation in human cells. The increased expression of Par-4 leads to apoptosis of cells through various pathways. However, not only is increased expression important, but increased activity is also important. Par-4 protein, mRNA, and activity levels are all regulated through multiple pathways in gastrointestinal cancers, some more than others. In colorectal cancer, Par-4 activity is downregulated through inhibition by Akt1 and 14-3-3. It is likely that Par-4 expression may also be regulated by epigenetic means, such as hypermethylation of the promoter and silencing through transcription factors, such as Twist, an EMT transcription factor that plays a role in breast cancer [66]. Par-4 activity can be upregulated pharmacologically by ISC-4 in colon cancer cells [36], and by withaferin A [67] and 3,3′ diindolylmethane [68] in other cancer cells, as well as other drugs targeting Akt. Par-4 activity can be upregulated pharmacologically by the Akt inhibitor, ISC-4 in colon cancer cells. Given the need for new treatments for GI cancers, the potential of Par-4 activation as a therapeutic strategy, and the paucity of studies in many GI cancers, further research is warranted. Specifically, Par-4 has not been reported as a player in the progression of gastric cancer, despite the strong implication of a role for Par-4 in this disease. Par-4 participation in gastric cancer and esophageal cancer needs investigations particularly as both of these cancers can be aggressive and lead to a poor prognosis. Finally, Par-4, or lack thereof, needs to be explored in cancers of the small bowel. All of these efforts may lead to a safe, targeted treatment in coming months to decrease the negative health effects of gastrointestinal cancers worldwide.

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Role of Par-4 in Radiation Sensitivity of Cancer

Seema Gupta, Amogh Narendra, Satvik Lolla, Muskan Qureshi, Muhammad Hamza Qureshi, and Mansoor M. Ahmed

Abstract

Radiation dose and fraction play an important role in dictating the pro-survival and pro-apoptotic effects. Pro-survival pathways that affect radiation response include NF-κB, Bcl-2, oncogenic K-RAS, whereas cytokines such as TRAIL and TNF-α influence the pro-apoptotic effects of radiation. Prostate apoptosis response-4 (PAR-4) plays an important role in abrogating pro-survival effects of radiation and synergizes radiation-induced pro-apoptotic effects. This chapter analyzes and explores distinct and unique pathways of PAR-4 that block the survival effects of oncogenic NF-κB, K-RAS, and Bcl-2 and synergize the effects of radiation. On the other hand, PAR-4 partners with radiation-induced cytokines such as TNF-α and TRAIL to augment radiation-induced apoptosis of cancer cells. Hence, preclinical studies demonstrate that PAR-4 protein and agonist of PAR-4 can be radiation sensitizers. These studies warrant clinical trial concepts with radiation and PAR-4 therapy.

Keywords

Par-4 · Ionizing radiation · Solid tumors · Apoptosis · K-RAS · Bcl-2 · NF-κB · TRAIL · TNF-α

1 Introduction

Ionizing radiation (IR) is an essential component of many treatment regimens for cancer. Presently, radiation therapy is utilized in approximately 50% of cancer cases as either a palliative treatment to reduce pain or, in most cases, as a mechanism to cure cancer [1]. IR generates high energy particles or waves that deposit in tissues that can directly or indirectly cause single and double-strand breaks in DNA or can cause damage to the cell membrane. Low LET radiation such as gamma radiation and X-ray radiation causes 60% of DNA damage through indirect action mediated by the generation of free radicals and 40 percent of DNA damage through direct action [2]. The lesions in DNA result in multiple cellular responses, including the arrest of cell cycle progression at certain cell cycle checkpoints and DNA repair. The accumulation of residual unrepaired or mis-repaired DNA can cause the death of the progeny of cells following several mitotic cycles in a process otherwise known as a mitotic catastrophe. The accumulation of DNA damage can also result in the induction of apoptosis, resulting in cell death [3].

Apoptotic cell death is a form of genetically programmed cell death and is modulated by several signaling molecules. One of such molecules is PAR-4 (prostate apoptosis response-4). This transcription modulator translocates to the nucleus following extrinsic or intrinsic apoptotic pathway activation and modulates chromatin transcription to inhibit pro-survival pathways and initiate apoptosis [4]. PAR-4 regulates apoptosis through its interactions with cytokines, RAS, and NF-κB pathway, including pro-survival proteins such as Bcl-2. Notably, while overexpression of PAR-4 or its ectopic expression can induce apoptosis, endogenous PAR-4 by itself does not induce apoptosis as it remains sequestered

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in the cytoplasm by 14-3-3 protein following binding and phosphorylation of PAR-4 by Akt1 [5]. However, endogenous PAR-4 is required for apoptosis induction by exogenous death signals such as IR. IR can induce several pro-survival pathways, such as NF-κB [6], by promoting apoptosis PAR-4 abrogates innate mechanisms of radiation resistance within cancer cells. This chapter will discuss the interaction of PAR-4 protein with IR-induced signaling pathways, particularly involving NF-κB, K-RAS, Bcl-2, TNF-α, and TRAIL pathways, leading to the radiation sensitivity of cancer cells.

2 Radiation-Induced Signaling Pathways

IR induces a complex network of cellular pathways leading to either cell survival or death [7]. IR results in the formation of reactive oxygen species (ROS) that generate DNA damage, causing changes in chromatin structure, resulting in the autophosphorylation of ATM, which promotes the formation of repair complexes and the substrate phosphorylation of p53 [8]. The activation of p53 results in the transient expression of p21, causing G1 arrest to prevent progression of the cell cycle with DNA damage accumulation, thus allowing repair and the cell survival. However, the accumulation of irreparable DNA damage is sensed by the p53 damage sensor, resulting in the activation of Bax, a pro-apoptotic protein that induces cell death [8]. IR induces MAP3K, PI3K, and MAPK8 in the cytoplasm, modulating cells' proliferation and survival. The activation of the epidermal growth factor receptor (EGFR) by IR results in a cytoprotective response through the MAPK and MAPK8 pathways [9]. Furthermore, IR-induced phosphorylation of EGFR results in the activation of RAS that induces the activation of PI3K [10]. PI3K under normal conditions is meant to protect cells from cell death caused by deprivation of growth factors. PI3K induces the activation of NF-κB via the PI3K/Akt axis, which results in the translocation of NF-κB to the nucleus promoting the transcription of pro-survival proteins such as Bcl-2 and Bcl-xL [11]. Furthermore, IR can also induce abscopal (distal) effects or bystander effects via the release of cytokines such as TNF-α and TRAIL. TNF-α binds to TNF receptors that either induce cell apoptosis via the activation of the caspase pathway or promote cell survival via the PI3K/Akt axis in bystander cells.

TRAIL binds to the DR4 or DR5 and can induce cell apoptosis via the caspase pathway in bystander cells [12]. Irrespective of intrinsic radiation sensitivity status, secretory and membrane-bound TRAIL induced after irradiation was found to mediate the translocation of PAR-4 to the nucleus, resulting in cell death by apoptosis [13]. These findings suggest that PAR-4 plays an essential role in defining cancer cells' radiation sensitivity by interacting with and regulating IR-induced signaling pathways, as discussed in the following sections.

2.1 NF-κB and PAR-4

NF-κB is a vital transcription factor complex that plays a critical role in regulating the expression of many genes, including pathways that control the immune response, inflammation, development, cellular growth, and apoptosis [14–16]. It is involved in cellular responses to stimuli such as stress, cytokines, free radicals, and radiation [17, 18]. When NF-κB is misregulated, it can lead to different disease states, including multiple cancer types [19]. In cancer, proteins that control NF-κB signaling are mutated or aberrantly expressed, leading to defective coordination between the malignant cells and the rest of the organism [17]. This is evident both in metastasis and the inefficient eradication of the tumor by the immune system.

The pro-survival functions of NF-κB are well established. They are mediated by upregulation of anti-apoptotic genes such as TRAF1, TRAF2, Bcl-2, and Bcl-XL, ultimately abrogating the caspases' activities, central to most apoptotic processes [20]. Accordingly, defects in NF-κB regulation and signaling results in increased susceptibility to apoptosis, leading to increased cell death. IR acts as a potent inducer of NF-κB [6, 18] and upregulates Bcl-2 in cells that lack wild-type p53, leading to enhanced radiation resistance [21].

PAR-4 has a nuclear localization sequence, NLS2, and can be translocated to the nucleus, a process facilitated by PKA phosphorylation [22]. However, PAR-4 can inhibit NF-κB activity both in the cytoplasm and in the nucleus (reviewed in 4). PAR-4 inhibits PKC activity in the cytoplasm, and atypical PKC, by preventing the ζPKC-mediated phosphorylation of IκB. Since active IκB is vital for nuclear translocation and NF-κB activity, PAR-4 inhibits NF-κB function through ζPKC blockade. Similarly, in the nucleus, PAR-4 inhibits the transcriptional activity of NF-κB, although the precise mechanisms of their interaction are not known. However, it has been suggested that PAR-4 can inhibit NF-κB, for example, by its interaction with co-repressors or direct DNA binding. Indeed, the overexpression of PAR-4 has been shown to decrease the DNA binding activity of NF-κB and its activation, leading to significantly reduced levels of Bcl-2 [23]. Together, these reports demonstrate that suppression of NF-κB activity plays a significant role in the pro-apoptotic functions of PAR-4 (Fig. 1).

Since PAR-4 is a pro-apoptotic gene that also abrogates oncogenic RAS or TNF-α-induced NF-κB activity, ectopic expression of PAR-4 represses the radio-induction of NF-κB and Bcl-2 proteins, leading to sensitization of the p53 null cancer cells to IR-induced apoptosis [17]. These findings

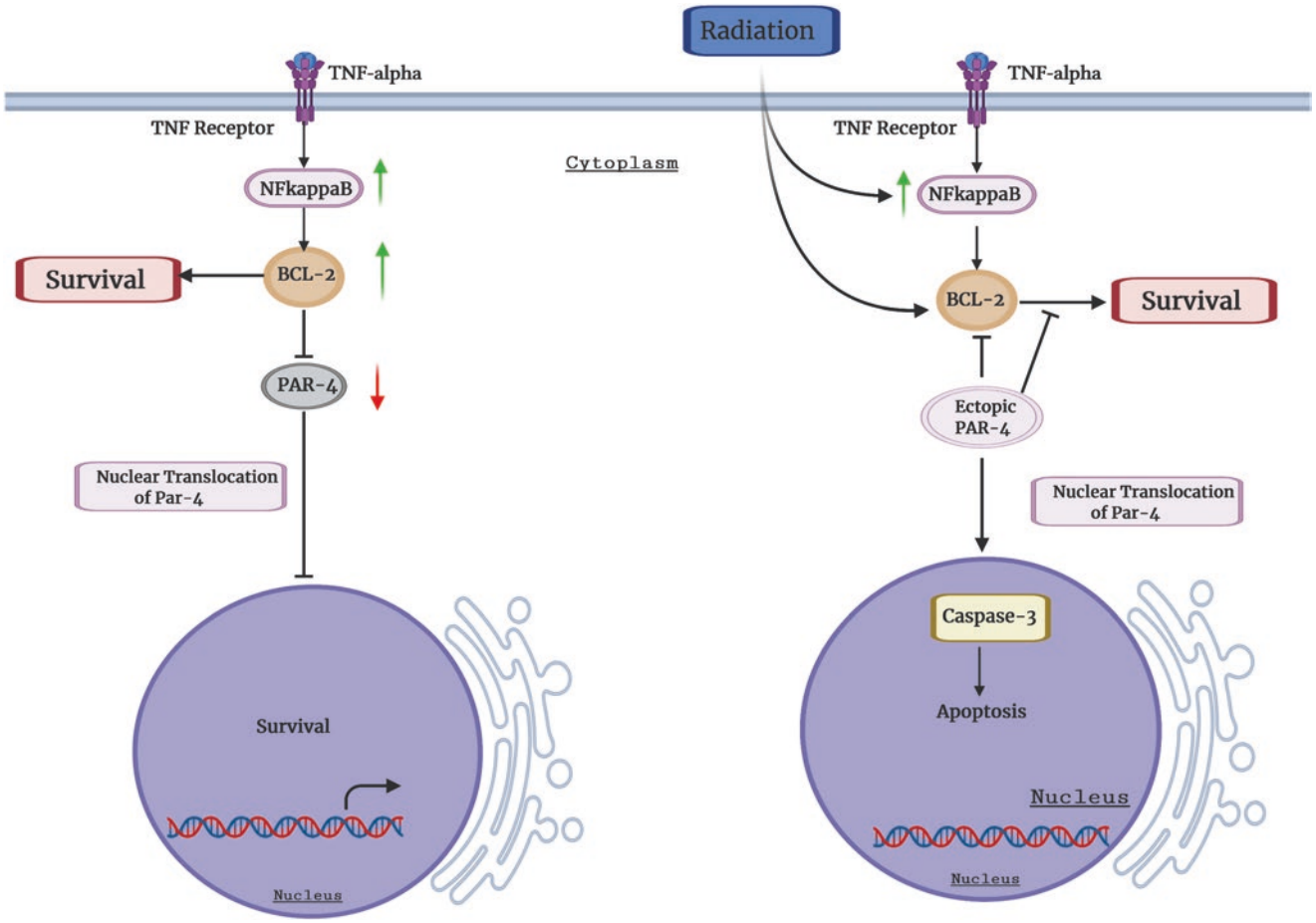

Fig. 1 PAR-4 inhibits NF-κB activity. Tumors with high NF-κB activity and Bcl-2 expression force downregulation of PAR-4 expression, providing a survival advantage (Left). Radiation activates NF-κB and Bcl-2 expression through TNF-α and will have an impact on function of endogenous PAR-4 protein. Hence, ectopic expression of PAR-4 can abrogate radiation-induced NF-κB and Bcl-2 expression and sensitize tumor cells to apoptosis (Right)

showed that enforced expression of PAR-4 could control p53 null radio-resistant tumors by inhibiting NF-κB activity and thereby inducing apoptosis in a p53-independent manner. Hence, PAR-4 gene therapy or PAR-4 agonist can play important translational roles in clinical settings.

3 Anti-Apoptotic Proteins and PAR-4

The relative levels of anti- and pro-apoptotic proteins regulate cell survival or death, respectively. One of the mechanisms through which anti-apoptotic Bcl-2 family members of proteins alter the cell death pathways is by blocking the function of pro-apoptotic proteins, such as Bax, Bid, Bad, and Bak. The Bcl-2 gene is responsible for preventing the Bax/Bak oligomerization, which would release pro-apoptotic molecules [24]. At the same time, Bax can also form a heterodimer with Bcl-2, accelerating apoptosis. Overexpression of Bcl-2 is documented in several cancers, more notably in prostate cancer. Previous studies showed that levels of Bcl-2 increase in androgen-independent prostate cancer, and there is a mutually exclusive interplay of levels between PAR-4 and BCL-2 in human prostate tumors [25]. Several studies had demonstrated that expression of Bcl-2 is regulated by several transcription factors, including p53, NF-κB, Wilms' Tumor 1 (WT1), and PAR-4 [20, 26, 27]. There may be several mechanisms that exist in terms of regulatory interplay between Bcl-2 and PAR4.

3.1 Bcl-2 and PAR-4

In one such mechanism, there is the role of WT1 in regulating the expression of PAR-4 and Bcl-2. The regulatory region of the *bcl-2* gene has two promoter regions: P1 and P2. The P1 promoter is the primary transcriptional activator, whereas the P2 promoter only has primary functions in specific tissues and can transrepress P1 when p53 is present [24]. WT1 has been shown to bind to the -1460 and -1807 sites on the *bcl-2* P1 promoter to both transrepress and transactivate the

bcl-2 transcription [27]. Interestingly, PAR-4 regulates the expression of Bcl-2 both by binding to the specific regions of *WT1* promoter as well as -1460 site of *bcl-2* P1 promoter. Upon binding to the exon 5-encoded domain of WT1, endogenous PAR-4 acts as a co-activator of the *WT1* gene, leading to increased Bcl-2 expression. On the other hand, increased PAR-4 in the nucleus (due to ectopic or over-expressed PAR-4) acts as a transcriptional repressor as it binds the zinc fingers of WT1, leading to reduced expression of Bcl-2. Furthermore, ectopic PAR-4 can bind to the -1460 site of *bcl-2* P1 promoter in a complex with WT1, transcriptionally repressing the expression of Bcl-2 [27]. When such ectopic PAR-4 complex localizes to the nucleus, it can bind to WT1 and the *bcl-2* P1 promoter. In this case, PAR-4/WT1 will limit the amount of Bcl-2 present in cancer cells by inhibiting the P1 promoter [27]. This will change the Bcl-2:Bax ratio, causing the cell to undergo apoptosis since, in the absence of low levels of Bcl-2, Bax binds to the cellular mitochondria and activates cytochrome C. This then causes apoptosis by activating APAF1 that cleaves caspase 9, releasing its activated form [28].

Interestingly, in prostate cancer cell lines, it has been shown that upon treatment with TNF-α, NF-κB transcriptionally activates Bcl-2 through its binding site on the P2 promoter of *bcl-2*, leading to cell survival [29]. This signaling event is further supported by a report that ectopic PAR-4 abrogates radiation-induced NF-kB (radiation is a potent inducer of TNF-α) and Bcl-2 induction to sensitize the effects of radiation in prostate cancer cells [18]. Hence, the role of PAR-4 in regulating the transcriptional activity of NF-κB needs to be explored. Nevertheless, it is established that PAR-4 is a unique pro-apoptotic gene that regulates the expression of anti-apoptotic molecule Bcl-2 and the activity of pro-survival transcription factor NF-κB. Based on the above mechanism, ectopic PAR-4 protein can have a significant translational impact.

4 RAS Signaling and PAR-4

The family of RAS genes (H-, K-, and N-RAS) are oncogenes that control cell growth, cell maturation, and cell death. In particular, K-RAS mutations are common in multiple cancers such as lung, pancreatic and colorectal cancer, mainly, K-RAS G12C mutation is extremely prevalent in small cell lung and pancreatic cancers [30]. Hence, oncogenic RAS is known to inhibit cell death and growth-inhibitory genes and activate pro-survival genes. The pro-apoptotic gene PAR-4 is a target of the RAS pathway and is reportedly downregulated by oncogenic RAS. In pancreatic tumors, low expression levels of PAR-4 correlated with the K-RAS mutational status. In this study, PAR-4 mRNA and protein expression were associated with prolonged survival in patients instead of tumors with null or low expression. This clinical observation is supported by in vitro studies whereby transient overexpression of oncogenic RAS in wild-type K-RAS cells significantly downregulated the endogenous PAR-4 protein levels and conferred accelerated growth. Hence, downregulation or loss of PAR-4 function by oncogenic RAS could provide a selective survival advantage for solid tumors by inhibiting the pro-apoptotic pathway mediated by PAR-4. Based on the functional role of PAR-4 in cell death and since several solid tumors harbor a high incidence of RAS mutations, it is well-reasoned that the downregulation of PAR-4 due to mutations in K-RAS will result in the impairment or dysregulation of apoptotic mechanism and thus render selective survival advantage for solid tumors [31].

4.1 K-RAS and PAR-4

Oncogenic RAS pathway has been found to cause DNA methylation in RAS mutant cell lines; notably, there is an association of K-RAS mutation to DNA methylation [32]. Such an increase in DNA methylation leads to PAR-4 downregulation rendering survival advantage (Fig. 2). Methylation of promoters is directly linked to DNA methyltransferase enzyme levels (DNMT).

Many reports have demonstrated elevated levels of DNMT expression and activity in tumors with oncogenic RAS mutations. Thus, the inactivation of pro-apoptotic and growth-inhibitory genes by oncogenic RAS may be directly linked to the elevated levels of DNMT that mediates the silencing of promoters through the epigenetic methylation process. Several agents can block DNA methylation and restore the expression of pro-apoptotic proteins. To establish the link with oncogenic RAS to elevated levels of DNMTs to PAR-4 downregulation, agents blocking oncogenic RAS function should influence the levels of PAR-4 protein. One such agent is the farnesyl-transferase inhibitor (FTI) that blocks the oncogenic K-RAS function. FTI was tested to restore TGF-β type RII expression as the RII promoter is methylated in pancreatic tumors [33]. It was found that FTI significantly downregulates the mRNA and protein levels of DNMT-1, and this led to re-expression of RII, suggesting that FTI-mediated inhibition of oncogenic RAS function will inhibit the DNA methylation process via downregulation of DNMT-1. Hence, in the context of PAR-4, the FTI can potentially restore PAR-4 protein and function via inhibition of DNMT-1 expression and further sensitize to the effects of IR (Fig. 2). Similarly, 5-Azacytidine can directly restore PAR-4 expression and cooperate with radiation effects to render apoptosis (Fig. 2) [30].

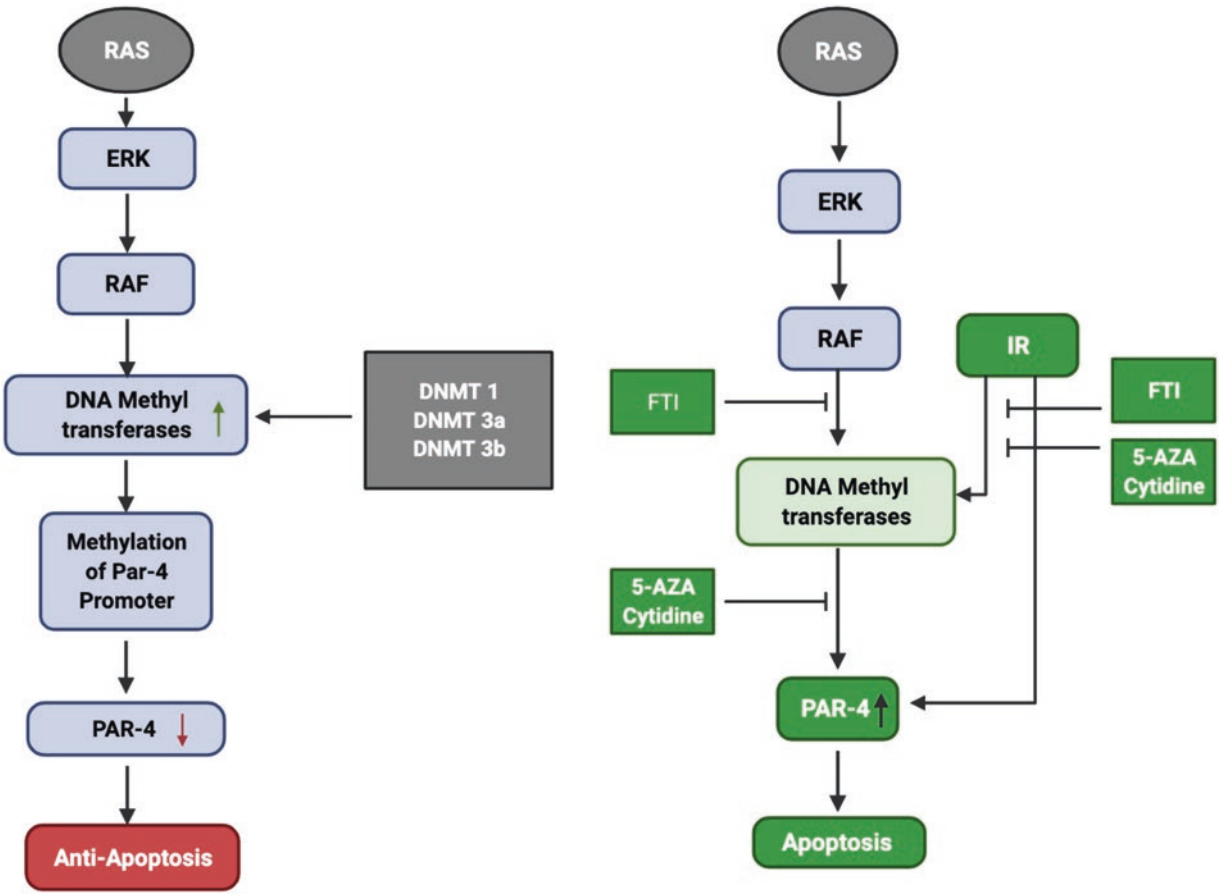

Fig. 2 Oncogenic RAS downregulates PAR-4. Oncogenic RAS inactivates pro-apoptotic proteins such as PAR-4 through DNMTs that are generally elevated in tumors with oncogenic RAS mutations, leading to survival (Left). Agents such as FTI can restore PAR-4 activity either by blocking the oncogenic K-RAS function or potentially inhibiting DNMTs (that silence promoters through epigenetic methylation) that are also induced by IR, leading to enhanced apoptosis (Right)

5 Cytokines and PAR-4

IR induces the upregulation and secretion of a wide variety of inflammation-related cytokines including TNF-α, IL-1, IL-6, IL-8, IFNs, G-CSF, VEGF, and EGFR within minutes to hours of irradiation through changes in gene transcription initiated by redox-sensitive transcription factors such as NF-κB, early growth response 1 (Egr-1), and AP-1 and in response to changes in chromatin structure. Control of redox-sensitive transcription factors is initiated by an increase in ROS caused by free radical formation induced by IR [34]. Besides, IR can induce the upregulation of inflammatory cytokine receptors such as TRAIL-R1 and TRAIL-R2, also known as DR4 and DR5.

The transcription of TRAIL-R1 and TRAIl-R2 is inducible via a p53-dependent DNA damage sensing pathway. The release of TRAIL is induced at high doses of IR [13]. Similarly, cytokine TNF-α can be induced at both low and high doses of IR and found to be regulated by NF-κB. The positive feedback loop of NF-κB→TNF-α→NF-κB occurs within irradiated cells and bystander cells not exposed to IR by binding of TNF-α to TNFR2 [35]. Apart from the effects of radiation on these cytokines, there exist discrete modulation of PAR-4 in response to cytokines, which is partly mediated by secondary messenger cascades initiated from the binding of the cytokines, TNF-α and TRAIL to the extracellular TNF receptors and DR-4/5, respectively. TRAIL is secreted by cells in response to cellular damage and acts as a paracrine and autocrine signaling mechanism.

The binding of TRAIL to either DR4 or DR5 results in the heterodimerization of c-FLIP and Procaspase-8 through FADD recruitment, creating the active Caspase-8 dimer. The active Caspase-8 dimer activates Caspase-3 by proteolytic cleavage. Caspase-3 is proposed to cleave 14-3-3, which is complexed to phosphorylated PAR-4. The cleavage of 14-3-3 results in the dissociation of PAR-4 from the phosphate group and 14-3-3, resulting in the active form of PAR-4. PAR-4 can then translocate to the nucleus and modulate DNA transcription in the nucleus to promote intracellular apoptosis (Fig. 3) [13]. TNF-α is also secreted by cells in

response to cellular damage and acts as a paracrine and autocrine signaling mechanism. TNF-α binds to either a TNF receptor complexed with FADD or TRAF2. FADD recruitment following TNF-α binding results in the heterodimerization of c-FLIP and Procaspase-8, creating an active form of Caspase-8, leading to the induction of PAR-4- and Caspase-3-mediated apoptosis. TRAF-2 induces inflammation or survival by the PI3K/Akt axis in response to TNF-α binding to the TNF receptor [36]. Akt is proposed to phosphorylate PAR-4 mediating the formation of the 14-3-3 phosphorylated PAR-4 complex, which inactivates PAR-4 minimizing the effect of PAR-4-mediated apoptosis (Fig. 3) [37]. The

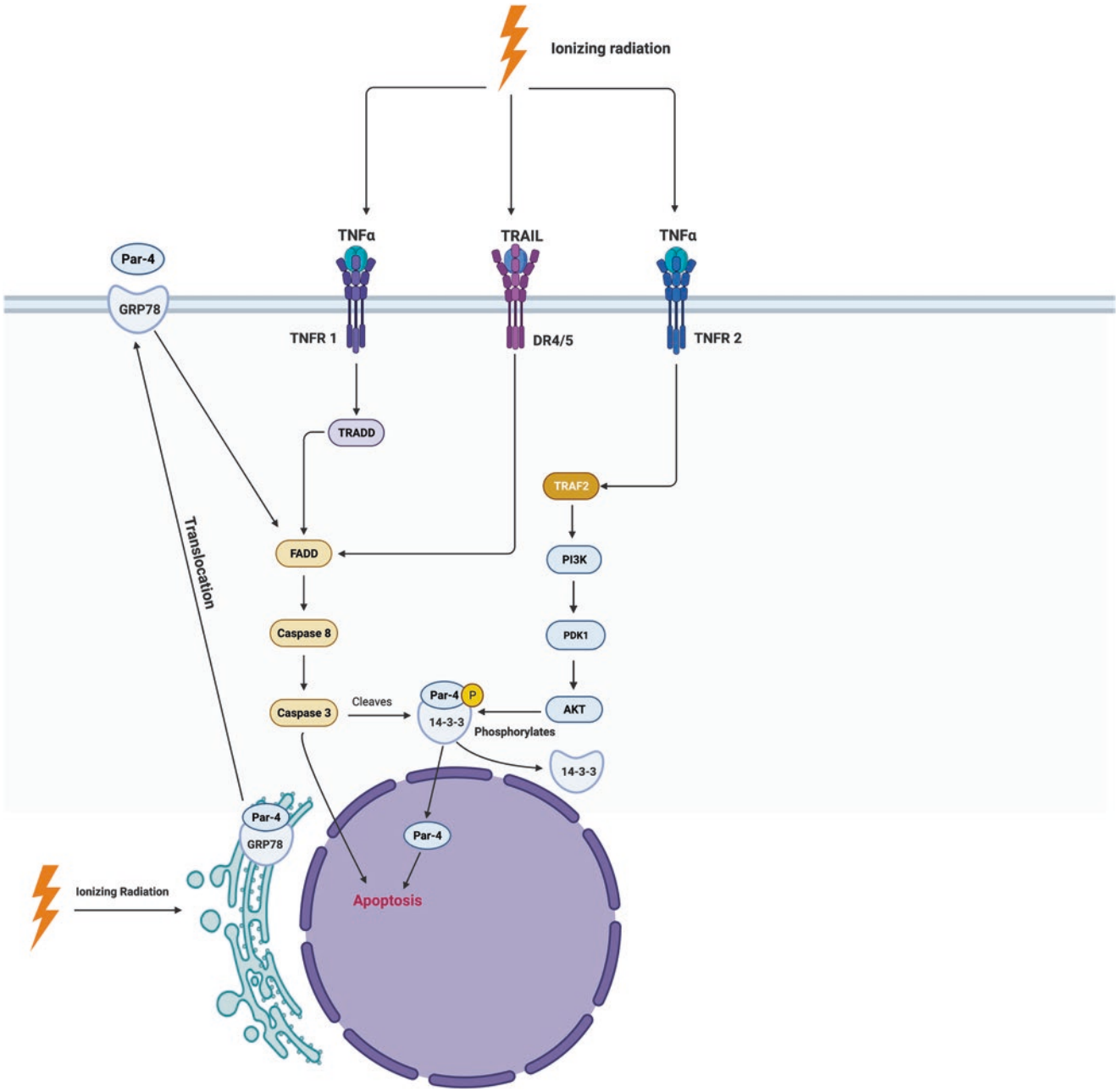

Fig. 3 Role of IR-induced cytokines in activation of PAR-4. IR induces the release of TRAIL and TNF-α from cancerous cells, endothelial cells, or immune cells, which bind to cancer cells. The binding of TNF-α to TNFR1 and TRAIL to either DR4 or DR5 results in the initiation of apoptosis via the Caspase-3 pathway. Caspase-3 has been demonstrated to cleave the PAR-4 and 14-3-3 complex resulting in the translocation of PAR-4 to the nucleus where transcription modulation occurs to promote apoptosis. The binding of TNF-α to TNFR2 initiates the activation of the PI3K/Akt axis which is purported to phosphorylate the PAR-4 and 14-3-3 complex inactivating PAR-4. IR can also directly induce the secretion of PAR-4 by the ER and translocates its receptor to the cell membrane initiating a positive feedback loop that further activates the Caspase-3 pathway in bystander cells

PI3K/Akt axis promotes the upregulation of NF-κB, enabling the transcription of pro-survival proteins. The duality of TNF-α as a cytokine is unique to the TNF family and has a dual indirect effect on the activity of PAR-4. In response to endoplasmic reticulum (ER) stress, PAR-4 and glucose-regulated protein-78 (GRP78), known as BiP, is translocated from the ER to the membrane and acts as a positive feedback loop that induces further activation of PAR-4 [38].

5.1 Radiation-Induced Cytokines and PAR-4

Radiation is a potent inducer of cytokines, including TNF-α, TRAIL, and TGF-β. Radiation dose-fraction dictates the extent of secretion of these cytokines. It is reported that compared to 2 Gy radiation dose (clinically relevant dose), doses greater than 10 Gy robustly induce several cytokines that have dramatic effects within and outside the tumor microenvironment. One study demonstrated that 10 Gy induced the transactivation function of EGR-1 to elevate TNF-α levels leading to concomitant dual effects. Secreted TNF-α exerts extrinsic apoptosis and clonogenic death in irradiated and non-irradiated cells. Simultaneously, there is increased activation of NF-κB, leading to pro-survival events in irradiated and non-irradiated cells. The ratio between these two events dictates the fate of cells. TRAIL is another cytokine that plays an essential role in utilizing the PAR-4 function for radiation effects. High dose of IR caused enhanced secretion of TRAIL, particularly the soluble form. The TRAIL caused PAR-4 mobilization to the nucleus through the TRAIL R1 receptor because both TRAIL forms activate this receptor. TRAIL signaling leads to activation of initiator and effector caspases that mediate the apoptotic events. Such mobilization of PAR-4 by TRAIL leads to the cleavage of 14-3-3 proteins by caspases, which mobilizes PAR-4 to the nucleus, analogous to the association of BAD with Bcl-xL.

It has been shown that PAR-4 can be secreted by normal and cancer cells, and exposure to the ER stress-inducing agents such as TRAIL further increased its secretion [38]. The secreted extracellular PAR-4 induced apoptosis by binding to the stress response protein, GRP78 on the surface via ER stress, and activation of the FADD/caspase-8/caspase-3 pathway (Fig. 3). It has been shown that IR-induced levels of either secretory or membrane-bound TRAIL mediate translocation of PAR-4 to the nucleus leading to apoptosis [13], suggesting that IR may facilitate the release of PAR-4 from ER (Fig. 3).

6 Summary and Future Directions

Overexpression of Bcl-2, constitutive NF-κB activation, and oncogenic K-RAS signaling are characteristics intrinsic hallmarks across several solid tumors. On the other hand, radiation dose-fraction is known to induce pro-survival pathways that utilize the functions of NF-κB, Bcl-2, and K-RAS signaling. Maintaining the PAR-4 function in the above characteristic tumor microenvironment can help curtail survival advantage in tumor cells. Furthermore, ectopic PAR-4 therapy can abrogate radiation therapy-induced pro-survival protein function and synergize the effects of radiation. This strong preclinical evidence warrants designing new clinical trial concepts with radiation and ectopic PAR-4 therapy in solid tumors that usually demonstrate higher levels of Bcl-2, increased activation of NF-κB and K-RAS oncogenic mutation.

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Prostate Apoptosis Response-4: a Therapeutic Target for Malignant Gliomas

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Abstract

Gliomas are the most common type of primary brain tumors and glioblastoma (GBM), the most lethal of gliomas accounts for more than 60% of all brain tumors in adults. Despite the advances in multimodal therapies over the last two decades, the prognosis is extremely poor. Various factors including glioma stem cells (GSC), genetic mutations, epigenetic modifications, and dysregulated pathways cumulatively render the GBM resistant to radiation and chemotherapies. While efforts are on to develop new drugs that can efficiently cross the blood–brain barrier (BBB), it has become important to identify novel strategies and molecular targets that reduce the tumor size, increase overall survival, progression-free survival and improve the quality of life. Prostate apoptosis response-4 (Par-4) is a unique tumor suppressor with the ability to selectively induce apoptosis in cancer cells but not kill the normal cells. The pro-apoptotic activity of Par-4 is exerted in an autocrine as well as paracrine manner. Furthermore, various inducers and secretagogues of Par-4 which have the ability to upregulate both intracellular and secretory Par-4 are being explored as strategies for cancer therapy. This chapter provides an overview of gliomas with a focus on GBM and the challenges in the development of drugs for the treatment of GBM. Also, we discuss the importance of microenvironment and the potential role of Par-4 in the highly interconnected signaling network thereby highlighting the importance of Par-4 as an exciting therapeutic target.

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Keywords

Gliomas · Glioblastoma (GBM) · Microenvironment · Dysregulation · Tumor suppressors · Prostate apoptosis response-4 (Par-4)

Abbreviations

AG	Anaplastic glioma
BBB	Blood–brain barrier
CNS	Central nervous system
CSC	Cancer stem cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
GBM	Glioblastoma/glioblastoma multiforme
GSC	Glioma stem cells
HAT	Histone acetyltransferases
HDAC	Histone deacetylases
HGF	Hepatocyte growth factor
HGG	High-grade gliomas
HIFs	Hypoxia-inducible factors
HIFα	Hypoxia-inducible factor alpha
IDH	Isocitrate dehydrogenase
LGG	Low-grade gliomas
MES	Mesenchymal
MGMT	(O[6]-methylguanine-DNA methyltransferase)
NSC	Neural stem cells
Par-4	Prostate apoptosis response-4
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B or Akt
PN	Proneural
PTEN	Phosphatase and tensin homolog
QOL	Quality of Life
RTK	Receptor tyrosine kinase
SHH	Sonic hedgehog

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TAM	Tamoxifen
TGF-β	Transforming growth factor-β
TIL	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
TMZ	Temozolomide
TTF	Tumor-treating field
VEGF	Vascular endothelial growth factor

1 Introduction and Classification of Gliomas

Brain tumors are uncontrolled growth of heterogeneous cell types in the central nervous system (CNS). De novo accumulation of mutations majorly in genes regulating cell cycle gives rise to primary brain tumors (low grade) which can advance to higher grades with additional mutations [1]. The highest incidence of CNS tumors is reported from China, the USA, and India [2]. Majority (>90%) of primary CNS tumors occur in the brain [3] and according to the National Brain Tumor Society, USA, there are more than 120 different types of brain tumors. The most prevalent brain tumors are intracranial metastases from systemic cancers, meningiomas, and gliomas. Other tumors categorized as brain tumors are astrocytomas**,** meningiomas, pituitary tumors, craniopharyngiomas, medulloblastomas, primary CNS lymphomas, and schwannomas [4].

Gliomas are the most common and predominant type of brain tumors and account for about 80% of all malignant brain tumors [5]. Gliomas originate from glial cells and may arise from neuroglial stem cells, progenitor cells, or differentiated astrocytes; however, the specific cell origin of gliomas remains a debatable issue [6]. The World Health Organization (WHO) has classified brain tumors into four grades based on the histological features, phenotypes of astrocytes and oligodendrocytes, and degree of malignancy [7]. Grade I gliomas are slow growing tumors with favorable prognosis and include pilocytic astrocytoma. Grade II are slow growing but invasive with infiltration into brain parenchyma; diffuse astrocytoma and oligodendroglioma are classified into this group. Grade III gliomas are highly proliferative with anaplastic features and have a propensity for recurrence; anaplastic astrocytoma and anaplastic oligodendroglioma are included in this group. Grade II and III gliomas are the most common glioma tumors and occur in young adults. Grade IV gliomas referred to as Grade IV astrocytoma or glioblastoma/glioblastoma multiforme (GBM) are highly heterogeneous and the most aggressive and malignant of gliomas [7–9]. Tumors in grades I and II are categorized as low-grade gliomas (LGG) and progressive tumors in grades III and IV that are rapidly proliferating are referred to as high-grade gliomas (HGG). Grade IV astrocytomas or GBM are characterized by excessive vascularization, are highly invasive in the brain regions but rarely metastasize to other organs. GBM is the only solid tumor that is defined as a high-grade tumor in the absence of any metastatic component [10]. GBM accounts for 16% of all primary brain tumors and 54% of all gliomas [11]. Despite the advancement in standard therapies in the past decades, the prognosis of GBM remains poor with the average survival period of 12–18 months; and only 25% of GBM patients survive more than 1 year and only 5% of patients survive more than 5 years [12].

GBMs are categorized into primary and secondary tumors based on the clinical and pathological features [13]. GBMs that arise de novo are known as primary GBM and those developing from lower grade tumors (WHO grades II and III) are referred to as secondary GBM. Majority of the GBM tumors (about 80%) are primary tumors [14–17] and occur in patients over the age of 50 in contrast to secondary GBM which is seen in patients below the age of 45 years [13]. Tumors from both the categories present similar clinical features; but show distinct genetic alterations and molecular pathways, tumor behavior and response to treatment [13, 16]. While genetic alterations in EGFR, CDKNA-p16, and PTEN are common features of primary tumors, secondary GBM tumors are characterized by mutations in the isocitrate dehydrogenase 1 (IDH 1) and TP53 [18].

The expanding applications of genomics, proteomics, bioinformatics, and epigenetics have provided valuable data for better understanding of different types of tumors. In-depth analysis using these technologies have aided in molecular profiling of tumors and identification of signaling pathways for revising classification of different tumors [19]. GBM has the distinction of being the first tumor to undergo comprehensive molecular characterization [20, 21]. Based on integration of large databases from TCGA, gene expression analysis, and transcriptome profiles, classification of GBM was restructured to include four subtypes, viz. proneural (PN), neural, classical, and mesenchymal (MES) [22, 23]. The MES and classical subtypes are highly aggressive GBM but patients in MES group have shorter overall survival (OS). The PN subtype is less aggressive with better prognosis. The GBM tumors of neural subtype display many genes related to neural development and function [21].

In the fifth edition series of the WHO classification (2016) of CNS tumors, the histological features of tumor as well as mutations were considered. Gliomas are categorized into two types based on their infiltrative behavior. Non-diffuse gliomas (non-infiltrating into the surrounding tissue including pilocytic and ependymoma) that include Grade I gliomas and can be almost completely treated by surgical resection. Diffused gliomas (capable of infiltrating in surrounding CNS tissues) represent highly infiltrative and malignant type of CNS tumors and are the most frequent types of gliomas, majorly occurring in adults. Histologically, astrocytoma and

oligodendroglioma with diffusely infiltrative behavior and GBM are categorized under diffuse glioma. All the diffused astrocytoma and oligodendroglioma tumors are grouped together as they share similar growth pattern and genetic mutation in isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) genes, which act as driver mutation for tumor incidence. Diffuse gliomas encompass Grade II, III (astrocytoma and oligodendroglioma), Grade IV GBM and diffuse glioma of childhood. Oligodendroglioma, under diagnostic procedure of oligodendroglioma and anaplastic oligodendroglioma with mutational status of IDH gene family and losses of whole arm of 1p and 19q (1p/19q co-deletion) are also included in this category. Based on IDH mutations, GBM is divided into three subtypes: (1) GBM-IDH-wild type represents 90% of cases that correspond to primary or de novo GBM and occurs at or above the age of 55 years. (2) GBM-IDH-mutant constitutes 10% of cases that correspond to secondary GBM because of progression of lower grade of diffuse glioma and is more common in younger patients. (3) GBM, NOS (not otherwise specified) comprises the category of GBM where status of IDH1 could not be evaluated [22].

2 Etiology, Epidemiology, and Clinical Symptoms

2.1 Etiology

The etiology of GBM is not completely understood, and there is limited evidence of causal links that are associated with increased risk of the disease. Ionizing radiation and genetic predispositions are the most commonly established GBM risk factors. In less than 5% of all GBM cases, there is a familial link but the cause remains unknown most of the time [24]. Population-based studies consistently demonstrate that incidence of gliomas varies significantly by sex. Most gliomas occur with a 30–50% higher incidence in males, and the male predisposition of glial tumors increases with age in adult glioma [25].

2.2 Epidemiology

In 2017, there were ~120,000 diagnosed incident cases of brain cancer in the eight major markets (the USA, the UK, France, Italy, China, Japan, Spain. and Germany), and the number is expected to grow to 151,067 cases by 2027. Urban China is expected to see the highest rate of growth at an annual growth rate of 4%, with the number of diagnosed incident cases of brain cancer growing to 84,374 cases. In 2017, adults aged 60–69 years accounted for the highest proportion of the diagnosed incident cases of brain cancer in the 8 major markets, accounting for 20.11% of cases, while adults aged 70–79 years accounted for 19.17% of cases [24].

As per American Brain Tumor Association (ABTA) 2020

- Over 700,000 Americans are living with a brain tumor today.
- Nearly 80,000 people diagnosed with a primary brain tumor this year.
- About 28,000 kids in the USA are fighting brain tumors right now.
- In the year 2020, nearly 16,000 died as a result of a brain tumor.

Of the estimated 80,000 people diagnosed with a primary brain tumor in 2020, approximately one third had malignant tumors, with 17,000 deaths. The median age at diagnosis for all brain and CNS tumors is 59 years. Gliomas account for 24% of all primary brain and CNS tumors; these tumors vary greatly in histology from benign ependymoma tumors to the most aggressive and deadly Grade IV GBM. The annual incidence of malignant glioma in the USA is ~5/100,000 [26].

2.3 Clinical Symptoms

The symptoms of glioma vary by tumor type, size, location, and rate of growth. The first symptom of a brain tumor of any type can be a headache. The reason that patients get headaches is that these brain tumors cause increased pressure in the brain leading to cerebral edema or hemorrhage. The headache associated with a brain tumor is frequently worse in the morning and may be associated with nausea or vomiting. The ten most prevalent symptoms of gliomas are: seizures (37%), cognitive deficits (36%), drowsiness (35%), dysphagia (30%), headache (27%), confusion (27%), aphasia (24%), motor deficits (21%), fatigue (20%), and dyspnea (20%). IJzerman-Korevaar et al. have also reported the prevalence of symptoms in a phase wise manner. The five most prevalent symptoms in the diagnostic phase are cognitive deficits (36%), seizures (35%), headache (31%), dizziness (24%), and motor deficits (22%). In the treatment and follow-up phase, the most prevalent symptoms are seizures (37%), nausea/vomiting (23%), cognitive deficits (18%), fatigue (14%), visual deficits (13%), and anorexia (13%). In the end-of-life phase, drowsiness (81%), fatigue (50%), aphasia (48%), seizures (45%), cognitive deficits (44%), and motor deficits (44%) are most prevalent [26].

3 Survival Rate

Long-term survivors of GBM are rare. Several variables including tumor size and location determine a patient's survival chances. Age at diagnosis is yet another important factor that determines survival chances of patients; where young patients receive more aggressive and multimodal treatment. Functional status is yet another factor, which has negative correlation with age. Similarly, histologic and genetic factors also influence survival [27]. The median survival time with GBM is 15–16 months in patients who undergo surgery, chemotherapy, and radiation treatment [28]. Delayed diagnosis is the major reason behind poor patient outcomes and a major unmet need in the pharma market. To date, GBMs remain incurable and most of the time the available treatment is palliative. Despite all the advancement in diagnosis, new research in brain neoplasms and multidisciplinary treatment approach, the overall 5-year survival rate is still approximately 5% [29] which is one of the huge unmet needs. Average survival usually is less than 1 year; and if the tumor recurs, the survival rate decreases, with tumor progression and death in most GBM patients [24].

4 Current Approved Treatment Options for GBM

4.1 Surgery

Surgery remains to be the first choice for tumor de-bulking and accessing tissue samples for pathology. Surgical resection of GBMs after diagnosis is used to (a) relieve mass effect, (b) confirm the diagnosis pathologically, and (c) decide on the course of treatment. If neurological functions are not compromised, maximal tumor resection may be beneficial. However, a fine balance between the aggressive removal of malignant tissue and minimizing the risk of worsening or inducing new neurologic deficits that may negatively impact outcomes is also very important. Furthermore, residual microscopic disease invariably results in disease progression and recurrence within months. To develop such an optimal balance, the neurosurgeon must assess patient prognostic factors, including tumor location and size to determine the extent of resection (EOR) that provides maximal survival and functional benefit. To date, the best data available which helps to establish this fine balance comes from large institutional retrospective analyses. To develop surgical strategies, the relationship between EOR, tumor resectability, involvement of critical brain areas and risk must be carefully elucidated. Usually, the EOR threshold remains identical at 80% in most of the scenarios [30]. However, because of the highly infiltrative and heterogeneous nature of GBM, surgical resection alone leads to median survivals of only 3–6 months. With the development of radiotherapy, postoperative survival has improved significantly to approximately 1 year. Currently, concomitant radiation and oral alkylating agent temozolomide (TMZ) extend the survival to 15–16 months [31].

4.2 Radiation Therapy

The post-surgery standard of care for patients 70 years or younger is partial-brain fractionated radiotherapy with concomitant administration of TMZ. For patients under 70 years, the optimal dose fractionation schedule for external beam radiation (EBRT) after surgery is 60 Gy in 2-Gy fractions administered over 6 weeks. For patients over 70 years, hypofractionated radiotherapy (HFRT) is recommended. Several recent studies suggest that the median OS of patients with HFRT increases to 20 months compared with conventional RT [31]. HFRT benefits patients in limiting tumor repopulation, increases cell kill and reduces the overall treatment time.

4.3 Chemotherapy

Due to the invasive nature of GBM, surgical resection rarely eliminates all tumor cells, and post-surgical treatment is usually necessary to prevent recurrence. Treatment varies depending on the age of the patient and stage of the disease. The most commonly prescribed GBM chemotherapy medications include (Fig. 1):

- TMZ, the current "gold standard" of care.
- Carmustine, another common medication for high-grade brain cancers.
- Bevacizumab, typically used as a second-line treatment for recurrent GBMs.
- Lomustine, which may help improve the efficacy of bevacizumab when both medications are administered at the same time.
- Cyclophosphamide.

4.3.1 Temozolomide (TMZ)

The standard-of-care TMZ is a DNA alkylating agent discovered in the 1970s and approved in 2005 by the FDA to treat newly diagnosed brain tumors. TMZ is an imidazotetrazine derivative. TMZ is not directly active but undergoes rapid non-enzymatic conversion at physiologic pH to its active metabolite 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide (MTIC). The cytotoxicity of MTIC is thought to be primarily due to alkylation of DNA. Alkylation (methylation) occurs mainly at the O6 and N7 positions of guanine. TMZ responsive patients have O[6]-methyl-guanine methyl transferase (MGMT) genes with methylated promoters. These patients showed higher survival rate as compared

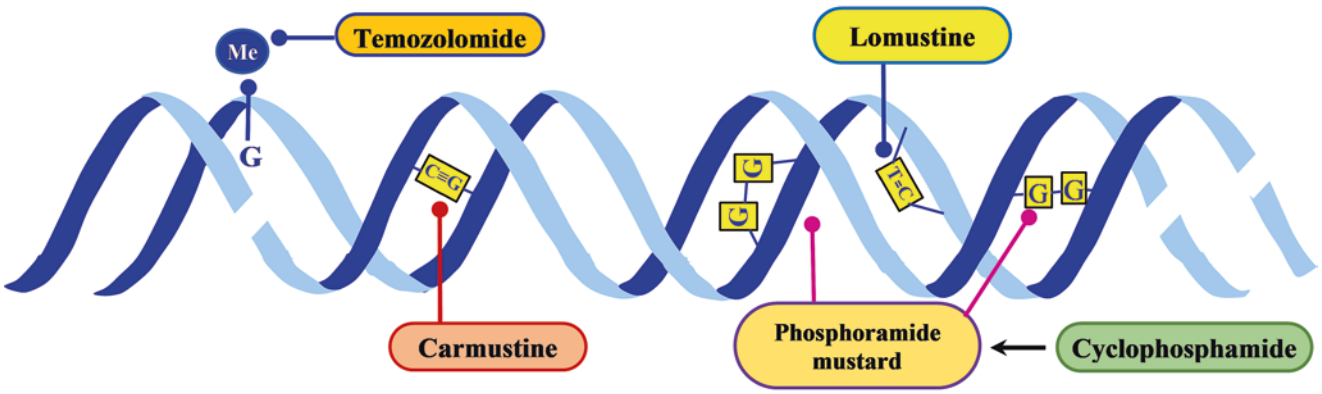

Fig. 1 Mode of action of alkylating agents approved for glioma. TMZ methylate DNA, at the N-7 or O-6 positions of guanine residues, damages the DNA and triggers the death of tumor cells. Carmustine and Lomustine alkylate DNA and inhibit several key enzymatic processes by carbamoylation of amino acids in proteins. Activated form of cyclophosphamide, a phosphoramide mustard irreversibly cross-links with DNA both in between and within DNA strands at guanine N-7 positions and causes cell death

to those with hypomethylated MGMT genes. MGMT is a DNA repair enzyme that repairs the N7 and O6 positions of guanine alkylated by TMZ. Hence, MGMT gene methylation status remains an important biomarker for GBM prognosis [31]. However, TMZ presents unwanted toxicity and does not eliminate the disease. The approved dosage regimen for newly diagnosed GBM is 75 mg/m^2 for 42 days concomitant with focal radiotherapy followed by initial maintenance dose of 150 mg/m^2 once daily for days 1–5 of a 28-day cycle of TMZ for 6 cycles. The recommended dose for TMZ as an intravenous infusion over 90 min is the same as the dose for the oral capsule formulation.

4.3.2 Carmustine and Lomustine

Carmustine and Lomustine are nitrosoureas used in the treatment of certain neoplastic diseases. While Carmustine is a 1,3-bis (2-chloroethyl)-1-nitrosourea, Lomustine is 1-(2-chloro-ethyl)-3-cyclohexyl-1-nitrosourea. Both Carmustine and Lomustine alkylates DNA and RNA. The drugs may also inhibit several key enzymatic processes by carbamoylation of amino acids in proteins. Due to their high lipophilicity and the relative lack of ionization at physiological pH, both drugs have high ability to cross the blood–brain barrier quite effectively. Studies have revealed that their concentrations in the CSF are ≥50% of those measured concurrently in plasma. The recommended dose of Carmustine as a single agent in previously untreated patients is 150–200 mg/m^2 intravenously every 6 weeks. This may be given as a single dose or divided into daily injections such as 75–100 mg/m^2 on two successive days. The recommended dose of Lomustine in adult and pediatric patients as a single agent in previously untreated patients is 130 mg/m^2 as a single oral dose every 6 weeks. In individuals with compromised bone marrow function, the dose should be reduced to 100 mg/m^2 every 6 weeks.

4.3.3 Bevacizumab

Bevacizumab, an anti-vascular endothelial growth factor (VEGF) monoclonal antibody, was first approved by the FDA in 2004 to treat metastatic colorectal cancer. Since then, it has been approved for several different types of cancer, including GBM in 2009. VEGF is a broad mediator of tumor neovascularization, and VEGF expression is linked with GBM tumorigenicity. Hence, Bevacizumab is indicated for the treatment of GBM as a single agent for adult patients with progressive disease following prior therapy. However, a phase III "Avaglio" trial conducted on 921 patients and another phase III trial RTOG0825 conducted on 637 patients showed no change in OS. Therefore, bevacizumab treatment is an option reserved only for patients with recurrent GBM.

4.3.4 Cyclophosphamide

Cyclophosphamide is a synthetic antineoplastic drug and is chemically related to the nitrogen mustards. The mechanism of action is thought to involve cross-linking of tumor cell DNA. Cyclophosphamide is biotransformed in the liver to its active alkylating metabolites. These metabolites interfere with the growth of susceptible rapidly proliferating malignant cells. One recent study suggested that cyclophosphamide improves survival in orthotopic GL261 GBM in mice by metronomic administration (every 6 days) [31]. The usual intravenous dosage regimen consists of 40–50 mg/kg given intravenously in divided doses over a period of 2–5 days. While, the oral dose is usually in the range of 1 mg/kg per day to 5 mg/kg per day for both initial and maintenance dosing.

4.4 Tumor-Treating Field

Tumor-treating field (TTF) is a relatively new treatment option for newly diagnosed and recurrent GBM. Optune, or the NovoTTF-100A System, was approved by FDA to treat recurrent GBM in 2011 and for newly diagnosed GBM in October 2015 [31]. TTFs are generated via electrodes on the scalp with unique array placement based on individual's MRI results. The tumor-treating field devices use low-intensity, intermediate-frequency, alternating electric fields to disturb the dividing processes in GBM cells. The mechanism of TTF is: (a) disturbing the formation of mitotic spindle fibres and (b) rupturing the cell membrane at the cleavage furrow during late mitosis by accumulating polar molecules at this site [31]. The results from a randomized phase III trial (EF-11) comparing the efficacy of TTF regarding extending OS and PFS in recurrent GBM in comparison to chemotherapy indicated superior health related to quality of life (HRQoL), pertaining to cognitive and emotional functioning as well as treatment-related toxicity. Another randomized phase III clinical trial (NCT00916409) for newly diagnosed GBM indicated superiority of TTF to TMZ alone in both OS and PFS [32].

5 Challenges and Unmet Need

One of the main challenges of an effective therapy lies in the heterogeneous and anaplastic character of this cancer. Rapid growth of cancer and a high rate of recurrence further complicates the matter. Further, the response to anticancer drugs is frequently inhibited by resistance to therapeutic agents [29]. The blood–brain barrier (BBB) represents another major hurdle to the development of new therapeutics, preventing most macromolecules from reaching the tumor site, thereby affecting the efficacy of the drug adversely. Thus, GBM treatment is an area of high unmet need leading to poor OS of GBM patients, the overall failure of marketed products for this indication, and more so the disappointing failures in the late-stage pipeline.

There are several new drugs targeting different pathways in tumorigenesis and angiogenesis in malignant gliomas currently being tested in clinical trials. Further, molecular and genetic profiling will be necessary to determine patients that are most likely to benefit from the specific treatment options. Although the high level of unmet need in this market creates ample opportunities for players with effective therapies, drug development has been exceedingly challenging. Most of the drugs that have shown promise in early-phase, single-armed trials unfortunately fail in larger, randomized studies. Currently, there are seven drugs in the late stages of development. These include Opdivo, DCVax-L, and depatuxizumab mafodotin, which have shown promising efficacy in Phase I trials.

The evaluation of tumor response criteria should also be improved to better examine whether new therapies are leading to a significant reduction in tumor size and improvement of quality of life (QOL). The GBM with the worst unmet need are those where tumors are large, centrally located, or have a "butterfly" shape. These GBM patients have the worst QOL and cannot be treated effectively. Even radiotherapy is difficult to administer and patients suffer from adverse neurological symptoms. Around 20% of patients fall within this category and never have any improvement in their QOL. Thus, agents that reduce the size of the tumor, rather than focus solely on extending PFS and OS, would be effective for these patients [24].

6 Cancer Stem Cells/Glioma Stem Cells

Cancer stem cells (CSC) were first identified in leukemia in 1997 and subsequently reported in tumors of breast, colon, lung, brain, and liver [32]. CSC-like cells in gliomas and other brain tumors are referred to as tumor-initiating cells, glioma stem cells (GSC) or glioma stem-like cells [33–36].

GSC constitute a small population of quiescent cells in tumors and play a key role in proliferation, progression, invasion and recurrence of tumors [37]. The ability of GSC to self-renew and differentiate into different lineages of tumor types in response to chemotherapeutic agents [38, 39] contributes to intratumor heterogeneity of GBM which is a striking feature of these deadly tumors.

GSC are proposed to arise from transformation of neural stem cells (NSC) or progenitor cells. When cultured under NSC conditions, GSC demonstrate a potential to form neurospheres and differentiate towards neuronal, astrocytic- and oligodendroglial-like cells. Gene expression studies in GBM have revealed similar profiles in CSC and NSC suggesting that CSC are malignant variants of NSC [40]. GSC derived from GBM are classified into PN and MES types [41–43]. While the PN type cells display characters of fetal NSC, MES are more like adult NSC [44, 45]. In comparison with PN-GSC that are present in Grade III and Grade IV (GBM) gliomas, MES type GSC reside in GBM and contribute to their highly aggressive nature of these tumors [44, 45]. A recent study by Guardia et al. reported that GSC cell lines of MES and PN subtypes depict differential expression patterns of genes regulating cell cycle, DNA repair, cilium assembly, and the splicing profiles correlated significantly with the survival of the patients. In GBM patients, high expression of multiple long noncoding RNAs in mesenchymal GSC is associated with poor survival [46].

CD133 (also known as AC133 and prominin-1) is a widely accepted biomarker for CSC and used for identification of tumor-initiating population in glioma tumors. There is no single marker or a single set of biomarkers that are

specific for GSC [47–49]. GSC share many genes associated with stem cells (Nestin, GFAP, A2B5, NANOG, OCT4, CD44, and KLF4) [50]. High-grade gliomas exhibit embryonic stem cells markers-NANOG, KLF4, OCT4, and SOX2. NANOG, a transcription factor that is involved in self-renewal and maintenance of undifferentiated state of pluripotent cells is associated with low- and high-grade astrocytomas and is a predictive marker for clinical outcome in this group of patients [50].

Many factors contribute to the aggressive nature of GSC. These factors may be intrinsic like those induced by genetic, epigenetic, and metabolic alterations, or they may be extrinsic factors that include components of microenvironment and host immune system [51]. TMZ, a point-of-care treatment for GBM expands the GSC population in glioma cell lines [52]. Vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) is expressed on the CD133+ve GSC and is important for viability, self-renewal, and tumorigenicity; the activity is mediated through VEGF-VEGFR2–Neuropilin-1 (NRP1) signaling axis [53]. GSC are responsible for resistance to radio- and chemotherapies and recurrence of tumors [54]. The mechanism(s) underlying drug resistance involves deregulation of signaling pathways through interaction between CSC and the protective niche of the tumor. Diverse signaling pathways are implicated in the growth and activity of GSC [55, 56]. Notch signaling is crucial for tumorigenesis, maintenance of GSC stemness, and resistance to radiotherapy [57]. The sonic hedgehog (SHH) signaling pathway plays a key role in cell survival and sustained growth of the tumor. In malignant gliomas, SHH controls stemness of GSC [58] and blocks tumor formation induced by intracranial injection of GSC. SHH pathway is also involved in tumorigenesis and TMZ chemoresistance in GBM [59]. Wnt signaling pathway is essential for a myriad of cellular functions such cell proliferation, embryonic development, cell polarity, and tissues homeostasis [60]. Wnt signaling is important for differentiation of GSC and deregulation of this pathway renders GSC resistant to chemo- and radiotherapy [61]. Bone morphogenetic proteins (BMPs) signaling regulates proliferation, differentiation, and apoptosis in NSC [61]. In GSC, this signaling pathway regulates differentiation of GSC towards astroglial lineage, inhibits its tumorigenic potential [62, 63], and sensitizes the cells to TMZ treatment through destabilization of HIF-1α and MGMT expression [64]. Transforming growth factor-β (TGF-β), a family of cytokines is crucial for morphogenesis and cell lineage specification during brain development [65, 66]. TGF-β maintains the stemness in GSC through expression of transcription factor SOX2 that is essential for expression of stemness features [67].

The phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB) signaling is crucial for cell growth and survival. Dysregulation of this pathway promotes proliferation, survival, invasion, and progression of tumors in different types of cancers [68]. Loss of PTEN increases the GSC population suggesting the involvement of PTEN-Akt signaling pathway in growth and survival of GSC [69]. In GSC, the PTEN/PI3K/Akt pathway is important in regulation of multidrug-resistant gene-ABCG2 [69].

Epigenetic modifications such as DNA methylation and histone modifications are crucial in programming of stem cells [70]; however, aberrant epigenetic alterations may lead to transformation of normal stem cells to cancer stem cells with the loss of differentiation [71, 72]. Histone modifications by phosphorylation, ubiquitination, acetylation, and methylation have an active role in initiation, growth, and progression of cancer [73, 74]. Aberrant histone modifications play a key role in GBM [75]. Global expression analysis of several histone modification markers in gliomas identified many distinct prognostic groups that were associated with significantly different survival time [76]. Recent studies have implicated cellular prion protein (PrPC) in progression of malignant phenotype of GSC, maintenance of multipotency, an increase in tumorigenesis and invasiveness of these cells [77].

7 Tumor Microenvironment (TME) in GBM

The highly heterogeneous nature of tumors confers resistance to radio- and chemotherapy in GBM. While epigenetic modifications, genetic aberrations and dysregulated signaling within the tumors are crucial in inducing heterogeneity in GBM tumors; recent studies have shed light on the importance of TME of these aggressive tumors. Accumulating data reinforce the involvement of TME components as key factors for tumor progression, invasion and therapeutic resistance, thus making them potential targets for therapy.

7.1 Tumor Vasculature

The TME of GBM is very rich in proangiogenic factors that promote poorly organized neovascularization in the tumor. The BBB formed by endothelial cells is a highly selective semipermeable border for exchange of ions and molecules from circulating blood to the nervous system. Proangiogenic factors also promote the dilation of the wall of endothelial cells and pericytes (tumor vasculature) and result in disruption of the BBB rendering it leaky [78]. In GBM, the leaky BBB increases intracranial pressure that leads to blockage of endothelial cells and disruption in availability of oxygen and nutrients to the tumor [79]. These conditions promote a hypoxic microenvironment which eventually makes the core of GBM to become necrotic [80]. Metabolic deprivation and reduced oxygen tension stimulate the release of HIFs in the necrotic core and promote neovascularization by upregulating tran-

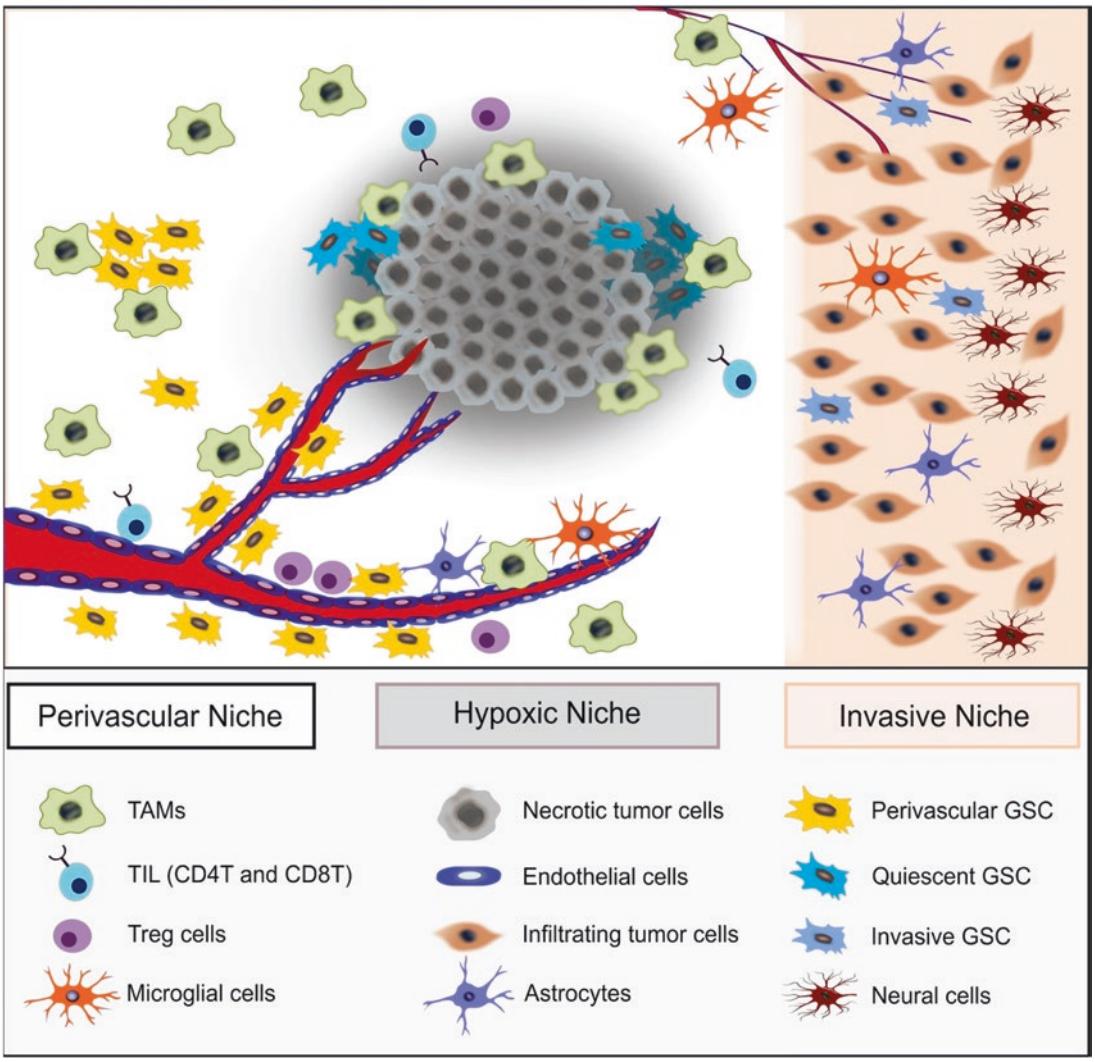

Fig. 2 Cellular heterogeneity in the microenvironment of GBM. Niche heterogeneity in GBM is majorly contributed by specific cell populations to meet their metabolic needs, maintenance of glioma stem cells, regulate immunosuppression and invasion. Due to poor vascularization, the core of GBM tumor is rich in quiescent GSC and attracts immune cells (TAM and TIL). The differentiated tumor in the deepest core undergoes necrosis to give rise to hypoxic/necrotic core. The cellular mass residing exterior of the necrotic region, known as perivascular niche is highly vascularized and rich in TAM which are crucial for promotion of proliferation, survival and differentiation of GSC. Perivascular zone is also enriched with TIL, T-regulatory (Treg) cells and microglial cells that play a role in immune suppression. Invasive niche is rich in microglial cells, astrocytes, neuronal cells and GSC. The GSC in the invasive niche differentiate towards mesenchymal subtype and facilitate invasion of tumor into the normal brain tissue and neo-vascularization

scription of VEGF [81, 82]. The HIFs in these poorly perfused areas of tumor attract immune cells (tumor-infiltrating lymphocytes and tumor-associated macrophages) which release proangiogenic factors and facilitate immune-evasion for tumor progression [83, 84]. Also, the hypoxic niche promotes self-renewal of GSC which subsequently contributes to neovascularization by secretion of VEGF [85, 86].

The TME contains various types of cells localized in different regions of the niche with a variety of activities. Spatially, the microenvironment of GBM can be divided into three zones: necrotic core, perivascular zone, and invasive edge. Each zone consists of various cellular and non-cellular components. The cellular components contain tumor population, GSC, and non-tumor cells such as endothelial cells, immune cells and neural cells (Fig. 2). The non-cellular constituents of the GBM microenvironment are cytokines, growth factors, interstitial fluid, and extracellular matrix (ECM). The highly heterogeneous microenvironmental niche plays a role in GBM progression by promoting activation of a cascade of cellular events (Table 1).

Table 1 Components of tumor microenvironment (TME) niches

Niche	Cellular components	Non-cellular components	Functions	Ref
Necrotic	Necrotic tumor cells, quiescent GSC, TAMs, and TIL	Proangiogenic factors, HIFs	Metabolic adaptation to nutrient deprivation, (aerobic glycolysis and glutamine-mediated fatty acid production)	[87, 88]
Perivascular	Proliferating tumor cells, GSC, TAMs, TIL, astrocytes, and microglia	Proangiogenic factors, IL-10, TGFß	Self-renewal and growth of GSCs, vascularization, immunosuppression	[89–92]
Invasive	Invasive tumor cells, GSC, microglia, neural cells, and astrocytes	Proangiogenic factors, IL-6, MMPs, L1CAM ECM, Galectin-1	Invasion of tumor cells to normal tissue	[93, 94]

7.2 Heterogeneity in the Microenvironment of GBM

7.2.1 Cellular Components

Glioma Stem Cells

Self-renewal and growth of GSC are mainly maintained by the perivascular niche. The endothelial cells promote the stemness phenotype of GSC through activation of NOTCH, sonic hedgehog, and nitric oxide-cyclic GMP signaling pathways [89–92]. Tumor-associated macrophages (TAMs) maintain the self-renewal capacity of GSC by secreting various cytokines in the perivascular niche. In the necrotic core of GBM, elevated HIF-2 activates stem cell signaling pathways to regulate expression of pluripotent markers, KLF-4, SOX2, and OCT4 [95, 96]. GSC of invasion edge exhibit higher invasion potential due to higher expression of L1CAM and ephrin-B2 [93, 94]. The NF-κB-STAT3 and TWIST signaling pathways regulating the epithelial to mesenchymal transition are also upregulated in GSC residing in the necrotic edge [93, 97, 98]. GSC migrating through the vasculature in the invading edge limit surgical resection of tumor and promote the recurrence of tumor following surgery.

Immune Cells

In GBM, about 30 or 40% of the tumor mass is composed of immune cells of which approximately 85% is contributed by infiltrating bone marrow-derived monocytes/macrophages, resident microglial cells, TIL, and nonmigratory dendritic cells account for 15% of the TME [99]. These different cell types create an immunosuppressive niche rendering the tumor cells resistant to immunotherapy. Additionally, the tumor cells also secrete immunosuppressive cytokines such as IL-6, IL-10, TGF-β, and prostaglandin-E that are responsible for inhibition of both innate and adaptive immune systems. These factors contribute to immune evasion by inducing T-cell apoptosis, suppressing T-cell activation and proliferation, downregulation of MHC expression, and suppression of NK cell activity. TAMs possess markers of M1 (immuno-permissive or proinflammatory) as well as M2 (immune-suppressive or anti-inflammatory) phenotypes. The TAM population is slightly skewed towards CD163 (hemoglobin scavenger receptor) and CD204 (macrophage scavenger receptor) expressing M2 phenotype which increases with the grade of tumor [100]. Macrophage polarization is dependent on the local microenvironment; while M1 macrophages are mostly present within normoxic tumor region, macrophages with M2 phenotype dominate the hypoxic region [101]. M2 type macrophages, devoid of expression of T-cell co-stimulatory molecules release immunosuppressive factors such as IL-10 to establish protumorigenic microenvironment within GBM tumors [100, 102]. Specific macrophage types are localized to specific regions, while monocyte-derived macrophages are concentrated in the tumor core, microglia-derived TAM are typically found at the tumor periphery [99]. Recent studies have reported the immunosuppressive role of distinct subpopulations within the immunoregulatory macrophage compartment of GBM tumors [103]. TAM attract Tregs to TME via CCL22. A small proportion of immune cells is contributed by TIL that are dispersed in GBM tumor and the brain parenchyma. Infiltration of TIL into the tumor is neutralized by an array of immune suppressive events such as downregulation of MHC to check antigen presentation, upregulation of programmed death ligand-1 (PD-L1) on monocyte, and promotion of Tregs recruitment in GBM [104]. Immunosuppressive Treg cells facilitate immune evasion by secreting immunosuppressive molecules, IL-10 and TGF [105, 106].

7.2.2 Non-cellular Components

Extracellular Matrix (ECM)

The ECM is formed by non-cellular components and provides physical scaffolding to cellular residents of the tissue. The ECM triggers a cascade of biochemical events contributing to differentiation, migration, EMT transition, and homeostasis [107, 108]. Composition of mesh-like scaffold of brain ECM is unique in comparison with various peripheral tissues; the fibrous proteins of ECM in brain are synthesized by neuronal cells whereas in other peripheral organs they are

synthesized and secreted by fibroblasts and mesenchymal cells. Mesh-like scaffold (perineuronal net) in ECM of adult brain is composed of glycosaminoglycans (GAGs), hyaluronic acid (HA), proteoglycans (e.g., lecticans), and glycoproteins (e.g., tenascins) [109–112] ECM of GBM is also highly enriched with tenascin-C and HA to provide rigid architecture to tumors [113, 114]. Stiffening of ECM provides advantage to sustain elevated fluid pressure due to edema and leaky BBB [115]. HA enhances stemness of the GSC by occupying the HA-specific cell surface receptors RHAMM128, CD44, and by activating the transcription of stemness modulators [116, 117]. HA also contributes to GSC stemness and maintenance by activating Toll-like receptor (TLR) 4-nuclear factor NF-κB signaling pathway. Tumor cells interact with components of ECM by secreting hyaluronidase or matrix metalloproteinases to facilitate migration and invasion of tumor. Galectins in ECM also hamper anti-tumor immune rejection by scavenging glycosylated cytokines such as IFNγ [118].

Interstitial Fluid

Interstitial fluid is a highly dynamic soluble compartment of TME. Interstitial fluid is secreted by stromal cells, tumor cells and intravascular components of tumor and contains metabolites like lactate, adenosine, galectin-1 (galactoside-binding lectin) and cytokines such as IL-10 [119–121]. Lactate and adenosine promote the development of a hypoxic environment which contributes to immune-suppression and tumorigenic progression [122–125]. These soluble factors are present abundantly in the TME and distributed throughout the tumor unequally causing a chemotactic gradient of interstitial fluid that promotes changes in cellular composition of the niche constantly. Galectin-1 is a key player in migration and invasion of GBM tumor cells; higher expression of galectin-1 is seen at the margin in comparison to core of GBM tumor. High expression of galectin-1 correlates with increased VEGF secretion and invasive behavior of GBM. Recent studies have identified the role of extracellular vesicles in invasion, angiogenesis, and drug resistance in GBM [126].

8 Epigenetic Alterations

Epigenetic alterations such as DNA methylation patterns, chromatin remodeling, and histone modifications induce changes in gene expression and contribute to tumorigenesis in GBM [127]. The changes are effected by silencing of tumor suppressor genes and/or the activation of oncogenic genes. MGMT is a potent DNA repair enzyme; it antagonizes the cytotoxic effect induced by alkylating agents such as carmustine or TMZ [128]. Epigenetic silencing by methylation of MGMT gene promoter is a strong prognostic marker for GBM and is associated with longer survival in GBM patients receiving radiotherapy and chemotherapy with alkylating agents. Methylation of MGMT promoter is found in about 45% of malignant gliomas (grades III and IV) and in about 80% of Grade II gliomas [129, 130]. More recent comprehensive analysis of GBM cells using wild type IDH1 and single cell gene expression profiling identified three distinct types of GBM viz. proneural, classical, and mesenchymal [131].

IDH mutation affects global DNA methylation pattern. Genome-wide DNA methylation profiling has revealed that CpG island methylator phenotype (G-CIMP)-positive phenotype is linked to IDH1 mutations and is associated with better survival compared to (G-CIMP) glioma-negative patients [132]. Further studies by quantitative methylation categorized IDH mutant type into two subsets—IDH-mutant/G-CIMP+ (G-CIMP-high) and the IDH-mutant/G-CIMP+ (G-CIMP-low) and demonstrated the association of high methylation in G-CIMP with better survival compared to low methylation G-CIMP. The findings suggested classification of gliomas based on IDH1 mutant that is independent of histology and grade of tumors [133].

Histones are subject to different post-translational modifications such as acetylation, methylation, and phosphorylation [134]. Acetylation of histones are well studied for their role in cancer initiation and progression. The activities of enzymes, histone acetyltransferases (HAT), and histone deacetylases (HDAC) together maintain the balanced state of acetylation [135]. In GBM, HDAC class IIa enzymes (HDAC 4, 5, 7, 9) play an important role in tumor progression, invasion, and response to drug and radiotherapy, thus making HDAC an attractive target for therapy [136, 137]. Other studies suggested that alterations in DNA methylation, histone modifications can affect growth in GBM by regulation of cell metabolism, especially glycolysis [138].

9 Genetic Mutations and Dysregulated Signaling Pathways

9.1 Dysregulated Pathways, Targeted Therapy, and Clinical Results to Date

GBM is an invasive tumor with hallmarks of uncontrolled cell proliferation, neoangiogenesis, resistance to apoptosis and intratumor heterogeneity, contributing to the poor prognosis. Also, a variety of genetic and epigenetic alterations have been identified in GBM that have an impact on patient prognosis. Despite this heterogeneity, studies on genetic aberrations in GBM identified three main signaling pathways that are commonly dysregulated, are activation of the receptor tyrosine kinase (RTK)/Ras/phosphoinositide 3-kinase (PI3K) pathway (88%), inhibition of p53 (87%),

and retinoblastoma protein (Rb) signaling pathways (78%). Hence, till date, drugs targeting many of these commonly observed alterations have been widely investigated as potential targeted therapies for GBM [139].

In the past two decades, advances in technologies have made it possible to evaluate genetic and epigenetic tumor changes at the genome-wide level. This has led to continuous influx of data describing genomic alterations in gliomas and particularly in GBM. Collectively, these data have created a better understanding of the glioma landscape and elucidated common pathways disrupted in this disease. Furthermore, significant contributions are made by focused consortium-based efforts and various individual laboratories. Leading among these is The Cancer Genome Atlas (TCGA), a US government-funded project with multiplatform data compilations for more than 520 GBM samples, including microRNA, messenger RNA, single-nucleotide polymorphism, DNA methylation aberrations, DNA copy numbers, coding and noncoding RNA expression, and exome sequencing data. Overall, these analyses established that deregulation of the core retinoblastoma (Rb)/p53, phosphoinositide 3-kinase (PI3K)/Akt/phosphatase and tensin homolog (PTEN)/mammalian target of rapamycin (mTOR), and receptor tyrosine kinase (RTK)/RAS/RAF/mitogen-activated protein kinase (MEK)/mitogen-activated protein (MAP; extracellular signal-regulated kinase [ERK]) pathways is essential event in majority of GBM tumors. Specific genetic aberrations in RTKs involved in RAS/RAF and PI3K/Akt signaling pathways, mutations in epidermal growth factor receptor (EGFR; 45%), platelet-derived growth factor receptor (PDGFR, 13%), and MET (4%). Moreover, disruption of the p53 pathway included mutations in p53, amplifications of murine double minute 2 (MDM2; 11%) and MDM4 (4%). Likewise, the most frequent event for RB pathway disruption was a deletion in the cyclin-dependent kinase inhibitor 2A (CDKN2A)/CDKN2B locus on chromosome 9p21 (55% and 53%, respectively) followed by amplification of the cyclin-dependent kinase 4 (CDK4) locus (14%). In parallel to these TCGA studies, in a high-throughput profiling study, Parsons et al. sequenced 20,661 protein-coding genes in 22 human tumor samples and concluded the similar alterations in genes alongside identified mutations in a metabolism-related gene, isocitrate dehydrogenase 1 (IDH1) [20]. Subsequently, various groups have attempted to use large-scale profiling data and sequencing results to sub-classify GBMs into subtypes that reflect common mechanisms of diseases [140].

9.2 Molecularly Targeted Therapies

The most frequent genetic alterations in GBM target pathways are involved in signal transduction, angiogenesis, cell-cycle control, tumor suppressors, invasion, and cell metabolism, indicating that these are potential targeted therapeutics towards pathology. Furthermore, despite the molecular heterogeneity of malignant gliomas, there exist common signal transduction pathways that are altered in many of these tumors. Homeostasis of pathways is maintained in a normal state through crosstalk between cytokines, growth factors, and hormones. However, in malignancies, mutation, deletions, or overexpression can occur in growth factor ligands and their receptors (e.g., EGF and EGFR), over and above in intracellular effector molecules (e.g., phosphatase and tensin homolog deleted on chromosome 10 [PTEN] and phosphoinositide-3-kinase [PI3K]/Akt). Genomic amplifications provide prevailing pathological mechanisms for upregulating various oncogenes, e.g., EGFR on chromosome 7, whereas deletions can target tumor suppressors, viz. PTEN on chromosome 10. This leads to constitutive activation of these growth factors and their downstream effector molecules resulting in uncontrolled cell proliferation, survival, and invasion [140].

9.3 Inhibitors of Receptor Tyrosine Kinases (RTKs)

RTKs play an important role in a signal transduction events and dysregulation of RTK signaling leads to a variety of human diseases, most notably, cancers. Aberrant RTK activation in human cancers is enabled by four principal mechanisms: gain-of-function mutations, genomic amplification, chromosomal rearrangements, and/or autocrine activation. It frequently occurs during glioma initiation and progression, in turn associated activation cascades may cooperate through multiple signaling crosstalks leading to malignant transformation of cells, tumor growth and progression, treatment resistance, and disease relapse.

The Cancer Genome Atlas project (TCGA-2008) reported significant alterations in three core signaling pathways, viz. RTK/RAS/PI3K (88%), p53 (87%), and retinoblastoma protein (78%), from patients with primary GBM, which may represent most human GBM. About 60% of the primary GBM harbors RTK amplifications and/or mutations. EGFR amplification occurs in approximately 50% of primary GBM samples and is often associated with the expression of a constitutively active, ligand-independent mutant form of the receptor, viz. EGFRvIII. EGFR and EGFRvIII overexpression perhaps enhances GBM cell growth and contributes to GBM pathogenesis through several mechanisms, such as increased signaling through RAS/RAF/MEK/MAP and mTOR pathways while simultaneously downregulating cell-cycle inhibitor proteins such as p27 leading to apoptotic resistance [141].

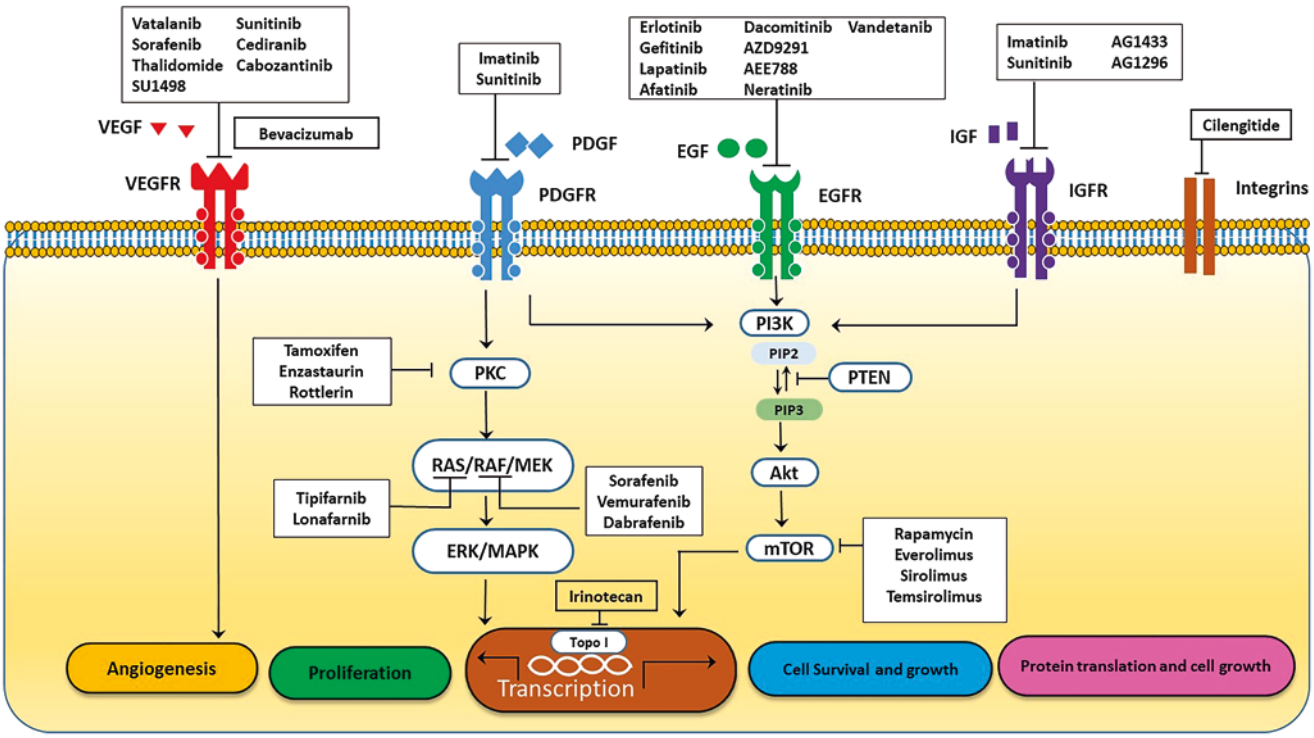

Fig. 3 Molecular targeted therapies for glioma and their therapeutic role
Diagramatic representation of key molecular pathways targeted for the development of therapies against glioma. RTKs are activated upon binding with respective ligands. Molecular treatments have been designed to inhibit signaling pathways at different phases of cellular responses. Line with black arrow head represents activation of intracellular pathway components and a line with blunted end represents inhibition of cellular processes. Specific inhibitors in the rectangular boxes depicts targets against RTKs and downstream pathway components

9.4 Epidermal Growth Factor Receptor (EGFR) Inhibitors

EGFR (also referred to as ERBB1 or HER1) receptor homo or heterodimerization takes place after the binding of a ligand to the ligand binding site of the receptor. In turn producing a conformational change that activates the intracellular tyrosine kinase domain. This results in autophosphorylation of the cytoplasmic tail and induction of a variety of downstream signaling pathways. EGFR amplifications and mutations are detected in 40–60% of GBM cases and are indicative of poor prognosis and correlated with decreased OS in GBM patients. The most common mutant form found in GBM is EGFRvIII, or de2-7EGFR, arising through an 801-base pair in-frame deletion from the extracellular domain. The overexpression or mutation of EGFR disturbs downstream signaling including mTOR/PI3K/Akt and RAS/MAPK. This primes to impairment of apoptosis, enhances proliferation, and angiogenesis contribute to GBM pathogenesis. Due to the high incidence of EGFR amplifications, a variety of EGFR inhibitors have been examined both preclinically and clinically (Fig. 3) [142].

9.4.1 Small-Molecule Inhibitors to EGFR

Small-molecule tyrosine kinase inhibitors are the most widely studied EGFR inhibitors in GBM. The first-, second-, and the third-generation EGFR inhibitors are studied in preclinical and clinical settings as a single agent and combination with SOC for GBM. Erlotinib, a first-generation EGFR inhibitor in preclinical studies showed inhibition of anchorage-independent growth of GBM cells in an EGFR expression-dependent manner and induced greater levels of apoptosis in more malignant GBM phenotypes. Moreover, in vivo studies showed that treatment with erlotinib reduced the tumor burden in GBM patient-derived xenograft (PDX) models. However, further studies using additional GBM PDX models demonstrated that tumors overexpressing EGFR were only sensitive to erlotinib if they also expressed PTEN. As PTEN expression is downregulated in 34% of GBM patients, indicating that erlotinib may not be a suitable treatment for most GBM patients overexpressing EGFR. Indeed, clinical trials demonstrated that erlotinib was not effective as a monotherapy in recurrent GBM patients and was only marginally beneficial following radiotherapy for non-progressive GBM patients. Interestingly, improved survival (19.3 vs. 14.1 months) was observed when combined with SOC TMZ and radiotherapy, suggesting that erlo-

tinib could be beneficial when combined with targeted drugs, instead of a monotherapy [142].

In contrast to erlotinib, gefitinib displays distinctive anti-tumor activity independent of the expression level of EGFR. Preclinical studies showed that in vitro gefitinib inhibits GBM cell migration, reduces proliferation of human glioma tumor-initiating cells, and in vivo enhances survival in an intracranial GBM mouse xenograft model. Thus, these preclinical studies indicate that gefitinib may be clinically beneficial. However, despite gefitinib reaching high concentrations in GBM tumor tissue (22-fold higher compared to plasma) alongside significant dephosphorylation of EGFR, limited clinical effects have been observed in Phase II trials. Several Phase I/II studies have demonstrated that even as the addition of gefitinib to radiotherapy is well-tolerated, it has no survival benefit. Lapatanib, another first-generation EGFR inhibitor, also showed modest anti-tumor activity in clinical trials either alone or in combination with TMZ [143].

Second-generation inhibitor afatinib and dacomitinib proved to have limited activity in a phase II clinical trial in recurrent GBM patients with EGFR amplification and had limited activity in combination with GBM.

The third-generation EGFR inhibitors, viz. AZD9291 and AEE788 are proved to have better activity and selectivity as demonstrated in preclinical models. AZD9291 overcomes primary resistance by continuously blocking ERK signaling in GBM, has a better capacity to inhibit proliferation and prolongs the survival of GBM cells. It is currently in phase I/II clinical trial. AEE 788, EGFR/Erb inhibitor also inhibits VEGFR. Phase I clinical trial results were disappointing due to the toxicity and modest efficacy. Neratinib is another inhibitor of EGFR investigated in clinical trials for GBM patients [143]. Overall, till date EGFR inhibitors had minimal activity in GBM patients and did not improve OS or PFS.

9.4.2 Monoclonal Antibodies to EGFR

While tumor immunotherapy has shown success for the treatment of cancers like melanoma and hematology, their relevance to GBM represents more of a challenge. Cetuximab (Erbitux) is a humanized monoclonal antibody that recognizes the extracellular domain of both EGFR and EGFRvIII. Preclinical studies have shown that treatment with cetuximab alone and in combination with radiotherapy improved survival in vivo and reduced tumors in EGFR-amplified PDX models. However, phase II clinical trial of cetuximab in patients with recurrent high-grade glioma showed that it was well-tolerated, but exhibited limited activity. Panitumumab, previously known as ABX-EGF, is a totally human high affinity IgG2 monoclonal antibody against human EGFR. Blocking EGFRvIII signaling with panitumumab was found to cause a rapid reversion to the hepatocyte growth factor (HGF)/c-MET signaling pathway, and this event could be inhibited by cotreatment with an HGF-neutralizing antibody like rilotumumab [143]. Conversely, HGF was shown to be able to transcriptionally activate EGFR ligands, leading to downstream activation of EGFR. Thus, crosstalk between c-MET and EGFR/EGFRvIII signaling pathways offers tumor cells to reduce their dependence on either RTK for critical downstream signaling which leads to chemoresistance. GBM tumors are considerably heterogeneous, and this intratumoral diversity represents a probable cause of anti-EGFR therapeutic resistance. Therefore, although overexpression of EGFR and mutations of EGFR are one of the most characteristic features of GBM. EGFR-therapeutic approaches are so-far disappointing [143].

9.5 Vascular Endothelial Growth Factor (VEGF) Inhibitors

Vascular proliferation is a hallmark of tumor survival and growth. Glioma cells produce many different proangiogenic factors, including VEGF, which is highly expressed. Highly vascularized tumors are associated with the poor prognosis than patients with less neovascularization. GBM tumors are largely hypoxic wherein increased expression of HIF-1 leads to activation of VEGF. Hence, VEGF and VEGFR have been targeted for the potential treatment of gliomas. Different strategies for targeting include VEGFR TKIs, VEGF antibodies, and protein kinase C (PKC)-β inhibitors.

Several small-molecule VEGF inhibitors, viz. vatalanib, sorafenib, tivozanib, and pazopanib have been studied for the treatment of GBM. Vatalanib (PTK787), an inhibitor of VEGFR2, PDGFR, and c-kit showed modest effect on GBM patients alone or in combination with SOC. However, the drug appeared to enhance the antiangiogenic activity [143]. Also, sorafenib, inhibitor of VEGFR showed little effect when used in combination with temsirolimus. Moreover, in recurrent GBM patients tivozanib and pazopanib exhibited limited anti-tumor efficacy and did not prolong PFS in phase II clinical trials. These results were rather disappointing. However, bevacizumab, a humanized monoclonal antibody to VEGF, interferes with ligand-receptor signaling and represents the most studied and successfully targeted agent for the treatment of GBM. In phase 2 trials of bevacizumab in recurrent GBM, radiographic responses were seen in 30–60% of patients, the median PFS was 4–6 months, and the median OS was 8–10 months. Based on these data, bevacizumab received accelerated approval by the FDA for use as monotherapy in progressive GBM in 2009. However, a meta-analysis of four clinical trials (607 patients) demonstrated that combination of bevacizumab with SOC either chemo- or radiation improved only PFS, with no improvement in OS. Furthermore, treatment-related adverse effects were increased, e.g., commonly observed decreased neurocognitive behavior. Bevacizumab impairs hippocampal synaptic plasticity and decreases dendritic spine number and length, raising the apprehensions about its utility for GBM [139].

9.6 Platelet-Derived Growth Factor (PDGF)

Platelet-Derived Growth Factor (PDGF) and its receptors play an important role in tumor growth and angiogenesis. PDGF and its cognate receptors are overexpressed in GBM and considered to be an attractive therapeutic target. Currently, many PDGF inhibitors are in preclinical development. Imatinib mesylate, a small-molecule inhibitor of PDGFR-α and β, c-kit, and the Bcr-Abl fusion protein is well studied in GBM. It showed some anti-tumor effects in preclinical studies, with minimal clinical benefit as monotherapy, and no significant changes in the tumor growth. The drug failed as a monotherapy as survival remained unchanged. However, imatinib mesylate in combination with hydroxyurea did show promising results. These initial results were supported in a phase II trial in patients with recurrent GBM, which reported a PFS-6 of 27% and a median PFS of 14 weeks, and 42% of patients had stable radiologic disease at a median follow-up of 58 weeks. The mechanism of action of this combination is still unknown, but it has been shown that imatinib decreases interstitial tumor pressure and may increase delivery of hydroxyurea, which gives a possible explanation for the initial success associated with this combination. Unfortunately, a combination phase III trial did not provide further evidence of efficacy compared to single treatment with hydroxyurea. Similarly, tandutinib, a PDGFRα inhibitor failed in the clinical trials of patients with recurrent GBM [139].

9.7 Other RTKs

RTKs, viz. IGF-1R and FGFR are also modulated in GBM and represent interesting targets. IGF-1R small-molecule inhibitors PQ401, OSI-906, and BMS-754807 showed good efficacy in preclinical in vitro and in vivo studies. Small-molecule inhibitors of FGFR are currently being studied [139].

Targeted therapy using RTK inhibitors represented a hope for GBM patients. However, multiple RTKs are coactivated in GBM tumors representing functional redundancy, which leads to minor results in clinical trials for monotherapy. Thus, the need for concomitant inhibition of multiple pathways with RTK-inhibitors together with radio-, chemo-, or immunotherapy could be a good solution.

9.8 Molecular Targeted Therapies for Glioma and Their Therapeutic Role

9.8.1 Targeting Downstream Intracellular Effector Molecules

Aberrant activation of the growth factor receptors in GBM leads to the recruitment of intracellular effector molecules to the cell membrane. The activation of second messenger protein PKC stimulates cell proliferation, invasion, and growth. Overlapping and crosstalk between the pathways that involve these messengers is the cause of great complexity of targeted therapies for malignant gliomas. Examples of crucial pathways in gliomas are Ras/MAPK, PI3K/AKT, and PKC.

9.8.2 PI3K/Akt/mTOR Pathway and Potential Clinical Inhibitors

The PI3K, a serine/threonine kinase signaling pathway, is activated in most GBM and plays a critical role in the regulation of signal transduction, and mediates a variety of cellular processes, including proliferation, survival, migration, and angiogenesis in GBM. This pathway is regulated by PTEN; loss of PTEN function results in constitutive activation of PI3K. Thus, genetic aberrations in GBM, viz. EGFR, PDGFR, PTEN, TP53, and PIK3CA, drive the dysfunction of signaling pathways, e.g., PI3K/Akt/mTOR, p53, and Rb1. Activation of pathway continues through a complex secondary messenger cascade that results in the activation of many downstream molecules including Akt, a serine/threonine kinase and is generally associated with negative prognosis in patients with GBM.

The PI3K/Akt pathway is typically initiated via the activation of RTKs or G protein-coupled receptors. This leads to conformational changes in the C-terminal kinase domain produced by autophosphorylation, thereby providing binding sites for the regulatory subunits of PI3K. It results into elevated lipid kinase activity of PI3K and activation of Akt. Despite the limited clinical efficacy of the previously described RTK inhibitors, as activation of each of these receptors leads to downstream activation of the PI3K/Akt pathway, it has therefore been suggested that PI3K pathway inhibitors may be beneficial in GBM. Several PI3K inhibitors have demonstrated preclinical efficacy in GBM and have entered clinical trials for GBM treatment. Buparlisib, a pan PI3K inhibitor, reduces GBM cell growth both in vitro and in vivo. Buparlisib is the most frequently used PI3K inhibitor in clinical trials for GBM treatment, as it is well-tolerated and BBB permeable. However, single-agent efficacy in Phase II trials in recurrent GBM has been minimal. The lack of clinical efficacy was explained by incomplete blockade of the PI3K pathway in the tumor tissue. Sonolisib is an irreversible wortmannin analog that demonstrates a more persistent inhibitor effect on PI3K than wortmannin. Sonolisib inhibits invasion and angiogenesis in GBM cell lines in vitro and extends survival benefit in orthotopic xenograft models in vivo. Despite these promising preclinical results, the response rate to sonolisib in a Phase II clinical trial in patients with recurrent GBM was low, and it failed to meet its primary endpoint. Due to many challenges, clinical studies do not favor PI3K inhibitors in GBM treatment, suggesting that targeting PI3K alone is not sufficient to treat GBM [144].

Akt regulates many central biological processes; however, it is extremely difficult to target directly, and hence the inhibition of upstream and downstream targets has been tried out. The main downstream target of Akt is the mammalian target of rapamycin (mTOR). mTOR, a protein kinase regulates processes such as transcription and protein synthesis as well as important cellular functions, including modulation of cell growth. Several mTOR inhibitors have been developed over the past few years and most studied are a synthetic analog of rapamycin and temsirolimus. Recent phase II trials of temsirolimus in patients with GBM have not shown much efficacy. Although a radiographic improvement was seen in 36% of patients, no real survival benefit was recorded, with a PFS-6 of just 7.8%. mTOR contains two distinct complexes named raptor/mTORC1 and raptor/mTORC2. Temsirolimus, however, only inhibits the mTORC1 complex, which increases the PI3K/Akt activity, thus negating the anti-tumor effect.

9.8.3 Ras/Raf/Mitogen-Activated Protein Kinase Pathway

The Ras superfamily of genes regulates many important cellular functions, including cell proliferation and differentiation, protein trafficking, and cytoskeletal organization. It is an important signal transduction effector of the EGFR and PDGFR. Ras mutations are nearly absent and a very small percentage of BRAF mutations are present in malignant gliomas. Therefore, the observed dysregulation of signaling pathway Ras-RAF-ERK is mainly attributed to its upstream positive regulators growth factor receptors, viz. EGFR and PDGFR, known to be highly active in many malignant gliomas. Overactivation of Ras is followed by the farnesylation of Ras, which catalyzes the recruitment of the Ras molecule to the plasma membrane. Then, downstream Ras and Raf molecules are activated, which triggers MAPKs, causing cytoskeletal organization, cell proliferation, and release of proangiogenic growth factors. To target Ras, farnesyltransferase inhibitors have been used.

The two most prominent farnesyltransferase inhibitors used to indirectly inhibit Ras are tipifarnib and lonafarnib. A phase II study of tipifarnib reported a PFS-6 of 12% in patients with GBM and 9% in patients with anaplastic glioma (AG) [145]. Also, a phase I study with lonafarnib and TMZ was performed in patients with recurrent GBM to overcome tumor resistance to TMZ. Using this therapy, 27% of patients who were previously resistant to TMZ treatment showed a partial response, and the PFS-6 was 33%. These data show promising possibilities, especially the potential improvement of patient treatment using TMZ in combination with lonafarnib [146].

Sorafenib is a multikinase inhibitor, it targets RAF-1, PDGFR, and VEGFR. It is probably the most promising and most extensively studied Ras-RAF inhibitor. In vitro studies showed that combination of sorafenib with rottlerin (PKC-δ inhibitor) potently inhibits proliferation and migration of human malignant glioma cells [147]. Furthermore, sorafenib and bortezomib (protease inhibitor; MG132) synergistically induced GBM apoptosis. Based on the FDA clinical trial database (http://clinicaltrials.gov), at least eight phases I and II clinical trials are being conducted to evaluate the effects of sorafenib in patients with malignant gliomas. Recently, published clinical trial data indicates that sorafenib in combination with temsirolimus demonstrated limited benefit in GBM. The authors concluded that significant dose reductions that were required in this treatment combination compared with tolerated single-agent doses may have contributed to the lack of efficacy [148]. Sun et al. [149] demonstrated that inhibition of Nrf2 might enhance the therapeutic benefit of TMZ by modulation of antioxidant and anti-apoptotic genes. These effects might be mediated by inhibition of Ras/Raf/MEK signaling pathway, leading to decreased cellular proliferation. Thus, inhibition of Nrf2 might be a new therapeutic approach for treatment of glioma [149].

9.8.4 Hepatocyte Growth Factor/c-MET Signaling and Potential Clinical Candidates

c-MET, a tyrosine kinase receptor binds to hepatocyte growth factor (HGF) and exerts its effect on proliferation, survival, migration, invasion, angiogenesis, stem cell characteristics and therapeutic resistance leading to recurrence of GBM. HGF is overexpressed in 1.6–4% of GBM patients, and via activation of c-MET, enhances tumor growth, and angiogenesis. Upon HGF binding, c-MET activates several downstream signaling cascades to induce EMT, primarily through PI3K/AKT, RAS/MAPK and Wnt/β-catenin pathways. Aberrant c-MET activity, arising due to either gene mutation or amplification, leads to development and progression of multiple human cancers, including high-grade gliomas. Therefore, combined targeted therapy for this pathway and associated molecules could be a novel and attractive strategy for the treatment of GBM. Over the last few decades, many antibodies or small-molecule inhibitors targeting c-MET or HGF have been examined in numerous preclinical and clinical studies.

Preclinical studies indicated that, SGX-523 a small-molecule inhibitor of HGFR/c-MET tyrosine kinase activity inhibits tumor cell growth, migration, and invasion in a panel of glioma cells in vitro and reduced tumor growth in a murine xenograft model of GBM. However, the two clinical trials registered for this agent for the treatment of solid tumors were terminated without available results (NCT00607399, NCT00606879). Furthermore, amuvatinib (MP470) a small-molecule inhibitor that acts on multiple tyrosine kinases, including c-MET, has been shown to radiosensitize GBM cell lines in vitro and in vivo studies. Another small-molecule inhibitor of c-MET kinase activity, crizotinib, inhibits

growth, sphere-forming capacity and expression of stem cell markers in a subcutaneous xenograft model of GBM using U87MG cell line. It effectively inhibits proliferation and survival of c-MET-positive GSCs, rather than c-MET-negative GSCs, and apparently prolongs the survival of mice bearing c-MET-positive GSCs [150]. However, in a subcutaneous xenograft model using Mayo39 and Mayo59 GBM cell lines, crizotinib was only effective in reducing tumor burden and vascular density when used in combination with the EGFR inhibitor erlotinib [151].

Several monoclonal antibodies against c-MET-HGF showed promising anti-tumor activity in preclinical studies but no evidence of clinical benefit. Rilotumumab (AMG102), a neutralizing antibody against HGF, has shown anti-tumor activity in U87MG tumor xenograft models in combination with bevacizumab. However, in clinical trial for recurrent malignant glioma, rilotumumab with bevacizumab did not significantly improve the objective response, as compared to that with bevacizumab alone. Moreover, adverse effects might impede the use of rilotumumab in combination with bevacizumab regimens [152].

Several targeted small-molecule inhibitors and monoclonal antibodies targeting receptor kinases (e.g., EGFR, PDGFRa and PI3K) have been evaluated as single agents or in combination with SOC for recurrent and newly diagnosed GBM. However, none of these trials have improved PFS or OS in GBM patients so far.

9.8.5 Immunotherapies

Immunotherapies have shown significant efficacy in many oncology indications and are attractive candidates also for GBM as they affect the TME rather than targeting the tumor. However, Opdivo, a programmed cell death protein 1 (PD-1) inhibitor, in a randomized Phase III trial for recurrent GBM patients failed and therefore the excitement of immune-checkpoint inhibitors as a monotherapy has faded for GBM patients. Instead, to improve the clinical outcome for GBM, currently immune-checkpoint inhibitors are being evaluated in combinations with other treatment modalities, such as chemotherapy, oncolytic viruses, and radiotherapy. Other immunotherapies in the GBM pipeline include autologous dendritic cell vaccines and oncolytic viruses. Vaccines have not shown promising efficacy in other oncology indications; however, many companies developing late-stage vaccine-based therapies have also identified various biomarkers which can be selective for most responsive patients, thereby increasing their chance of success. AbbVie's biomarker driven strategy involving the antibody drug conjugate, depatuxizumab mafodotin, targets EGFR amplification-positive patients, increasing the chance of a signal in GBM patients. Other targeted agents involve proteasome inhibitors and novel chemotherapy agents or radiosensitizing agents to improve the effect of chemo-radiotherapy in GBM [24].

9.8.6 Other Dysregulated Pathways

GBM are associated with high rates of mortality due to their intrinsic resistance to conventional therapies. Experimental evidence suggests that cell invasion and proliferation in glioma cells are mutually exclusive events, with proliferating cells being less migratory while rapidly migrating cells divide more slowly. Highly migratory cells escape surgical resection and invade the surrounding brain tissue, giving rise to satellite lesions that lead to tumor recurrence. The intrinsic resistance to various treatments manifested in GBM has been attributed to the presence of a small subpopulation of cells-CSC. GSC harbor exclusive self-renewing and tumor-initiating potential, they are believed to be the tumor driving force in this fatal disease and play a significant role in tumor progression, maintenance, and recurrence after therapeutic intervention. Several studies have shown that these GSC display enhanced invasive behavior in in vitro and in vivo. Specifically, increased expression of Wnt5a and TGF-β2 has been found to enhance the invasion capacities of GSCs [153]. Moreover, human studies have demonstrated that TGF-β is overexpressed in malignant glioma tissues but undetectable in normal brain tissues, suggesting that TGF-β contributes to glioma development. Studies have been conducted to analyze the expression of TGF-β1 and TGF-β2 expression in 159 GBM tumor specimens and Kaplan–Meier and multivariate analyses were used to correlate expression with OS and PFS [234]. Higher expression of TGF-β1 significantly conferred a strikingly poorer OS and PFS in newly diagnosed patients, thus, it could serve as a prognostic biomarker and patient follow-up [153]. Several small-molecule TGFβRI kinase inhibitors, e.g., LY-2157299 are being evaluated in preclinical studies for GBM. Notably, the combination of TGF-β signaling inhibitors with US FDA-approved immune-checkpoint blockade agents, such as anti-PD1, anti-PD-L1, and anti-CTLA4 antibodies, most likely would improve clinical outcomes over targeting a single pathway, especially as these antibodies have recently been shown to have efficacy in murine models of glioma [154, 155]. Likewise, Wnt signaling pathway is important for differentiation of GSC. Aberrant Wnt signaling in GSCs renders them resistant to conventional chemo- and radiotherapy. It is suggested that a combinatorial approach which allows cellular targeting of Wnt signaling such as CAR-T mediated drug delivery may be considered to address the challenge [61].

Taken together, there has been a rapid increase in our knowledge of the molecular, genetic, and epigenetic pathogenesis of GBM. This has led to the development of different therapeutics against these aberrant multiple signal transduction pathways and also there are many innovative chemotherapeutic strategies developed for the treatment of GBM. However, the clinical outputs for the use of many of these agents are largely disappointing. Nevertheless, there are several potential reasons that the current molecular targeted therapies have been largely ineffective. It would be

very difficult to find therapeutically efficient molecularly targeted drugs because of the promiscuous character of GBM cells [140, 156].

Thus here, we have described the major pathways that are highly mutated or deregulated in GBM as well as some newly developed therapies targeting those pathways. With the current knowledge of tumor biology, we believe that the combination of inhibitors of multiple pathways alongside targeting TME through modulation of tumor suppressors like p53/Rb/Par-4 could provide insight into potentially active drug combinations for future treatment.

10 Tumor Suppressors and Par-4

Mutations in oncogenes, tumor suppressor genes, and genes involved in DNA damage repair process promote cancer initiation in humans. Growth factor receptors, protein kinases, G-protein-signaling molecules, and transcription factors in selected signal transduction pathways are common targets for oncogene mutations. Tumor suppressor genes commonly contribute to maintain the fidelity of the cell-cycle replication process. They act as negative regulators for oncogenes, cell-cycle checkpoints, or gene products that supply the appropriate nutrients or components to complete a faithful cell-cycle division in the absence of stress. Deletions, nonsense mutations, frame-shift mutations, insertions, or missense mutations that inactivate functional activity of a protein are observed in tumor suppressor genes processes.

Most cancers are noted to contain a number of oncogene and tumor suppressor gene mutations. An inclusive definition of tumor suppressor gene as put forth by Haber and Harlow states that suppressor genes are genes that sustain loss-of-function mutations in the development of cancer, regardless of their presumed functional role processes.

The products of tumor suppressor genes have diverse functions. The following table classifies certain functions and their related tumor suppressor genes (Table 2).

The inheritance of a mutated form of these tumor suppressor genes can initiate the formation of a specific set of tumors, commonly at a much younger age than those arising from spontaneous somatic mutations.

10.1 Tumor Suppressor as a Therapeutic Target for GBM

Current research focuses on defining the biochemical factors that govern the interplay between cell growth and cell death in GBM. The concept of tumor suppressor genes is examined, with an emphasis on the functional studies of the role of the *p53, p16, Rb,* and *PTEN/MMACI* genes in gliomas. Moreover, recent advances linking tumor suppressor genes, apoptosis, and cell-cycle control pathways in brain tumors are reviewed. The ability to detect mutations in tumor suppressor genes plays an important role in cancer diagnosis and prognosis. Perhaps of greatest significance has been the realization that tumor suppressor genes may provide novel targets for the development of specific anticancer therapies for brain tumors.

10.2 p53/RB/CDKN2A

p53 is a well-known tumor suppressor and transcription factor that coordinates cell responses to a diverse array of cellular stresses by regulating genes that are involved in processes such as apoptosis, DNA repair, neovascularization, and metabolism. Disruption of this gene is common in a diverse array of human cancers. According to the TCGA 2008 publication, p53 was found to be mutated in 37.5% and 58% of untreated and treated GBM samples respectively. The p53 signaling pathway can also be misregulated by inactivation of CDKN2a. This gene encodes two distinct proteins (p16INK4a and p14ARF) that are tumor suppressors and act as negative regulators of the cell cycle. They are deleted in approximately 55% of GBMs. One of the encoded protein products, p14ARF, was found to promote the degradation of the p53 repressor (MDM2) and thus lead to the stabilization and accumulation of p53. Therefore, a loss of p14ARF (via CDKN2a deletion) results in suppression of p53 and provides a mechanism for tumorigenesis. CDKN2a also encodes p16INK4A, a protein that inhibits the association of CDK4/6 (cyclin-dependent kinase) with cyclin D. This association would otherwise form a complex that functions to promote the G1/S transition through activation of downstream mediators. Specifically, this complex phosphorylates a tumor suppressor protein, retinoblastoma protein (pRb), and this facilitates the release of bound E2F, a G1/S transcription factor. The loss of p16INK4a, therefore, allows CDK4/6 and cyclin D association and subsequently promotes the G1/S transition. Thus, p16INK4a acts as a tumor suppressor. According to TCGA data, 77% of samples harbored aberrations in the Rb pathway, with the most prevalent being a deletion at the CDKN2A/CDKN2B locus (55% and 53%, respectively). The relevance of this locus to disease progression is also underscored by studies using mice models that have shown that homozygous deletions of p16(INK4a) and p14(ARF) are tightly associated with progression to higher grade gliomas. A phase II trial (NCT01227434) currently recruiting participants will test a small-molecule inhibitor of CDK4/6 (Palbociclib isethionate [PD 0332991]) in patients with Rb-positive recurrent GBM [157].

Table 2 Tumor suppressor genes with protein functions

Sr #	Tumor suppressor genes	Function of protein
1	p53	Transcription factor
2	Rb	Negative regulator of transcription factor
3	APC	Ubiquitin ligase activator
4	ARF	Ubiquitin ligase inhibitor
5	NF1, TSC1, TSC2	GTPases
6	BRCA1, BRCA2, ATM, MSH1, MLH1	DNA repair functions
7	LKB1	Protein kinases
8	PTEN	Protein kinase inhibitor
9	MEN2	Histone modifiers
10	E-cadherin, α-catenin, RASSF1, NF2	Cytoskeletal and adhesion compounds

10.3 Par-4 as a Tumor Suppressor

The tumor suppressor protein, Par-4 is known to be ubiquitously expressed in different tissues across different species. Par-4 can selectively cause apoptosis in a wide variety of cancers leaving normal cells unaffected. Par-4 knockout mice develop spontaneous tumors in various tissues. The Par-4 gene is located on an unstable region 12q21 of the human chromosome that is often deleted in gastric and pancreatic cancer [158]. Par-4 is known to be downregulated in over 70% of renal cancers, neuroblastoma, acute, and chronic forms of leukemia [158].

10.3.1 Discovery

In order to identify the genes induced by effectors of apoptosis in androgen-dependent and -independent prostate cell types, Rangnekar et al. performed differential hybridization on a cDNA library prepared from an androgen-independent prostate cancer cell line, AT-3, exposed to ionomycin. Prostate tumors are hybrid and have a mixed population of androgen-dependent and -independent cells. While androgen-dependent prostate cancer show high intracellular Ca^{2+} levels upon androgen ablation, the androgen-independent prostate cancer does not exhibit such a behavior making the androgen-independent cells refractory to androgen-ablation therapy leading to relapse of aggressive prostate cancer [159]. Forced elevation of intracellular calcium, using the ionophore ionomycin, can cause apoptosis in androgen-independent cells [160]. Five distinct cDNAs representing ionomycin-inducible genes, designated prostate apoptosis response (Par)-1, -2, -3, -4, and -5, were identified. Of these Par-4 and Par-5 were novel. Further characterization of these genes revealed that induction of Par-4 is apoptosis specific and is not induced by effectors of growth stimulation, oxidative stress and necrosis, or growth arrest in prostate cells [159].

10.3.2 Par-4 Protein Structure

Par-4 is a 40 kDa multi-domain protein composed of 340 amino acids (Fig. 4 and Table 3). The key domains include a leucine zipper domain (LZ) at the carboxyl terminal region, two nuclear localization sequences (NLS1, NLS2), nuclear export sequence (NES) and a Selective for Apoptosis of Cancer Cells (SAC) domain, which is unique to Par-4 [158]. Functions of certain other domains in Par-4 have yet to be elucidated. These include the casein kinase 2 (CK2) phosphorylation site, an ATP-GTP binding motif, protein kinase C (PKC) phosphorylation site, also sites for N-glycosylation and N-myristoylation [158].

Par-4 exhibits two nuclear localization sequences NLS1 and NLS2 at the N-terminal region.

The NLS1 (24–29) sequence is relatively shorter in length (six amino acids long), and its function is yet not known.

The NLS2 (147–163) domain is important for the translocation of Par-4 from the cytoplasm to the nucleus, which is essential to cause apoptosis. The function of NLS2 was confirmed by using a Par-4 deletion mutant lacking the NLS2 sequence that was unable to translocate to the nucleus, thereby losing its ability to block NF-κB activity and induce apoptosis.

Par-4 contains five leucine repeats at its carboxyl terminus (300–340). The primary function of a leucine zipper domain is to allow protein–protein interactions. The leucine zipper domain contains about 40 amino acids found in the form of heptad repeats, where the fourth position of each repeat is occupied by a leucine. The ability of Par-4 to form homo- and hetero-dimers with almost all its binding partners is through its leucine zipper domain [158].

The SAC domain is unique and indispensable for the pro-apoptotic activity of Par-4. SAC is a core domain that is 59 (146–203) amino acids in length. The SAC domain includes the NLS2 domain and the Thr residue, which is the target for PKA phosphorylation. The SAC domain thus gives Par-4 the ability to selectively kill cancer cells, while leaving normal cells unaffected. This cancer selectivity of SAC and Par-4 is due to the higher endogenous PKA levels exhibited by cancer cells. The basal level of PKA in normal cells is insufficient to cause T163 phosphorylation, thereby making normal cells resistant to Par-4-mediated apoptosis. It is also seen that the endogenous level of Par-4 does not by itself cause apoptosis, unless accompanied by another stimulus [158].

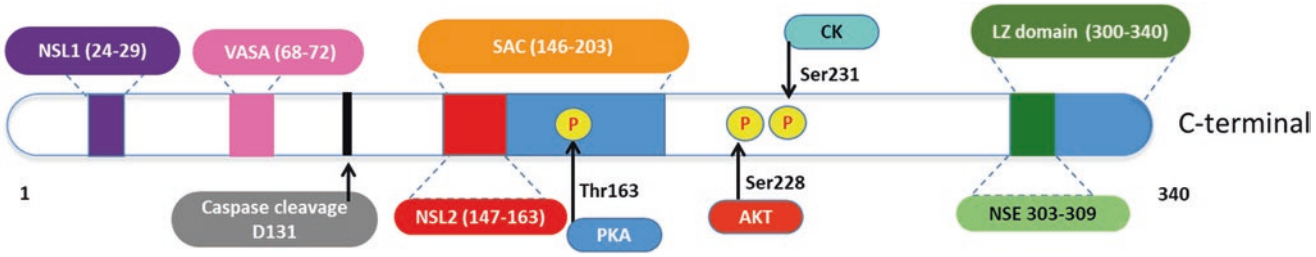

Fig. 4 . The structure and key functional domains of human Par-4

Table 3 Par-4 functional domains with known functions

Par-4 Domain	Amino acid sequence	Functions
NSL1	24–29	No known regulatory function
VASA	68–72	Foxo45 binding for ubiquitination and proteasome degradation
Caspase site	D131	EEPD131G caspase cleavage site
NSL2	147–163	Nuclear translocation
NES	303–309	No known regulatory function
LZ domain	300–340	Binding site for proteins
Phosphorylation by PKA	Thr163	Activation and nuclear translocation of Par-4 for pro-apoptotic activity
Phosphorylation by CK	Ser231	Activation and nuclear translocation of Par-4 for pro-apoptotic activity
Phosphorylation by Akt	Ser228	14-3-3 chaperone-mediated sequestration

10.4 Intrinsic, Cellular, and Secretory Par-4

Par-4 is known to cause apoptosis selectively in cancer cells. Depending on the nature of stimulus, apoptosis can occur via two different pathways, extrinsic and intrinsic (Fig. 5) [158].

10.4.1 Intrinsic Par-4

Various intracellular stimuli including oxidative stress, endoplasmic reticulum stress, hypoxia, DNA damage, and higher levels of protein kinase A leads to Par-4-mediated selective apoptosis in cancer cells [158]. Par-4 protein possesses several sites for phosphorylation by protein kinases A and C (PKA and PKC). These sites play an important role in regulation, localization, dimerization, and post-translational modification of Par-4 and thus are essential for Par-4 mediated activity. The intrinsic mechanism involves the PKA/PKC-mediated phosphorylation of Par-4 in its SAC domain followed by its nuclear translocation. The nuclear translocation is followed by inhibition of NF-κB-mediated cell survival mechanisms including genes belonging to the Bcl-2 family [158]. Par-4 is known to modulate NF-κB function both in nucleus and cytoplasm. In the cytoplasm, Par-4 represses NF-κB-dependent gene transcription by inhibiting the TNFα-induced nuclear translocation of the p65 (Rel A) subunit by blocking the atypical protein kinase C (aPKC), or IκB kinase (IKKβ)-mediated phosphorylation of the NF-κB inhibitory protein IκB [161]. The PKA-mediated phosphorylation of Par-4 at T163 is also essential for trafficking of Fas/FasL to the membrane. The Fas/FasL interacts with Fas-dependent death domain (FADD) thereby inducing the formation of the death-inducing signaling complex (DISC) causing activation of the Fas/FasL-FADD-Caspase 8 apoptotic death pathway [162]. Par-4 is known to upregulate the tumor suppressor activity of Wilms' Tumor-1 (WT-1) and also sequester topoisomerase 1 (TOP1) causing attenuation of its ability to relax supercoiled DNA thereby preventing DNA unwinding making it unavailable for transcription [162].

10.4.2 Secretory Par-4

Normal and cancer cells spontaneously secrete Par-4. Agents which induce ER stress cause upregulation in Par-4 secretion via brefeldin-A pathway. Par-4 transgenic mice have elevated levels of secretory Par-4 and show high resistance to spontaneous tumor generation. Par-4 is reported to localize in the endoplasmic reticulum and plasma membrane and its secretion can be inhibited by brefeldin-A, indicating that secretion of Par-4 takes place via the conventional ER-Golgi secretory pathway [161]. ER stress leads to upregulation of an ER chaperone protein glucose-regulated protein-78 (GRP78) which is an important binding partner of Par-4 and an essential component required for exhibiting the paracrine activity of Par-4. Intracellularly, GRP78 promotes cell growth, survival, and is an anti-apoptotic factor. GRP78 is overexpressed in multiple cancers, and elevated levels are associated with tumor severity and chemoresistance. GRP78 expression is also detected on the surface of cancer cells. Hence, the paracrine effect of Par-4 is restricted to cancer cells while the normal cells are spared from this effect. This effect of Par-4 can be attributed to GRP78 which under normal conditions is an endoplasmic reticulum-resident protein which functions as a chaperone involved in protein folding and a regulator of ER stress signaling. Under conditions of ER stress, intracellular Par-4 binds to GRP78 and facilitates its translocation from the ER to the plasma membrane, where GRP78 acts as a receptor for Par-4 at the cell surface. Once it is extracellular, Par-4

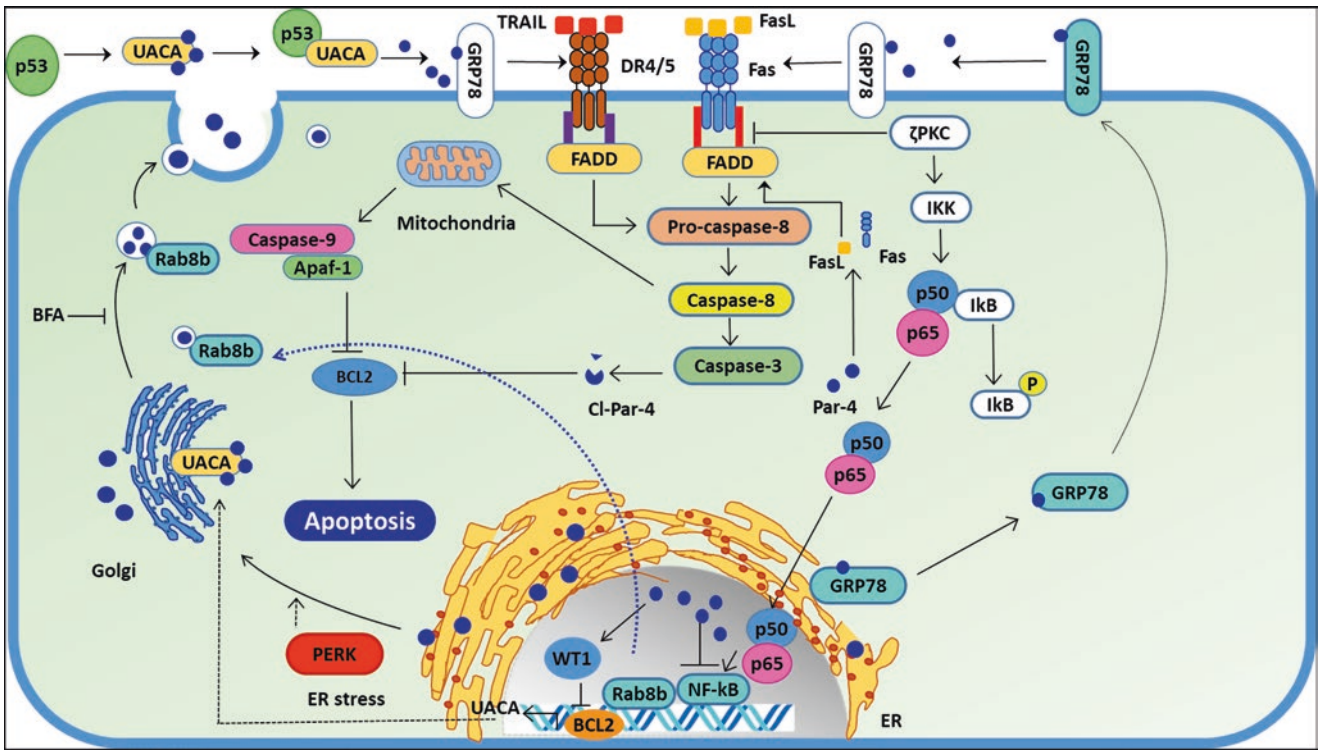

Fig. 5 Intrinsic and extrinsic pathways of Par-4-mediated apoptosis. Under the influence of apoptosis signals, Par-4 undergoes nuclear translocation via its SAC domain followed by inhibition of NF-κB facilitated cell survival. Within the nucleus, Par-4 also inhibits TOP1 enabled DNA unwinding. ER stress leads to translocation of Par-4 or GRP78-Par-4 complex to the plasma membrane leading to activation of Fas-FasL or TRAIL-mediated FADD/Caspase-8-dependent apoptosis

binds to GRP78 and uses FADD as the adaptor via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to recruit caspase-8 to the membrane. Activated caspase-8 then triggers the basic apoptotic machinery involving caspase-3 and other downstream effector proteins [163].

10.5 Expression of Par-4 in Normal Cells/ Tissues and in Cancers

Par-4 is ubiquitously expressed by normal cells and tissues and exhibits both intra- and extracellular pro-apoptotic functions. However, Par-4 is often downregulated in cancer. Previous studies have indicated that Par-4 downregulation in cancer occurs during cellular neoplastic transformation by the Ras oncogene through the Raf/mitogen-activated protein kinase-extracellular signal-regulated kinase (ERK) kinase/ERK pathway [161]. Moscat et al. have shown that Par-4 inhibits cell survival and tumorigenesis in vitro, and its genetic inactivation in mice leads to reduced lifespan, enhanced benign tumor development, and low frequency carcinogenesis. According to their studies, the loss of Par-4 dramatically enhances Ras-induced lung carcinoma formation in vivo. Par-4 is highly expressed in normal lung, but is reduced in a significant proportion of human non-small cell lung carcinomas, strongly suggesting that Par-4 is a relevant tumor suppressor in lung cancer [164]. The levels of Par-4 have been shown to be severely decreased in human renal cell carcinoma when compared to normal tubular cells [165]. Decreased Par-4 expression has also been demonstrated in other cancers, including neuroblastoma [166] pancreatic tumors [167], gastric cancer [168], breast [169], endometrial carcinomas [170], triple negative breast cancer (TNBC) [171], Wilms' tumor [172] and gliomas [173].

A couple of contrasting observations have come forth with respect to the expression of Par-4 in cancer and normal cells. Cohen et al. have shown that Par-4 mRNA level is not significantly different between healthy and cancer ovarian cells. Further, immunohistochemistry on ovarian tissue showed that ovarian cancer cells are positive for PAR-4 nuclear and cytoplasmic staining whereas ovarian healthy cells are negative for Par-4 nuclear staining. The Par-4 present in the ovarian cancer cells was shown to induce cell apoptosis and relocation of GRP-78 from endoplasmic reticulum to the cell surface of ovarian cancer cell line (SKOV-3 cells) [174]. In yet another contrasting study in chronic lymphocytic leukemia (CLL), Bondada et al. showed that cells from CLL patients and from Eμ-Tcl1 mice constitutively express Par-4 in greater amounts than normal B-1 or B-2 cells. Also knockdown of Par-4 in human CLL-derived

Mec-1 cells results in a robust increase in p21/WAF1 expression and decreased growth due to delayed G1-to-S cell-cycle transition. This Par-4 expression in CLL cells was dependent on constitutive expression of B-cell receptor (BCR) signaling. Inhibition of BCR signaling caused a decrease in Par-4 messenger RNA and protein, and an increase in apoptosis [175].

10.6 PAR-4 Modulation/Overexpression/Knockdown/Knockout in Animal Models

The prominent role of Par-4 to selectively induce apoptosis in tumor cells without affecting normal cells has gained wider attention to expedite its potential as a therapeutic tool. In animal models, the modulatory effect of Par-4 is elaborately studied to discern its novel molecular mechanism. The pro-apoptotic activity of Par-4 is largely established in in vitro systems, and experimental animal models have clearly demonstrated anti-tumor activity of secretory Par-4. Particularly, the role of Par-4 as a negative regulator of NF-κβ pathway has been deciphered in Par-4 knockout and knockdown mouse models [176]. Studies clearly indicate inverse relation of Par-4 and NF-κB in various tumor suppression models [176]. Transgenic mice models ubiquitously expressing Par-4/SAC domain are reported to show resistance to the growth of spontaneous and inducible tumors [177]. The secretory Par-4 induced pro-apoptotic activity in susceptible mice when Par-4 from cancer-resistant transgenic mice was transferred by bone marrow transplantation. Moreover, inoculation of recombinant Par-4 or SAC protein into mice inhibited metastasis of cancer cells. In addition, Par-4/SAC core domain-expressing adenovirus when intratumorally injected into xenografts in nude mice, caused rapid inhibition of tumor growth [178]. The secreted Par-4 in serum from transgenic mouse model affected apoptosis through extracellular surface stimuli upon binding to GRP78, thereby inhibiting the growth of tumor cells grown in vitro [179]. Burikhanov et al [180] reported that normal cells can be triggered to induce p53-dependent Par-4 secretion in systemic circulation. The secreted Par-4 remains functionally active in serum for an extended period and promotes ex vivo apoptosis in tumor cells sparing normal cells. Interestingly, PTEN heterozygous mice with Par-4 deletion led to the development of invasive prostate carcinoma [181]. Syngeneic mice (C57/BL6) with higher systemic levels of Par-4 levels are reported to hinder the growth of LLC-1-derived metastatic lung cancer [182]. Furthermore, ectopically expressed Par-4 inhibited development of lung nodules in tail vein metastatic models and reversed EMT in BXPC-3/CDDP cells [176]. Withaferin as Par-4 inducer is reported to upregulate E-cadherin and inhibit expression of mesenchymal marker β-catenin and vimentin in prostate cancer model [183].

10.7 PAR-4 Knockout (KO) Models

Knockout of Par-4 in the experimental model system has clearly demonstrated a tumor-prone phenotype exhibiting neoplastic transformation of multiple tissues types like lungs, liver, urinary bladder, endometrium, and prostate cancer. Among these, endometrium and prostate cancers are vulnerable to loss of Par-4, signifying a prominent role of Par-4 in hormone dependent tissues. Besides spontaneous tumor development, Par-4 KO models are prone to chemical or hormone-induced lesions [161]. The immunological profile of Par-4 null mice represented increased proliferative response of peripheral T cells that leads resistance to apoptosis with increased IL-2 levels, dysregulated NF-κB activity, and abrogation of JNK activity [184]. Embryo fibroblasts from Par-4 KO mice showed high levels of NF-κβ activation and reduced stimulation of JNK, in turn inhibiting cell survival [184, 185]. Par-4 deficient mice harbor higher levels of activated Akt in lung and prostate epithelial cells, and as is the case for NF-κB, this activation is mediated by PKCζ [186]. Par-4 null mice show decreased survival over wild type due to high incidence of tumor formation [187]. Interestingly, Par-4 null mice have shown symptoms of depression and adverse stress revealing possible role of Par-4 due to reduced expression [188]. PKCζ-deficient mice displayed impaired B-cell proliferation and Th2 differentiation [189]. Par-4 null mice revealed B-cell proliferation and overproduction of Th2 cytokine IL-4 by T cells in vitro and ex vivo [184]. These two KO mouse models have clearly unveiled opposite immunological phenotypes. Female C57BL6 mice with Par-4 null trait developed endometrial hyperplasia at 9 months of age from their development and interestingly none of the wild types showed similar alterations. Further, endometrial carcinomas were confirmed in 36% of moribund Par-4 null females after 12 months. Mechanistic studies in hyperplastic mice have revealed high levels of the anti-apoptotic protein XIAP. The basis of increased levels of XIAP was attributed to negative regulation of Par-4 with ζPKC–NF-κB pathway [187]. Moreover, similar findings were reported in Par-4 null male mice with incidence of prostate hyperplasia, prostatic intraepithelial neoplasia (PIN), and increased sensitivity towards testosterone-induced prostate hyperplasia. Chloroquine (CQ) induced Par-4 levels in Par-4 $^{+/+}$ as against Par-4 $^{-/-}$ mice and prevented distant migration of EO771 tumor cell in lungs, thus demonstrating anti-metastatic role of Par-4 [190].

10.8 Models with Overexpression of Par-4

Transgenic mice model overexpressing Par-4 are resistant to the growth of spontaneous or oncogene-inducible tumors. Par-4 overexpression sensitizes several cancer cell lines to apoptosis induced by endogenous tumor surveillance ligands, such as TNF-related apoptosis-inducing ligand, and chemotherapeutics. Role of Par-4 in inducing cell death and proliferation is widely known in multiple solid cancers; however, B-cell-specific leukemic mouse model of CLL also associated with increased Par-4 expression. TCL1 leukemic model has considerably shown delay in disease progression and conferred significant survival benefit. B-cell specific knockout mouse model with lack of Par-4 expression resulted in accelerated disease progression and abbreviated survival in the TCL1 model [191].

10.9 Signaling Pathways and regulation of Par-4

Par-4 expression and its interactions with multiple pathways components make it selective and multifaceted signaling crosstalk protein involved in cell survival signaling networks. Notably, these interactions occur at both sites, cytosol and nucleus mainly through protein–protein interactions, intracellular trafficking, and post-translational modifications (PTMs) with the help of two putative nuclear localization sequences (NSL 1 and 2) and nuclear transport sequence (NSE) [158]. A leucine zipper (LZ) domain at C-terminus end recognizes and binds with several Par-4 interacting proteins. Moreover, Par-4 phosphorylation by different kinases is regarded as an important regulatory mechanism depending on site and nature of kinase which can activate, inhibit, or substantiate pro-apoptotic activity. Structure-function analysis of Par-4 has distinctly identified SAC domain to selectively mediate apoptosis in cancer cells [192]. Thus far, altered expression of Par-4 is known to regulate varied anticancer mechanisms including metastasis, senescence, and autophagy. The translocation of Par-4 into the nucleus is a prerequisite for suppression of NF-κB-dependent transcription activity which is a significant mechanism involved in Par-4-mediated cellular toxicity (Fig. 6).

10.9.1 PTEN/Akt/mTOR

Par-4 has an integral co-operation with PTEN-mediated signaling pathways. The deficiency of Par-4 with PTEN haploinsufficiency in mice led to tumor progression in prostate cancer while concomitant deficiency of both tumor suppressors activated Akt and synergistically stimulated NF-κB promoting cellular growth, survival, inflammation, and angiogenesis [193]. Akt phosphorylate Par-4 at Ser228 and result in 14-3-3 sequestration in cytoplasm and blocks apoptosis [176]. PKCζ phosphorylates Akt as a direct substrate at Ser124 to regulate basal Akt activity and promotes sufficient phosphorylation of Akt at two other key residues Ser473 and Thr308, which are essential for full activation of Akt. Modulation of Par-4 is a common step in the regulation of the Akt and NF-κB pathways. First, PI3K-Akt pathway leads to Par-4 dependent apoptosis. Thus, Par-4 is essential for PTEN inducible apoptosis, and inactivation of Par-4 by Akt promotes cancer cell survival, Secondly, Akt1 acts as a critical cell survival protein that binds, phosphorylates, and promotes sequestration of Par-4 in the cytoplasm. It is also demonstrated that Par-4 is transcriptionally upregulated following treatment with multiple drugs targeting the PI3K-Akt-mTOR signaling pathway.

10.9.2 RAS/Raf/MAPK/ERK

PAWR gene is downregulated by oncogenes such as Ras, Raf, or Src [194]. However, treatment with azadeoxycytadine restores Par-4 mRNA and protein levels indicating that downregulation of Par-4 is mediated through promoter methylation. Abrogation of RAS/MEK/ERK by an inhibitor of MAP kinase - PD98059, restores Par-4 levels.

10.9.3 FOXO Pathway

Forkhead Box O3a (Foxo3a) transcription factor acts as mediators of Par-4 upregulation where it directly binds to the Par-4 promoter and activates its transcription following inhibition of the PI3K-Akt pathway [183]. Constitutive expression of Foxo3a is known to induce Par-4 whereas overexpression of Akt or silencing of Foxo3a expression inhibits the process.

10.9.4 Tumor Suppressor p53

Uveal autoantigen with coiled-coil domains and ankyrin (UACA), a Par-4 binding protein negatively regulates Par-4 secretion and sequesters Par-4 in ER and prevents it from translocating GRP78 to the cell surface. p53 can directly bind to a consensus motif on UACA and inhibit UACA expression in NF-κB independent manner and elevate Par-4 levels [180]. Similarly, study has also shown that p53 activation in normal cells induces Par-4 secretion, leading p53-dependent paracrine apoptosis in p53-deficient cells. Similarly, in response to chloroquine (CQ) treatment, p53 binds to promoter region of *Rab8b* (GTPase Rab family member) to promote Par-4 secretion [190]. p53 adenovirus E1B 19 kDa interacting protein 3 (BNIP3) is downstream target of Par-4 during ceramide-mediated autophagic cell death.

10.9.5 Casein Kinase 2 (CK2)

Par-4 is a novel substrate of the Caseins kinase CK2 and phosphorylation by CK2 impairs Par-4 pro-apoptotic functions. A serine/threonine kinase CK2 phosphorylates Par-4 at Ser231 and thus impede Par-4 mediated apoptotic activity [195].

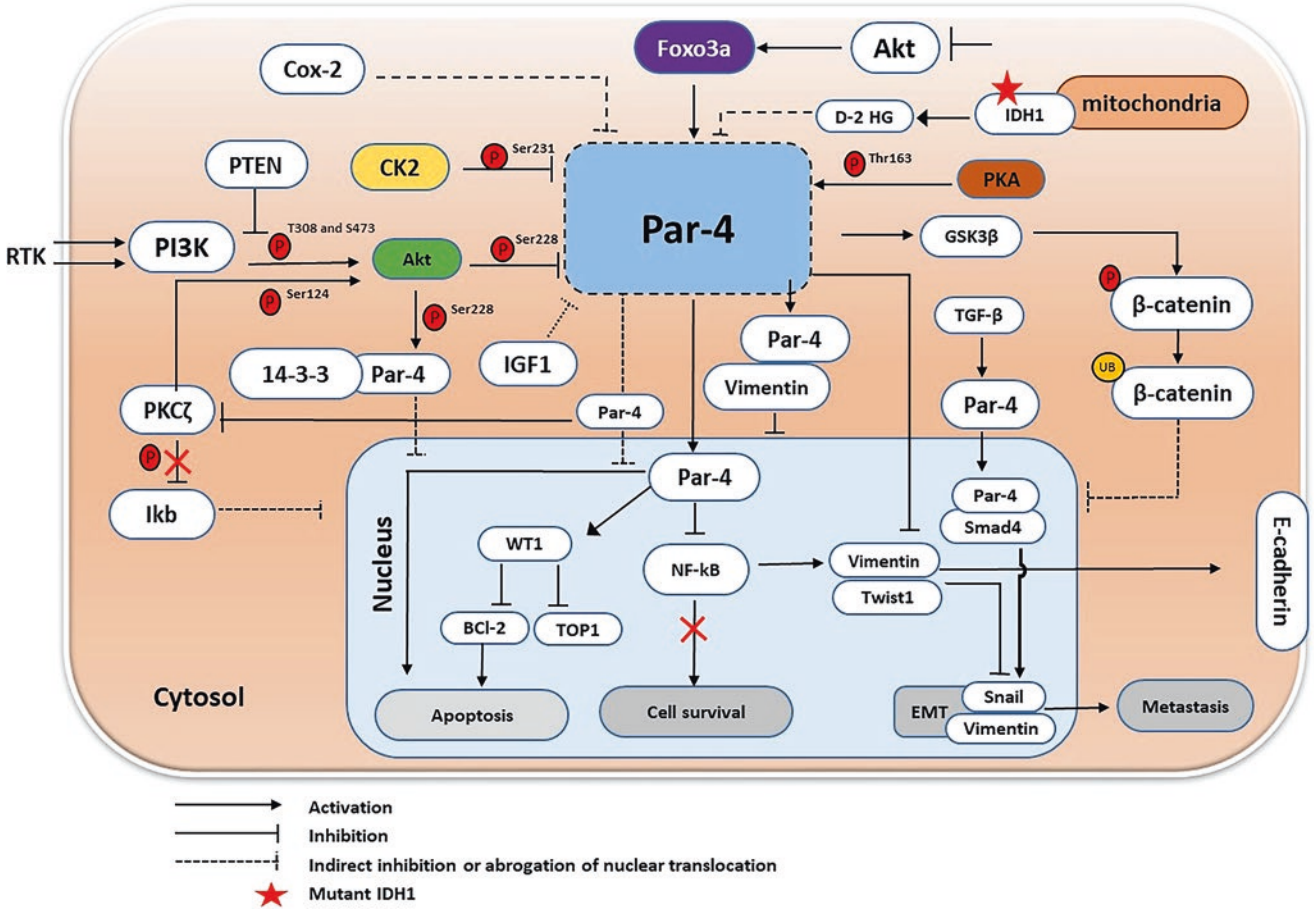

Fig. 6 Role of Par-4 in response to several external and internal stimuli.
Nuclear translocation of cytosolic Par-4 is essential for inhibition of NF-κB through p65 to regulate cellular toxicity. PKA mediated phosphorylation of human Par-4 at Th163 is essential for nuclear translocation. Akt-mediated phosphorylation of Par-4 at Ser228 sequesters Par-4 in cytoplasm and thus abrogates apoptosis. PTEN, as tumor suppressor gene regulates dephosphorylation of PIP3 to PIP2 to regulate Akt mediated dysregulation. CK2 impairs apoptotic properties of Par-4 by triggering the phosphorylation at residue S231. Vimentin sequesters Par-4 and prevents its extracellular function. Intracellular Par-4 induces GSK3β activation leading to phosphorylation of β-catenin and attenuates its nuclear translocation. TGF-β activates Par-4 expression and translocates to the nucleus to bind with Smad4 which leads to activation of epithelial-to-mesenchymal (EMT) markers - Snail and vimentin. IGF1 is indirectly associated with reduced expression of Par-4. PKCζ inactivation by Par-4 facilitates inhibition of NF-κB and pro-apoptotic survival pathway. Par-4 interacts with PKCζ and inactivates kinase through conformational changes and prevents phosphorylation of Iκb, a crucial step for translocation of NF-κB proteins P50/P65. Par-4 interacts with transcription factor WT1 and inhibits activity of Bcl2 and topoisomerase TOP1. Mutant IDH1 enzymatic activity accumulates 2 hydroxyglutarate (2-HG) and negatively affects regulation of Par-4 by unidentified mechanism

10.9.6 Protein Kinase a (PKA)

PKA-mediated phosphorylation at Par-4 at Thr163 is crucial for nuclear translocation of Par-4 which is missing in normal cells and this explains why normal cells are resistant to PKA-directed apoptosis by ectopic Par-4 [196].

10.9.7 Protein Kinase C (PKC)

Par-4 interacts with PKCζ and thus inactivates kinase though conformational changes and thus blocks PKCζ-directed NF-κB activity. Phosphorylation of IκB through PKCζ also blocks NF-κB mediated pro-survival signaling [197].

10.9.8 Autophagy

Recent literature has revealed that Par-4 overexpression can induce autophagic cell death. Wang et al., observed simultaneous modulation of autophagy and apoptosis in hypopharyngeal cancer [198]. Similarly, curcumin induces ROS-dependent overexpression, inducing autophagic cellular toxicity in human malignant glioma [199]. Par-4 is involved in autophagy via a p53-BNIP3-dependent manner where p53-dependent Bcl-2/adenovirus E1B 19 kDa protein 3 (BNIP3) acts as a downstream target of Par-4-induced autophagy after ceramide treatment in human malignant glioma cells. Activation of Par-4-P53-BNIP3 axis was reported in autophagy inducers like ceramide and arsenic tri-

oxide suggesting significant role of Par-4-mediated tumor suppression [200]. Curcumin induced autophagic cell death in human malignant glioma cells is associated with ROS-dependent Par-4 and ceramide generation [199].

10.9.9 Apoptotic Pathway

Par-4-induced extrinsic apoptosis occurs through a unique mechanism that involves cell death facilitated by various death ligands such as tumor necrosis factor-α (TNFα), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) to their cognate receptors of the pro-death FasL–Fas–FADD–caspase-8 pathway and inhibit NF-κB pro-survival pathway. Caspase-dependent post-translational regulation of Par-4 involves two fragments, a 15 KDa amino terminal fragment—PAF (Par-4 amino terminal fragment) and 25 kDa carboxy terminal fragment cleaved Par 4 (cl-Par-4). The cl- par-4 fragment translocates into the nucleus and initiates apoptosis. The intrinsic pathway is mediated through mitochondrial membrane permeabilization and depolarization leading to the release cytochrome c, AIF, Smac/DIABLO, etc. These factors promote caspase signaling cascade and bring about the cell death process. Apoptosis is tightly controlled through a balance of pro-apoptotic and anti-apoptotic proteins. The net result is balance between Bcl-2 family member of both pro-apoptotic such as Bad, Bid, Bax, Bak, Bcl-Xs, Bim and anti-apoptotic Bcl-2, Bcl-xL, Mcl-1 that determines whether a cell will undergo apoptosis. Apoptosis of cancer cells by Par-4 is dependent on anti-apoptotic markers, such as Bcl-xL, or Bcl-2 and the status of p53 or PTEN.

10.9.10 Senescence

Par-4-dependent p53 induction has a key role in thymoquinone-induced senescence in glioma cells. Markedly, Par-4 overexpression increased senescence in glioma cells, wherein Par-4 inhibition through shRNA or siRNA, drastically reduced senescence [201]. Induction of Par-4 increased senescence markers p53, p21, and Rb, and decreased expression of lamin B1, cyclin E, and cyclin-dependent kinase-2 (CDK-2). Also, Par-4 promoted senescence in cardiac mouse fibroblast [202].

10.9.11 Metastasis

Par-4 has a crucial role in EMT as it significantly inhibited mesenchymal-epithelial (MET) marker—vimentin and twist-related protein (Twist-1) and restored levels of E-cadherin as EMT marker in metastatic pancreatic cell line [203]. It is hypothesized that Par-4-NF-κB-mediated axis has negative impact on Snail and Twist 1 as these transcription factors promote vimentin and repress E-cadherin [204]. Secondly, Par-4 promotes ALK2/Smad4 signaling which is essential to maintain Par-4-mediated E-cadherin levels [205]. Vimentin is also known to target Par-4 through sequestration in cytoplasm and inhibit Par-4 expression [206]. Par-4 expressed through external stimuli inhibits EMT markers like β-catenin and vimentin [183]. A negative modulation of Par-4 by microRNA (miR-17-3p) leads to increased levels of CEBPB (CCAAT enhancer binding protein beta), focal adhesion protein (FAK), N-cadherin, vimentin and reduced expression of E-cadherin. Du et al [202] has reported that Indolylkojyl methane analog (IKM5) potentially inhibits invasion of breast cancer cells via diminution of GRP78 expression. Interestingly, modulation of oncogenic β-catenin through GSK3β activation is another important phenomenon by which Par-4 mediates cell migration and metastasis [207]. Besides, extracellular Par-4 secreted by brefeldin-A (BFA) pathway inhibits tumor invasion and angiogenesis via negative regulation of MMP2 thus targeting TME [208]. Contrary, Par-4 induced by TGF beta promotes metastasis due to Snail and vimentin expression through smad4-mediated NF-κB-XIAP axis [188].

10.9.12 Cytoskeleton Proteins

Par-4 interacts with cytoskeletal intermediate filament protein—vimentin which reduces its expression. However, arylquins (AQ) which belongs to the class of Par-4 secretagogues, binds with vimentin and enhances Par-4 secretion to efficiently induce paracrine apoptosis in tumor cells [206].

10.9.13 Metabolic Pathways

Activated PI3K/Akt/mTOR regulates activity of glucose-6-phosphate dehydrogenase (G6PD) via SREBP-c which is actively involved in cellular transformation. G6PD which is downstream of PI3K pathway supply pentose for the synthesis of nucleic acid to support tumor growth and stabilization of NADP/NADPH—equilibrium crucial for antioxidative defence. Different approaches were proposed for targeting of Par-4 which selectively inhibits apoptosis and G6PD, that provides nutrient supply to tumor cells [209].

10.10 Induction of Par-4 in Normal Cells and Cancer Cells

In cancer cells, the downregulation of Par-4 activity can either be due to a decrease in mRNA levels in the cell or due to the inhibition of its activity. This inhibition of Par-4 activity can be attributed to (1) preventing its phosphorylation, (2) sequestration of Par-4 in the cytoplasm by association with Akt1, or (3) reducing caspase-3 mediated Par-4 cleavage and preventing apoptosis [174]. However, it is also known that Par-4 sensitizes cancer cells for apoptosis. Studies have indicated that an intermediate filament protein, vimentin, and UACA are the intracellular binding partners of Par-4 which sequesters Par-4 within the cells. Further, inhibition of UACA by p53 activation and/or inhibition of NF-κB activity

results in elevated secretion of Par-4. Secretion of Par-4 via this pathway is dependent on downregulation of UACA, a functional target of p53 [190]. Because the baseline levels of Par-4 secreted by normal cells are inadequate to cause massive apoptosis in cancer cell cultures, secretogogues (small molecules that upregulates secretions) that upregulate the release of Par-4 by displacing Par-4 from its intracellular binding partners, viz. vimentin or UACA therefore constitute an important therapeutic category. Several such secretogogues have been identified till date including chloroquine (Rangnekar et al.). Chloroquine has been shown to cause secretion of Par-4 from normal cells in mice and cancer patients as observed during clinical trials. Similarly, 3-arylquinoline derivative, Arylquin-1 has also been identified as a potent secretagogue able to induce Par-4 secretion in cell cultures and in mice [210]. Jagtap et al. examined the sensitivity of glioma-derived stem cell line (HNGC-2) and primary culture (G1) derived from glioma tumor samples that express neural stem cell markers to various drugs including lomustine, carmustine, UCN-01, oxaliplatin, TMZ, tamoxifen (TAM), and the co-relation of Par-4 with drug-induced apoptosis. Of the various drugs studied, TAM significantly upregulated Par-4 and induced cell death. Further, Par-4 knockdown protected the cells from TAM-induced apoptosis [173].

11 Role of Par-4 in Gliomas

Par-4 is an endogenous tumor suppressor expressed in normal and cancer cells [211]. The significance of Par-4 in tumor cells is attributed to its pro-apoptotic function. In various types of cancers including gliomas, Par-4 is silenced or downregulated at protein or transcript level [161, 212]. Analysis with TCGA and REMBRANT databases revealed low levels of Par-4 and correlated with low survival period in GBM but not in astrocytomas and oligodendrogliomas suggesting low *PAWR* expression as a predictive risk factor in GBM [173]. High level of Par-4 is associated with longer median survival time in high-grade gliomas patients with wild type-IDH1 phenotype while patient-derived gliomas with mutant-IDH1 display low Par-4 expression [213]. The downregulation was partly shown to be due to D-2-hydroxyglutarate (2-HG), the metabolic product of mutant IDH1 that suppresses Par-4 transcription in vitro via inhibition of promoter activity as well as enhanced mRNA degradation. Further studies by the same group demonstrated Par-4 as an effective sensitizer of gliomas to apoptosis regardless of IDH1 status and highlighted the significance of induction of Selective for Apoptosis induction in Cancer cells (SAC) domain of Par-4 against glioma cells and in patient-derived GSC from orthotopic xenografts [214].

Endogenous Par-4 is essential for sensitization of cells to diverse apoptotic stimuli and is important for inducing apoptosis in cancer cells [215–217]. Besides its intracellular localization, Par-4 also exists in extracellular form as a secretory protein and induces apoptosis by binding to GRP-78 on tumor cells [179]. Upregulation or induction of Par-4 by apoptotic stimuli such as tumor necrosis factor alpha (TNF α), TRAIL [218] and Fas [161] induce cytotoxicity in cancer cells. Overexpression of Par-4 enhances the activity of anticancer drugs [217, 219] and induces radio-sensitivity in human prostate cancer cells [220].

Recent studies reported chloroquine (CQ), an antimalarial drug as a strong inducer of secretory Par-4 in normal cells in mice and in cancer patients. CQ-induced secretory Par-4 triggered apoptosis of cancer cells and inhibited metastasis in tumors. The Par-4 activity was dependent on p53 and involved Rab8b-a GTPase protein transport regulator for translocation of Par-4 to the plasma membrane prior to secretion [190]. Zhuang et al. established a link between cell-cycle phase, expression of PrPc, and response to TMZ in human glioma cell lines. The G1/S phase cells displayed lower expression of PrPc and were sensitive to TMZ induced apoptosis while cells in G2/M phase were resistant to apoptosis. The mechanism underlying resistance to TMZ induced apoptosis involved interaction of PrPc and SAC domain of Par-4 that resulted in inhibition of pro-apoptotic activity of Par-4 [221]. Chemotherapeutic drugs kill most of the proliferating cells while CSC survives and further expansion of this population results in enrichment of these GSC in tumors and contribute to drug resistance and recurrence in malignant gliomas [222, 223].

TAM is a well-established anti-estrogen drug widely used for the treatment of estrogen-dependent breast cancers [224]. Recent studies have demonstrated its anti-tumor activity in non-estrogen receptor tumors including gliomas [225–227]. TAM is reported to sensitize glioma cell lines to radiation therapy by inhibition of PKC-ι activity [227]. 4-hydroxytamoxifen(4-OHT), an active metabolite of TAM induces autophagy mediated death in glioma cell lines [228]. Jagtap et al unravelled the role of Par-4 in human GSC cell line-HNGC-2 (human Neuro-Glial cell line-2) [229] and in primary cultures (G1) derived from high-grade GBM tumor. The cell lines were resistant to a panel of drugs including TMZ, lomustine, carmustine, UCN-01, and oxaliplatin but were sensitive to TAM induced cytotoxicity and apoptosis. Exposure to TAM upregulated the intracellular Par-4 level, induced secretory Par-4, and enhanced GRP-78 expression in these cells. The study concluded that TAM induced apoptosis in GSC was dependent on the intracellular as well as secretory Par-4 and activation of Akt and ERK 42/44 pathways [173].

There have been continued efforts to improve strategies for better understanding of glioma biology with the goal towards developing new and effective drugs for improved

survival of patients. Most in vitro studies in this direction are conducted using monolayer cultures which do not actually reflect the structure, architecture, or behavior of tumors [230, 231]. On the other hand, 3D-multicellular spheroids (MCS) overcome many of these limitations and mimic tumor conditions with acquisition of multidrug resistance, and therefore serve as ideal experimental model for drug development program [232, 233]. Studies by Jagtap et al demonstrated that MCS generated from HNGC-2 and G1 cell lines displayed high expression of chemoresistance genes compared to monolayer and were resistant to TAM-induced cytotoxicity [235]. Par-4 expression was lower in MCS suggesting an association between chemoresistance and Par-4 level. TAM effectively induced secretory Par-4 in conditioned medium (CM) of cells cultured as monolayer but not in the MCS. The 3-D cultures were rendered sensitive to TAM-induced cytotoxicity by exposure to conditioned medium (CM)-containing Par-4 (derived from TAM-treated monolayer cells). A combination of TAM and inhibitors to PI3K inhibitor (LY294002) or PKCζ triggered secretion of Par-4 and cell death in MCS. The study concluded that Par-4 is a potential indicator for screening and evaluating anti-tumor agents in CSC.

12 The Road Ahead: Future Perspectives

GBM remains an incurable disease with a depressing prognosis and few therapeutic options over the past two decades. Multiple genome-wide analysis has yielded a wealth of knowledge and uncovered dysregulation of key cellular signaling pathways that constitute attractive targets for therapy. Targeting single dysregulated pathway using either small-molecule inhibitors and antibodies alone or in combination showed varying clinical response in GBM. However, none of these trials have improved PFS or OS in GBM patients so far. Moreover, it is important to understand that the tumor cells are highly heterogeneous and therefore, it may be more valuable to target multiple signaling pathways for the better prognosis of GBM.

Many therapies failed to show beneficial effects due to the BBB and the presence of active efflux pumps which prevent drug entry into the brain. This leads to sub-optimal concentration, which is below therapeutic concentration of the drug in the target cells which in turn adversely affect efficacy of the drug. An alternative reason for the failure of precision therapy could be that many of the dysregulated signaling pathways currently targeted are imperative for the initiation and/or initial growth of the tumor and are subsequently overridden by secondary pathways and mechanism of late tumor progression. Agents that can induce the secretion of Par-4 from the normal as well as cancer cells therefore would make for attractive drug candidate/s. With these many combinations, there is an immense potential for Par-4 in targeted therapies that remain to be explored in GBM. With the knowledge that we gained over the past two decades about molecular pathogenesis of GBM, we believe that the combination of inhibitors of multiple pathways alongside targeting tumor microenvironment through modulation of tumor suppressor like p53/Rb/Par-4 could provide insight into potentially active drug combinations for future treatment of GBM.

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Involvement of Par-4 in Breast Cancer

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Abstract

The normal mammary gland's development, maintenance, and function depend on a complex signal transduction system orchestrated by integrins, growth factors, and steroid hormones. Changes in these cell signal transduction pathways have been associated with breast cancer development and progression. Despite technological advances in diagnostic methods and molecular classification, breast cancer morbidity and mortality rates remain unchanged. A significant number of patients die due to the formation of metastases, resistance to treatment, and disease recurrence. Thus, one of the main objectives of research in breast cancer is the identification and characterization of new biomarkers, which can be used as prognostic and predictive factors of response or indicators of therapeutic resistance, preventing disease recurrence. Par-4 (Prostate Apoptosis Response 4; also known as PAWR, pro-apoptotic WT1 regulator) is a tumor suppressor gene that encodes a pro-apoptotic protein expressed ubiquitously among the various tissue types, and for which altered expression patterns are already described in many types of cancers, including breast cancer. In this chapter, we review the possible roles of Par-4 in breast cancer, providing insights into the potential role of this tumor suppressor gene as a novel prognostic and predictive biomarker of this complex and heterogeneous disease.

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Keywords

Par-4 · Breast cancer · Prognosis · Biomarker · Apoptosis · Recurrence · Chemosensitivity

1 A Brief Overview of Mammary Gland Development, Function, and Maintenance

The mammary gland is an exclusive organ of mammals whose primary function is to produce and secret milk to provide food for the newborn. Like other mammalian species, the human mammary gland is a bilateral organ formed by epithelial and mesenchymal components, which undergoes constant structural changes and has a great capacity for remodeling and regeneration from development in intrauterine life to postmenopausal involution. This complex and important organ changes in size, shape, and function, including two main phases, development and differentiation, during growth, puberty, pregnancy, and lactation [1, 2].

The development of the human mammary gland begins in the fourth week of pregnancy, and its initiation depends on specialized mesenchymal cells called the mammary adipose pedicle, which emit signals to the epithelial cells, which migrate to the adipose pedicle and form the primary ducts with little branched [3]. After birth, the mammary gland remains in its rudimentary form until puberty. Changes in the hormonal environment, especially in the levels of steroid hormones, estrogen and progesterone, which act as stimulating the growth and development of the mammary gland, induce the elongation, proliferation, and extension of the terminal lobular ducts, namely lobes type 1 and 2 [3, 4]. In fact, the mammary gland is a unique organ, having the ability to involve and reorganize itself between the stages of pregnancy and lactation, which involves the action of steroid hormones, growth factors, and different cell signaling pathways (Fig. 1).

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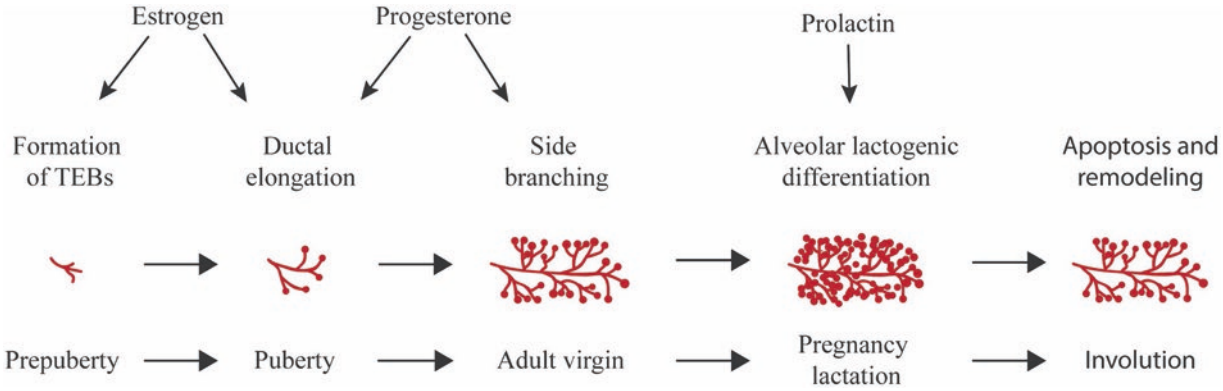

Fig. 1 Schematic illustration of mammary gland developmental stages from prepuberty to pregnancy, lactation, and involution

The stages of branching, lactation, and involution involve the complex cross-talk between different signal pathways and biological processes such as proliferation, differentiation, and apoptosis [5, 6]. Estrogen has a mitogenic effect on the epithelium resulting in duct proliferation and development, and progesterone acts on cell differentiation resulting in the development of lobules. Type 1 and 2 lobular ducts are present in nulliparous adult women. During pregnancy, the mammary epithelium experiences the largest and fastest growth phase, and lobes 1 and 2 progress to lobules of type 3 and 4; this occurs in response to the hormones of the corpus luteum (estrogen and progesterone), placenta (estrogen, progesterone, and somatotrophin), pituitary (prolactin), and adrenal glands. During the third trimester of pregnancy and lactation, the lobular ducts go through extensive ramification, alveolar formation, and differentiation. After the breastfeeding period, the involution phase follows, with apoptosis of the secretory epithelium and lobes regression for types 2 and 3 and regression for lobe 1 in the postmenopause [7]. Nulliparous women have a large percentage of lobes types 1 (50–60%) and 2 (30–35%), while women with at least one full-term pregnancy have a predominance of type 3 lobe (80–100%). These data corroborate the epidemiological data that show that a history of pregnancy at a young age is a protective factor for breast cancer, as these women have a lower relative risk of breast cancer development when compared to nulliparous women, a factor attributed to the differentiation degree of the mammary epithelium [8].

The mammary gland's complex structure is essentially composed of two components, the glandular one that is involved in the production and transport of milk, and the stromal and connective tissue composed of fibroblasts, extracellular matrix (ECM), and fat cells. To maintain functionality, epithelial cells must adequately receive signals from the bloodstream (growth hormone, estrogen, progesterone, prolactin) and stroma (growth factors, such as FGF10, IGF1, and EGF), whose action and integration depend on cell–cell and cell–ECM interactions involving the action of multiple genes [4]. In vitro and in vivo studies have highlighted the potential role of cell death during the development and involution of the mammary gland. The balance between proliferation and cell death rates is critical for the development and maintenance of the mammary gland [9]. On the other hand, cells' ability to evade apoptosis or cell death is one of the essential changes in cell physiology, which impacts tumor progression and resistance to chemotherapy and radiation therapy [10, 11]. Maintaining the structure and function of the normal mammary gland depends on a complex signal transduction system. The signaling pathways orchestrated by integrins, growth factors, and steroid hormones are fundamental in maintaining growth homeostasis and differentiation of the mammary gland. Changes in these cell signal transduction pathways have been associated with breast cancer development [12, 13].

2 Breast Cancer

GLOBOCAN 2018 estimated a total of 18.1 million new cases and 9.6 million cancer deaths worldwide in 2018. Among the various types of cancer, those with the highest annual incidence in the world are lung (2.093 million cases), breast (2.088 million), colon (1.096 million), prostate (1.276 million), and stomach (1.033 thousand) [14]. Worldwide, breast cancer is the most common malignancy and the leading cause of morbidity and mortality in the female population. In addition to the high incidence, the disease's social and economic impact is enormous and makes breast cancer an important public health problem.

Despite technological advances and improvement of diagnostic methods, such as mammography, which allows the early detection of a greater number of breast tumors, the mortality rates associated with breast cancer remain unchanged with a significant number of patients dying due to the formation of metastases, resistance to treatment and dis-

ease recurrence. Thus, one of the main objectives of research in the area of breast cancer is the identification and characterization of new biomarkers, which can be used as prognostic and predictive factors of response or indicators of therapeutic resistance, preventing disease recurrence. However, such research is hampered due to the high complexity and heterogeneity of this disease.

Data from epidemiological and experimental studies allowed to elaborate the proposal of a linear model for the natural history of breast cancer, which involves progression through clinical and pathological stages, starting with hyperplastic epithelial atypia, progressing to carcinomas in situ, invasive carcinoma, and finally the development of metastatic disease [3, 15, 16]. However, epithelial atypia (AS), atypical ductal hyperplasia (ADH), and carcinoma in situ (DCIS) are not considered mandatory precursor lesions for the development of invasive and metastatic ductal carcinoma (ICD), which represents 80% of tumors diagnosed [17, 18].

Breast cancer has a complex etiology, and both genetic and environmental factors play an important role in the disease development, and the penetrance of genetic factors can be influenced by a variety of genetic and non-genetic risk factors [19, 20]. Several factors are associated with an increased risk of developing breast cancer. Each factor confers a different degree of risk. The main risk factors for breast cancer include family history, age, geographic variation, age at menarche and menopause, age at first pregnancy, number of deliveries, obesity, sedentary lifestyle, diet, hormone replacement therapy, use of oral contraceptives, and alcohol consumption [21–24]. Lifestyle impacts breast cancer development and some of these risk factors can be modified, and not every woman with one or more associated risk factors will develop the disease. Identifying modifiable risk factors can contribute to primary and secondary prevention leading to a reduction in the incidence of the disease [25].

Early menarche and nulliparity influence the time of steroid hormone exposure, especially estrogen and progesterone, which is considered one of the dominant factors in breast cancer's etiology and development [22, 26, 27]. As already described, estrogens play a critical role in the development, maintenance, and function of the mammary gland and are also associated with breast cancer development and progression [19]. Most of the complex biological functions regulated by estrogens are mediated by estrogen receptors, ERα and ERβ, through transcription of ER target genes and non-genomic mechanisms [28, 29]. Estrogen actions can be partially blocked by selective estrogen receptor action modulators (SERMs), such as tamoxifen and raloxifene, or by selective estrogen receptor reducers (SERDs), such as fulvestrant ("faslodex", ICI 182,780), which is a pure antagonist, which binds to the ER leading to its destabilization and degradation [30–32]. In addition, third-generation agents or aromatase inhibitors (AIS), anastrozole and letrozole, which inhibit estrogen production, can be combined with fulvestrant to overcome resistance and improve patients' response to hormone therapy [33, 34]. The presence or absence of ERα determines whether a patient's breast cancer can be classified as a positive or negative estrogen receptor, respectively. About two-thirds of breast tumors express ER and are considered hormone-dependent. In fact, ER is considered a good biomarker for breast cancer, not only because it is associated with the disease development and progression but also because its presence can predict breast cancer patients' response to anti-estrogen treatment. However, a subgroup of patients with ER-positive breast tumors does not respond to hormone therapy or become resistant to it [32, 34]. Several molecular techniques that allow for large-scale expression analysis have been used to identify the gene expression signature associated with hormone resistance therapy that can improve our understanding of ER-positive breast cancers and select the most appropriate therapies for each patient [35, 36].

The most important risk factor for breast cancer, however, is a family history, that is, the presence of breast cancer in first-degree relatives. Approximately 5–10% of breast and/or ovarian cancer cases are identified in families as a result of high penetration mutations inherited in an autosomal dominant manner [37, 38]. Breast cancer is defined as familial when the disease manifests in at least two first-degree relatives. Epidemiological data show that first-degree relatives of affected individuals have a significant increase in the relative risk of developing breast cancer, being this risk increased by young age at diagnosis, the development of bilateral tumors, and the number of affected family members by the disease [39–42].

Genetic linkage studies in families with breast and familial ovarian cancer led to the identification of the BRCA1 and BRCA2 genes [43–45]. A significant number of mutations in these genes have already been identified, with the majority of these mutations, around 70%, of the "frameshift" or "nonsense" type resulting in the formation of a non-functional truncated protein product [46, 47]. Germline mutations in the BRCA1 and BRCA2 genes are highly penetrating, giving an estimated risk of more than 80% for the development of breast cancer, being responsible for 15–20% of breast tumors in the hereditary form [48, 49]. Mutations in other genes associated with other inherited cancer syndromes, such as *TP53*, *PTEN*, *STK11*, and *RAD51* also confer high breast cancer risk, others such as *ATM*, *CHEK2*, *BRIP1*, *PALB2* confer moderate risk, and others such as *FGFR2*, *TNRC9*, 2q35, 8q24 confer low risk for the development of familial breast cancer [49–51].

About 90–95% of breast cancer cases do not show a pattern of familial association and are considered sporadic. The development of breast cancer occurs within the ducts and lobules of the mammary gland and, as well as other types of

tumors, raise from genetic and epigenetic changes. The accumulation of genetic and epigenetic changes, which occurs during the development and progression of cancer, leads to the acquisition of new hallmarks typical of malignant transformation, such as unlimited replicative potential, self-sufficiency of growth factors, insensitivity to antiproliferative factors, evasion to apoptosis, induction, and maintenance of angiogenesis, invasion and metastasis formation [10, 52]. Genetic and epigenetic changes associated with the process of tumorigenesis result in differential gene expression profile between normal tissue and the tumor and among tumors from different patients. Identifying and characterizing genes differentially expressed in cancer can lead to the discovery of new markers for diagnosis, prognosis, and new strategies for treatment of the disease.

Historically, the classification of breast cancer was based solely on clinical and pathological characteristics, such as tumor stage and lymph node involvement, as well as morphological characteristics, such as histological type, proliferative status, and histological and nuclear grade [53, 54]. However, breast cancer is a complex and heterogeneous disease in relation to genetics, histopathology, and clinical course of the disease, and its histological classification alone is not sufficient to predict disease behavior and the adequate clinical management of patients [55–57].

Large-scale analysis of gene expression allowed the classification of breast cancer according to intrinsic molecular subtypes [58, 59]. The existence of four distinct intrinsic molecular subtypes for breast cancer is widely accepted: luminal A, luminal B, ERBB2-enriched, basal or triple-negative (Fig. 2).

Tumors of luminal subtype A, which have the best prognosis, are ER-positive, ERBB2 negative, and show expression of genes regulated by estrogen in luminal epithelial cells (such as cytokeratins 7, 8, 18, 19, GATA 3, GREB1, XBP1). Tumors classified as luminal subtype B, which present a worse prognosis relative to luminal A, are ER and ERBB2 positive and have a low or moderate expression of genes expressed in luminal cells. Tumors classified as belonging to the ERBB2 overexpression subtype are ER-negative and ERBB2-positive, which is an important prognostic and predictive biomarker of response in breast carcinoma. The basal or triple-negative subtype's tumors are ER, PR, and ERBB2-negative and have the expression of genes expressed by the progenitor cells or basal/myoepithelial cells. This subtype presents an aggressive phenotype with a worse prognosis and has no known therapeutic targets being treated by platinum-based chemotherapy with platinum and PARP inhibitors [60]. The data generated by the studies by Perou et al. [58] showed the potential of large-scale expression analysis techniques in revealing the molecular basis of the biological heterogeneity and clinical behavior of breast tumors and has generated a series of new studies for the validation and expansion of these data [56, 61, 62].

Different prognostic multigenic tests, such as MamaPrint, Oncotype, Mammostrat, and Prosigna have been developed and are commercially available. Despite their limitations, there is a growing consensus that multigenic signatures provide standardized and complementary information to histopathological variables, including tumor size, lymph node status, and histological grade [62–64]. The high cost of analyzing the gene expression profile has limited its incorporation in most clinical trials. On the other hand, several groups have developed the immunophenotypic evaluation, that is, the analysis of markers by immunohistochemistry as a valid method to determine the molecular subtypes. It has been suggested that ER, PR, Ki67, HER2, AURKA, basal cytokeratins (CK 5/6, CK14), and EGFR determined by immunohistochemistry can be used in the clinic for the classification of different breast cancer subtypes defined by the gene expression profile [65–68]. Estrogen and growth factor receptors with tyrosine kinase activity (such as HER2) are recognized as important mediators of cell signaling pathways associated with breast cancer development and progression [57]. The expression analysis of estrogen receptor (ER) and progesterone receptor (PR) by immunohistochemistry (IHC), and of HER2, both by IHC and fluorescent in situ hybridization (FISH), are used to determine the status of these receptors and are prognostic and predictive biomarkers to identify patients likely to benefit from endocrine or anti-HER2 therapies [69–72].

The development and application of high-throughput sequencing techniques have allowed a substantial improvement in our understanding of genomic, epigenomic, transcriptomic, and proteomic changes associated with breast cancer [73]. A series of detailed studies using the integration of different genomics and transcriptome platforms allowed the identification of the profile of somatic changes associated with high heterogeneity of breast cancer [73–75]. From 2012, with the publication of the sequencing data from The Cancer Genome Atlas Network [73], concerning a large number of samples of different types of tumors, including breast cancer, it was possible to unveil the extensive set of genomic changes underlying the pathogenesis of breast cancer [74–76]. In addition to confirming the role of several previously identified mutations, such as PIK3CA, PTEN, AKT1, P53, CDH1, GATA3, RB1, MAP3K1, MLL3, and CDKN1B, many other genes and CVNs (copy number change) have been cataloged [74, 76, 77]. It is estimated that more than 30 genes associated with different signaling pathways and biological processes are associated with the development and progression of breast cancer. However, only a few are altered in a significant portion of breast tumors, such as PIK3CA, TP53, MYC, CCND1, and HER2 [78]. Also, the real clinicopathological significance of these alterations alone or in

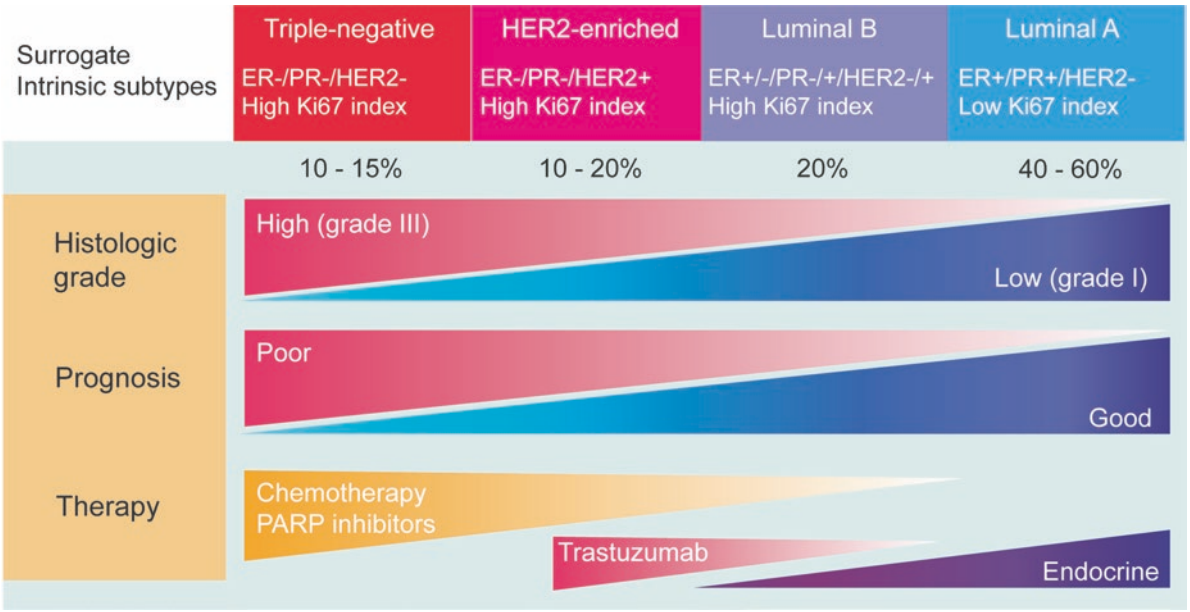

Fig. 2 The distribution of the four major molecular subtypes of breast carcinomas. The surrogate intrinsic subtypes clinically used are based on the immunohistochemistry expression of: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and the proliferation marker (Ki67)

combination is not fully defined, and several of these alterations or combinations of alterations have different frequency and clinical relevance depending on the molecular or histological subtype [52, 78, 79].

Despite advances in the molecular classification of breast cancer subtypes, the disease's high heterogeneity is still the most significant obstacle in clinical oncology, which has an impact on the diagnosis, accurate prognosis, and treatment of patients [55]. In this context, several studies using a large number of samples aiming to determine the landscape of genomic and transcriptomic changes of different breast cancer subtypes are being developed aiming at the identification of biomarkers for diagnosis, prognosis, drug targets, and predictors of therapeutic response [36, 80–82].

Intratumoral heterogeneity is the main obstacle to the effective treatment of breast cancer and personalized medicine. The spectrum of genetic and epigenetic alterations associated with the disease's intratumoral heterogeneity has been revealed, and several technologies have been developed for its characterization. However, very few predictive molecular biomarkers have been identified for making treatment decisions in patients with metastatic breast cancer. The main biomarkers of prognosis and predictors of therapeutic response in breast cancer remain the receptors for steroid hormones (ER and PR) and the HER2 oncoprotein. The functional and clinical characterization of new biomarkers and the definition of mutational processes that act during the different stages of disease development and progression, which allow the identification of clinically relevant prognostic and predictive factors for therapeutic response, are among the areas that still need to be addressed in breast cancer. In addition, understanding more about the molecular pathophysiology and the functional role of potential biomarkers in the biology of breast epithelial cells and breast cancer is extremely important for a better understanding of the changes associated with the process of breast tumorigenesis, identification and characterization of biomarkers, development of targeted therapies and to improve the prediction of response and/or resistance to conventional hormonal and chemotherapy treatments.

3 Role of Par-4 as a Biomarker in Breast Cancer

As described above, despite the great utility and impact of the molecular classification on our understanding of the physiopathology and natural history of breast cancer, a major challenge remains, that of breaking the heterogeneity found in tumors, bringing an adequate distinction of breast cancer intrinsic subtypes. Identifying putative biomarkers of clinical outcomes in breast cancer is crucial for selecting patients who are the most likely to benefit from a specific therapeutic approach. For this reason, a wide range of new possible biomarkers has been described in the literature. Par-4 (Prostate Apoptosis Response 4; also known as PAWR, pro-apoptotic WT1 regulator) is a tumor suppressor gene that encodes a pro-apoptotic protein expressed ubiquitously among the various tissue types. Altered expression of Par-4 is observed in many types of cancers, including breast cancer [83–85].

The study by Zapata-Benavides et al. evaluated the expression of Par-4 in 59 breast tumors showing negative immunostaining for Par-4 in 64.4% of the samples pointing out its role as a tumor suppressor in breast cancer [85]. Nagai et al. evaluated the expression of Par-4 on tissue microarrays containing 1161 samples of primary breast tumors [84]. In that study, low expression of Par-4 was found in 57% of the samples, and this low expression was associated with the worst prognosis (short disease-free survival and overall survival) of breast cancer patients, especially patients in the luminal subtype A group. In the same year, a study carried out in Mexico also associated the negative expression of Par-4 with the unfavorable prognosis in 67 primary breast tumors, further confirming the prognostic value of Par-4 in breast cancer [83]. Interestingly, in our study, it was also possible to observe the relationship between Par-4 expression and other markers already well established in breast cancer literature. Patients with tumors positive for EGFR and HER-2 receptors and positive for pAkt kinase, when associated with decreased expression of the Par-4 protein, represented a group with a worse prognosis [84]. Together, these results showed for the first time the Par-4 role as a tumor suppressor gene in breast cancer. Supporting the tumor suppressor role of Par-4 in breast cancer, Satherley et al. demonstrated that both Par-4 mRNA and protein down-regulation are associated with poorer overall survival rates. Moreover, they provided further information that *Par-4* overexpression reduces cancer cell adhesion, invasion, and growth in breast cancer cells [86].

Alvarez et al. [87], using transgenic mouse models and data from breast cancer patients, confirmed the results from previous studies [83, 84] and identified down-regulation of Par-4 as an important mechanism for tumor cell survival and local or distant recurrence after chemotherapy and targeted therapy. These authors also showed that low Par-4 expression was an independent predictor of poor recurrence-free survival in breast cancer patients. They also found low Par-4 expression in the highly aggressive, estrogen receptor negative (ER−), basal-like, and high-grade (grade 3) breast tumors. This study provides important insights into the value of Par-4 as a potential biomarker to predict recurrence and therapy response for breast cancer patients.

So far, few works on the relationship between the expression of Par-4 and prognosis of breast cancer has been done. However, a growing number of studies, in vitro and in vivo, provide important evidence of the role of Par-4 in proliferation, apoptosis, and chemosensitivity in breast cancer. In the next sections, we describe a series of experimental data providing evidence that Par-4 shows potential to be a biomarker of great value in the prognosis and prediction of breast cancer patients' therapy response.

4 Role of Par-4 in Breast Cancer Proliferation and Apoptosis

Par-4 (Prostate Apoptosis Response 4) is a tumor suppressor gene that encodes a pro-apoptotic protein expressed ubiquitously among the various tissue types [88–90]. The majority of its actions are related to the apoptotic pathways' activation with the direct or indirect inhibition of proliferative cascades. However, it is sometimes difficult to determine if the final direct outcome of Par-4 action is related to cell death or cell growth mechanisms since some intermediate molecules are redundant, that is, they participate in both processes. Moreover, evidence raise that Par-4 is able to bind more than one partner at a time and thus could function as a hub linking the functions of several proteins [91].

Some studies suggest that Par-4 expression increase is related to apoptosis while its decreased levels are related to cell growth arrest as a consequence of the no activation of Par-4-mediated apoptosis pathways [87, 92]. Nevertheless, the data about the direct interference of Par-4 on cell proliferation are controversial to some extent. For example, it was demonstrated that in BT474 breast cancer cells, both Par-4 knockout and cell with Par-4 ectopic expression grew at equal rates. However, after treatment with PI3K-Akt-mTOR pathway inhibitors (MK-2206, BKM120, BEZ235, and Torin1), the Par-4 knockout cells did not show any differences in their viability at early time-points (three days). In turn, the long-term (31 days) treatment with Lapatinib or MK2206 led to a dramatic selection for Par-4 knockout cells, which were able to resume proliferation after drug removal [92]. These findings in cell models are consistent with the results obtained in mouse primary tumors in which Par-4 knockdown does not affect the growth and survival of untreated cells [87]. Despite that, it was demonstrated that the Par-4 knockdown in MCF7 breast cancer cells led to increased proliferation ratios [93].

Par-4 activation is triggered by a myriad of apoptotic stimuli such as the withdrawal of growth factors and estrogens, tumor necrosis factor, intracellular calcium elevation, ionizing radiation, chemotherapeutic drugs, or those involved in neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, and stroke [94, 95]. In the MCF7 breast cancer cell line that has a hormone-responsive phenotype (ER+/PR+), the estrogen withdrawal has an inhibitory effect on cell proliferation along with the activation of apoptotic pathways [96]. These effects could be related to the Par-4 expression increase observed in these cells when cultured under estrogen-free conditions [94].

Interestingly, Par-4 expression level increase *per se* is sufficient to induce apoptosis in hormone-independent breast and prostate tumor cell lines, while in normal and hormone-dependent cells, the first apoptotic stimulus increases the

cells' sensitivity to apoptosis, and the Par-4-induced cell death is carried out by the occurrence of a second apoptotic signal (Fig. 3) [89, 97].

To better understand the Par-4 action in cell survival, it is important to have in mind the basic structure of Par-4 protein. *Par-4* gene (12q21) encodes a 332-amino acid (aa) protein composed of two putative nuclear localization sequences (NLS) corresponding to the aa residues 20–25 (NLS1) and 137–153 (NLS2), a leucine zipper (LZ) domain spanning aa 292–332 and a nuclear export sequence in the carboxi-terminal region [95]. The minor aa sequence, unique and sufficient to induce apoptosis specifically in cancer cells, comprises the residues 137–195 and is named SAC (Selective for Apoptosis in Cancer cells) domain. The SAC domain encompasses the NLS2 and is followed by a linker domain that connects it to the carboxi-terminal coiled-coil (CC, aa254–332) motif that includes a nuclear export sequence

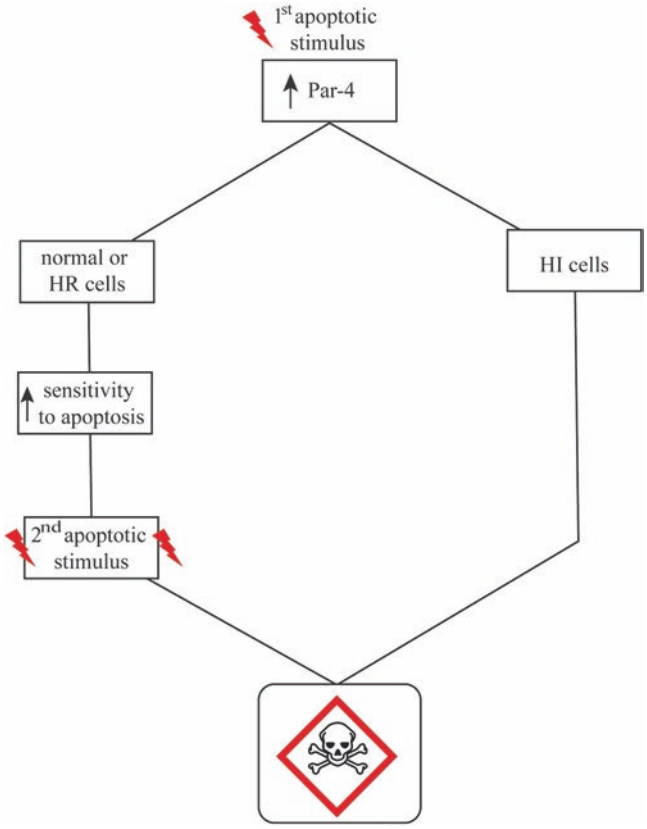

Fig. 3 Par-4 apoptotic action timing in hormone-responsive (HR), hormone-independent (HI) and normal breast cancer cells. Upon an apoptotic stimulus, the increase in Par-4 expression level is sufficient to induce apoptosis in HI breast tumor cell lines (e.g., MDAMB-231 cells) while in normal (e.g., MCF10A cells) and hormone-dependent cells (e.g., MCF7 cells), the first apoptotic stimulus increases the cells sensitivity to apoptosis and a second apoptotic signal is necessary to accomplish Par-4-induced cell death. 1st, first; 2nd, second; , cell death, ↑, increase

(NES, aa 295–301) [98, 99]. Noteworthy, Threonine 155 (T155) and Serine 249 (S249) are the residues more frequently described as phosphorylated by important molecules such as PKA and AKT, respectively (Fig. 4) [91].

The CC and LZ motifs are described as the mediators of the majority of protein interactions involving Par-4 already identified, including its homodimerization [99, 100]. Three exceptions for this rule can be made in relation to Twist [101] and Foxo3a [92] interactions that take place in the Par-4 promoter region, and GRP78 binding that occurs in the SAC domain [88]. Interestingly, the NES seems to be masked upon Par-4 homodimerization and heterodimerization with its partners, indicating a potential mechanism for Par-4 nuclear localization maintenance [99]. In this context, it is interesting to mention that Par-4 is accepted as an intrinsically disordered protein (IDP) because of its interaction with a great number of proteins. In general lines, IDPs are proteins with great medical potential characterized by multiple interactions due the lack of a stable three-dimensional structure and the presence of a CC motif in ordered regions [100].

Par-4 aa sequence contains potential non-typical Caspase cleavage sites, raising the possibility that following an apoptotic stimulus, Par-4 could be cleaved by caspases [98, 102]. According to this model, during apoptosis induction, the full-length cytoplasmic Par-4 is cleaved by Caspase-3 at D131↓G, generating a 24 kDa "activated" fragment that includes, at its amino-terminus, the SAC domain and the NLS2 (Fig. 4). These Par-4 fragments display increased apoptotic activity and can translocate to the nucleus or be accumulated in the cytoplasm [98]. In the cytosolic compartment, the Par-4 fragment is able to inhibit IκBα (an NF-κB inhibitor) phosphorylation with the consequent blockage of NF-κB nuclear translocation and reduction of the cellular levels of some NF-κB–targets, such as the anti-apoptotic proteins Bcl-2 and FLIP [98]. Moreover, the occurrence of Par-4 cleavage is dependent on Caspase-3 expression and can be related to specific stimuli and cell types. For example, in MCF7 cells that are Caspase-3-deficient, the Par-4 cleavage is not observed, being that the induction of Caspase-3 ectopic expression is sufficient to produce a decrease in the full-length Par-4 levels, due to the appearance of the cleaved fragments [98]. The Par-4 cleavage mediated by Caspase-3 was also observed in HeLa cervical tumor cells and A2780 ovarian tumor cells following treatment with cisplatin [98]. Additionally, knockdown of Par-4 in HeLa cells reduces Caspase-3 activation and apoptosis induction while the overexpression of wild-type Par-4, but not the Par-4 D131A mutant, sensitizes them to cisplatin-induced apoptosis [98].

Guo et al. demonstrated that Caspase-8-induced Par-4 cleavage results in the nuclear accumulation of the C-terminal fragment. The transient expression of Par-4 and the C-terminal Par-4 fragment leads to a reduced expression of cIAP1 in TNBC cells (BT-20 and MDA-MB-468). They also

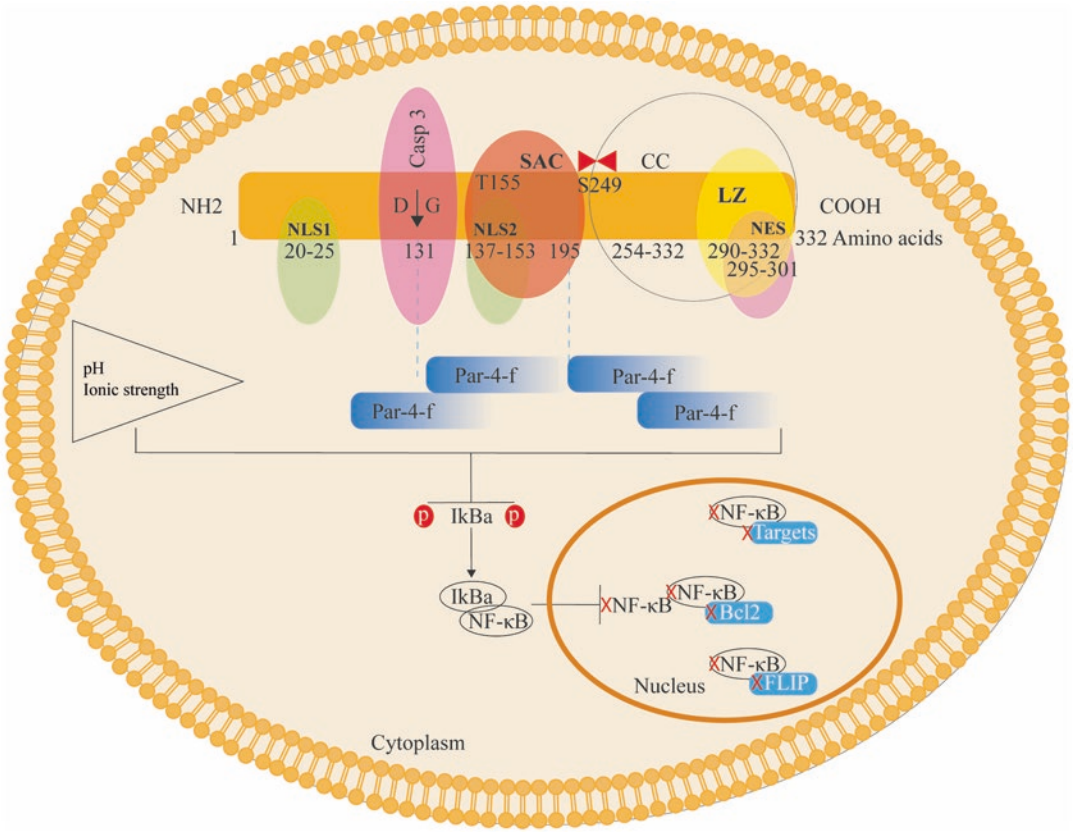

Fig. 4 Par-4 protein structure and the Caspase-3-dependent mechanism of cleavage. Par-4 is composed of two nuclear localization signals (NLS1 and NLS2), a leucine zipper sequence (LZ), and a nuclear export signal (NES). Functionally, the minor portion sufficient to induce apoptosis in cancer cells is named SAC (Selective for Apoptosis in Cancer cells) domain encompassing the NLS2 that and followed by the coil-coiled (CC) domain that, in turn, encloses the LZ and NES domains. The CC domain, together with LZ domain, is the site where the majority of Par-4 protein interactions take place. T (Threonine) 155 and S (Serine) 249 are the most studied sites for Par-4 phosphorylation. The presence of one non-typical Caspase-3 cleavage site (D↓G), N (amino)-terminal to SAC domain, allows the generation of Par-4 fragments (Par-4-f) with a Caspase-3-dependent apoptosis role. These fragments act in both, nucleus and cytoplasm. It is believed that it is necessary to form Par-4-f tetramers in special pH and ionic strength conditions for their efficient function. One described pro-apoptotic action of the Par-4 fragments tetramers is the inhibition of IκKα phosphorylation that in turn keeps the NF-κB/IκKα interaction with the consequent sequester of NF-κB in cytoplasm and reduction of the transcription of NF-κB-dependent targets, like Bcl-2 and FLIP. ⋈, SAC to CC linker; **X**, blockage; COOH, carboxi-terminal protein end; Par-4-f, Par-4 fragment; NH2, amino-terminal protein end; p, phosphorylation

found that cIAP1 overexpression can protect BT-20 cells from apoptosis and Caspase-8 activation, and loss of Par-4 in TNBC cells completely prevents cIAP1 depletion induced by genotoxic stress and results in drug resistance [103]. Moreover, these authors found that down-regulation of cIAP1 with RNAi or Smac (second mitochondrial-derived activator of caspases) mimetics developed to target IAPs overcomes chemo-resistance induced by loss of Par-4 and restores Caspase-8 activation [103]. To add complexity to this novel mechanism, it was showed that the apoptotic action of the generated fragments seems to be dependent on their tetramerization in specific conditions of ionic strength and pH (Fig. 4) [104].

As mentioned, Pereira et al. demonstrated the impact of Par-4 on cell proliferation in MCF7 breast cancer cells [93]. In this study, the cell proliferation ratios, measured by MTT assay, were significantly inhibited in cells overexpressing Par-4 compared with the control transfectants being that this proliferative restraint seems to involve the inhibition of the Ras/Raf/MEK/ERK pathway since MCF7 cells with increased Par-4 expression exhibited a significant reduction in ERK phosphorylation. Additionally, it was also demonstrated that MCF7 cells overexpressing Par-4 display a greater proportion of cells in the sub-G1 population compared with control cells what is indicative of increased apoptosis. Nevertheless, the question remains: is the Par-4 effect on proliferation direct or the result of Par-4 action on key pro-apoptotic molecules that interfere in proliferative pathways?

In in vivo studies, the overlap of apoptotic and proliferative events have also been observed. For example, Par-4 and SAC-transgenic mice are resistant to the growth of spontaneous tumors, whereas GFP-transgenic mice and littermate control mice develop spontaneous tumors in the endome-

trium, prostate [105], liver/spleen [106], and lungs [107]. In a mouse model of Ras-induced lung tumorigenesis, Par-4 knockout was accompanied by increased Ki67 staining in both, normal alveolar or tumor tissues, and enhanced NF-κB activation resulting in higher cell survival during the oncogenic transformation process [107]. Additionally, in a mouse HER2/neu-induced mammary tumor formation model, it was showed that primary tumors expressing ectopic Par-4 grow at rates similar to control tumors but have an increased latency period for tumor recurrence. This suggests that Par-4 expression does not alter primary tumor growth but could have an important role in the survival of the residual cells responsible for tumor recurrence [87].

Given the complexity of Par-4 pro-apoptotic action, it is possible to infer that the mechanisms triggered upon its activation are dependent on the cell type and the apoptotic stimulus that originate the response [98, 104]. Moreover, the Par-4-mediated effects on cell survival involve not only Par-4 intracellular levels variations but also the Par-4 molecules that are secreted to the extracellular microenvironment under some conditions. The next session will describe the Par-4 intracellular and secretory mechanisms in more detail.

5 Par-4 Intra and Extracellular Possible Mechanisms of Action in Breast Cancer

Par-4 is a very interesting pro-apoptotic molecule for a number of reasons: (1) Tumor cells seem to be more sensitive to its action than normal ones, (2) it is involved in key pathways related to the development of the majority of tumor types, namely NF-κB, PI3K-AKT, Ras, and GRP78, and (3) it can act not only in the cells that received the apoptotic stimuli (intracellular effects) but it can also sensitize neighboring cells through its secretion (extracellular effects).

The first studies describing Par-4 functions were focused on Par-4 intracellular roles and showed its interference in the NF-κB pathway. According to this model, Par-4 is activated by an apoptotic stimulus that is received by different cellular receptors such as growth factor receptors, estrogen receptors, namely in case of hormone depletion, GPCR (G-protein coupled receptor), and ionic channels. These stimuli lead to Par-4 upregulation that in turn promotes the NF-κB inhibition and the co-parallel trafficking of Fas/FasL to membrane activating the Fas-associated death domain—Caspase-8 pro-death pathway. Interestingly, this mechanism seems to be independent on the levels of Bcl-2 and Bcl-xL anti-apoptotic protein and activity of p53 and PTEN [108].

Additionally, the PI3K pathway's central molecule, the AKT, was showed to physically interact with and phosphorylate Par-4 inhibiting its apoptotic function [109]. In this context, PTEN, an important negative regulator of PI3K/AKT activation, can induce apoptosis by a Par-4-dependent mechanism. Since cancer cells generally express activated AKT as a result of PTEN loss, oncogenes upregulation, and increased growth factor signaling, the Par-4 apoptotic action linked to PTEN is lost, leading to NF-κB activation. However, in cells that have an active PTEN, the AKT phosphorylation is inhibited, allowing the Par-4-mediated apoptosis [109].

Par-4 upregulation in response to PI3K-AKT-mTOR pathway inhibition was observed in Her2-amplified breast cancer cells (BT474 and SKBR3) and breast cancers with activating PI3K mutations, which together constitute nearly half of breast cancer cases. In this context, AKT seems to be crucial for Par-4 apoptotic actions in a way that AKT inhibition is essential for Par-4 upregulation following Her2 inhibition [92]. One of the Par-4 effects observed upon PI3K-AKT-mTOR pathway inhibition was the restriction of the residual cells survival [92], positioning Par-4 as an important target to circumvent the post-treatment survival of residual cells responsible for tumor recurrence and metastasis.

Another molecule described as a Par-4 regulator is PKA. The PKA activity is dependent on AMPc levels, and its expression is increased in various transformed and cancer cells compared to corresponding nontransformed normal or immortalized cells [110]. According to this model, cells with elevated PKA activity can have high levels of Par-4 phosphorylation at T155. The T155 phosphorylated Par-4 translocates to the nucleus where inhibits NF-κB activity and induces apoptosis. The high PKA activity in transformed cells can explain, in part, the higher sensibility of these cells to Par-4 mediated apoptosis in comparison to normal ones. However, it is curious to mention that in cells with the concomitant high PKA and high AKT activities, if Par-4 is also phosphorylated by AKT, the 14–3-3 protein binds to Par-4 blocking its nuclear translocation. This is one more evidence of AKT importance to Par-4 action fate [109, 111].

The Ras oncogenic pathway, another important oncogenic driver, is also involved in Par-4 regulation. When activated, this pathway is related to Par-4 down-regulation through two main mechanisms: (1) AKT phosphorylation in S473 and (2) ERK2 activation [107, 112]. Interestingly, in this context, Par-4 is also a negative regulator of AKT by promoting the ERK1/2 and PKCζ down-regulation being the PKCζ regulation made through a direct binding [107, 112]. PKCζ is an AKT kinase (S473) also required for the nuclear translocation of NF-κB [107]. Therefore, the Par-4-mediated apoptosis, beyond dependent on stimuli origin and cell type, could be more or less effective concerning the activated pathways in a determined cell context [113].

Additionally, to AKT and PKCζ interactions, other molecules that showed to have direct interactions with Par-4 in different cellular models were Twist, Dlk/ZIP kinase, WT1, Bcl-2, TOP1, and THAP1. Twist is an important transcrip-

tion factor related to Epithelial to Mesenchymal Transition (EMT) and was described to induce histone modifications to repress Par-4 transcription in recurrent tumors [101]. The coexpression of Dlk/ZIP kinase and Par-4 in rat embryo fibroblasts is related to the relocation of Dlk from the nucleus to the actin filaments in the cytoplasm leading to cytoskeleton reorganization with morphological signals of apoptosis [114]. The Par-4 interaction with WT1, in the HEK293 human embryonic kidney cell line, is related to the inhibition of the transcriptional activation with the enhancement of the transcriptional repression activity of WT1 rescuing, even partially, the growth suppression mechanism induced by WT1 [115]. It was also shown, in prostate cancer cell lines, that Par-4 and WT1 proteins binding to Bcl-2 promoter decreasing its transcription [116]. The Par-4 direct binding to TOP1 in normal and tumor prostate cells leads to the suppression of DNA relaxation abrogating cell cycle progression and transformation [117]. Finally, the Par-4/THAP1 interaction, however little explored, could have important effects on the activation of the apoptotic pathways coordinated by the promyelocytic leukemia protein (PLP) as shown in human endothelial and cervical cancer cells [118].

The Par-4 apoptotic mechanisms aforementioned refer to its intracellular (cytoplasmic/nuclear) levels (Fig. 5). However, in last years, the Par-4 secretion by normal and tumor cells has emerged as an important mechanism by which Par-4 *per se* becomes an apoptotic stimulus. The first work describing the secretory Par-4 pathway was published in 2009 [88] and showed that Par-4 protein is spontaneously secreted by normal and tumor cells and that Par-4 transgenic mice have this protein in the serum. The Par-4 secretion occurs by the classical pathway involving the ER (endoplasmic reticulum) to Golgi network in a BFA-sensitive way. The Par-4 secretion and reception by adjacent cells are mediated by the stress response protein GRP78 followed by FADD/Caspase-8/Caspase-3 apoptotic pathway activation as will be described ahead.

At first glance, the GRP78 involvement in apoptosis could be sound contradictory since GRP78 exists primarily as an endoplasmic reticulum (ER) protein with intracellular chaperone functions, that is upregulated in response to ER stress in order to diminish inhibitory growth signals and promote cell survival [119]. Nevertheless, according to the proposed mechanism, the ER stress induced by extracellular insults promotes the Par-4/GRP78 binding and translocation from the ER to the plasma membrane. Interestingly, unlike most of the previously described protein–protein interactions of Par-4 that are mediated by its coiled-coil domain, including the leucine zipper domain, the binding of Par-4 to GRP78 is mediated by the SAC domain [88].

The accumulation of Par-4/GRP78 complexes in the membrane leads to Par-4 secretion. The Par-4, now in the extracellular microenvironment, binds to cell surface GRP78, from the same cell or the neighboring cells, inducing more ER stress and activating the loop for translocation of GRP78/Par-4 to the plasma membrane. Moreover, ER stress also involves upregulation of the ER components, such as PERK, and activation of Caspase-8 in an FADD-dependent manner. Collectively, the secretory Par-4 reception and other molecules activate under ER stress, turn on the events that lead to activation of Caspase-3 and apoptosis (Fig. 6) [88]. This mechanism was originally described in prostate cancer cells and recapitulated in cervical and lung cancer cells [88, 120, 121].

As we can conclude from what has been exposed so far, the induction of Par-4-dependent apoptosis is complex and involves a myriad of up/downstream molecules, through direct/indirect regulation, that is related to different pathways that converge in some crucial points. It is also important to have in mind that the Par-4 action is consonant with the stimulus received by the cell, the cell type, the cell genetic background, and all of these aspects are associated with the cell compartment in which Par-4 is located. This bi-compartmental function of Par-4 constitutes a challenge in the interpretation of the mechanisms of Par-4 action and increases the therapeutic significance of this cancer cell-selective apoptotic molecule. In this context, some studies correlate the Par-4 expression and activity, especially in the intracellular environment, with the breast cancer cells response to chemotherapy, as will be reported hereafter.

6 Par-4 in Breast Cancer Chemosensitivity

The vast majority of cancer-related mortality in solid tumors is associated with the capacity of cancer cells to invade and colonize nearby or distant vital organs forming metastasis [122]. A line of investigation suggests that tumor recurrence and metastization is due to a population of residual cells that survive the surgery, radiation, and adjuvant therapy and serve as a reservoir for cancer recurrence [87, 92]. These residual cells are capable of leaving their primary location, disperse into the bloodstream, endure pressure in blood vessels, escape immune response, and acclimate to new cellular environments in secondary sites [123]. Consistent with this, as reviewed by Bednarz-Knoll et al. [124], up to 40% of breast cancer patients presented disseminated tumor cells in the bone marrow at diagnosis, and their presence and number following adjuvant therapy is a strong independent predictor of recurrence risk. Recurrent breast cancer, in turn, affects nearly 25% of patients, and is frequently resistant to the drugs used to treat the primary tumor.

The development of new therapies that can eliminate residual tumor cells or prevent their emergence is conditioned by the scarce understanding of the mechanisms under-

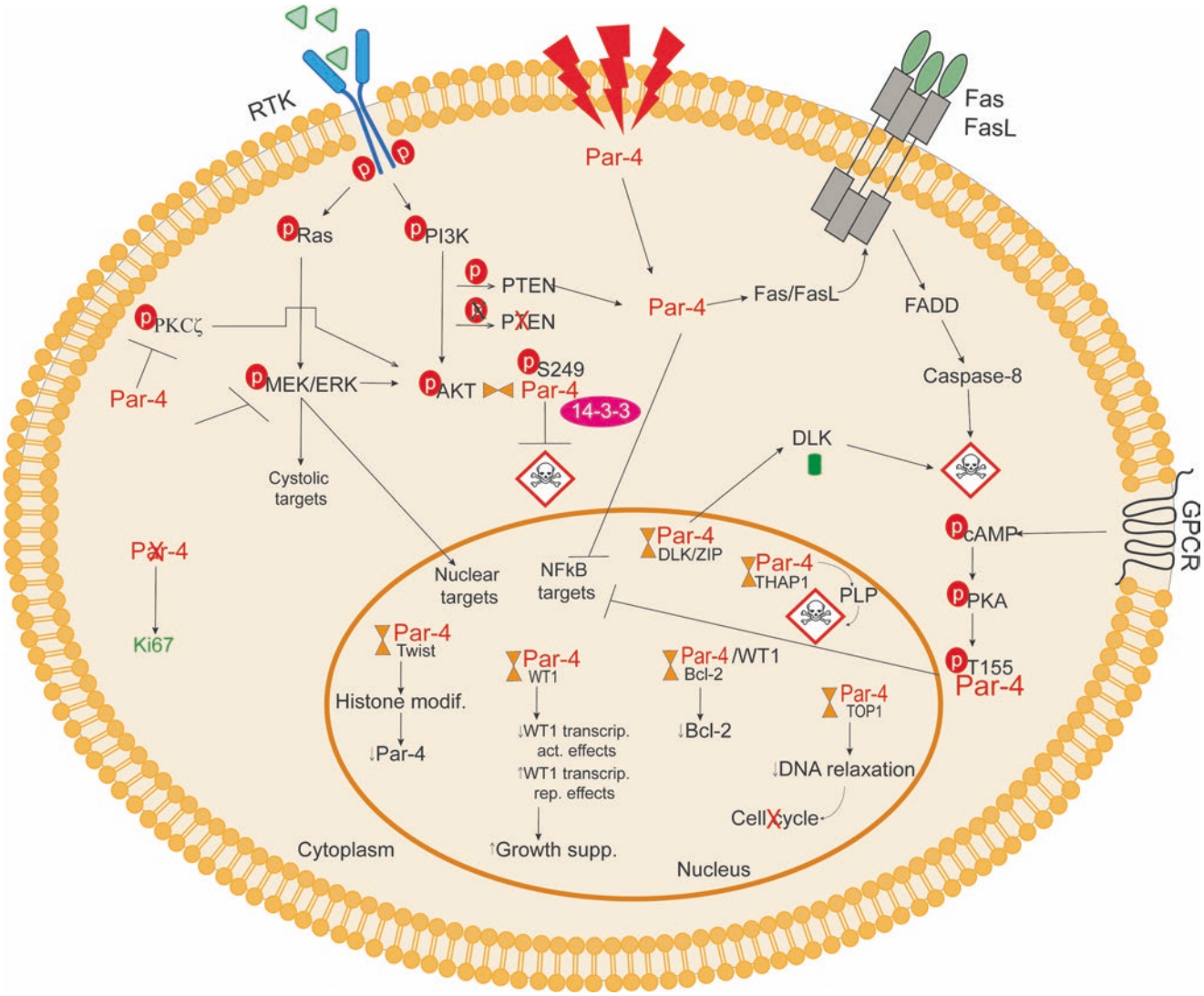

Fig. 5 Par-4 intracellular mechanisms. Upon an apoptotic stimulus, Par-4 is upregulated and can act in many different ways, consonant cell type and genetic background. The Par-4 upregulation can activate the Caspase-8 death pathway by inducing Fas/FasL translocation to the cytoplasmic membrane and/or interfering in proliferative cascades such as ERK pathway, reducing its activation. PKA activity is related to Par-4 phosphorylation and apoptotic activity. On the contrary, oncogenic molecules, like AKT, can physically bind to Par-4, impeding its nuclear translocation and apoptotic signalization. The AKT activity is enhanced when PTEN function is lost. However, in normal conditions, PTEN can regulate the AKT phosphorylation and induce the Par-4-dependent apoptosis. The effects of Par-4 direct interaction with some nuclear molecules are shown. The DLK/ZIP, WT1, Bcl-2, TOP1, and THAP1 interactions are related to tumor suppression acting in actin filaments organization, growth suppression, and apoptosis inclusive with the coordinate action with PLP (Promyelocytic Leukemia Protein) in human endothelial and cervical cancer cells, while Twist binding is related to oncogenic behavior due the negative effect in Par-4 transcription. Par-4 knockout cells present elevated ki67 activation that could be related to an increased proliferation index. , actin filaments; , apoptotic stimulus; , binding; , cell death; **X**, blockage; Act, activation; GPCR, G-protein coupled receptor; Prolif., proliferation; RTK, Receptor Tyrosine Kinase; Rep., repression; Supp., suppression; Transcrip., transcription

lying the long-term survival of these cells following treatment [92]. Interestingly, it was observed that in breast cancer patients treated with neoadjuvant chemotherapy (NAC), low Par-4 expression in primary tumors is associated with increased residual cancer burden [87]. Moreover, it was also showed that during breast tumor progression, Par-4 down-regulation seems to be a crucial mechanism for recurrence and resistance to chemotherapy [87, 92]. These statements identify Par-4 as a potent-negative regulator of residual cell survival following therapy and suggest that approaches to enforce Par-4 expression in breast cancer cells may prevent residual cell survival, recurrence, and chemo-resistance.

Human cancers undergoing targeted therapies and models of oncogene-induced mammary tumors in mice follow the

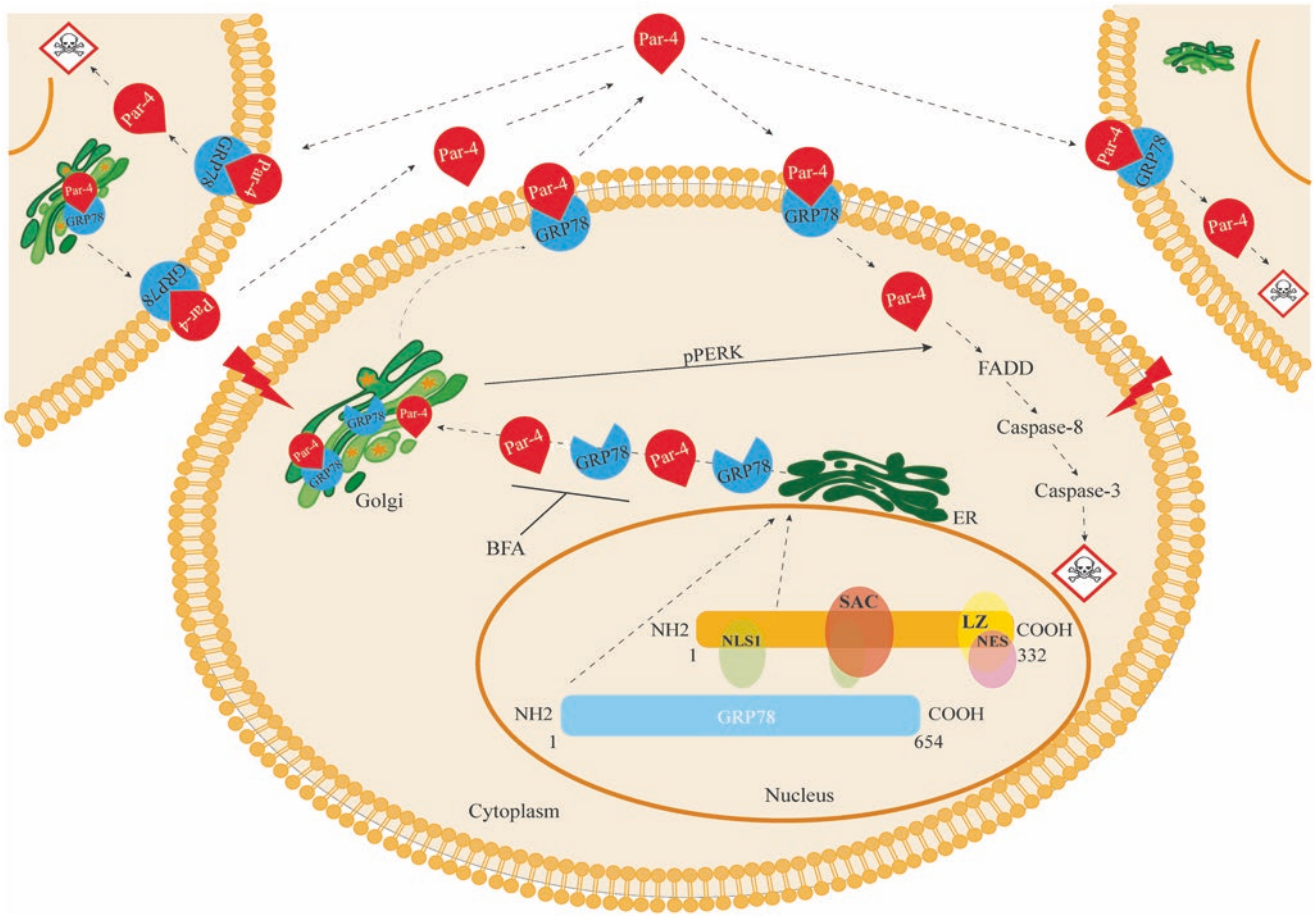

Fig. 6 Par-4 extracellular mechanisms. Extracellular insults can cause the endoplasmic reticulum (ER) stress, promoting the Par-4/GRP78 binding and translocation from ER to cytoplasmic membrane. However, this process could be inhibited by Brefeldin A (BFA) treatment that blocks the protein transportation from ER to Golgi. The accumulation of the complexes in the plasma membrane leads to Par-4 secretion. The secreted Par-4 could bind to the GRP78 from neighboring cells, including those ones that not receive any stimulus so far (upper right). In cells already stimulated, the secretory Par-4 induces more ER stress and activates the loop for translocation of GRP78/Par-4 to the plasma membrane. The Par-4 received from the extracellular microenvironment activates the FADD/Caspase-8/Caspase-3 apoptotic pathway. Interestingly, the ER stress also leads to the upregulation and phosphorylation of some ER components, such as PERK, that could boost the activation of the FADD-Caspase-8 pathway. However, the mechanisms involved in these collective events for Caspase-3 and apoptosis should be better studied. , apoptotic stimulus; cell death; ER stress; p, phosphorylation

oncogene addiction phenomenon that predicts the regression of the majority of tumors to a non-palpable state upon oncogene down-regulation. However, preexisting cells with low Par-4 expression preferentially survive during tumor regression, and following a variable latency period recur activating alternative growth and survival pathways.

An interesting work by Damrauer et al. [92] in mice models showed that the inhibition of the PI3K-Akt-mTOR pathway, but not the Ras-MAPK pathway, is sufficient to induce Par-4 upregulation in Her2-amplified breast cancer, including tumors with PIK3CA mutation. According to this model, inhibition of Akt (by MK2206) is required for Par-4 upregulation following Lapatinib treatment and suggests that mTORC1/2 inhibition (by Torin 1) functions downstream of Akt to induce Par-4 upregulation. This upregulation is mediated by Foxo3a direct binding to the Par-4 promoter region and acts to limit the long-term survival of residual cells. The Par-4 upregulation following Lapatinib treatment was also observed in the breast cancer cell lines BT474 (Luminal B) and SKBR3 (HER2+) [92]. Another elegant study by Alvarez et al. [87] showed that Par-4 is down-regulated during tumor recurrence in mice and is a key event for this process in a way that elevated Par-4 expression in recurrent tumor cells might be incompatible with cell survival.

In the same line of thought, Par-4 down-regulation was observed in mice breast tumors that recur following chemotherapy (adriamycin plus cyclophosphamide followed by paclitaxel) and in human breast cancer cells that survive chemotherapy. In women with breast cancer, low Par-4 predicts an increased risk of recurrence and a decreased response to

chemotherapy. Therefore, Par-4 levels assessment prior therapy could be an important marker of response and restoring its expression in recurrent tumor cells could result in growth arrest and apoptosis [87].

The Par-4 ectopic expression in human and mouse breast cancer cells that recur and were treated with targeted agents (e.g., Lapatinib) or chemotherapeutic agents (e.g., adriamycin) results in the multinucleation of cells that undergo p53 activation and show a ZIPK-dependent increase in MLC2 phosphorylation. The MLC2 phosphorylation increase disrupts the precise temporal and spatial control of MLC2 action that is required for the successful completion of cytokinesis leading to growth arrest and apoptosis [87]. In MCF7 breast cancer cells was demonstrated that Par-4 overexpression modulates the expression of genes involved in the Wnt pathway after Docetaxel (Taxotere) exposure [125]. The already described roles of Wnt and its downstream effectors in breast development [126–128] combined with the possibility of Par-4 interference in this pathway could be an important factor in the modulation of the cellular response to docetaxel treatment [125].

Moreover, it was described that upon re-expression, using an epigenome editing approach, Par-4 bind to the protein phosphatase PP1, leading to widespread changes in phosphorylation of cytoskeletal proteins, and cooperated with microtubule-targeting drugs (adriamycin/cyclophosphamide plus paclitaxel, docetaxel, and vincristine) to induce mitotic defects [101].

Par-4 has also been described as involved in response to inflammatory signals from the tumor microenvironment. The inflammation emerged as the seventh hallmark of cancer, presenting a new target for chemotherapeutic agents. In breast cancer, the tumor-associated macrophages constitute about 50% of the tumor mass and play an essential role in cancer progression and therapeutic response by secreting multiple inflammatory cytokines and growth factors that assist tumor cell malignant behavior in many ways [129]. In this context, piroxicam and sulindac sulfide are non-steroidal anti-inflammatory drugs (NSAID) that decrease the incidence and progression of several types of cancer. In MCF7 and MDA-MB-231 cells, the conditioned media (CM) from human monocytes caused a significant increase in cell survival through a significant increase in Ras expression, which resulted in upregulation of inflammatory mediators, oncogenic expression, angiogenic and metastatic markers and down-regulation of Par-4. Treatment with NSAIDs produced a time- and concentration-dependent growth inhibition of CM-stimulated cells by inhibiting *Ras*, Bcl-2, VEGF-A, MMP2, and MMP9 expression, and activating the apoptotic machinery through upregulation of Par-4 and Caspase-3 [130].

Drugs, that are mainly used to treat other diseases, as 1,1-dimethylbiguanide hydrochloride (biguanide metformin), also affect Par-4 expression. Metformin is a hypoglycemic agent that is widely used in Type 2 diabetes treatment and was found to be associated with a lower risk of cancer. It was suggested that metformin has an anticancer and antiproliferative effect and affects apoptosis by activating the AMPK and inhibiting the mTOR. In the MCF7 breast cancer cells, the exposure to metformin led to a dose-dependent increase in the Par-4 mRNA expression levels in comparison to the control group [131].

The Par-4 response has also been analyzed in the context of ayurvedic medicine, an ancient Indian medical system based on past writings that rely on a "natural" and holistic approach to physical and mental health [132]. The Withaferin A, which is a naturally derived steroidal lactone from *Withania somnifera*, has been found to prevent angiogenesis and metastasis in diverse tumor models [133]. The 3-azido Withaferin A (3-AWA), a semisynthetic analog of Withaferin A has emerged as a compound with anticancer potential [134]. It was shown that in cervical (HeLa), prostate cells (PC3), and breast cells (MCF7), the exposure to 3-azidoWA induces the Par-4 secretion in conditioned media, by classical BFA-sensitive pathway, resulting in cellular effects related to CTNNB1 [132] and MMP2 [121] roles. Relative to effects on CTNNB1, the 3-AWA treatment of MCF7 and DU145 cells consistently sequestered nuclear CTNNB1 and increased its cytoplasmic pool reducing its transcriptional activity. Moreover, 3-AWA treatment triggered robust induction of intracellular Par-4, decreased AKT activity, and rescued pGSK3b expression promoting CTNNB1 destabilization [132]. Concerning MMP-2, Par-4 secretion induced by 3-AWA treatment in HeLa and PC3 cells abolished the secretory MMP-2 expression and activity, decreasing the pERK and pAKT expression in a dose-dependent manner, and substantially inhibited the angiogenesis process in a mouse model [121]. The Par-4 interference in pathways involved with breast cancer chemosensitivity is illustrated in Fig. 7.

7 Conclusions and Future Perspectives

As can be concluded from the aforementioned studies, the Par-4 upregulation in cancer cells has important effects on tumor progression inhibition in response to chemotherapy. In line with these observations, Kim et al. [135] have engineered the Par-4 protein giving raise to the Par-4Ex protein entity, produced using the *Escherichia coli* expression system suitable for large-scale production. The Par-4Ex fully retains the pro-apoptotic activity of Par-4 protein with a substantial improvement in the biological half-life. Moreover, in vivo assays confirmed that Par-4Ex protein is more potent in suppressing metastatic tumor growth.

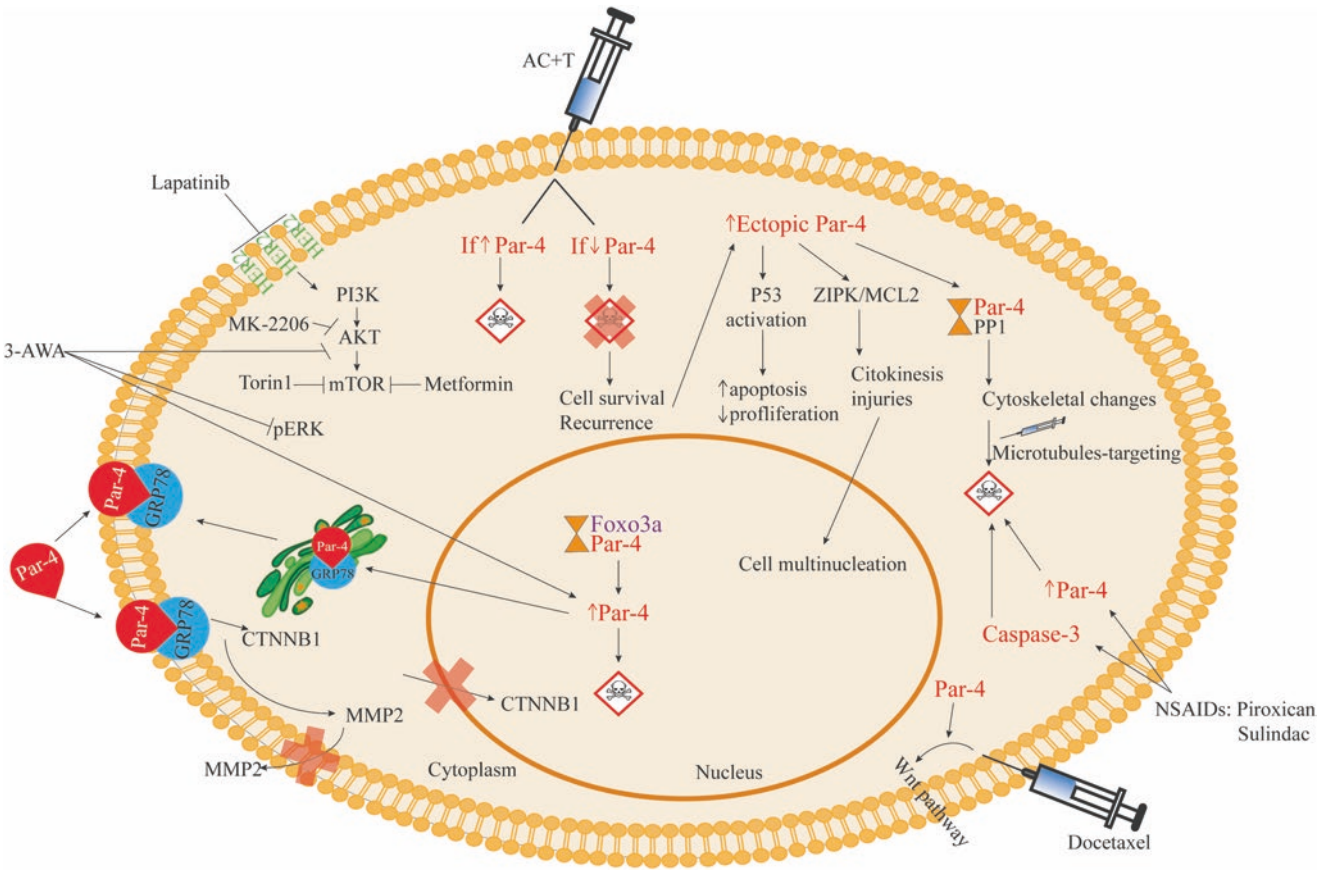

Fig. 7 Par-4 interference in pathways involved with breast cancer chemosensitivity. The Par-4 expression before the beginning of breast cancer treatment is inversely related to response and recurrence ratios. Upon AC + T (adriamycin plus cyclophosphamide followed by paclitaxel) therapy regimen, if Par-4 is upregulated, the treatment tends to be more effective while Par-4 down-regulation can be correlated with survival of residual cells and recurrence. However, the induction of ectopic Par-4 expression in recurrent cells is able to improve treatment through different mechanisms concerning the cellular genetic background and the therapeutic target. Some targets of Par-4 ectopic expression are p53, cytokinesis events, and PP1. Drugs related to other diseases, like metformin and the non-steroidal anti-inflammatory drugs (NSAID, namely Piroxican and Sulindac), are related to Par-4 activation with consequent anti-tumoral effects. A natural and ayurvedic compound, 3-AWA (3-azido Withaferin A), also shows anti-tumoral effects interfering in ERK activation and Par-4 intracellular levels and secretion. The Par-4 secretion stimulated by 3-AWA supplementation occurs via the classical ER/Golgi/GRP78 pathway and is related to CTNNB1 retention in the cytoplasm, reducing its transcriptional activity in the nucleus and MMP2 secretion abrogation. The therapy targeted to HER2+ tumors with the use of Lapatinib in combination with inhibitors of AKT (MK2206) and mTOR (Torin 1) leads to an increase in nuclear Par-4 upregulation, mediated by the binding of the transcription factor Foxo3a, and culminating with apoptosis. Additionally, it is speculated that Par-4 modulates the Wnt pathway interfering in the breast cancer cell's response to Docetaxel treatment. , apoptotic stimulus; , binding; , blockage; , cell death; , ER stress; , therapy regimen; p, phosphorylation

Summarily, Par-4 up- or down-regulation in different cell types interfere in key pathways as NF-κB, Ras, PI3K, Bcl-2, GRP78, thus showing a certain consistency on the mechanisms involved in its outcomes. However, the Par-4 actions are not only related to fluctuations in its expression levels but also to the direct/indirect interaction with cell partners, whose availability is dependent on the cell type and genetic background, and to the cell compartment where the scene takes place. In this context, the Par-4 function as a potent death sensitizer in tumor cells associated with cell-specific variables dictates the type and extension of Par-4 actions. In terms of genetic therapy, these particularities could be used to drive Par-4 expression in a cell type and tumor stage-specific manner.

Therefore, despite the complexity of Par-4 roles, a better understanding of its mechanisms of action in different cancer types is a central question. The Par-4 selective action in cancer cells is of great benefit for disease management since the side effects in normal cells can be considerably reduced. Moreover, the use of Par-4 as a therapeutic approach can bring immeasurable benefits related to chemoresistance circumvention in diverse cancer types. The way to reach this is not easy, but Par-4 may be the joker molecule for cancer pre-

vention, prognosis, treatment, and maintenance of remission.

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Role of Par-4 in B-Cell Hematological Malignancies

Sunil K. Noothi, Mary K. McKenna, Sara S. Alhakeem, James P. Collard, J. T. Greene, Natarajan Muthusamy, Vivek M. Rangnekar, and Subbarao Bondada

Abstract

Prostate apoptosis response-4 (Par-4) was identified as a tumor suppressor protein that is silenced by promoter methylation in various cancers and has been shown to induce apoptosis selectively in cancer cells but not in normal cells. Par-4 interacts with a variety of partners in cells to mediate various cellular responses and appears to have a pro-apoptotic role in non-hematological tumors. Here, we summarize the literature on the role of Par-4 in hematological cells that is in contrast to its classic pro-apoptotic role. Par-4 is expressed basally in various hematopoietic cells and malignancies at the mRNA and protein level, but is predominant in the early stages of B-cell maturation and specifically in chronic lymphocytic leukemia (CLL). CLL B cells express higher levels of Par-4 than normal B-cell subsets and constitutively active B-cell receptor signaling (BCR) maintains high Par-4 levels in these cells, suggesting a novel regulation of Par-4 through BCR signaling. CLL cell growth is dependent on BCR signaling-mediated Par-4 expression, which is in part due to downregulation of p21 by Par-4. Bcl2 and NF-κB pathways cause differential regulation of apoptotic genes in contrast to non-hematological cancers, and Par-4 may also play a significant role in tumor microenvironment. Thus, Par-4 appears to have unique roles in hematological malignancies.

Keywords

Par-4 · B cells · B-cell receptor · Lymphoma · Chronic lymphocytic leukemia · Microenvironment · Tcl1 · Splenectomy · Stromal cells · p21

1 Introduction

One of the original hallmarks of cancer is to evade apoptosis and many cancers master this skill by down-regulating tumor suppressors and pro-apoptotic factors [1]. Prostate apoptosis response-4 (Par-4) is a tumor suppressor that is found to be downregulated in many cancers including renal cell carcinoma [2], breast cancer [3], neuroblastoma [4], and also in about 40% of all endometrial cancers, where about 32% of

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those cases were due to Par-4 promoter hypermethylation and occasionally due to silencing mutations [5, 6]. Early studies in lymphoid cells established that Par-4 expression is deregulated with decreased frequency of expression in immature or less differentiated populations and that an inverse expressional pattern exists between Par-4 and Bcl-2 in leukemic cell lines and acute lymphocytic leukemia (ALL) [6, 7]. Par-4 was originally identified by Sells and colleagues by its upregulation during ionomycin-induced apoptosis of androgen-independent and -dependent rat prostate cancer cells in 1994 [8]. Shortly after, using a yeast two-hybrid assay and HEK-293 mammalian cells, Johnstone et al. discovered that Par-4 interacts with the Wilm's Tumor-1 protein, a transcriptional suppressor [9]. Additional early studies found that Par-4 also interacts physically with atypical protein kinase c (aPKC) and overexpression of Par-4 in NIH 3T3 fibroblasts led to an apoptotic morphological change [10]. These initial studies defined Par-4 as a pro-apoptotic factor and tumor suppressor primarily in non-hematopoietic cells and suggested a similar role in hematopoietic cells. In fact, the first study to identify Par-4 as a pro-apoptotic protein in lymphatic cells showed that overexpression of Par-4 *per se* in the Jurkat leukemia cells is not sufficient to induce apoptosis but markedly increased their sensitivity to apoptosis with different chemotherapeutic agents [11].

The human Par-4 gene is located on chromosome 12q21 and contains 7 exons, encoding a 340 amino acid, 43-47 kDa protein [12, 13]. Par-4 is ubiquitously expressed in tissues of different species and Johnstone et al. found that mouse Par-4 shows 83% and 91% identity to human and rat Par-4, respectively [12]. Interestingly, the leucine zipper domain, carboxy terminal region, and nuclear localization sequences (NLS) exhibit 100% conservation across species [14]. The leucine zipper domain allows Par-4 to interact with other proteins as either a homo- or a heterodimer. The nuclear localization sequences suggest that Par-4's function is dependent on nuclear translocation; however, in normal tissues, Par-4 is localized mostly to the cytoplasm [15]. The NLS2 sequence is very interesting in Par-4 as it is sufficient to allow nuclear translocation alone, but it is also part of a domain that is necessary for the apoptosis-inducing properties of Par-4, termed selective for apoptosis of cancer cell (SAC) domain [16]. SAC is a core domain of 59 amino acids in length and includes a threonine residue that is the site of phosphorylation via Protein Kinase A [17]. Activation of Par-4 through phosphorylation indicates that its function is tightly regulated by post-translational modification. PKA, a broad spectrum serine/threonine kinase regulated by cAMP signaling, is associated with cell proliferation, and is frequently overexpressed in cancer cells [17]. Par-4 is able to utilize PKA upregulation in cancer cells to specifically induce apoptosis of cancer but not normal cells [17]. This selective ability of Par-4 makes it an attractive therapeutic target. Additionally, Par-4 is negatively regulated by AKT activity through phosphorylation at serine 249, which is located between the SAC domain and leucine zipper region [13]. Phosphorylation of Par-4 via Akt is required for cancer cell survival, as phosphorylation of Par-4 by Akt leads to binding of the chaperone 14-3-3, retaining[18] Par-4 in the cytoplasm [19]. Many studies have linked the pro-apoptotic activity of Par-4 to its ability to inhibit NF-κB transcriptional activity. Activated Par-4 prevents PKC-ζ from phosphorylating IκB, which is necessary for RelA translocation to the nucleus [10, 18, 20]. Another mechanism of NF-κB inhibition is due to a direct repressive effect of Par-4 in the nucleus but the exact mechanism still needs to be elucidated.

Seminal studies investigating Par-4 function led to the discovery that Par-4 is secreted from most cell types and can induce apoptosis of neighboring cells [21]. PC-3 (prostate cancer) cells transfected with GFP-labeled Par-4 or SAC domain-GFP undergo apoptosis, but GFP-negative cells were also dying as measured through caspase-3 activation [21]. Par-4 secretion is independent of apoptosis. Par-4 secretion occurs through the classical ER-Golgi pathway as inhibition of the network with brefeldin A (BFA) blocked secretion [13, 21]. Par-4 secretion is associated with the ER stress response and was also found to associate with GRP78, a member of the heat shock protein family 70 (HSP70) that works to facilitate proper protein folding, prevent intermediate aggregates, target misfolded proteins for degradation, bind calcium, and serve as an ER stress signal regulator [22]. Burikhanov et al. showed that Par-4 and the SAC domain bind to GRP78 at the plasma membrane in response to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). TRAIL is a known ER stress-inducing factor and treatment of PC-3 cells with TRAIL led to increased GRP78/Par-4 at the cell surface and induced apoptosis [23]. Initiation of extracellular Par-4-mediated apoptosis results in a feedback loop that promotes more translocation of Par-4 and GRP78 to the surface of the cell. More recently, Hebbar et al. have reported that an N-terminal 15 kDa fragment of Par-4 generated by caspase-3 cleavage is pro-apoptotic to cancer cells and released by cancer cells treated with chemotherapy agents [24]. With respect to hematopoietic cells at least two studies reported in 2004, before the discovery of the role of secreted Par-4, have demonstrated that expression of Par-4 in Jurkat cells promotes a complex interplay between the intrinsic and extrinsic pathway of apoptosis through molecules such as Apaf-1 and survivin [25]. Par-4 was also shown to sensitize neoplastic lymphocytes to ligation of a death receptor CD95 in the extrinsic pathway, thereby activating initiator caspases 8 and 10 which are able to directly activate executioner caspases 6, 7, and 3 [26]. We describe the unique role of Par-4 in normal and cancerous hematological cells in the following sections.

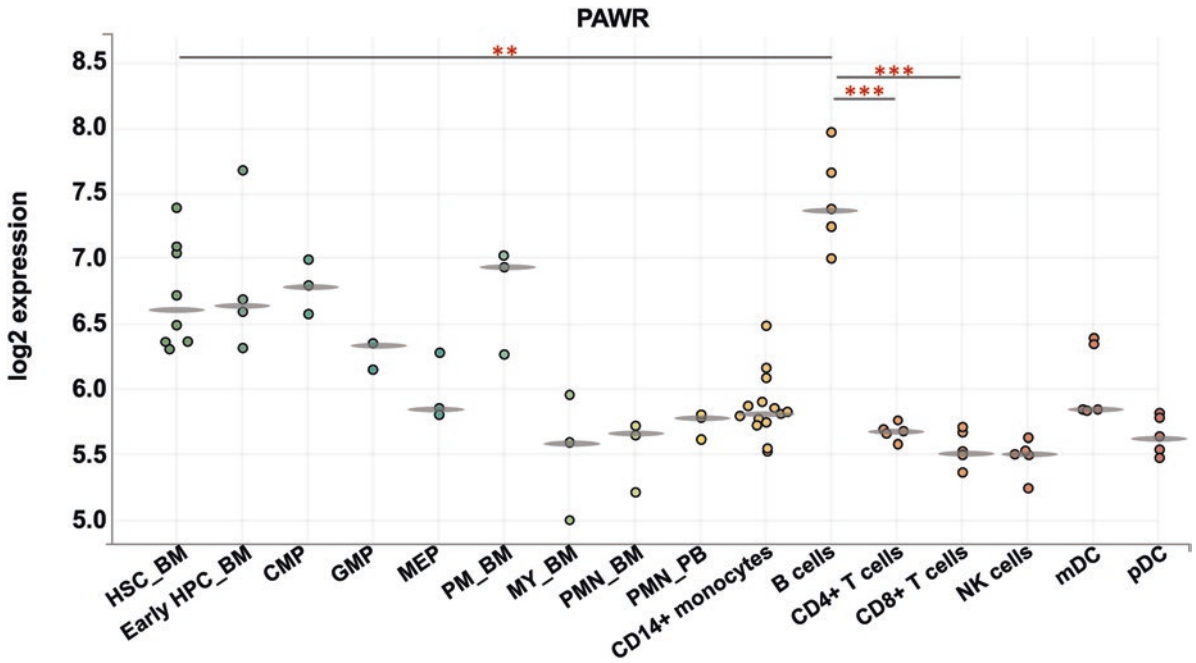

Fig. 1 *PAWR* expression in normal hematopoietic cells according to BloodSpot Database, Normal Human Hematopoiesis (Hemaexplorer). Log2 expression of the *PAWR gene* in subsets of immature and mature immune cells. Significance was determined by student *t*-test (**$p < 0.01$; ***$p < 0.0001$) (HSC_BM:Hematopoietic stem cells from bone marrow; early HPC_BM: Early hematopoietic progenitor cells from bone marrow; CMP: Common myeloid progenitor cell; GMP: Granulocyte monocyte progenitors; MEP: Megakaryocyte-erythroid progenitor cell; PM_BM: Promyelocyte from bone marrow; MY_BM: Myelocyte from bone marrow; PMN_BM: Polymorphonuclear cells from bone marrow; PMN_PB: Polymorphonuclear cells from peripheral blood; mDC: CD11c+ myeloid dendritic cells; pDC: CD123+ plasmacytoid dendritic cells)[27]

2 Par-4 Expression and Function in Immune Cells

There are now a number of gene expression data sets available from various sequencing studies utilizing normal and malignant hematopoietic cells for data curation of individual genes. We investigated the expression of *PAWR* (Par-4) gene in normal hematopoietic cells (Bloodspot.eu: Hemaexplorer) [27] and found that it was highly expressed in immature and progenitor stem cells compared to mature counterparts with the exception of B cells (Fig. 1). *PAWR* expression was significantly higher in B cells compared to other mature lymphoid cells including CD4 and CD8 T cells as well as HSCs (**$p < 0.01$; ***$p < 0.0.001$). Consistently, *PAWR* was found to be most highly expressed in B cells within the hematopoietic hierarchical differentiation tree (Fig. 2). The elevated expression of Par-4 in B cells suggests it may play a unique role in B-cell neoplasms that we will discuss further in detail in Sects. 2, 3 and 4.

Similar to its role in non-hematological malignancies, Par-4 in hematological cells is involved in several protein–protein interactions including but not limited to protein kinase A (PKA) [17], atypical protein kinase C [28], Wilm's tumor 1 (WT-1) [9], death-associated protein (DAXX), DAP-like kinase/ZIP kinase (DLK/ZIPK) [29, 30] and THAP-domain protein 1 (THAP1) [31]. Par-4 interactions with PKA, PKC, and WT-1 are similar to those in non-hematological cells and have been described in above cited references. Analysis of 62 untreated CLL patient peripheral blood and bone marrow samples showed a positive correlation between Par-4 and both DAXX and ZIPK proteins [29]. Phosphorylation of Par-4 and DAXX by ZIPK is involved in the nuclear pathway of apoptosis in promyelocytic leukemia (PML) oncogenic domains (PODs, nuclear domains that exist in all nucleated mammalian cells) through caspase activation. Simultaneous overexpression of DAXX, Par-4, and ZIPK proteins leads to more than a six-fold increase in apoptosis [32]. It has also been shown that a nuclear pro-apoptotic factor THAP-1 co-localizes with Par-4 in PML nuclear bodies (NBs) and that Par-4 is a component of PML NBs in blood vessels, which is a major site of PML expression in vivo [33]. PML nuclear bodies (PML NBs) are discrete membraneless subnuclear domains organized by the promyelocytic leukemia protein, PML, a tumor suppressor, with other client proteins. PML NBs function in promoting apoptosis by recruiting various pro-apoptotic proteins such as DAXX [34, 35] and p53 [36]. PML NBs were discovered through their disorganization in acute promyelocytic leukemia (APL) and arsenic therapy-induced reorganization has been directly implicated in its eradication [37]. PML NBs like nucleoli and Cajal bodies are a type of liquid-like droplets of biomolecules, which self-assemble within another liq-

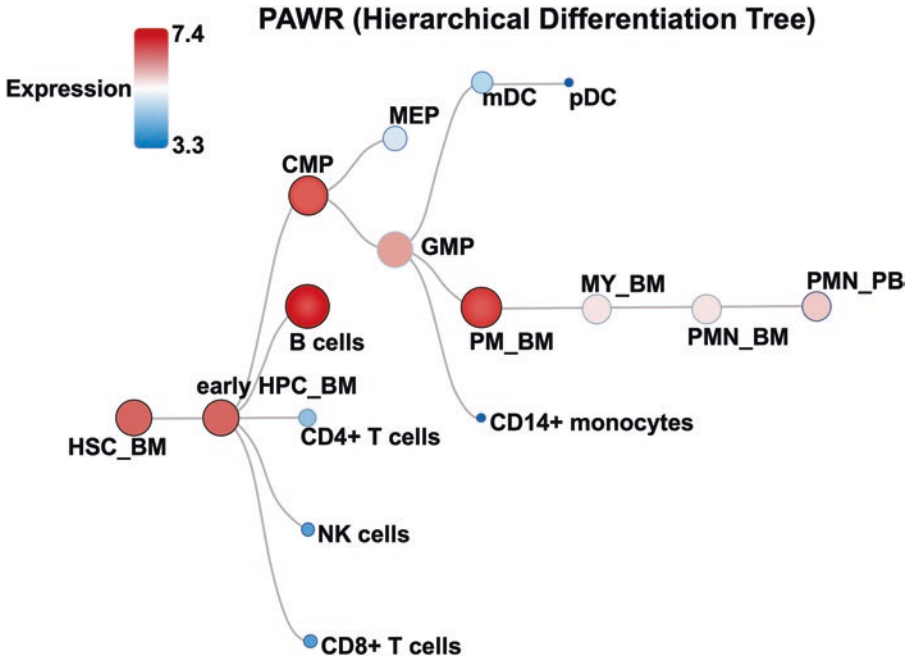

Fig. 2 PAWR expression as in Fig. 1 but depicted in hematopoietic hierarchical differentiation tree [27]. See Fig. 1 legend for abbreviations

uid—the cytoplasm or nucleoplasm—and arise from a physicochemical process known as liquid-liquid phase separation, sometimes also called coacervation [38]. At the molecular level, weak, transient interactions between different proteins/RNA molecules with multivalent domains or intrinsically disordered regions are a driving force for this phase seperation [38]. Par4 is well known to interact weakly and transiently with a variety of proteins including various pro-apoptotic proteins including DAXX and p53 [39] which home into PML NBs. The structure of Par-4 has shown that it is basically an intrinsically disordered protein [40–42]. All these divergent properties of Par-4 should suggest an emerging theme of molecular interactions in organelles that do not have an enclosing membrane such as PML NBs to remain coherent structures that can compartmentalize and concentrate specific sets of molecules to orchestrate their function.

Par-4 and THAP1 have also been shown recently to form a protein complex by the interaction of their carboxyl termini and this complex competitively with Notch modulated alternative pre-mRNA splicing of cell cycle and apoptosis regulator 1 (CCAR1) inducing cellular apoptosis in Jurkat cells, a human T-ALL cell line [31]. Genome-scale sequencing has revealed that more than 70% of the genome is transcribed into RNAs that do not produce protein. These RNAs are called noncoding RNAs (ncRNAs). Within the last decade, by integrating transcriptome profiles with chromatin state maps, many previously unreported T-ALL-specific lncRNA genes were identified. Notch-regulated LUNAR1 [43] and ARIEL (ARID5B-inducing enhancer-associated) long noncoding RNAs [44] are a few that have garnered interest in T-ALL recently. A novel lncRNA, T-ALL-R-LncR1, discovered with whole-transcriptome deep sequencing from the Jurkat leukemic T-cell line was shown to be markedly expressed in neoplastic T lymphocytes of children with T-ALL. Further studies revealed that knockdown of this T-ALL-R-LncR1 facilitated the formation of a Par-4/THAP1 protein complex, resulting in apoptosis [45]. This suggests a novel role of Par-4 in lncRNA-mediated escape of apoptosis in T-ALL.

To investigate the physiological role of Par-4, Garcia-Cao et al. generated a whole body *Par-4* knockout mouse. The average lifespan of Par-4 null mice is 18mo compared to 25mo for Par-4 WT animals with a 87% propensity to develop tumors [18]. These mice also exhibited normal B- and T-cell development but do have a slight increase in total number of lymphocytes leading to an increase in spleen size [46]. The proportions of B and T cells were not changed in young mice lacking Par-4, nor were the memory subsets in each lymphocyte population suggesting that Par-4 does not play a role in B- or T-cell differentiation. Interestingly, the proliferative responses to BCR and TCR cross-linking were increased in Par4$^{-/-}$ compared to WT animals with increased B-cell proliferation associated with an increase in PKC-ζ activity. The lack of Par-4 in these mice led to hyperactivation of atypical protein kinases, blocking JNK signaling in CD4 + T cells that resulted in increased IL-4 production and skewed the null mice towards a Th2 response [46]. Of note, Par-4 deficiency in both CD4+ and CD8+ T cells resulted in increased IL-2 secretion post-TCR stimulation without changes in CD25 expression suggesting Par-4$^{-/-}$ T cells have enhanced functional activity. These observations by Lafuente and colleagues suggest that Par-4 plays a role in regulating B and T lymphocyte function. Par-4 is abundantly expressed in various leukemic/lymphoma cell lines and THP1, a human

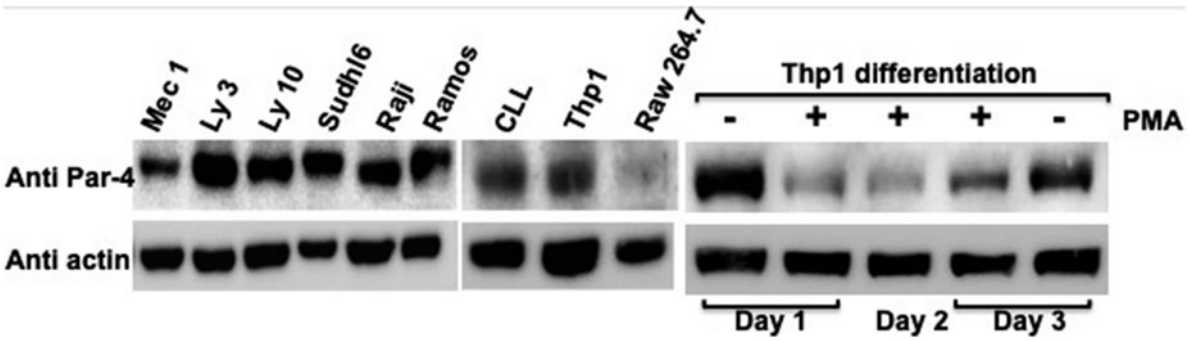

Fig. 3 Expression of Par-4 protein in various hematological cells; Mec1(human CLL); Ly3 and Ly10 (Diffuse Large B-Cell Lymphoma); SUDHL6 (Diffuse Histiocytic Lymphoma); Raji and Ramos (Burkitt's lymphoma); CLL (Eμ-Tcl1 mouse); Thp1 (human monocytic leukemia); Raw264.7 (murine macrophage cell line). Thp1 monocytes were differentiated into macrophages by treating with PMA which was accompanied by a decrease in Par-4 (Western blot on the right side)

monocytic leukemia cell line (Fig. 3, left part). However, expression of Par-4 is dramatically decreased with differentiation into macrophages by phorbol-12-myristate-13-acetate (PMA) treatment (Fig. 3, right). When Par-4 is overexpressed in *Mycobacterium tuberculosis* (Mtb strain: H37Ra) infected macrophages, intracellular survival of Mtb H37Ra was significantly reduced, in part due to increased apoptosis [47].

3 Par-4 in B-cell Malignancies

B-cell malignancies encompass both lymphomas and leukemias. According to the 2016 SEER database, leukemia is the ninth most common cancer in the USA contributing to 3.8% of all reported cancer deaths [48]. As a cancer of the blood, abnormal leukemic cells accumulate and do not die, suppress the function of normal immune cells, and eventually outpopulate other hematopoietic cell types resulting in anemia. Leukemia may be classified as chronic (slow progression of mature cells) or acute (rapid growth of primarily immature cells) and can affect both the myeloid and lymphoid white blood cells. Patients that are diagnosed with acute leukemia will normally start treatment as soon as possible while patients with chronic leukemia may be placed under a "wait and watch" status until symptoms progress. Subtypes of leukemia include: acute lymphoblastic leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, and chronic myelogenous leukemia (CML). Specifically, CLL is the most common adult leukemia in the Western world and like all leukemias, patients are grouped into fast or slow progressing disease based on prognostic indicators. Many patients with CLL may live a relatively normal life without symptoms, while others may only survive months to years after diagnosis or treatment initiation. Patients with CLL do have an 82% 5-year survival rate but experts in the field classify CLL as incurable [49].

CLL is a highly heterogeneous disease in terms of clinical course as some patients may live decades past initial diagnosis and likely die from other complications such as infections, while others may progress more rapidly. This heterogeneity can be attributed in part to mutations found within the variable gene segments of the BCR [50]. CLL can be classified into mutated (M-CLL) and unmutated (U-CLL) forms, the latter resulting in increased BCR signaling, more aggressive disease, and worse prognosis. This BCR signaling pathway is a desirable target as it is required for the survival of malignant B cells and is constitutively activated in many CLL cases and B-lymphomas [51, 52]. Additionally, the microenvironment has been found to play a key role in promoting the growth of B-cell malignancies, including CLL, by providing proliferative signals and promoting drug resistance [53, 54]. BCR signaling and microenvironment make CLL a very complex disease to study and treat but also allows for new targets to be explored for therapeutic potential.

Primarily, Par-4 has been characterized in the context of a diseased state rather than healthy tissues but a few studies have investigated its expression in lymphoid cells. Boehrer et al. reported the expression pattern of Par-4 mRNA and protein levels in healthy donor peripheral mononuclear cells compared to patients with ALL and CLL. Par-4 protein expression was detected in 100% of the healthy mononuclear cells and CLL samples [7]. Conversely, Par-4 protein levels were detected in 50% and 70% of pro-lymphocytic leukemia (PLL) and ALL samples, respectively, suggesting that Par-4 protein is downregulated in less differentiated cells comprising PLL and ALL compared to more mature cell populations of peripheral mononuclear cells and CLL [55]. In the same study, the authors also reported that sorted B and T cells expressed the Par-4 protein consistent with the Par-4 mRNA expression patterns from gene expression databases summarized above. Analysis provided by Bloodspot database using the leukemia MILE study shows *PAWR* to be highly expressed

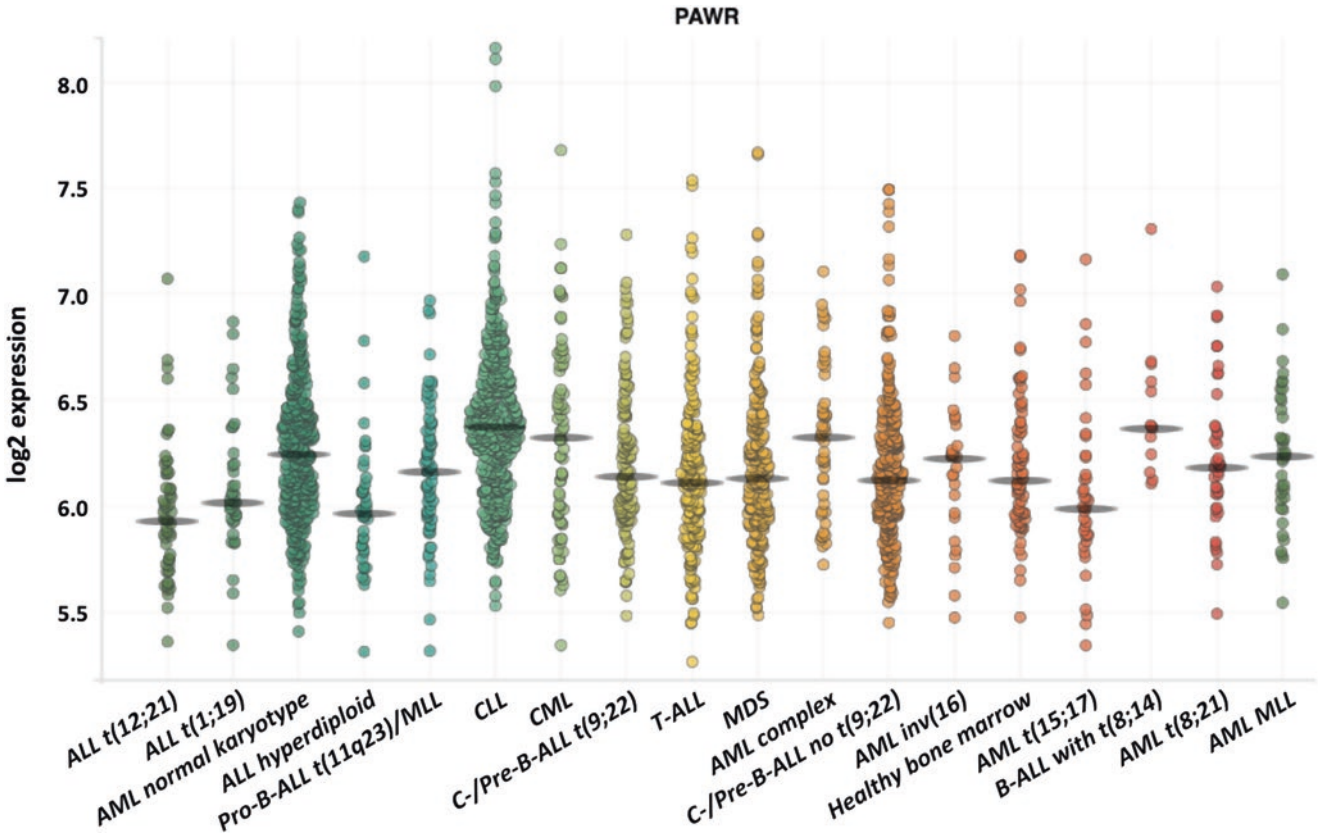

Fig. 4 PAWR log2 expression in various hematological diseases with indicated mutations. Figure is adapted from Bloodspot.eu analysis of leukemia MILE study [27]. (ALL: Acute Lymphocytic leukemia (ALL); AML: Acute Myeloid Leukemia; CLL: Chronic Lymphocytic Leukemia; CML: Chronic Myeloid Leukemia; MDS: Myelodysplastic syndrome; T-ALL: T cell ALL; B-ALL: B cell ALL; t: translocation; inv: inversion; c:common; pre:precursor)

in CLL samples compared to other types of leukemias as well as normal bone marrow (Fig. 4).

Several studies have examined Par-4 levels in CLL leading to prognostic predictions. Initially, Boehrer et al. examined the levels of Par-4 in normal and neoplastic lymphocytes and found that all patients with CLL ($n = 30$) expressed Par-4 protein, but only 63% were positive for Par-4 mRNA expression suggesting that there may be a difference in Par-4 regulation in different types of leukemias [7]. Bcl-2 is a well-characterized protooncogene initially identified at the chromosomal breakpoint of t(14;18) bearing B-cell lymphomas. Overexpression of Bcl-2 is to be considered a crucial event in leukemogenesis/lymphomagenesis and is aberrantly overexpressed in CLL, follicular lymphoma (FL), mantle cell lymphoma (MCL), Waldenstrom macroglobulinemia (WM), and one-third of diffuse large B-cell lymphoma (DLBCL) [56]. Bcl-2 is unique among protooncogenes in that it is localized to mitochondria as a key regulator of the intrinsic, mitochondrial apoptotic pathway for specifically blocking apoptosis rather than promoting proliferation [57]. Bcl2 family of proteins are also important in inducing drug resistance by many of the chemotherapeutic agents including the most recently approved Bcl2 inhibitor drug Venetoclax [56]. Bcl-2 is directly antagonistic to the actions of Par-4. It is evident that in non-hematopoietic cancer cells Par-4 is consistently downregulated and as such the effects of Par-4 on Bcl2 might be straightforward with respect to the intrinsic mitochondrial pathway of apoptosis. However, in hematopoietic cancer cells with a robust Bcl2 activity, the levels of Par-4 required to counteract it need to be different and this shifts the rheostat of pro- and anti-apoptotic mechanisms intracellularly. Hence, these cancer cells expressing high Bcl2 might express more Par-4, but this amount of Par-4 is not amenable to apoptosis without additional stimuli. It is not surprising that synergistic and antagonistic drug combinations within a single lymphoma model led to uncorrelated levels of Bcl2 and Par-4 [58]. Previous studies had indicated that Par-4 and Bcl-2 are inversely correlated [59], but there was no relationship found between the expression of Par-4 and Bcl-2 protein expression in CLL patients. Next, Chow and colleagues found that CLL patients that lacked the

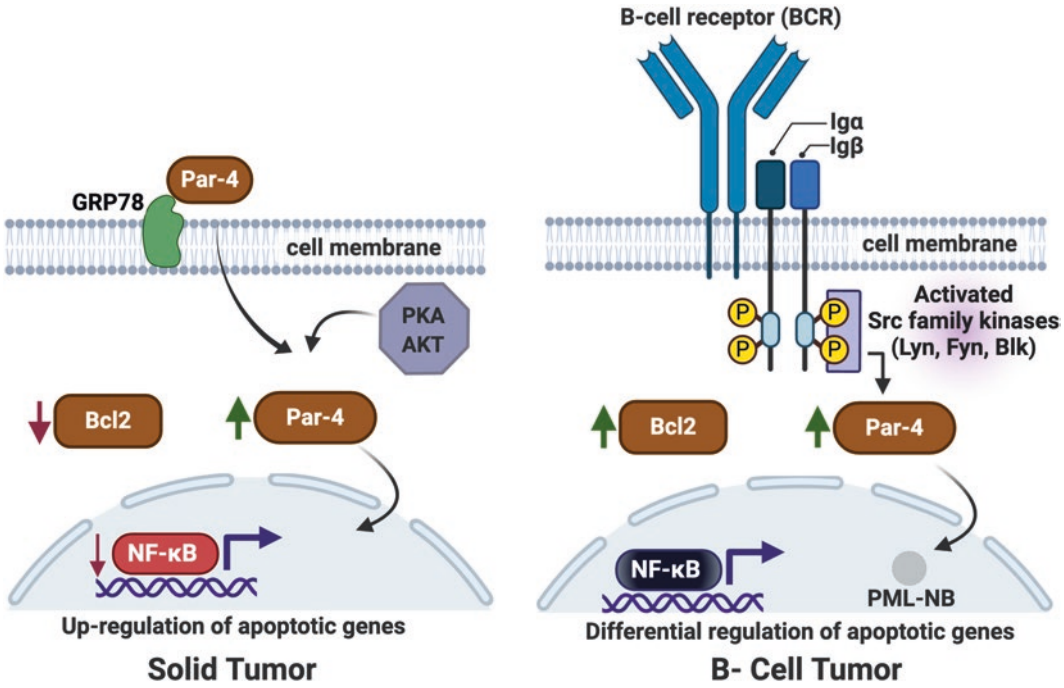

Fig. 5 Cartoon depicting the differences in the role of Par-4 in non-hematological vs hematological malignancies (Created with BioRender.com)

Imatinib targets BCR-ABL, C-Kit and PDGFR were still sensitive to Imatinib treatment and that, the response correlated with Par-4 expression [30]. Additionally, this study confirmed Boehrer et al. findings that there was no relationship between Par-4 expression and Bcl-2 in patients that did or did not respond to Imatinib treatment. Par-4 was also downregulated in the course of the treatment with Imatinib when cells underwent apoptosis after caspase-8 and -3 activation [30]. Lastly, Bojarska-Junak and colleagues assessed the expression of Par-4 in CLL B cells and found a positive correlation of Par-4 with Bcl-2, which is opposite of what is observed in non-hematopoietic cells (Fig. 5).

Par-4 was also positively correlated with DAXX (death-associated protein), and ZIPK (zipper-interacting protein kinase) expression in CLL patients [29]. Additionally, Par-4 was found to positively correlate with LDH (lactate dehydrogenase) serum concentrations and was more highly expressed in CD38+ CLL patients who have a more aggressive form of CLL disease [50, 60]. These initial studies suggested that Par-4 in CLL may be regulated differently. Importantly, rather than being downregulated as shown in other cancers, Par-4 was found to be expressed in 30/30 human CLL patient samples [7]. These results were also confirmed in another study showing increased Par-4 expression in peripheral blood mononuclear cells (PBMCs) of human CLL patients, compared to healthy donor PBMCs [61]. These surprising discoveries suggested that aberrant Par-4 expression in CLL is unique and is further discussed below.

4 Intrinsic Role of Par-4 in CLL

4.1 Par-4 Expression in CLL

Even with the incredible discoveries made in the field of chronic lymphocytic leukemia over the last few decades, the cellular origin of the disease is still debated today [62, 63]. CLL cells distinctively express CD19, CD5, CD23, as well as surface Ig molecules [60]. CD19 is a surface antigen that is expressed on both normal and neoplastic B cells and is critical for intrinsic B-cell signaling through BCR interactions as well as BCR-independent signaling [64]. CD5 is also a cell surface molecule that is expressed on thymocytes, mature T cells, and B1 cells but not on conventional B2 population [65]. Previous studies have also indicated that CD5 is found on some activated human B cells that are autoreactive [66]. CD5 is thought to be a negative regulator to mitigate signaling in order to prevent over activation of signaling downstream of the TCR or BCR [67].

The co-expression of low IgM and IgD levels on the surface of CLL cells originally suggested that these cells arise from naïve antigen-inexperienced B cells [68]. Further studies classified CLL into two subgroups, M-CLL and U-CLL defined by mutations in the variable gene segments of the BCR indicating that 50–60% of CLL cells had undergone somatic hypermutation (M-CLL), leading to hypotheses that suggest CLL cells are derived from two cellular origins [69, 70]. Seifert and colleagues suggest that U-CLL cells are derived from unmutated mature CD5+ B cells as their IgV

sequence is less than 2% different from germline, whereas M-CLL cells are derived from a distinct CD5 + CD27+ post-germinal center B cell [62]. An additional study investigating phenotypic markers found that CLL cells express more of an activated state (CD69 + CD25 + CD71+) independent of their Ig mutational status when compared to normal CD5+ B cells in humans, suggesting that CLL cells are mature antigen-experienced cells [68]. Antigen-experienced cells can be derived from cells that undergo somatic hypermutation within a germinal center or from extrafollicular responses, but may also develop in a T cell-independent manner which may account for CLL cells that have unmutated Ig variable regions [70, 71]. Further support for antigen-experienced B cells to be CLL precursors comes from studies that have examined the BCR repertoire in multiple CLL samples. It is well accepted that CLL cells have constitutive BCR signaling, but CLL cells also respond to antigen [72, 73]. Recent studies have found that 30% of BCR immunoglobulins within the CLL patient population are quasi-identical resulting in a stereotypy of BCRs [72, 74, 75]. This indicates that the malignant B cells from unrelated patients recognize similar antigens suggesting that there are a few common epitopes which activate CLL cells. Not many antigens have been identified to stimulate CLL cells, but one potential candidate is non-muscle myosin heavy chain IIA which is an intracellular protein that interacts with actin to provide cellular movement and therefore is considered a self-antigen [76]. This is interesting as B1 cells are thought to be self-reactive and respond to autoantigens supporting the idea that CLL cells are derived from B1 cells [70]. Cell autonomous signaling was identified in some CLL patients that express specific immunoglobulin variable regions which also associate with greater severity of the CLL disease. This is thought to be due to homotypic interactions of B-cell receptors with specific V region mutations [77, 78].

B1 cells are primarily found within the peritoneal cavity of mice but are also present in the spleen, albeit at a lower level [79]. As mentioned before, B1 cells express self-reactive BCRs but respond poorly to BCR cross-linking to prevent against self-activation that is suggested to be mediated through CD5 [67, 75, 79, 80]. B1 cells also express restricted BCRs with a predominance of V_H12 promoting B1 phenotype [81] and are known to produce antibody quickly in response to infection, primarily IgM, independent of T-cell help (similar to U-CLL) [82]. Additionally, B1 cells are divided into B1a (CD5+) and B1b (CD5-) subsets where B1a cells are the primary source of natural IgM production and B1b cells respond to antigens in mice [63, 79, 83]. It has been suggested that B1a cells serve as the normal counterpart for CLL cells [61]. Elegant studies by Rajewsky and colleagues, where conditional ablation of BCR signaling was combined with conditional activation of candidate downstream signaling pathways of the same cell in vivo, led to the novel revelation that mature B-cell subsets may differ in their dependence on specific signaling pathways. Specifically genetic ablation of canonical NF-κB signaling in mature B cells in mice severely impaired development of marginal zone B cells, but had only mild effects on follicular B cells [84]. Thus, Nf-κB is a critical transcription factor for both B1 cells and CLL cells.

Adoptive transfer studies of young/early B1a populations into immunocompromised recipient mice led to the development of CLL-like disease [85]. CLL development in this study was independent of oncogene expression but a follow-up study was able to confirm that early B1a cells expressing the oncogene, T-cell leukemia 1 (Tcl1), also led to the development of CLL in recipient mice [86]. These authors did note that not all B1a cells result in CLL development, but were restricted to specific BCRs that were later identified to promote CLL growth [85, 86]. Additional studies favoring the B1a population serving as CLL normal counterpart provide evidence that both B1a cells and CLL cells secrete significant amounts of the cytokine Interleukin-10 that works to suppress the immune response [80].

Controversy regarding the normal counterpart of CLL has been focused on the inability to identify a human B1 population that is similar to mouse B1 cells [87]. Recently, reverse engineering has allowed researchers to identify a human B1 cell population within the umbilical cord blood and adult peripheral blood [88]. Rothstein et al. summarized evidence showing that mouse and human B1 cells share similar phenotypes and also express autoreactive antibodies that protect against infections. Seifert and colleagues compared normal CD5+ B cells from healthy human donors with both populations of CLL cells, M-CLL and U-CLL, and confirmed that CD5+ B cells are the normal B-cell subset that are most similar to CLL [62]. Additionally, a recent review also summarizes evidence that identifies B1 cells as the origin of CLL [89].

Interestingly, a case report involving a 65y male with stage IV CLL identified a “Side Population” of CLL cells identified through flow cytometry that were proposed to be precursors to leukemic development [90]. Ablation of these cells through vaccination after CD40L stimulation diminished the bulk of the disease 12 months after treatment. The “side population” of cells were CD5 and CD19 positive and thought to be similar to the cancer stem cell population characterized in other types of tumor models [91]. True identification of this “side population” would be of great benefit to determine if the likely B1 cells give rise to the malignant counterpart.

Intriguingly, one study investigated if CLL cells could be generated from self-renewing adults HSCs [92]. HSCs from CLL patients developed monoclonal or oligoclonal B cells that frequently expressed CD5. According to the Bloodspot database in Figs. 1 and 2, HSCs, B cells and CLL cells

express high levels of Par-4 and it would be of interest to further investigate the role of Par-4 in B-cell development.

Because of these studies, McKenna and colleagues compared Par-4 expression in CLL cells to normal B1 and B2 subsets. Utilizing the Eμ-Tcl1 mouse which is considered to be the most representative model of human CLL [93–97], they measured Par-4 protein and mRNA levels in mouse CLL cells compared to wildtype (WT) mouse B cells. WT B1a cells expressed more Par-4 compared to the other B-cell populations in WT mice but only ~33% of the levels observed in CLL. Par-4 mRNA expression was also elevated in CLL cells compared to B-cell subsets, mirroring the levels of Par-4 protein expression. We further analyzed the B-cell subsets in the Eμ-Tcl1 mouse to confirm that Par-4 expression was not dependent on the overexpression of the Tcl1 oncogene. We isolated different B cell subsets from 2mo old Eμ-Tcl1 mice that had no detectable levels of CLL in the peripheral blood and measured Par-4 levels compared to WT B cell subsets (Suppl. Figure 1C in ref. 61). B1a Eμ-Tcl1 cells expressed higher Par-4 protein levels compared to B1b and conventional B2 Eμ-Tcl1 cells. This finding was similar to what was observed in WT B cell subsets which continues to suggest B1a cells exhibit characteristics similar to CLL cells. Importantly, elevated levels of Par-4 were also detected in human CLL samples compared to normal B cells which are consistent with studies presented by Boehrer and colleagues in that most CLL samples have detectable Par-4 protein levels [7].

Furthermore, CLL is known to be more common in elderly with an average age of CLL patients being 71. Interestingly, we found that B cells from aged mice express more Par-4 than those from young mice (unpublished). This was unique to B cells since there was no such age-related increase in other tissues such as liver and heart but also consistent with other reports that B cells express more Par-4 than other cell types (Fig. 1).

4.2 BCR-Mediated Par-4 Regulation in CLL

High expression levels of Par-4 in CLL led to the investigation of its regulation. In spite of original observation about the increase in Par-4 upon ionomycin treatment, there are very few studies that examine signaling pathways that induce Par-4 expression. Since CLL cells have been shown to have elevated tonic BCR signaling [98, 99], McKenna and colleagues tested the hypothesis that Par-4 expression may be regulated by BCR signaling. The BCR pathway is required for the survival of both normal and malignant B cells despite their oncogenic activation, making it a therapeutic target in B-cell malignancies [51, 98, 100]. Kinase inhibitors targeting Src family kinases (SFK) [101], Syk [102], BTK [103], and PI3K [104] have all been proven effective in the treatment of CLL as each inhibit the required downstream survival signals. Anti-CD20 monoclonal antibodies such as rituximab have also been proven efficient with combination of other chemotherapies [105]. We therefore utilized FDA-approved therapies to target BCR signaling and examined their effects on Par-4 expression. Treatment with dasatinib (SFK inhibitor), fostamatinib (SYK inhibitor), and ibrutinib (BTK inhibitor) all led to a decrease in Eμ-Tcl1 CLL cell survival accompanied by a reduction in Par-4 expression [61]. Par-4 mRNA levels decreased after SFK and BTK inhibition, suggesting regulation at the transcript level. Additionally, Par-4 protein downregulation was replicated in primary human CLL samples after treatment with dasatinib and fostamatinib indicating that this is not unique to mouse CLL cells. shRNA knockdown of Lyn, the most prevalent SFK in B cells, also led to a decrease in Par-4 expression. The most compelling evidence that Par-4 is regulated by BCR signaling is by targeting Igα or CD79a which confirmed that Par-4 is downstream of BCR activation and regulated through this signaling pathway. Additional studies investigating the levels of Par-4 after ERK inhibition in CLL cells showed that Par-4 is further downstream of the BCR signaling cascade. These results provide evidence that a well-defined survival signaling pathway is regulating the expression of Par-4 specifically in B cells since downregulation of Par-4 was not observed after ERK inhibition in PC-3 cells as shown by McKenna and colleagues [61].

4.3 Role of Par-4 in the Regulation of CLL Growth Kinetics

The aberrant expression of Par-4 in CLL and regulation through BCR signaling leads to the question of the true role of Par-4 in CLL. Par-4 knockdown studies in two CLL cell lines (Mec-1 and OSUCLL) resulted in a reduced growth rate in vitro and in xenograft in vivo studies [61]. Par-4 knockdown in these cells lead to increased Akt phosphorylation and reduced Bcl2 levels concordant with previous literature [7, 106] and to promote prosurvival signaling and anti-apoptotic pathways. Studies investigating the reduced growth rate in Par-4 knockdown cells revealed fewer cells entering S phase but more cells in G1 phase suggesting a halt in the G1 to S transition and a unique increase in p21 expression. p21 is involved in different phases of the cell cycle, but primarily works to control the transition from G1 to S [107].

The reduced CLL growth with the loss of Par-4 was also confirmed by crossing the Eμ-Tcl1 mouse with a Par-4$^{-/-}$ mouse. CLL development was significantly delayed in Par-4$^{-/-}$EμTcl1 mice compared to Par-4$^{+/+}$EμTcl1 mice leading to an overall improved survival [61]. Indeed, Par-4$^{-/-}$EμTcl1 spleen cells expressed higher p21 protein levels compared to Par-4$^{+/+}$EμTcl1 spleen cells providing in vivo confirmation of p21 upregulation observed in vitro using Par-4 knockdown

cell lines. In order for p21 to execute its function to block the cell cycle from G1 to S phase, p21 must be found in the nucleus of the cell [108]. Nuclear and cytoplasmic fractions of Par-4$^{+/+}$EμTcl1 and Par-4$^{-/-}$EμTcl1 spleen cells were examined and was found that Par-4$^{-/-}$EμTcl1 cells had greater nuclear p21 levels compared to Par-4$^{+/+}$EμTcl1 cells, further confirming that Par-4 knockout led to increased levels of functional p21 [61]. This novel finding in CLL is clinically relevant as a study investigating the expression of p21 in CLL cases and patients with Richter's syndrome found that 80% of CLL cases did not express detectable levels of p21 [109]. Forty-three percent of patients with Richter's syndrome did express detectable levels of p21. Cobo et al. analyzed the sequence of p21 in three CLL patients and 6 Richter's syndrome patients to find a germline configuration in all of them indicating that it was not mutated. Sequencing of the *p21* gene in the Par-4 knockdown and knockout cells to confirm the mutation was not done, but an increase in nuclear p21 levels in Par4$^{-/-}$Eμ-Tcl1 CLL cells was observed suggesting that p21 is still able to translocate to the nucleus and function in the regulation of the cell cycle that occurs in the nucleus [61].

Greene and colleagues investigated the effect of overexpression of Par-4 in CLL leukemogenesis in the Eμ-Tcl1 mouse [110]. They generated a B cell-specific human Par-4 overexpressing mouse and crossed it to the Eμ-Tcl1 mouse resulting in reduced accumulation of CD5 + CD19+ CLL cells. They went on to determine that Par-4 overexpression impedes Tcl1-driven NF-κB signaling with reduced nuclear translocation of p65. This finding aligns well with the role of Par-4 and its known interactions with NF-κB [111] and emphasizes the distinct roles of physiological versus increased intracellular levels of Par-4. It also provides further evidence of the pleiotropic roles that Par-4 may play in the development of B cell-specific CLL as well as in its surrounding microenvironment.

5 Par-4 in the Tumor Microenvironment

The original hallmarks of cancer proposed by Hannahan and Weinberg have been expanded to include the tumor microenvironment that promotes growth of cancer cells by avoiding apoptosis and evading immune suppression [112]. However, the dependence of cancer cells on a protective niche is a very old concept dating as far back as 1889 with Stephen Paget's "seed and soil hypothesis" [113]. Both solid and hematologic tumors are very heterogeneous and comprise of multiple different cell types such as stromal cells, endothelial cells, tumor infiltrating macrophages, and lymphocytes accounting for more than half of the total tumor cell mass. These accessory cells produce vascular growth factors and various cytokines and chemokines that support cancer cell growth [114]. Compelling evidence exists that recognizes the importance of the BCR signaling pathway, Chemokine (C–X–C motif) Receptor 4 (CXCR4) and Chemokine (C–X–C motif) Ligand 12 (CXCL12) axis, which are key pathways of CLL microenvironment cross talk [115]. The role of tumor microenvironment in the form of bone marrow or secondary lymphoid organs that can provide a unique niche for CLL proliferation is based on the following:

(a) Primary CLL cells do not proliferate or survive in long-term in vitro cultures, but undergo spontaneous apoptosis even when conditions that support the growth of other B-cell lines are provided [116].
(b) CLL cells proliferate primarily in secondary lymphatic tissues, where they form characteristic "proliferation centers," sometimes also referred to as "pseudofollicles" [117].
(c) Deuterium (^{2}H) labeling in patients with CLL demonstrated that lymph nodes are the principle site of proliferation compared to bone marrow or blood [118].
(d) The unique gene expression profile along with Ki67 staining of CLL cells isolated from lymph nodes compared to blood and bone marrow-derived CLL cells [119].
(e) BCR signaling targeted therapies as a drug class effect, induce "redistribution lymphocytosis" causing a rapid shrinkage of primarily lymph nodes with a transient increase in blood leukemic cell counts [120, 121].

The actual site of proliferation and the CLL microenvironment is still debated in the field. This could be because CLL cells are found within the peripheral blood, bone marrow, and other secondary lymphoid organs in which the malignant cell comes into contact with a variety of accessory cells depending on their location. Although studies in human samples find that the lymph node is the site of CLL proliferation; questions are still raised based on the dramatic splenomegaly observed in mouse models [118, 122]. Splenomegaly is indeed observed in human CLL patients at later stages of the disease but role of spleen as a secondary lymphoid organ during earlier stages of human CLL is not known, as it is not amenable to surgical interventions.

The CLL tumor microenvironment provides a physical location supporting the cross talk between malignant cells and accessory cells that inhibit apoptosis and also provide resistance to drug treatment [123]. CLL is a slow progressing disease and was originally thought to simply be an accumulation of cells with defective apoptosis, but recent studies using deuterium labeling have determined that CLL cells proliferate at a rate of 0.1–1% per day suggesting that CLL is a dynamic disease involving cell proliferation [124]. Pseudofollicular proliferation centers that are found throughout infiltrated tissues are the source of newly generated CLL

cells [125]. Within this area, CLL cells depend on stimulation through a functioning BCR as discussed above. It is well appreciated that some CLL cells may be activated through antigen-dependent manner and the microenvironment may be the source of antigen/stimulus [126]. Theses antigens are not specifically defined, but may include microbial antigens, natural antibodies, and autoantigens expressed by dying cells. As noted above cell autonomous signaling due to homotypic interactions of BCR may be involved in a subset of CLL patients.

The CLL microenvironment promotes cell-to-cell interactions with a variety of different cell types. Direct interaction between B-CLL cells and T cells via CD40 on B cells and CD40L on T cells provides a proliferative stimulus [127]. CD40 signaling in B cells induces expression of anti-apoptotic molecules and proliferative signaling through AKT, ERK, TRAF, and NF-κB. T cells also secrete cytokines such as IL-4, TNFα, and IL-2 that support CLL proliferation. Alternatively, the CLL microenvironment also supports immune evasion allowing CLL cells to dampen the immune function of cytotoxic T cells by secreting immunosuppressive cytokines like TGFβ and IL-10 [127, 128].

Stromal cells derived from bone marrow or other secondary lymphoid tissues support the survival and proliferation of CLL cells [129]. This interaction provides a bi-directional cross talk that promotes the growth of both CLL and stromal cells. In cell culture, CLL cells actually migrate beneath bone marrow mesenchymal cells, a process known as pseudoemperipolesis, suggesting that this interaction is dependent on cell contact in order for CLL cells to survive. Cells known as nurse-like cells (NLC) can be found in the peripheral blood of patients that are derived from monocytes and become adherent in culture systems [130]. These cells express stromal cell-derived factor-1 (SDF-1) that binds to CXCR4 on CLL cells to prevent spontaneous apoptosis and promotes resistance of CLL cells to chemotherapies. CXCL12 is also secreted by NLCs as well as mesenchymal-derived stromal cells that attract CLL cells via CXCR4 towards proliferation centers within the secondary lymphoid compartments [131]. The phenomenon of "redistribution lymphocytosis" with BCR signaling inhibitors in CLL where mobilized CLL cells, devoid of their nourishing microenvironment in lymph nodes, die gradually has led researchers to propose a novel mechanism of action called "death by neglect" [121]. Similar to CLL cells dying in vitro, CLL cells detached from their supportive tissue microenvironment leads to anoikis, a form of programmed cell death [132]. It is interesting to note that BCR signaling inhibitor, specifically the Bruton tyrosine kinase (Btk) inhibitor, ibrutinib inhibits not only the BCR signaling in the CLL cells but also signaling of other cell surface receptors including chemokine receptors and adhesion molecules [133, 134]. This has been proposed to be the mechanism of redistribution lymphocytosis which is also seen in the other classes of BCR signaling inhibitors such as phosphoinositol 3 kinase (PI3K) inhibitor, idelalisib, and inhibitors of SYK and PI3Kδ which are involved in signal transduction pathways of chemokine receptors and adhesion molecules [135, 136]. Redistribution lymphocytosis does not cause any adverse symptoms and resolves over time. Ibrutinib is also known to cause redistribution lymphocytosis in mantle cell lymphoma (MCL) [137], Waldenstrom macroglobulinemia (WM) [138] and DLBCL. However, activated B-cell-like (ABC) subtype of DLBCL cells are exquisitely sensitive to ibrutinib but not GCB-DLBCL. This is interesting because ABC-DLBCL are known to have chronic active BCR signaling [139] and use an amplified prosurvival NF-κB signaling [140].

There are very few studies looking at the role of Par-4 in tumor microenvironment of hematological malignancies. In solid tumors, the role of secreted Par-4 is gaining increased attention since it was discovered to be secreted extracellularly and to act exclusively on cancer cells in a paracrine manner and preventing metastasis [141–144]. Cancer-associated fibroblasts (CAFs) in contrast to normal fibroblasts were modified through an miRNA-dependent (mir-7) pathway to dramatically reduce the secretion of Par-4. Inhibition of mir-7 expression in CAFs induced them to convert back to normal fibroblasts [145].

Chronic active BCR signaling results in constitutive activation of NF-κB and PI3K pathways, both of which are regulated by Par-4 as discussed initially [61, 110]. Antigen-independent tonic BCR signaling supports survival of malignant B cells primarily through the PI3K–AKT–mTOR pathway which is also closely linked to Par-4. These pathways are again involved in regulating homing of malignant cells and retention of proliferating cells in a supportive niche as evidenced by novel BCR inhibitors causing "redistribution lymphocytosis." Hence, it is not over-arching to hypothesize that aberrant levels of Par-4 in hematological malignancies like CLL alter the balance required to inhibit tumorogenic signals.

As summarized above, CLL cells overexpress Par-4 compared to its levels in normal B-cell subsets [61]. Additionally, CLL cells secrete Par-4 that can induce apoptosis of other cancer cell lines. This led us to question if Par-4 from CLL cells is able to manipulate the microenvironment's ability to promote or delay CLL growth. Studies in our laboratory have confirmed that the spleen is the primary site of CLL tumor growth in the primary Eμ-Tcl1 mouse model of CLL as well as in adoptive transfer recipients as splenectomy dramatically delayed the development of CLL (manuscript in preparation). We have previously described the difference in CLL development between the Par-4$^{-/-}$EμTcl1 and Par-4$^{+/+}$EμTcl1 mice [61]. Par-4$^{-/-}$EμTcl1 mice exhibited an improved lifespan compared to Par-4$^{+/+}$EμTcl1 suggesting that the lack of Par-4 intrinsically and/or extracellularly

reduced the aggressiveness of the disease. And since elimination of the primary site of CLL growth with splenectomy results in delayed CLL development, we splenectomized Par-4$^{-/-}$ mice to see if the lack of spleen and Par-4 may contribute to changes in CLL growth. Interestingly, we find that absence of the spleen in the Par-4 null background allows for faster growth of CLL cells elsewhere (unpublished), suggesting that Par-4 plays a significant inhibitory role extracellularly in the tumor microenvironment.

6 Summary and Future Outlook

In this chapter, we summarized the expression pattern of Par-4 in normal and malignant immune cells. Unlike its well-established tumor suppressor role in solid cancers, the role of Par-4 in hematological malignancies is complex. In CLL, we have shown that constitutive BCR signaling leads to high levels of Par-4 and a cell intrinsic prosurvival role for Par-4 in CLL cells. This is consistent with a variety of BCR signaling inhibitors that have been shown to be effective in control of CLL disease in patients. Our studies showing an inverse relation between Par-4 and p21 expression suggested that drugs affecting cell cycle could affect CLL despite their low proliferation index. Indeed, Dinaciclib, a CDK inhibitor, has been found to have beneficiary effects in refractory and relapsed CLL patients [146]. This approach may be important for CLL patients with Chr17 deletion (del (17p)) who have a more aggressive form of CLL disease, with a poor prognosis. This deletion leads to the absence of p53, which is known to upregulate p21. Currently, there are no therapies that specifically target this pathway [147]. We have highlighted here the absence of the inverse relation between Par-4 and Bcl2 as well as differential regulation of NF-κB in leukemias, which is in contrast to that seen in non-hematological malignancies (Fig. 5).

Like most cell types, CLL cells secrete Par-4 but are resistant to cytotoxic effects of secreted Par-4. On the other hand, we have discovered that Par-4 has a profound effect on CLL microenvironment. Our studies have shown a unique role for splenic microenvironment for CLL growth. Absence of Par-4 in the microenvironment of splenectomized mice enables better CLL growth. Presently, it is known that chemokines like CxCL12 play a critical role in the interaction between CLL cells and the microenvironment. However, effects of Par-4 expression on these critical chemokines required for CLL homing and survival in secondary lymphoid organs is at present unknown. Interestingly, Par-4 has been linked to the Wnt signaling pathway [148] in breast cancer cells and its overexpression led to downregulation of Frizzled, a Wnt ligand linked to cell proliferation. Expression of Wnt family members is elevated in CLL [149] and frizzled-6 has been shown to be required for CLL growth [150]. Future studies regarding Par-4-mediated gene expression in the CLL microenvironment may enable better Par-4-based treatment strategies for CLL.

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The Par-4 Tumor Suppressor Protein in TCL1-Induced Leukemogenesis

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Abstract

Prostate apoptosis response 4 (Par-4) is a pleiotropic tumor suppressor protein that is downregulated in numerous solid tumors to evade its pro-apoptotic and cytostatic effects. Par-4 is thought to respond to dysregulation of various signaling pathways during oncogenic transformation by promoting expression of pro-apoptotic proteins and inhibiting transcriptions factors vital to proliferation. It has been shown to be a faithful indicator of response to chemotherapy in breast cancer and is being explored in other cancers as a factor in response to multiple classes of therapeutics. Despite this, its role in hematological malignancies has been little described. Studies in leukemia have shown that Par-4 expression levels correlate with known prognostic indicators and may factor into treatment response and disease progression. These reports also indicate that dysregulation of the pathway differs between different forms of leukemia, and the significance of Par-4 activity may vary between chronic, acute, lymphoid, and myeloid forms of the disease. Par-4 expression levels are also variable within different forms of leukemia and correlate with incidence of other genetic lesions that impact prognosis and treatment outcomes. Follow-up, in vitro studies have provided some mechanistic insight into how Par-4 functions in different forms of leukemia and suggest relevance to the apoptotic response of leukemia cells to therapeutics. Additionally, animal models of chronic lymphocytic leukemia (CLL) have revealed that extrinsic and intrinsic Par-4-mediated effects may both play a role in leukemogenesis, adding more complexity to the collective understanding of this protein's role in hematologic disease. Given the relevance of Par-4 activity in treatment of solid tumors, further exploration of this pathway in heme malignancies may be clinically relevant. This chapter contains an analysis of literature regarding Par-4 in leukemia, recently published data suggesting a role for leukemogenesis in CLL, and future prospects for utilizing Par-4 to therapeutic benefit in patients.

Keywords

Par-4 · Leukemia · Apoptosis · Lymphoid · Myeloid · Tumor suppressor · Oncogene · Heme malignancy · NF-κB · TCL1 · PKC · Chemotherapy

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1 Introduction

1.1 Leukemia

Leukemia is blood cancer that arises from production of abnormal white blood cell populations in the bone marrow, leading to accumulation of malignant cells in the peripheral blood and invasion of tissues in later stages of disease. They

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can be categorized as lymphocytic or myeloid based on whether the malignant cells exhibit morphology and biological characteristics of lymphocytes or myeloid blasts. Both forms are further classified as acute or chronic determined by molecular features that contribute to unique pathologies. Together they make up 3.4% of new cancer cases annually [1].

Lymphocytic leukemia results from production of abnormal B or T cells that proliferate in the lymphoid organs due to abnormal signaling events that result in defective apoptosis and proliferative responses. Acute lymphocytic leukemia (ALL) accounts for 80% of leukemia diagnoses in children, whereas the median age of onset for chronic lymphocytic leukemia is 72 [2, 3]. Around 80% of ALL cases are caused by abnormal B cell production (B-ALL), which results from chromosomal translocations that cause aberrant transcription factor activation, survival signaling, or epigenetic changes [4]. In the 20% of ALL cases that result from abnormal T cell development (T-ALL), over 50% contain mutations in the NOTCH1 and CDKN2 genes, which contribute to uncontrolled proliferation [5, 6]. Both types of ALL can be caused by mutations in several other genes at lower frequencies, which factor into prognosis. Chronic lymphocytic leukemia (CLL) is caused by abnormal B cell production. Chromosomal abnormalities are a hallmark of CLL, as is chronic B cell receptor signaling which promotes survival of CLL cells [7]. Reliance of CLL on B cell receptor signaling has heavily influenced treatment strategies, which target this pathway through inhibition of lynchpin signaling complexes essential to CLL survival (Fig. 1) [3]. Treatment for lymphocytic leukemias depletes the body of malignant lymphocytes and involves treatment with a combination of chemotherapy, small molecule inhibitors, and immunotherapy (Table 1). Depletion of these cells followed by bone marrow transplant is the only curative treatment.

Myeloid leukemia is characterized by expansion of malignant myeloblastic cells in patient bone marrow, which accumulate in the peripheral blood [8]. Myeloid leukemia can develop from a more benign form of the disease called myelodysplastic syndrome (MDS), in which immature blood cells in the bone marrow fail to mature into populations of healthy blood cells [9]. Myeloid leukemia occurs when immature myeloid blasts begin to proliferate out of control and rapidly accumulate in the bone marrow. Chronic myeloid leukemia (CML) is caused by a chromosomal translocation that causes the ABL1 gene on chromosome 9 to become fused with the breakpoint cluster region (BCR) on chromosome 22, resulting in production of an oncogenic fusion protein [10]. Inhibition of this pro-

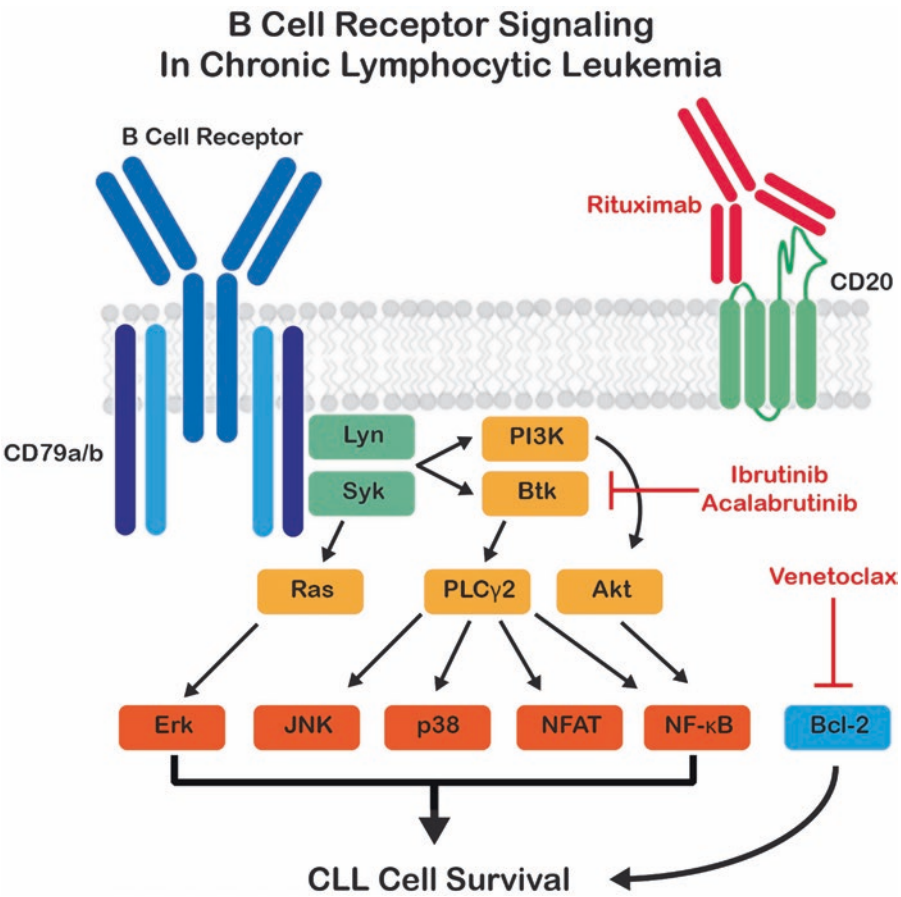

Fig. 1 Signaling downstream of B cell receptor activation in CLL and therapeutic interventions that target it. The signaling cascade is initiated by kinases Lyn and Syk at the membrane. This induces formation of a signaling complex that includes signal transducers PI3K and Btk. The cascade results in activation of Ras, PLCγ2, and Akt signaling pathways. Activation of transcription factors Erk, JNK, p38, NFAT, and NF-κB, in addition to Bcl-2 activity, contribute to the survival of CLL cells. Therapeutics targeting these pathways and the membrane-associated protein CD20 are shown in red

Table 1 Current therapies for leukemia

Leukemia type	Current treatments
Acute lymphocytic leukemia (ALL)	*4-Phase regimen including intrathecal chemotherapy*: Induction, consolidation, intensification, maintenance Gluccocorticoid (all phases) Vincristine (induction, intensification, maintenance) Asparaginase (induction, consolidation, intensification) Nucleotide analogs (consolidation, maintenance) Anthracyclines (induction, intensification) Methotrexate (consolidation, maintenance) Allogeneic bone marrow transplant in high-risk patients Anti-CD22 and anti-CD19 antibodies (dependent on expression) Anti-CD19 CAR T cell therapy (dependent on expression)
Chronic lymphocytic leukemia (CLL)	*Front line therapies*: Fludarabine-cyclophosphamide-rituximab (anti-CD20 antibody), FCR therapy Bendamustine + rituximab Chlorambicil + Obinutuzumab (anti-CD20 antibody) Ibrutinib (BTK inhibitor) Acalabrutinib (BTK inhibitor) Acalabrutinib + Obinutuzumab Venetoclax (Bcl-2 inhibitor) + Obinutuzumab *Relapsed disease/salvage therapy* Ibrutinib Acalabrutinib Venetoclax Venetoclax + rituximab Allogeneic bone marrow transplant
Acute myeloid leukemia (AML)	*2-Phase chemotherapy regimen:* Induction, consolidation Nucleotide analogs (induction, consolidation) Anthracyclines (induction) Midostaurine if FLT3 mutated (Induciton, consolidation) Bone marrow transplant in relapsed or unresponsive patients *High-risk patients are commonly enrolled in clinical trials* *Based on response to therapy and genetic features of the disease* *Examples of experimental compounds*: Venetoclax (Bcl-2 inhibitor) for unresponsive and NPM1 mutated patients Anti-CD200 antibody for RUNX1 mutated patients Anti-CD33 antibody for WT1 mutated patients Syk inhibitors for TP53, NPM1 and MLL mutated patients FLT3 inhibitors for FLT3 mutated patients IDH1/2 inhibitors for IDH mutated patients
Chronic myeloid leukemia (CML)	*Front line therapies*: Imatinib (BCR-ABL and tyrosine kinase inhibitor) Nilotinib (BCR-ABL and tyrosine kinase inhibitor) Dasatinib (Src family kinase, BCR-ABL, and tyrosine kinase inhibitor) If disease is accelerated, chemotherapy consistent with induction for AML Bone marrow transplant in unresponsive patients *Unresponsive patients or relapsed disease/salvage therapy*: Dose-escalation of inhibitor therapy Switching to another inhibitor Chemotherapy as with front line for accelerated disease

Treatment differs by leukemia type, disease severity, whether disease is treatment naïve. Individual mutations in patients are also taken into account. Radiotherapy has been omitted from this table, as it is rarely used and on a case-by-case basis

tein with BCR-ABL inhibitors, such as imatinib, is the primary route of therapeutic intervention for CML [11]. Acute myeloid leukemia (AML) is caused by a number of recurrent genetic lesions and chromosomal translocations, with RAS, FLT3, and NPM1 being some of the most common [8]. However, the genetic landscape of AML is diverse, making it difficult to treat. Induction treatment typically beings with chemotherapy, but the regimen varies based on the molecular phenotype of the cancer in individual patients. Remissions are shorter in AML than other types of leukemia and attempts to lengthen them often include bone marrow transplant.

1.2 Par-4

Par-4 has been the subject of in-depth study in solid tumors due to its unique ability to induce apoptosis in a cancer-selective fashion. Its ability to sensitize malignant cells to apoptosis while not contributing to cell death in the tissue from which the cancer originates has peaked interested in its utility for the purposes of targeted cancer therapy [12–14]. Cancer selectivity results from regulatory phosphorylation sites targeted by kinases that are commonly upregulated in cancer, such as PKA (T163) and CK2 (S231) [15, 16]. The selective for apoptosis in cancer domain (SAC), which has been shown necessary and in certain cases sufficient to induce apoptosis, also appears to specifically target cancer cells even though it does not contain any of the aforementioned phosphorylation sites [14]. Expression and activity of the protein are regulated by signaling events that are, again, commonly dysregulated in cancer cells. These include Ras signaling, endoplasmic reticulum stress, upregulation of PKA through cAMP production, PKC activation through various mechanisms, and others [15, 17–21].

Overexpression of Par-4 sensitizes cell lines established from several different solid tumors to apoptotic insult, including apoptosis inducing compounds used for chemotherapy, and has been shown to impact response of pancreatic cancer to treatment with small molecule inhibitors [15, 22]. Some of these compounds directly activate the Par-4 pathway, indicating that Par-4 can influence drug sensitivity intrinsically [23–25]. In fact, a significant correlation is documented between low Par-4 expression and relapse after chemotherapy in breast cancer patients [26]. This is interesting because Par-4, which is typically thought of as a tumor suppressor, can apparently act as an oncogene in some cases. Studies on Par-4 in hematological malignancies has produced data linking Par-4 to prognosis and disease have progression. Studies performed using patient-derived cells have indicated that the Par-4 pathway is prone to dysregulation through various mechanisms that are unique to the disease and patient group. Mechanistic work interrogating the potential for targetability of the pathway are ongoing. This chapter discusses those studies examining Par-4 in myeloid and lymphoid leukemogenesis and the clinical significance of their conclusions, with an emphasis on known pathway constituents and in vivo studies demonstrating the influence of Par-4 on leukemia progression.

2 Initial Observations in Lymphoid and Myeloid Leukemias

The pro-apoptotic WT1 regulator (PAWR) gene, which produces the protein prostate apoptosis response-4 (Par-4), has been shown to be downregulated in several tumor types, regardless of tissue of origin [27–30]. It was initially established as a tumor suppressor in mouse model experiments where knockout was shown to increase incidence of a cancer across various tissue types, and was verified in human tumors through gene expression analysis [26, 30–32]. This trend prompted study into its regulation in leukemias using cells from patient blood. Expression of Par-4 was found to be downregulated in some forms of the disease and upregulated in others, with expression varying between patient groups within each cancer. This suggests that different cancers develop unique adaptations that result in Par-4 dysregulation through various mechanisms. These results have created a picture of Par-4 as a highly specialized, pleiotropic tumor suppressor that imposes anti-neoplastic regulation through several mechanisms, imposing selective pressure on cancers to develop various adaptations to evade its activity.

B cell chronic lymphocytic leukemia (CLL) was one of the first leukemias in which the Par-4 pathway was examined for any therapeutic benefit. Contrary to what was observed in the majority of solid tumors examined, expression of Par-4 appeared to be upregulated in CLL cells relative to healthy donor B cells [29, 33]. Cells from patients with the acute form of the disease, acute lymphocytic leukemia (ALL), showed downregulation of the Par-4 protein, indicating that the mechanism by which the pathway is circumvented differs between cell types of origin and modes of malignant transformation [34]. The mechanism of downregulation in B cell ALL (B-ALL) was later shown to be methylation of the PAWR promoter, which is observed in multiple solid tumors [35]. Downregulation of Bcl-2 by Par-4 is documented in several solid tumors and thought to be one of the principal mechanisms by which Par-4 induces apoptosis. It does so by binding to the transcription factor Wilm's tumor protein (WT1), thereby modulating its activity [36]. This relationship is demonstrated in cells from ALL patients, but not in those from CLL patients, likely due to the absence of WT1 expression in CLL. This further suggests that the pathway is compromised through different mechanisms in these two forms of lymphocytic leukemia [34].

Further study of Par-4 in CLL revealed that protein levels correlate with response to apoptosis inducing compounds, expression of apoptosis regulators, and prognostic indicators. The response of primary CLL B cells to the tyrosine kinase inhibitor Imatinib was shown to be influenced by Par-4 protein levels, with higher levels contributing to more robust induction of apoptosis [23]. Interestingly, Par-4 levels were also shown to correlate with response of primary B-ALL cells to treatment with prednisolone, a synthetic glucocorticoid [37]. The effect in CLL was further shown not to depend on Bcl-2 levels, in concurrence with previously generated data suggesting that Par-4 does not downregulate Bcl-2 in CLL. In fact, Par-4 expression in CLL cells shows a positive correlation with Bcl-2 expression [33]. Statistically significant, posi-

tive correlations have also been demonstrated with apoptosis regulators DAXX and ZIPK, as well as serum levels of the prognostic marker lactate dehydrogenase. Additionally, CD38-positive and ZAP70-positive CLL B cells, which are known to be more aggressive, were shown to express Par-4 at higher levels than negative cells. This is contrary to what is observed in solid tumors and other hematopoietic malignancies, but does indicate that Par-4 expression may be determinant of disease progression in CLL. Together these data qualify the Par-4 pathway as a potential prognostic indicator and possibly a druggable target that could be leveraged in therapy for lymphocytic leukemias.

Par-4 has also been associated with disease etiology in acute myeloid leukemia (AML). In AML cells, Par-4 expression is downregulated relative to normal CD34+ cells, but the level of Par-4 expression correlates with mutation of the prognostic marker isocitrate dehydrogenase (IDH) [38–40]. IDH1 and IDH2 mutations result in epigenetic changes and are associated with poor prognosis and reduced relapse-free survival in AML [41]. Par-4 levels are higher in IDH mutated cells relative to unmutated. Another recurrent genetic lesion in AML is rearrangement of the EVI1 gene [42, 43]. This lesion is also associated with higher Par-4 expression and poor prognosis [40]. Finally, elevated Par-4 protein levels have been found in a patient who progressed from myelodysplastic syndrome (MDS) to AML [44]. Together, these data indicate that Par-4 upregulation contributes to aggressive disease progression and poor patient outcomes in AML, providing further evidence that Par-4 may play an oncogenic role in some malignancies. Exploration into the specific mechanisms is still ongoing (Table 2).

Table 2 Summary of observations made on the Par-4 pathway made in primary leukemia cells from CLL, B-ALL, and AML patients

	B-Cell chronic lympohcytic leukemia (CLL)	B-Cell acute lympohcytic leukemia (B-ALL)	Acute myelogenous leukemia (AML)
Par-4 expression	↑	↓	↓ Overall, ↑ with IDH Mutation and EVI1 Rearrangement
Bcl-2 expression	↑	↑	↑
Reciprocal expression of Par-4 and Bcl-2	No	Yes	Unknown
Par-4 expression correlated with prognostic factors	Positive Correlationswith LDH, CD38, Zap70	Unknown	Positive correlations with IDH mutation, EVI1 rearrangement, and serum LDH
Par-4 expression results in drug sensitivity	Imatinib	Prednisolone	Unknown

Average Par-4 expression is known to be increased in CLL patients and AML patients with IDH and EVI1 rearrangements relative to healthy donors, while expression is decreased in B-ALL and non-mutated AML patients. Bcl-2 expression is increased in all three forms of leukemia, but a relationship between Bcl-2 and Par-4 expression has only been demonstrated in B-ALL cells. However, Par-4 expression is correlated with prognostic marker expression in both CLL and AML. Elevated Par-4 levels result in increased sensitivity to Imatinib in CLL and Prednisolone in B-ALL

3 Mechanistic Studies

The activity of Par-4 is regulated by activating and inhibitory phosphorylation sites that influence the protein's ability to associate with its binding partners. The pathway is roughly divided into extrinsic and intrinsic components that have unique mechanisms triggered by different stimuli, but both result in apoptosis. The extrinsic pathway is triggered by ER stress and induces Fas trafficking at the plasma membrane. Par-4 is shuttled from the endoplasmic reticulum (ER) to the cell membrane through interaction with GRP78 [18]. The intrinsic pathway is triggered by activation of Par-4 through phosphorylation of the T163 residue (T155 in the murine isoform) by protein kinase A (PKA) or DAP-like kinase (DLK) [15, 45]. Nuclear localization and association with various binding partners then occur; resulting in compromised mitochondrial membrane potential, and inhibition of NF-κB signaling contributing to pro-apoptotic gene expression changes [14, 34, 46]. Ectopic expression of Par-4 protein encourages these interactions in various cancer cell lines to a sufficient degree that apoptosis is induced. The ability of Par-4 to induce apoptosis in multiple cancers has prompted rigorous interrogation of its activity and the mechanisms by which it facilitates apoptosis induction in response to different apoptotic stimuli.

Current work in hematological malignancies is attempting to parse out the pieces of this complex and malleable pathway that are misregulated. Early studies in Jurkat cells, an acute T cell leukemia (T-ALL) cell line, provided evidence that Par-4 effects apoptosis in response to treatment with cytarabine and doxorubicin. Increased expression of Par-4 results in enhanced caspase cleavage after exposure, conferring increased sensitivity of the cells to treatment with these chemotherapeutics [34]. The mechanism was determined to involve downregulation of anti-apoptotic proteins cIAP1, XIAP, and Bcl-2. These proteins prevent loss of mitochondrial membrane potential (MMP) and assembly of the apoptosome. Their downregulation through Par-4 overexpression results in compromised MMP and activation of caspases 3, 6, 7, and 9 after treatment with cytarabine and

doxorubicin. Treatment of Par-4 overexpressing ALL cells with (TNF)-related apoptosis inducing ligand (TRAIL) produced similar results.

TRAIL-induced apoptosis was found to rely on inhibition of cIAP1, cIAP2, and XIAP and activation of caspases 6, 7, and 9. Bcl-2 levels were not reduced and MMP was not compromised in the case of TRAIL-induced apoptosis after Par-4 overexpression, suggesting that TRAIL and the chemotherapeutics cytarabine and doxorubicin induce apoptosis through separate pathways that each include Par-4 (Fig. 2).

Continued study revealed that Par-4 regulates the expression of several other genes that influence apoptosis in ALL cells. It regulates transcript levels of the pro-apoptotic gene CCAR1 by occupying its promoter in a complex with THAP1. This displaces NOTCH3, a transcriptional inhibitor of CCAR1, and increases CCAR1 transcript levels [47]. Expression of CCAR1 promotes sequestration of anti-apoptotic 14-3-3 proteins, resulting in apoptosis induction. The Par-4/THAP1 complex was also found to inhibit the association of splicing factors with CCAR1 pre-mRNA to influence production of alternative splice forms. SRp40 and SRp55 associate with CCAR1 pre-mRNA in the presence of Notch3 to promote exon skipping, producing a truncated form of CCAR1, with a missing DNA binding domain, which does not promote apoptosis as effectively in ALL cells. Additionally, the Par-4/THAP1 complex was found to form upon knockdown of the long non-coding RNA (LncR) T-ALL-R-LncR1 in T-ALL cells [48]. This precipitated upregulation of pro-apoptotic Smac protein and activation of Caspase-3. It is not clear whether upregulation of Smac and Caspase-3 activation are reliant on the activity of CCAR1. The opposing influences of Par-4 expression and modulation of pro-apoptotic gene expression by Notch3 and T-ALL-R-LncR1 could be of clinical significance in the treatment of T-ALL (Fig. 3).

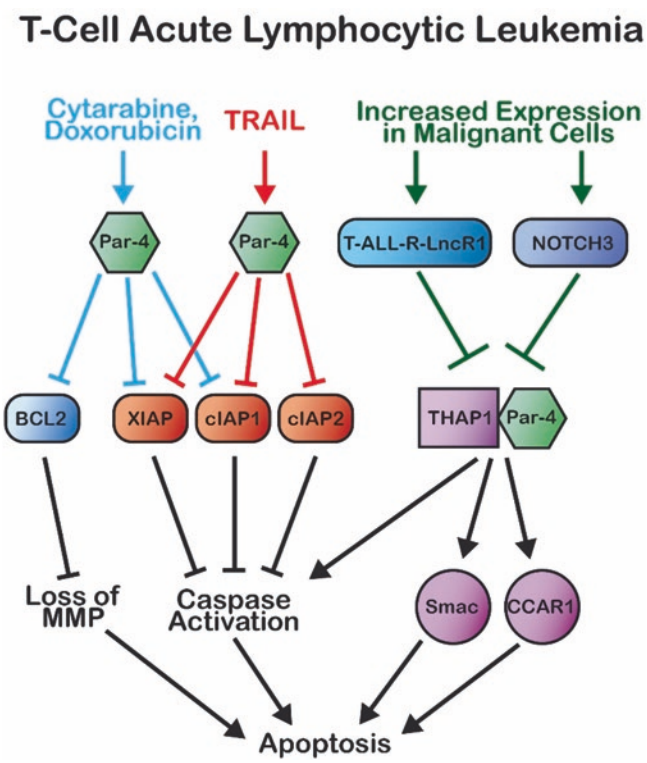

Fig. 2 The composition of the Par-4 pathway as it has been determined in T-ALL cell lines. Par-4 overexpression in T-ALL cell lines results in differential sensitivity to apoptosis inducing compounds. Cells become sensitive to cytarabine and doxorubicin upon overexpression of Par-4, which inhibits Bcl-2, XIAP, and cIAP1 to induce apoptosis through loss of mitochondrial membrane potential (MMP) and caspase activation. Par-4 overexpression also results in sensitivity to TRAIL through inhibition of XIAP, cIAP1, and CIAP2 and downstream caspase activation. Par-4 induced caspase activation also occurs when T-ALL-R-LncR1 and Notch3 expression increase in T-ALL cell lines, in addition to activation of Smac and CCAR1

The Par-4 pathway in myelogenous cells seems to bear little resemblance to that of lymphoid cells. Critical gene expression changes in response to Par-4 activity differ, along with potential for clinical utility. In the erythroleukemic cell lines K562 and HEL, DAXX and Bcl-2 expression are both upregulated by Par-4 overexpression, as well as pro-caspases 8, 9, and 10 [46, 49]. Despite the increase in pro-caspase expression, neither cell line exhibits an increase in basal apoptosis rates or sensitivity to chemotherapeutics, TRAIL or FAS; suggesting that these cell types are resistant to Par-4-mediated apoptosis [46]. In K562 cells however, Par-4 overexpression did sensitize cells to the effect of the BCR-ABL inhibitor imatinib and histone deacetylase (HDAC) inhibitors [49]. This is likely due to the presence of BCR-Abl protein and its anti-apoptotic effect in K562 cells [50]. Studies performed in murine, IL-3-dependent cell lines made factor independent by BCR-Abl expression found that Par-4 exerts an influence on BCR-Abl-mediated signaling. Interestingly, Par-4 overexpression resulted in downregulation of Bcl-2, c-myc, Akt, and STAT5 in 32D myeloid cells, but protein levels were not modulated in Ba/F3 lymphoid cell lines [51]. However, p38, a mitogen-activated protein kinase with pro-apoptotic functions, was activated at higher levels via phosphorylation in Ba/F3 cells. The factor-independent proliferation and colony forming capacity of the Ba/F3 and 32D cell lines were completely compromised by Par-4 overexpression due to inhibition of RAS, an effect which is also seen in rat prostate cancer cells [17].

These studies and the previous retrospective descriptions of Par-4 in patient cells suggest that the Par-4 pathway behaves differently not just in lymphoid and myeloid malignancies, but in acute and chronic forms of leukemia. Par-4 appears to exhibit an anti-neoplastic effect in ALL cells and myeloid leukemia cell lines that express BCR-Abl. While mechanistic data in AML and CLL is lacking, it does appear Par-4 expression may be a negative prognostic indicator in AML. Given the influence of Par-4 protein levels on imatinib

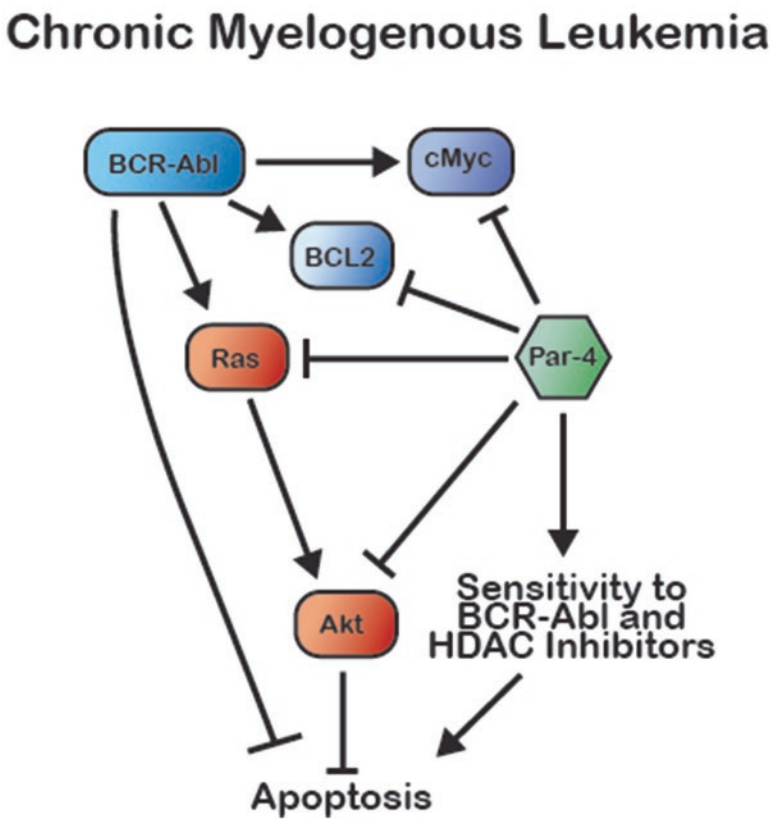

Fig. 3 The composition of the Par-4 pathway as it has been determined in CML cell lines. Par-4 overexpression in CML cell lines results in increased sensitivity to BCR-Abl and HDAC inhibitors, likely due to inactivation of RAS by Par-4. Par-4 also reduces expression of cMyc, Bcl-2, and Akt in CML cell lines to induce apoptosis

response in CLL, which does not express the BCR-Abl fusion protein, it is likely that Par-4 influences signaling pathways that are affected by c-Abl inhibition or the off-target effects of imatinib treatment. These pathways may therefore be targetable themselves with selective inhibitors, and Par-4 would likely influence response. These conclusions would suggest that further study of Par-4 in heme malignancies could lead to novel therapeutic approaches.

4 Par-4 in the TCL1 Chronic Lymphocytic Leukemia Model

Recent work in the T cell leukemia 1 (TCL1) leukemia model has suggested a pleiotropic role of Par-4 with respect to its effect on the development of CLL. These animals overexpress the TCL1 transgene under control of the Eμ-enhancer region, which regulates expression of immunoglobulin heavy chain, restricting expression to mature B cells [52]. These mice develop a CLL-like disease that manifests as lymphocytosis of a clonal population of CD5+ B cells in the primary lymphoid compartments and peripheral blood. Interestingly, it has been discovered that Par-4 protein levels increase in these cells as the disease progresses and CD5+ B cells become more abundant, mirroring what is seen in patient CLL cells [53]. Studies examining the role of Par-4 in leukemogenesis that utilized this model have revealed that the protein, while capable of inhibiting proliferation and resulting in less aggressive disease in a B cell-specific overexpression model, also contributes to proliferation and whole-organism knockout also results in a less aggressive disease. This suggests that Par-4 may contribute to leukemogenesis through both intrinsic and extrinsic mechanisms.

McKenna et al. showed that complete Par-4 knockout in the TCL1 model results in reduced accumulation of leukemic B cells and improved survival time. Par-4-deficient leukemia cells in these animals were found to upregulate the cell cycle regulator p21, causing reduced proliferation and slowing the rate of leukemogenesis. p21 is a cyclin-dependent kinase (CDK) inhibitor that controls progression of the cell cycle into the G2/M phase by inhibiting CDKs 2,4, and 6. Its expression is controlled by p53, which is located in a region of chromosome 17 that is commonly deleted in CLL patients, preventing p21-mediated cell cycle arrest [54]. In vitro experiments using the human, EBV-transformed cell lines OSUCLL and Mec1 showed that impairment of cell cycle progression by p21 also occurs in human CLL cells. Knockout of Par-4 reduced proliferation of cells in vitro and after engraftment in immunocompromised mice and was shown to be accompanied by a decrease in p21. McKenna et al. then showed that Par-4 knockout causes accumulation of cells in the G1 phase of the cell cycle. This indicates that p21 upregulation as a result of Par-4 knockout prevents cells from cycling past the restriction point in the G1 phase. This

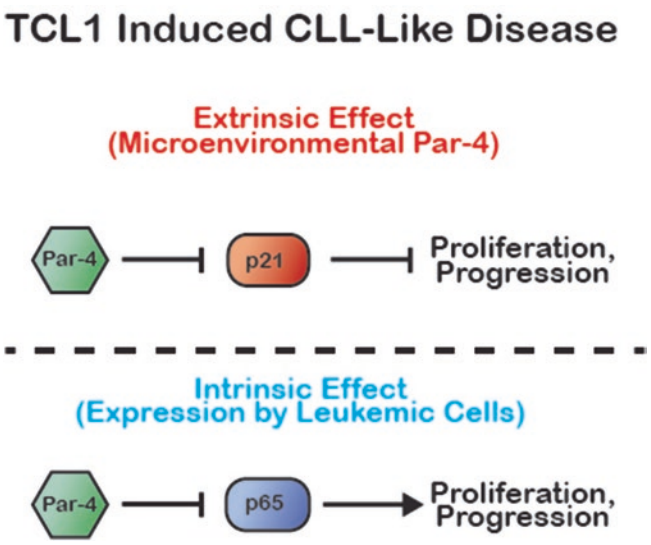

Fig. 4 Extrinsic and intrinsic effects mediated by Par-4 in the TCL1 murine leukemia model. Whole-organism knockout of Par-4 followed by transplant of TCL1 CLL-like cells results in less proliferative disease. This is due to hyperactivation of p21, which is inhibited by microenvironmental Par-4. When Par-4 is overexpressed in the B cells of TCL1 mice, the disease is also less proliferative due to an intrinsic mechanism that includes inhibition of p65

effect is also demonstrated in human CLL cells treated with the cereblon inhibitor lenalidomide. In a study by Fecteau et al., it was shown that treatment of CLL cells with lenalidomide can impede proliferation that is induced by CD154 through upregulation of p21 and G1 arrest [55]. The effectiveness of lenalidomide in maintaining progression-free survival in CLL and the mechanistic similarities it shares with Par-4 knockout lead to consideration of Par-4 inhibition as a potential therapeutic avenue (Fig. 4).

Par-4 is also known to be a potent tumor suppressor protein, which led our group to explore the effect of overexpression on leukemogenesis in the TCL1 model. We found that B cell-specific overexpression of the protein stifles disease progression, with Par-4xTCL1 mice exhibiting slower accumulation of leukemic CD5 + CD19+ B cells in the peripheral blood and a survival advantage relative to TCL1 littermates. Interestingly, human Par-4 overexpression on its own did not appear to affect B cell development or function and was shown not to influence p21 expression. Normal proportions of developing B cell subsets were observed in the spleen, bone marrow, and peritoneal cavity, and response to antigen was found to be normal in terms of proliferation and immunoglobulin secretion. However, in the leukemic B cells, it was found that Par-4 overexpression inhibits TCL1-driven NF-kB signaling as determined by nuclear translocation of the transcription factor p65. This phenomenon has been demonstrated in cell lines that overexpress Par-4. Investigation into the regulatory signaling events immediately upstream of p65 translocation revealed that Par-4 overexpression does not alter phosphorylation of IkBα or p65 itself. This indicates that the mechanism through which Par-4 inhibits p65 translocation is novel and as of yet, undescribed. Considering the effectiveness of CLL therapies, such as Ibrutinib, that inhibit NF-kB signaling and the consistency with which CLL patients on these therapies are relapsing with clones that carry mutations for drug resistance, it would seem prudent to study the mechanism of Par-4-mediated NF-kB inhibition more thoroughly in primary cells to shed light on its potential therapeutic use [56, 57].

5 Future Work and Prospects for Therapy

Much of the work published on Par-4 in leukemias is observational, but in ALL the pathway has been mechanistically described and has shown promising potential. In these cells, Par-4 has been shown to be an effective inducer of apoptosis and mediator of drug sensitivity. A study published in 2017 by the Rangnekar group at the University of Kentucky capitalized on these same characteristics in the treatment of solid tumor cell line transplants through use of a molecular decoy that prevents degradation of Par-4 through the action of Fbxo45, resulting in upregulation of Par-4 protein and sensitivity to chemothotherapy [58]. Such a strategy may be of use in ALL, which has been shown sensitive to the effects of Par-4 activity. It may also be of use in CLL, where increased Par-4 expression has been shown to inhibit NF-kB activity to reduce proliferation. Upregulation of Par-4 in patient cells may provide an opportunity for utilizing its activity in treatment. Further study in primary cells would yield information on the effectiveness and feasibility of this therapeutic strategy in leukemia.

Study of Par-4 in lymphoid and myeloid leukemias has mostly been through retrospective gene expression analysis of patient data that correlates Par-4 expression with prognostic markers, with only a few studies performed in cell lines and animal models [34, 38, 39, 44, 53]. A partial explanation is that patient cells from these diseases are very difficult to culture and there are few cell lines established from patient cells that can be used to model these diseases in vitro. Progress has been made recently towards overcoming these obstacles, providing more tools for researchers studying these diseases [52, 59, 60]. The potential targetability of Par-4 demonstrated by studies with primary ALL cells and in the TCL1 CLL model indicates that further investigation could lead to therapeutic advancements that utilize the pro-apoptotic activity of Par-4 to benefit patients.

The relationship between Par-4 and Bcl-2 expression is a central component to the apoptosis induction mechanism, and a potential avenue of translation in leukemia. Bcl-2 targeting therapies are already in development for CLL and

other cancers, with the goal of effecting apoptosis in a cancer-specific fashion [61–63]. This targeted approach shows an obvious benefit over traditional chemotherapy regimens prescribed for leukemia, which by comparison are relatively destructive to the body. Given the characteristic cancer-selective activity of the Par-4 pathway, its targeting is perhaps a strategy by which Bcl-2 levels could be modulated in cancer with minimal collateral damage to normal cells within the same tissue. The same is true of therapies that target Akt and the PI3K pathway. There is a plethora of drugs aiming to inhibit the pro-survival signals created by hyperactive Akt and PI3K [64–67]. Par-4 has been shown to inhibit Akt in cancer cells and induction of this interaction could provide another means of disrupting pro-survival signals created by Akt/PI3K with minimal off-target effects [68].

The Par-4 pathway includes several other proteins that have been shown relevant to survival and proliferation in leukemia cells. Complex formation with WT1 promotes Par-4-mediated downregulation of Bcl-2, the primary mechanism of apoptosis induction in this pathway, and could have other effects on gene expression [12]. WT1 is not expressed at appreciable levels in CLL but is overexpressed in AML and displays several prognosis-relevant mutations [69–71]. Despite the role of WT1 in leukemia development, its interaction with Par-4 in these cells has not been studied. PKCζ is another interacting protein that may be therapeutically beneficial. This atypical protein kinase C transduces signals from several immune cell receptors, such as Toll-like receptors (TLR) and B cell receptors, making it critical to the function of healthy B cells and contributing to pro-survival signaling in leukemia cells [72–75]. These features of PKCζ suggest a significant role in disease and its study as part of the Par-4 pathway in leukemia could lead to therapeutic strategies that target survival signaling.

Many of the proteins within the Par-4 pathway have been studied in hematological malignancies and found to be clinically relevant, but there have been very few studies examining the direct influence of Par-4 itself. Bcl-2 and WT1 are examples of survival regulators that have been linked to leukemogenesis and disease progression which are central to the function of Par-4 as an apoptosis inducer. AKT and PKCζ transduce signals that activate NF-kB in a pathway that is known to contribute to leukemogenesis and survival of malignant cells, but the involvement of Par-4 has been little described in leukemia despite known interactions within this pathway. Par-4 has already been shown to influence responses to therapy in solid tumor models and further study in leukemia could yield similar advancements. Some of these proteins have already been targeted with novel therapeutics and Par-4 may be influencing patient responses through unknown mechanisms that require further mechanistic description. Cancer-selective apoptosis induction is the desired outcome of all cancer therapies and is the defining feature of the Par-4 tumor suppressor pathway. Considering that the few studies of Par-4 in leukemia have shown involvement in survival and proliferation mechanisms, further exploration into this pathway in hematological malignancies would likely reveal novel strategies to enhance response to therapies and lead to significant advancements in cancer treatment.

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Potential of PAR-4 as a Therapeutic Target for Pancreatic Cancer

Md. Hafiz Uddin, Asfar S. Azmi, and Ramzi M. Mohammad

Abstract

Pancreatic cancer is a lethal malignancy with an increased incidence. The disease lacks biomarkers and actionable molecular targets. KRAS, P53, SMAD, and CDKN2A are known prevalent mutations in this disease. Prostate apoptosis response-4 (PAR-4) is involved with these signaling and all of them negatively regulate PAR-4. So far, targeting NF-κB and BCL-2 with chemopreventive agents and small molecule inhibitors has shown to induce apoptosis in pancreatic cancer via the upregulation of PAR-4. Unfortunately, the role of *PAR-4* gene in the biology of pancreatic cancer is largely ignored despite being adequately described in various cancer types. Pancreatic cancer is believed to be largely driven by KRAS mutation which is inversely correlated with PAR-4 expression. A relationship exists between PAR-4, drug resistance, survival signaling molecules including NF-κB and BCL-2. In this chapter, we highlight the role of PAR-4 and its significance as a molecular target in pancreatic cancer treatment.

Keywords

PAR-4 · Pancreatic cancer · KRAS · P53 · NF-κB · BCL-2 · Chemo preventive agents · Small molecule inhibitors · Nuclear export · KPT-185 · Exportin 1 (XPO1) · Chromosomal region maintenance 1 (CRM1)

1 Introduction

Pancreatic cancer is one of the most lethal malignancies and the third leading cause of cancer related deaths in the United States [1]. Attempt at curative resection after diagnosis is possible in less than 20% of patients. Of those patients who are eligible for surgery, only 20% survive for more than 5 years despite improvements in adjuvant chemotherapy. Treatment of advanced disease is palliative with a limited number of options. Single agent gemcitabine produces a response rate (RR) of 5–10% with median overall survival (OS) of 6 months [2]. A combination of gemcitabine and epidermal growth factor receptor (EGFR)-targeted agent erlotinib showed a marginal 2-week improvement in median OS [3]. In the phase III MPACT trial, gemcitabine in combination with nab-paclitaxel showed some benefits, an increase of OS for 1.8 months compared to gemcitabine monotherapy [4]. Though a combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) provided survival advantage (median OS 11.1 months vs 6.8 months) over gemcitabine, but it was associated with significant grade 3 or 4 toxicities [5, 6]. Lately other targeted agents such as poly (ADP-ribose) polymerase (PARP) inhibitor olaparib [7] and neurotrophic tyrosine receptor kinase (NTRK) inhibitor larotrectinib and entrectinib have been introduced [8]. Aberrant regulations of many signaling pathways contribute to pancreatic cancer progression. Approximately 90% of pancreatic cancers harbor Kirsten rat sarcoma (KRAS) mutation with dysregulated KRAS signaling [9]. The rare KRAS-g12c mutation can be targeted using covalent inhibitors [10, 11] and clinical trials with MRTX849 are ongoing (NCT03785249; NCT04330664). Frequent deregulations also occur in NOTCH, Sonic Hedgehog (sHH), transforming growth factor beta (TGF-β), EGFR, and nuclear factor kappa B (NF-κB) signaling [12, 13]. The invasive potential of pancreatic cancer is largely influenced by upregulated NOTCH signaling and its cross-talks with EGFR and NF-κB signaling [13]. Therapeutic targeted agent OMP-54F28 that binds to the WNT ligands is in phase I clinical trial (NCT02050178). Overexpression of sHH pathway molecule smoothened protein (SMO) in pancreatic ductal adenocarcinoma (PDAC) results in cancer metastasis [12, 14]. Although it seems promising, sHH SMO inhibitor vismodegib in combination with gemcitabine showed unsatisfactory results in phase II

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clinical trial (NCT01064622) [15]. Because of small incremental advantages of different targeted agents, there is an urgent necessity for better and less toxic options.

PRKC, apoptosis, WT1, regulator or PAWR also known as prostate apoptosis response-4 (PAR-4) is a tumor-suppressor protein that induces apoptotic cell death in cancer cells, but not in normal cells [16, 17]. It was identified first in the apoptotic cells of rat prostate cancer [18]. The importance of PAR-4 has been demonstrated in prostate and other cancer types; however, its significance in pancreatic cancer has not been fully evaluated [19]. Tumor progression and acquired chemoresistance of pancreatic cancer is influenced by oncogenic KRAS, BCL-2, TGF-β, and NF-κB signaling [20–23]. PAR-4 is involved with these signaling molecules, and all of them negatively regulate PAR-4. So far, targeting NF-κB and BCL-2 with chemopreventive agents and small molecule inhibitors has shown to induce apoptosis in pancreatic cancer via the upregulation of PAR-4. Moreover, ectopic expression of PAR-4 sensitized pancreatic cancer cells to cytotoxic agents with subsequent apoptotic cell death. Based on current evidence, PAR-4 is a promising candidate for targeting in this deadly disease [24].

2 PAR-4 and Its Significance in Pancreatic Cancer

The chromosomal location of *PAR-4* gene is 12q21 that is frequently deleted in pancreatic cancer [25] and associated with poor prognosis [26]. Kimura et al. observed deletion of a region between D12S81 and D12S1719 at 12q21 location at a frequency of 67.5% by microsatellite analysis of 40 pancreatic tumors and 19 pancreatic cancer cells lines [25]. PAR-4 is under the regulation of several genes including *KRAS*. The occurrence of somatic mutations of *KRAS* in pancreatic cancer is most frequent among different cancer [27]. The mechanism of oncogenic KRAS regulation of PAR-4 [24] is an interesting topic and will be discussed in the following section.

PAR-4 shows differential subcellular localization in normal cells and most cancers [28–31]. In normal cells, PAR-4 is typically found in the cytoplasm whereas in cancer cells it is present both in cytoplasm and in nucleus based on cancer cell lines and clinical specimens [32]. Tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), cytotoxic agents, or ionizing radiation-induced apoptosis may be prevented by the inhibition of PAR-4 with a variety of approaches including application of antisense oligonucleotides, a dominant negative leucine zipper domain, or RNA interference, suggesting an important role of PAR-4 in diverse apoptotic cell death pathways [33]. Furthermore, the spontaneous tumor development in liver, lung, endometrium, and prostatic epithelium in PAR-4 knockout mice indicates its crucial role in apoptosis [34]. Very recently, it has been demonstrated that Caspase 3 cleaves PAR-4 at D131 position generating a C-terminal activated fragment of 24 kDa. This fragment translocates to the nucleus, inhibits pro-survival genes, and promotes pro-apoptotic processes in cancer cells [35].

Overexpression of PAR-4 can induce apoptosis selectively in cancer cells, but not in normal cells [36] and has important implication in therapy. Cancer cells generally express elevated level of protein kinase A (PKA) [37] which phosphorylates the T155 residue of PAR-4, a critical step to initiate apoptosis [33]. Normal cells are resistant to PAR-4-mediated apoptosis; however, forced expression of PKA using cAMP-doxorubicin or vincristine induces apoptosis suggesting an important role of PKA in cancer cells [33]. Besides, PAR-4 activates FAS death receptor signaling pathway along with the inhibition of NF-κB leading to the caspase cascade [38]. Importantly, apoptotic cell death caused by ectopic PAR-4 is independent of P53 or phosphatase and tensin homolog (PTEN) [39]. It has been shown in diabetic mice, islet beta cells undergo apoptosis if PAR-4 is overexpressed. Elevated PAR-4 binds to telomerase reverse transcriptase (TERT) and inhibits its activity. The binding of PAR-4 to TERT occurs via nuclear localization signal (NLS) and leucine zipper domains which recently demonstrated by biological film interference experiments. Such binding allows the complex to be inside the nucleus and exert its apoptotic functions [40]. Preclinically, in subcutaneous or orthotopic tumor model systems overexpressed PAR-4 was able to induce apoptosis. The inhibition of tumor growth indicates PAR-4 as a potential therapeutic target. Inside the cancer cells, endogenous PAR-4 remains in the cytoplasm in its inactivated state its nuclear translocation is necessary for the induction of apoptosis [39, 41]. Cancer cells avoid apoptosis by keeping PAR-4 in the cytoplasm through binding with AKT1 as well as 14-3-3-mediated cytoplasmic localization [41]. Overall role of PAR-4 has been illustrated in the Fig. 1. How frequently mutated KRAS affect PAR-4 is being discussed in the next section.

3 Oncogenic Mutations and PAR-4

Among oncogenic mutations, *KRAS* is the most important one that regulates PAR-4. KRAS is a GTPase and an upstream player of several signaling pathways [42]. A single substitution mutation causes constant activation of KRAS which results in various malignancies including pancreatic ductal adenocarcinoma [43–47]. Oncogenic KRAS promote tumorigenesis partially by suppressing pro-apoptotic genes including PAR-4 [48, 49]. It has been shown that transfection of mutated KRAS (V12) downregulates PAR-4 expression in rat epithelial cells. The study further showed that such a

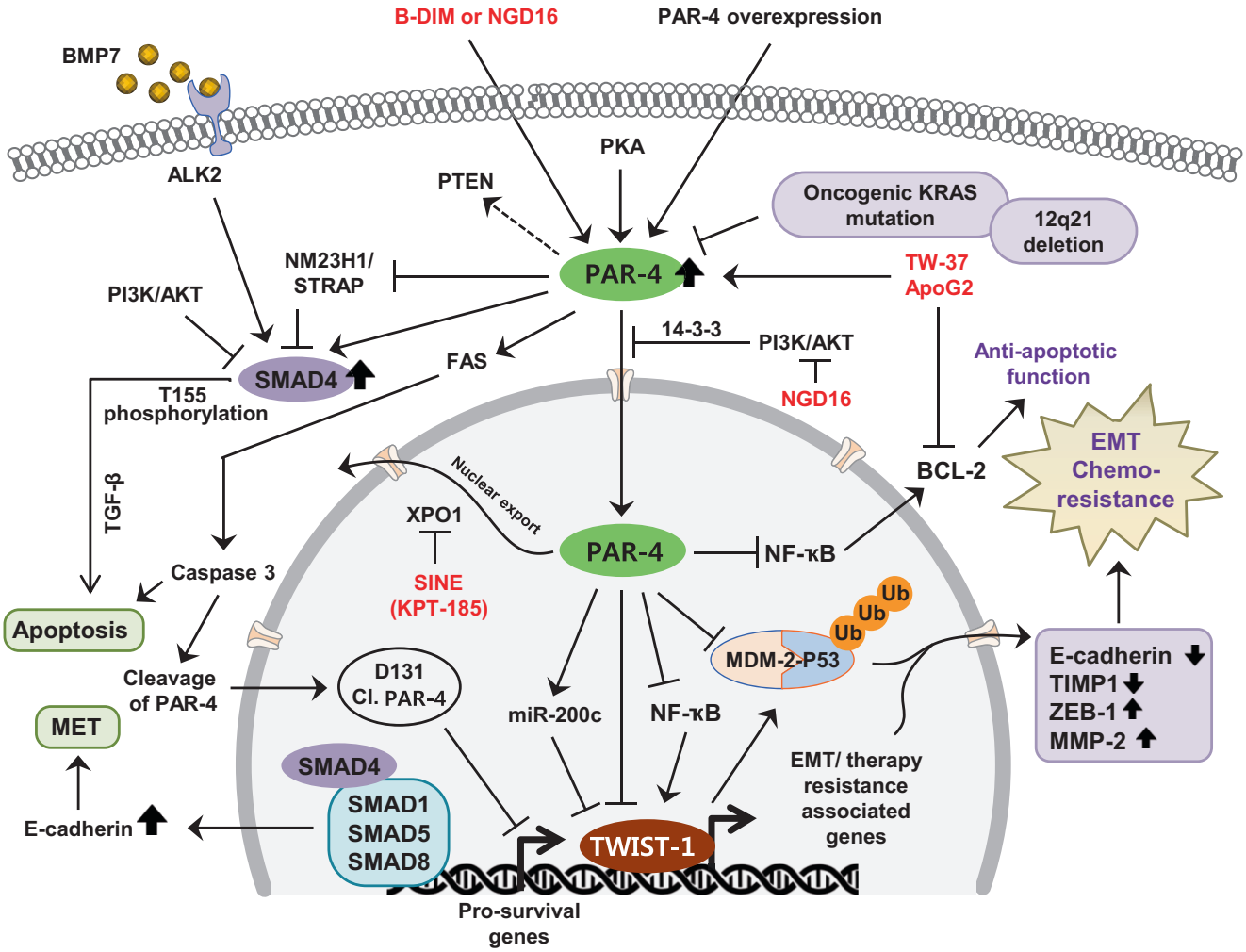

Fig. 1 Schematic diagram illustrating the role of PAR-4 in pancreatic cancer. Oncogenic KRAS and 12q21 deletion frequently suppresses PAR-4 in pancreatic cancer. The PI3K/AKT signaling interferes with the translocation of PAR-4 to the nucleus via 14-3-3 protein. Expression of PAR-4 either by PKA, pharmacological inducer (B-DIM, NGD16, TW-37, and ApoG2) or ectopic overexpression affects multiple signaling pathways in pancreatic cancer cells including translocation of PAR-4 in the nucleus. Elevated PAR-4 induces apoptosis via direct induction of FAS death receptor, SMAD4 signaling or disrupting SMAD4-negative regulators NM23H1 and STRAP's interaction. BMP7 can activate SMAD4 signaling via ALK2. Upon nuclear translocation SMAD4 activates transcription of MET-associated genes. In the nucleus, PAR-4 can inhibit TWIST-1 transcription (directly or via induction of miR-200c), MDM-2-mediated P53 degradation and NF-κB resulting disruption of EMT and anti-apoptotic activity by ZEB-1, BCL-2, and other molecules. Blocking nuclear exporter XPO1 by KPT-185 can enhance nuclear accumulation of PAR-4. In the cytoplasm, activated Caspase 3 can cleave PAR-4 generating a 24 kDa fragment which can inhibit pro-survival gene transcription in the nucleus. The arrows indicate positive regulation, a blunt line indicates inhibition and dotted arrow indicates possible induction. Abbreviations: ALK-2, activin receptor-like kinase-2; ApoG2, apogossypolone; BCL-2, B-cell leukemia and lymphoma 2; B-DIM, bioavailable 3,3′-diindolylmethane; BMP7, bone morphogenetic protein 7; EMT, epithelial-mesenchymal transition; KRAS, Kirsten rat sarcoma; MDM-2, murine double minute-2; MET, mesenchymal-epithelial transition; miR, microRNA; MMP-2, matrix metalloproteinase-2; NF-κB, nuclear factor kappa B; NGD16, 1,1′-β-D-glucopyranosyl-3,3′-bis(5-bromoindolyl)-octyl methane; NM23H1, NME/NM23 nucleoside diphosphate kinase 1; PAR-4, prostate apoptosis response-4; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PTEN, phosphatase and tensin homolog; SINE, selective inhibitor of nuclear export; SMAD-4, mothers against decapentaplegic homolog 4; STRAP, serine-threonine kinase receptor-associated protein; TGF-β, transforming growth factor beta; TIMP1, TIMP metallopeptidase inhibitor 1; TWIST-1, twist family bHLH transcription factor 1; XPO1, exportin 1; ZEB-1, Zinc finger E-box-binding homeobox 1

decrease of PAR-4 is associated with promoter hypermethylation of PAR-4 in a mitogen-activated protein kinase (MEK)-dependent manner [50]. Barradas et al. studied mutated KRAS in NIH3T3 mouse fibroblast cells and speculated the involvement of oncogenic RAS products in PAR-4 downregulation through modulation of RAF/PKC-MEK pathway without P53 and P16/19 dependency [51]. From these two studies, it can be assumed that PAR-4 downregulation by

mutated KRAS might be dependent on cell types and/or mutational status.

Ahmed et al. analyzed PAR-4 expression and KRAS mutational status in a retrospective study utilizing 4 pancreatic cancer cell lines, 10 normal pancreatic cells, 44 frozen and 25 paraffin-embedded tumor tissue samples by quantitative PCR, western blotting, immunohistochemistry, and allele-specific oligonucleotide hybridization [52]. They observed a correlation between KRAS mutation and PAR-4 expression. The study demonstrated a lower expression of PAR-4 when oncogenic KRAS transiently transfected in BxPC-3 pancreatic cancer cells which harbor wild-type KRAS. About 70% of the frozen tissue and paraffin-embedded tissue samples showed significant downregulation of PAR-4 and a correlation with KRAS mutational status [52]. Our study on the baseline expression of PAR-4 in a panel of pancreatic cancer cell lines is in agreement with Ahmed et al. findings where higher expression of PAR-4 BxPC-3 cells was detected [19, 53].

4 Regulation and Interaction of PAR-4 with Other Proteins

The B-cell leukemia and lymphoma 2 (BCL-2) oncogene promotes the survival of cancer cells mainly through is anti-apoptotic function [54]. In normal prostate basal epithelia, it has been shown that PAR-4 is co-expressed along with BCL-2 [55]. However, in mice prostate cancer xenograft and in acute lymphocytic leukemia PAR-4 and BCL-2 have an inverse correlation [32, 56]. In vitro data from NIH3T3 mouse fibroblasts and PC3 prostate cancer cells demonstrated that ectopically expressed PAR-4 downregulates BCL-2 expression [56]. In pancreatic cancer, BCL-2 overexpressed and considered as a promising target [57, 58]. In pancreatic cancer mice model, the BCL-2 family member BCL-XL was shown to regulated by EGFR signal via the activated transcription factors NF-κB and signal transducer and activator of transcription 3 (STAT3) [59]. It has been demonstrated that BCL-2 can activate NF-κB via inhibitor of nuclear factor kappa B kinase subunit beta (IKKβ) involving RAF/MEK signaling in pancreatic cancer [60]. The inverse relationship between BCL-2 and PAR-4 indicates a beneficial role of BCL-2 inhibition in PAR-4-induced apoptotic cell death. These studies suggest that BCL-2 plays multiple roles in pancreatic cancer in suppressing PAR-4 beyond its classical anti-apoptotic function.

Recently, co-immunoprecipitation and subsequent mass-spectrometry identified tripartite motif containing protein 21 (TRIM21) as a novel regulator of PAR-4 in colon and pancreatic cancer cells. TRIM21 has been demonstrated as predictive and prognostic marker for pancreatic cancer [61]. Further analysis showed that endogenous interaction between PAR-4 and TRIM21 occurs through the PRYSPRY domain. TRIM21 shown to exert platinum resistance might be through the suppression of PAR-4 expression upon cisplatin treatment in colon and BxPC-3, MIA PaCa-2, AsPc-1 pancreatic cancer cells. Forced expression of PAR-4 has been shown to sensitize pancreatic cancer cells to cisplatin [61].

PAR-4 binds to endoplasmic reticulum (ER) stress inducer glucose-regulated protein 78 (GRP78) via SAC domain independently of its carboxyterminal leucine zipper domain [36] and plays an important role in the alteration of indolylkojyl methane analog 5 (IKM5)-induced modulation of GRP78-TIMP metallopeptidase inhibitor 1 (TIMP1) complex. Therefore, it is likely that GRP78 inhibition can free intracellular PAR-4 which may assist its nuclear import to exert apoptotic function or blockade of NF-κB activity [62–64]. In cancer cells, downregulated NF-κB could suppress both twist family bHLH transcription factor 1 (TWIST-1) and TWIST-2 immediately that directly prevents apoptosis [65]. This is very important as TWIST-1 can repress E-cadherin transcriptionally by binding to the E-boxes 2/3 in the promoter region [66]. Such tumor suppressive role of PAR-4 suggests its therapeutic potential. In fact, in metastatic pancreatic cancer cells, pharmacological overexpression of PAR-4 shown to enhance activin receptor-like kinase-2/mothers against decapentaplegic homolog 4 (ALK-2/SMAD-4) signaling resulted in downregulation of epithelial-mesenchymal transition (EMT) markers along with the upregulation of mesenchymal-epithelial transition (MET) [67]. These findings strongly suggest a crucial role of PAR-4 in pancreatic cancer cell proliferation, invasion, and progression of disease (Fig. 1).

5 Role of PAR-4 in Pancreatic Cancer Drug Resistance

Platinum resistance is an impediment to successful treatment in pancreatic and other cancers. However, the mechanism(s) involved in the process remains largely unknown [68]. A study showed that cisplatin-resistant BxPC-3/CDDP cells have low levels of PAR-4 both at transcriptomic and proteomic levels. Overexpression and knockdown of PAR-4 confirms its association with cisplatin resistance both in vitro and in vivo BxPC-3 xenograft model system [69]. Mechanistically, downregulated PAR-4 allows significant activation of phosphoinositide 3-kinase (PI3K)/AKT signaling and cisplatin resistance. This finding was validated using PI3K/AKT inhibitor LY294002 in PAR-4 siRNA-transfected cisplatin-resistant BxPC-3/CDDP tumor model system [69]. Above findings warrant further investigation to determine the role of PAR-4 in the resistance of cisplatin and other cytotoxic drugs [69]. Similar mechanism of cisplatin resistance also observed in colon and pancreatic cancer cells as

described above possibly involving TRIM21 for the downregulation of PAR-4 expression [61]. Therefore, targeting PAR-4 to reverse cisplatin resistance may be of major clinical importance.

Gemcitabine, a deoxycytidine analog that inhibits DNA replication is used since 1997 as first-line of chemotherapy for advanced pancreatic cancer [70, 71]. Unfortunately, the progression-free survival (PFS) is very short largely because of de novo and acquired resistance [72]. Role of extra cellular matrix (ECM) remodeling were recognized by some studies through interference with uptake of gemcitabine by tumor cells [73, 74]. Moreover, EMT-induced chemoresistance in pancreatic cancer has been suggested in recent years [75] which adds further complexity to acquired drug resistance. The human homolog of murine double minute-2 (MDM-2) overexpression is associated with EMT phenotypes of the cancer cells which is under the regulation of PAR-4 [67]. Role of MDM-2 in EMT in relation to PAR-4 will be discussed in the later part of this chapter. Though MDM-2 protein is not a direct target of gemcitabine-based chemotherapy, it induces the expression of MDM-2 in advanced PDAC [76–79] possibly through the off-target effects. A study demonstrated in vitro and preclinical mouse model system that gemcitabine resistance in PDAC was associated with MDM-2 overexpression and induction of PAR-4 and that the use of NGD16 reduced such resistance [80].

6 Role of PAR-4 in Epithelial-Mesenchymal Transition (EMT)

TGF-β plays a differential role in the process of oncogenesis and tumor progression [81, 82]. In the early phase of pancreatic cancer development, TGF- β promotes apoptosis and inhibits cell cycle progression. However, in later phase it promotes tumor growth and metastasis though the regulation of genomic instability, neo-angiogenesis, immune evasion, and cell motility [83]. In the presence of TGF-β, Panc-1 cells with PAR-4 homozygous deletion fail to induce SMAD4 significantly compared to wild-type. Inversely, ectopic expression of PAR-4 restored SMAD4 activity and stabilizes TGF-β/SMAD4 axis in the same cell line by the abrogation of NME/NM23 nucleoside diphosphate kinase 1 (NM23H1) and serine-threonine kinase receptor-associated protein (STRAP) interaction [83]. The PAR-4 associated EMT was also observed in BXPC-3 pancreatic cancer cells [69]. In BxPC-3 cells, downregulated PAR-4-mediated EMT has been shown to be dependent on PI3K/AKT pathway. Study revealed that one of the mechanistic possibilities is the inability of reduced PAR-4 to inhibit PI3K/AKT signaling. In lung cancer, elevated PAR-4 inhibits AKT via the regulation of protein kinase C, zeta (PKCζ) [84, 85]. Another possibility is the negative regulation PI3K/AKT through PTEN as both PAR-4 and PTEN are mutually induced in the cancer cells [86]. The downregulated PAR-4-mediated EMT though observed in pancreatic cells, opposite results were observed in A549 lung cancer cells [87] and no effect observed in breast cancer cells [88]. These findings suggest that PAR-4-mediated EMT may be cancer cell type specific. With the reduced basal levels of PAR-4 seen in different pancreatic and other cancer cell lines, it is worthy to explore the EMT phenotype in such cells. Ahmed et al. (2020) observed reduced expression of PAR-4 and enhanced EMT processes in Panc-1 cells resistant to gemcitabine. They demonstrated that ectopic overexpression of PAR-4 or induction of PAR-4 by pharmacological modulator NGD16 reversed EMT by attenuating MDM-2. Higher level of PAR-4 results in the downregulation of EMT markers TWIST-1, and Zinc finger E-box-binding homeobox 1 (ZEB-1) along with the elevation of E-cadherin, P53, and TIMP1. They also observed inverse effect when PAR-4 was silenced by RNAi technique. Moreover, through immunoprecipitation experiment, this study confirmed the role of PAR-4 in the disruption of P53-MDM-2 interaction, further supporting its importance in EMT [80].

Involvement of bone morphogenetic proteins (BMPs) in the downregulation of TGF-β1-induced EMT in developmental stages is well recognized [89–91]. One of the BMPs member, BMP7 was demonstrated to induce MET by stabilizing positive expression ratio of E-cadherin and Vimentin [92, 93]. BMP7 also reported to upregulate ALK2 during the induction of MET [94]. Interestingly, pharmacological inducer of PAR-4 has shown to increase the expression of ALK2 both in Panc-1 and MIA PaCa-2 cells. Elevated PAR-4 also modulates several EMT and MET-associated proteins including vimentin, TWIST-1 and SMAD4 in these cells [90, 92, 93]. BMP7 also has been shown to play an antagonistic role on zinc finger protein SNAI1 (SNAIL) and enhance E-cadherin expression [95]. In a nutshell, induction of PAR-4 in cancer cells with SMAD4+/+ background poses potential prospect in development of anti-metastatic therapy.

Recently, role of microRNA (miR) has been discovered involving PAR-4 in the EMT process in pancreatic cancer cell lines. Katoch et al. (2020) showed that miR-200c not only regulates cellular proliferation but also EMT in Panc-1 cells and in vivo syngeneic mouse pancreatic cancer model system [96]. miR-200 family promotes metastasis by deregulating EMT possibly through the negative interaction with ZEB-1 protein in several cancers [97, 98]. Suppression of tumor promotion and invasion by miR-200c is evident in pancreatic cancer from earlier studies [99]. A study observed concomitant augmentation of miR-200c in PAR-4 transfected cells which delays the expression of crucial mesenchymal markers including TGF-β1, TGF-β2, ZEB-1, and

TWIST-1 and enhance the expression of E-cadherin. The pharmacological induction of PAR-4 with NGD16 also increases miR-200c and downregulates ZEB-1. Silencing of PAR-4 reverses the effect on EMT markers as well as miR-200c [96]. This finding indicates intricate relationship of PAR-4 and miR in the EMT process in pancreatic cancer and this area requires further exploration (Fig. 1).

7 Chemopreventive Agents as an Inducer of PAR-4

7.1 3,3′-Diindolylmethane

Chemoprevention is the use of natural, synthetic, or biological agents to suppress, reverse, or prevent the cancer progression. A study has demonstrated that chemopreventive agent 3,3′-diindolylmethane (DIM) or its bioavailable formulation, B-DIM upregulates PAR-4 in L3.6pl and Colo-357 pancreatic cancer cells even in very low concentration (20 μmol/L). DIM was effective in inducing apoptotic cell death, growth inhibition might be through the induction of PAR-4. In addition, DIM was able to sensitize cancer cells to standard chemotherapeutic drug gemcitabine via overexpression of PAR-4 [53].

7.2 NGD16

It has been reported that 1,1′-β-D-glucopyranosyl-3,3′-bis(5-bromoindolyl)-octyl methane (NGD16), an N-glycosylated derivative of DIM induce PAR-4 and abrogates EMT. This study further demonstrated that NGD16 treatment differentially affect the expression of epithelial and mesenchymal markers such as E-cadherin, Vimentin, and TWIST-1 via induction of PAR-4 both in vitro and in vivo models. Moreover, in pancreatic cancer cells MET was triggered by NGD16 in a PAR-4-dependent manner through augmentation of ALK2/SMAD4 signaling [67]. Involvement of PAR-4 in the MET process has been confirmed by endogenous PAR-4 silencing approach. The study observed a diminished E-cadherin expression in PAR-4 silenced cells. An intact SMAD4 is necessary for PAR-4-mediated regulation of E-cadherin. The regulation of E-cadherin by PAR-4 was further modulated by TWIST-1 promoter activity in the pancreatic cancer cells [67].

Anti-malarial drug chloroquine (CQ) and its derivatives hydroxychloroquine (HCQ)-induced PAR-4 in preclinical and clinical setting [100, 101] in lung and prostate cancer. Mechanistically, CQ induces PAR-4 via the activation of P53- RAS-related protein Rab-8B (RAB8B) pathway both in mice and cancer patient's normal cell. The studies have indicated that secretion of CQ or HCQ-induced PAR-4 by normal cells can cause paracrine apoptosis to tumor cells and can inhibit lung metastasis in vivo [100, 101]. Though it is likely CQ and HCQ possibly will induce PAR-4 in pancreatic cancer but has not been tested yet.

8 Small Molecule Inhibitors and PAR-4

8.1 BCL-2 Inhibitors

It has been demonstrated that non-peptidic small molecule inhibitors of BCL-2 family proteins apogossypolone (ApoG2) and TW-37 induce growth inhibition and apoptosis PAR-4 dependently. A good correlation (r = 0.92 and R^2 = 0.95) exists between apoptotic sensitivity and PAR-4 expression which is supported by siRNA-mediated silencing approach [19]. Nuclear localization PAR-4 is considered a requirement for cellular apoptosis. Treatment of Colo-357 and L3.6pl cells with ApoG2 cause nuclear retention of PAR-4 which was confirmed by 4′,6-diamidino-2-phenylindole staining. These BCL-2 inhibitors sensitized cancer cells to gemcitabine via induction of apoptosis [19].

8.2 Selective Inhibitor of Nuclear Export (SINE)

SINE compounds including KPT-185, KPT-127, KPT-205, and KPT-227 enhance growth inhibition and apoptosis in pancreatic and other cancer cells [102, 103], but not in normal human pancreatic ductal epithelial cells [103]. This differential effect may be associated with a number of tumor suppressors including PAR-4, forkhead box protein O (FOXO), P73 and P27. Our earlier study reported nuclear accumulation of PAR-4 upon KPT-185 treatment which inhibits the binding of exportin 1 (XPO1) or chromosomal region maintenance 1 (CRM1) with PAR-4 that prevent nuclear export. We have confirmed the consistent nuclear accumulation of PAR-4 along with cytoplasmic reduction by western blotting, immunofluorescence, and immunoprecipitation techniques. Moreover, we have shown mechanistically that mutated XPO1 at Cys-528 or transient RNAi-mediated knockdown of PAR-4 abolish the activity of KPT-185 suggesting their crucial role in apoptotic cell death. SINE compound KPT-330 was able to disrupt the XPO1 and PAR-4 interaction in both mice subcutaneous and orthotopic xenograft tumor model [103].

9 Targeting PAR-4 in Pancreatic Cancer in Clinical Settings

HCQ a robust inducer of PAR-4 in plasma that correlates with apoptosis in cancer cells [101]. HCQ is usually given at 200 mg twice daily dose for 2 weeks and shown to be safe in clinical settings. A clinical trial with HCQ in prostate cancer (NCT03015324) is ongoing to determine the impact of HCQ on plasma level of PAR-4 and efficacy in preventing tumor relapse [101]. The initial encouraging findings led to expansion to phase 2 clinical trial with long-term use of HCQ for the prevention of tumor recurrence [101]. Currently, directly targeting PAR-4 agent is not in clinical trials in pancreatic cancer patients. However, clinical study with pancreatic cancer patients treated with selinexor and other chemotherapeutic agents is currently assessing PAR-4 as an apoptotic marker (NCT02178436). Targeting PAR-4 in pancreatic cancer patients is an underexplored area and requires prospective investigation.

10 Conclusion and Future Perspectives

PAR-4 is a tumor suppressor with predominant pro-apoptotic function that inhibits malignant progression. Induction of apoptosis by PAR-4 in cancer cell-specific manner is remarkable. Currently, targeting regulator of PAR-4 in combination with conventional chemotherapy is a rational approach. Research on direct targeting of PAR-4 to treat cancer patients is still in its infancy. Recombinant PAR-4 peptide as a therapeutic option for pancreatic cancer is feasible; however, early experiments with in vivo model demonstrated limited serum persistence of PAR-4 protein [104]. The modified extended PAR-4 peptide PAR-4Ex showed approximately a seven-fold improved biological half-life and significant pro-apoptotic activity [104]. Improvement of PAR-4 recombinant protein warrants further investigation in clinical setting.

Intra tumor heterogeneity in part gives rise to intrinsic or acquired resistance to therapy. It has been shown that PAR-4 amino-terminal fragment (PAF) is released by various therapy-sensitive cancer cells following therapy which causes apoptosis and checked tumor growth [105]. Both PAF and PAR-4 contain VASA segment which binds with ubiquitin ligase F-box protein 45 (FBXO45) resulting degradation. Excessive production of PAF competitively binds with FBXO45 and protects PAR-4 from degradation [105]. Introduction of PAF in the tumor microenvironment may act as a preventive strategy to overcome therapy resistance. A study demonstrated apoptosis of normal pancreatic islet beta cells in type 2 diabetes which is mediated by elevated PAR-4. The study revealed the interaction of PAR-4 and TERT via nuclear localization signal and leucine zipper domains [40]. These findings indicate the importance of optimum endogenous level of PAR-4 in the cells which may help in optimizing future research on targeted therapy.

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Developing Quinoline-based Secretagogues for Prostate Apoptosis Response-4 Protein (Par-4) as Potential Antineoplastic Agents

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Abstract

Secretagogues bearing a 3-arylquinoline scaffold-induced secretory events in normal cells that released the tumor suppressor protein, prostate apoptosis response-4 protein (Par-4) sequestered by the intermediary filament protein, vimentin. The secretion of the Par-4 protein and its binding to a selective, cell-surface receptor GRP78 subsequently triggered paracrine apoptosis in cancer cells. These findings provided a rationale for the study of Par-4 secretagogues as potential agents for the inhibition of tumor growth. Developing secretagogues with these scaffolds, determining vimentin as the biomolecular target, using molecular dynamics to model arylquin binding to vimentin, and understanding the secretion of Par-4 and its apoptotic effects held promise as a new approach for small-molecule interventions as potential treatments for cancer.

Keywords

Apoptosis · 3-Arylquinolines · Intermediate filament proteins · Molecular dynamics · Prostate Apoptosis Response-4 protein (Par-4) · Secretagogues · Tumor suppressor protein · Vimentin

Abbreviations

Aq-1 or Arylquin-1	3-(2-Fluorophenyl)-N^7,N^7-dimethylquinoline-2,7-diamine
BFA	brefeldin A
CM	cell-culture conditioned medium
CQ	chloroquine
CYP	cytochrome P450 monooxygenases
DMF	Dimethylformamide
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EMT	Epithelial to Mesenchymal transition
ERES	Exit-site-from-the-endoplasmic-reticulum
ERGIC	Endoplasmic Reticulum-to-Golgi Intermediate Compartment
ER	Endoplasmic reticulum
Et_3N	Triethylamine
FIDAS agents	(*E*)-4-(2,6-Difluorostyryl)-*N,N*-dimethylanilines
GDP	guanosine diphosphate
GRP78	Glucose-regulated protein-78
GTP	guanosine triphosphate
HOBt	1-Hydroxybenzotriazole hydrate
LMP	lysosomal membrane permeabilization
MAT-2	methionine S-adenosyltransferase-2
MDM2	minute-double-minute-2 protein, an E3 ligase
NSAID	non-steroidal anti-inflammatory drug

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Nutlin-3a	4-[(*4S,5R*)-4,5-Bis(4-chlorophenyl)-2-(2-isopropoxy-4-methoxy-phenyl)-4,5-dihydro-imidazole-1-carbonyl]-piperazin-2-one
Par-4	Prostate Apoptosis Response-4 Protein
PEG	polyethylene glycol spacer $(CH_2CH_2O)_n$
SAC	Selective-for-Apoptosis-Induction-Cancer domain [104, 105]
SAR	Structure–activity relationships

1 Protein Secretion

Protein secretion drives mechanisms that maintain the extracellular matrix and that provide signaling to other cells. The traditional, conventional secretory pathway involves protein synthesis in the rough endoplasmic reticulum (ER), exit sites from the ER, translocation through an ER-to-Golgi intermediate compartment to the Golgi complex, and post-Golgi vesicles to convey these proteins to their final destination [1–4]. The organization of the participating organelles and compartments of this pathway accommodate the complexity of the process and provide an environment that ensures production of the properly folded and post-translationally modified proteins destined for secretion. Beyond the syllogism of ER-to-Golgi-to-plasma membrane that defines conventional, protein secretion [5, 6] lie the unconventional secretory mechanisms [7–10] that are reliant on the so-called leaderless proteins without an established, secretory-signal sequence and non-vesicular proteins that bypass the Golgi apparatus. In addition to these distinctions based on utilization or circumvention of the Golgi, newly synthesized proteins may undergo either *constitutive* secretion involving rapid, direct export to the extracellular matrix or *regulated* secretion involving the capture and sequestration of these proteins, typically in vesicles [11] but occasionally by sequestering, decoy biomolecules, until such time as needed. The distinction here is a temporal one in which the time frame for a response is limited, favoring regulated secretion that requires the capture of secreted proteins, or lengthy, favoring constitutive secretion that requires the synthesis and intracellular transport of secreted proteins. The intended roles for the secreted proteins drive the sorting mechanisms that govern either constitutive or regulated secretion in response to an appropriate stimulus [1].

The original identification of prostate apoptosis response-4 protein (Par-4) in prostate cancer cells undergoing apoptosis [12] led later to its classification as a tumor suppressor protein [13–15]. Consistent with this classification, Par-4 null mice developed spontaneous tumors in the prostate, liver or lungs or uterus or developed carcinogen-induced tumors in the bladder and uterus [16]. The Par-4 protein experiences post-translational modification in the Golgi but lacks a conventional, N-terminal signal sequence [17] that normally drives trafficking events leading to secretion *via* the conventional pathway [18, 19]. The absence of a conventional, signal sequence might suggest that Par-4 follows an unconventional pathway for secretion, but brefeldin A [20] (BFA) that blocks secretion *via* the conventional pathway, inhibits Par-4 secretion. Specifically, BFA inhibits a guanine nucleotide GDP-GTP exchange reaction that catalyzes the activation of a small GTPase, Arf1p [21–23], and that, in turn, recruits vesicular coat proteins necessary for anterograde ER-Golgi trafficking. Furthermore, deletion of the N-terminal, 25 amino acids [24] in Par-4 from rats prevents Par-4 secretion and suggests that the N-terminus may possess an atypical, secretory sequence that nevertheless drives conventional secretion. On the other hand, an unconventional pathway often emerges as a consequence of a disease process that induces cellular stresses at either the cellular or organismal level. A tumor suppressor may utilize a conventional secretory pathway for export to the extracellular matrix, but in stressed circumstances requiring immediate secretion, a tumor suppressor may follow an unconventional pathway, just as in the case of leaderless cytokines secreted in response to inflammation [25]. In summary, despite the presence of an atypical, N-terminal sequence, the influence of brefeldin A favors secretion by a regulated pathway involving an initial cellular "waystation" prior to its conventional secretion. The development of agents that release Par-4 from its waystation and thereby promote secretion is the focus of this particular chapter.

2 Small-Molecule Secretagogues

Man-made secretagogues function as compensatory agents that provide a corrective means to promote the otherwise disabled secretion of biomolecular signaling agents. Secretagogues, for example, that promote the secretion of either growth hormone [26, 27] or insulin [28, 29] possess a rich history as molecular tools that have important applications in medicine. These secretory agents typically function by elevating deficient levels of a protein with a specific signaling function. Still other antisecretory agents serve to depress protein secretion, as for example, in the case of brefeldin A [22, 23]. Inventive methodology [30] designed to screen small-molecules, including natural products (*i.e.*, secondary metabolites) and drugs, promises to identify additional inhibitors [31] of protein secretion. The interest in identifying inhibitors of protein secretion arises, quite understandably, out of an interest in understanding fundamental aspects of signaling and

finding agents that control the signaling mechanisms driving cell proliferation in cancerous tissues.

The converse of this process, also driven by an interest in antineoplastic drug development, focuses on *augmented* production and secretion of tumor suppressors [32, 33]. Under normal circumstances, these tumor suppressors inhibit cell division, induce apoptosis, affect DNA-damage repair, and/or inhibit metastasis. The sophistication of these activities requires an equally sophisticated series of events between gene expression and the aforementioned cellular events, including the sequestration of tumor suppressors that is one focus of this chapter. Failure to produce these tumor suppressors through defects in gene expression [34], abnormal ubiquitination and proteasomal degradation, or failure to secrete tumor suppressors from normal cells underlies a principal cause for cancer development that is beyond the scope of this chapter. Under normal circumstances, non-cancerous cells generate tumor suppressors and sequester them until an external signal reverses their sequestration and promotes their secretion. The work described in this chapter on the retro-sequestration of Par-4 and its subsequent secretion follows in the footsteps of a rich history of other small molecules that promote protein secretion for which the archetypical example is the secretion of insulin by sulfonylurea secretagogues [35]. In this chapter, we will restrict our discussion to those secretagogues that augment the secretion of Par-4 and that are best characterized as man-made small-molecules with molecular masses less than 1 kDa.

3 Protein Sequestration by Filament Proteins

The selective and efficient sequestration of specific proteins require interactions either with other monomeric or aggregated proteins or with non-protein biomolecules. Among the potential partners in sequestration events are the filament proteins, including the microfilaments, intermediate filaments, and microtubules. Intermediate filaments that possess a length intermediate between the microfilaments, such as actin, and the microtubules, such as tubulin, provide structural integrity. Unlike the microfilaments and microtubules that are polymers of single types of proteins, the intermediate filaments are heteropolymers with a range of proteins in different cell types and with sequences defining their classification into six groups (*i.e.*, type I–VI). Like the microfilaments and microtubules, the intermediate filament proteins interact with a host of other proteins as part of their primary role in maintaining cellular, structural integrity.

Among the type III intermediate filament proteins are desmin that is found in sarcomeres of muscle cells, peripherin that is found in peripheral neurons, glial fibrillary acidic protein that is found in astrocytes and glia; and vimentin that is found in fibroblasts, macrophages, neutrophils, leukocytes, endothelial cells lining blood vessels, and renal tubular cells. Vimentin plays either a direct or indirect role in many facets of the metastatic cascade. The role of vimentin evolved from the maintenance of the cytoskeleton of cells and tissue integrity [36] to its interaction with a large number of proteins and participation in various cellular functions including other processes such as the formation of signaling complexes with cell signaling molecules and other adaptor proteins [37].

Vimentin is now recognized as an essential player in many aspects of cancer, including initiation and progression, tumorigenesis, epithelial-to-mesenchymal transition, and the metastatic cell migration and invasion. Because vimentin is constitutively expressed in mesenchymal cells, it serves as a marker of cells undergoing EMT, a process that is activated during cancer progression and contributes to the metastatic spread of cancer. Although the role of vimentin in tumorigenic events associated with EMT and in cancer progression is not fully understood, its overexpression in cancer correlates well with accelerated tumor growth, invasion, and poor prognosis. Increased vimentin expression in tumor cell lines and tissues includes prostate cancer [38–42], breast cancer [43–45], lung cancer [46], tumors of the central nervous system [47–49], malignant melanomas [50, 51], and gastrointestinal tract tumors [52] that include pancreatic [53–56], colorectal [57–60], and hepatic cancers [61, 62].

Targeting filament-forming proteins with compounds that either inhibit filament growth or hyperstabilize filaments represents a successful strategy for the development of chemical probes and potential therapeutics. Among the prominent examples of such compounds are swinholide that inhibits actin filament growth [63]; taxol that stabilizes tubulin filaments [64]; vinblastine that inhibits tubulin filament growth [65–66], and withaferin A that covalently modifies Cys328 in the 2B subdomain of vimentin rod domain and causes aggregation [67]. Both actin and tubulin are globular proteins as monomers, composed of both β sheets and α helices, and both possess cavities that are suitable for binding small molecules. On the other hand, a vimentin monomer is structurally distinct from actin and tubulin because of its linear rather than globular structure. The N- and C- terminal regions of vimentin lack secondary structure, but its core, rod domain consists of α-helices connected by flexible linker regions [68]. In contrast to actin and tubulin monomers, a vimentin monomer has a fairly dynamic structure and consequently, is not a classical, druggable target.

4 Tumor Suppressors

Tumor suppressors represent one evolutionary mechanism for handling aberrant cellular behavior. The activation of an oncogene forces normal cells and their descendants through unrelenting mitotic cycles, and tumor suppressors provide an equally elaborate array of growth-constraining elements [69]. The balance between these hyperactive, growth-promoting genes (*i.e*., oncogenes) and normally inactive versions of the growth-constraining genes (tumor suppressors) dictates cellular fates. Tumor suppressor genes, often delineated as "caretaker genes" that stabilize the genome, "gatekeeper genes" that generate protective proteins that prevent the growth, and "landscaper genes" that encode proteins that provide a conducive environment for cell proliferation when mutated. The functions of tumor-suppressor proteins include a suppressive role for genes that regulate cell cycle progression and an integrative role that couples cell cycle progression and DNA damage and absent successful DNA repair that initiates apoptosis. The hypothesis that drove our investigations asked if the protective mechanism afforded by a specific tumor suppressor, namely Par-4, could be amplified through augmented secretion and marshaled to effect cancer cell apoptosis in situations where this normal, protective mechanism alone (*i.e*., without amplification) would be temporally insufficient to rescue cancer cell proliferation.

Articles enumerating the challenges in drug development [70, 71] often ignore a factor that governs any human activity: serendipity. No single factor dominates the circumstances that play a role in drug discovery in an academic setting more than proximity. Where industry may segregate functions in different locales, academia's limited space forces proximity among faculty with quite different interests. This accidental proximity leads to communication across interdisciplinary lines that, when coupled with intellectual curiosity and shared interests in hypothesis-driven research, often leads to unexpected outcomes. This chapter describes one such tale led an exploration of substituted 3-arylquinolines, *soi-disant* "arylquins," as a solution to the problem of promoting the secretion of Par-4 as a possible treatment for prostate cancer.

5 Secretagogues for Par-4

5.1 Introduction

Cellular homeostasis [72] requires vigilance and requires timely mechanisms for responding to internal dysfunction and external environmental threats. Among the mechanisms for responding to unrestrained growth, the release of sequestered, tumor suppressors represent an immediate means for responding to this problem and offers a more rapid response than the time-dependent process of transcription and translation. Although naturally occurring, biological ligands in signaling cascades may trigger the release of tumor suppressors, the development of unnatural, small-molecule ligands represents an attractive approach for inducing the release and secretion of tumor suppressors from a sequestered environment in normal cells (Fig. 1). Cancer cells, as might be expected, may inhibit the biosynthesis of tumor suppressors and their cell-surface receptors, bring about their rigorous sequestration, or effect inactivating post-translational modifications or proteolysis in addition to activating the pro-survival, unfolded protein response [73]. Suppression in normal cells would be expected to tilt in favor of sequestration, and prior to the initiation of our efforts, secretagogues such as nutlin-3a [74, 75] (Fig. 2a) were available to promote the secretion of the tumor suppressor, *Par-4* [17, 76, 77] . Nutlin-3a promoted Par-4 secretion at concentrations of 10 μM in cell culture and at 10 mg/kg in mice, and as we will discuss shortly, these relatively high concentrations left open the possible development of other secretagogues for Par-4 that were active at concentrations below those for nutlin-3a.

Nutlin-3a emerged as an effective agent that prevented the proteosomal degradation of another, tumor suppressor protein, cellular tumor antigen p53 that normally protected against carcinogenesis involving DNA damage by inducing cell cycle arrest and activating the DNA repair mechanism. DNA damage promoted the phosphorylation of p53 and minute-double-minute-2 (MDM2) protein. These phosphorylation events prevented p53-MDM2 interactions and thereby stabilized the otherwise short-lived p53 [78]. If subsequent events failed to achieve DNA repair and thereby released the cell cycle arrest, p53-induced apoptosis. Countervailing this protective mechanism provided by p53, MDM2 acted in the absence of DNA damage as a p53-specific, E3 ubiquitin ligase that led to continuous degradation of the non-phosphorylated p53 protein. Inhibition of the p53-MDM2 interaction rescued this degradation of p53 and provided augmented levels of the tumor suppressor protein p53, and this inhibition emerged as a principal target for cancer therapy.

Nutlins [74, 75] were among the early MDM2 antagonists [79–81] designed to inhibit p53 degradation, but unfortunately, nutlin-3a [75, 82–84] and subsequent, related antagonists [85–89] also led to drug-resistant, p53-mutated cancer cell lines. Lung cancer cells with p53 mutations or deletions often develop resistance to chemotherapy and radiation therapy [90–92]. These p53-deficient, cancer cells lacked the p53-driven apoptotic pathway but proved susceptible to apoptosis by the pro-apoptotic tumor suppressor, Par-4, that induced apoptosis in diverse cancer cells but not in normal cells. As a consequence, cancer cells rigorously controlled

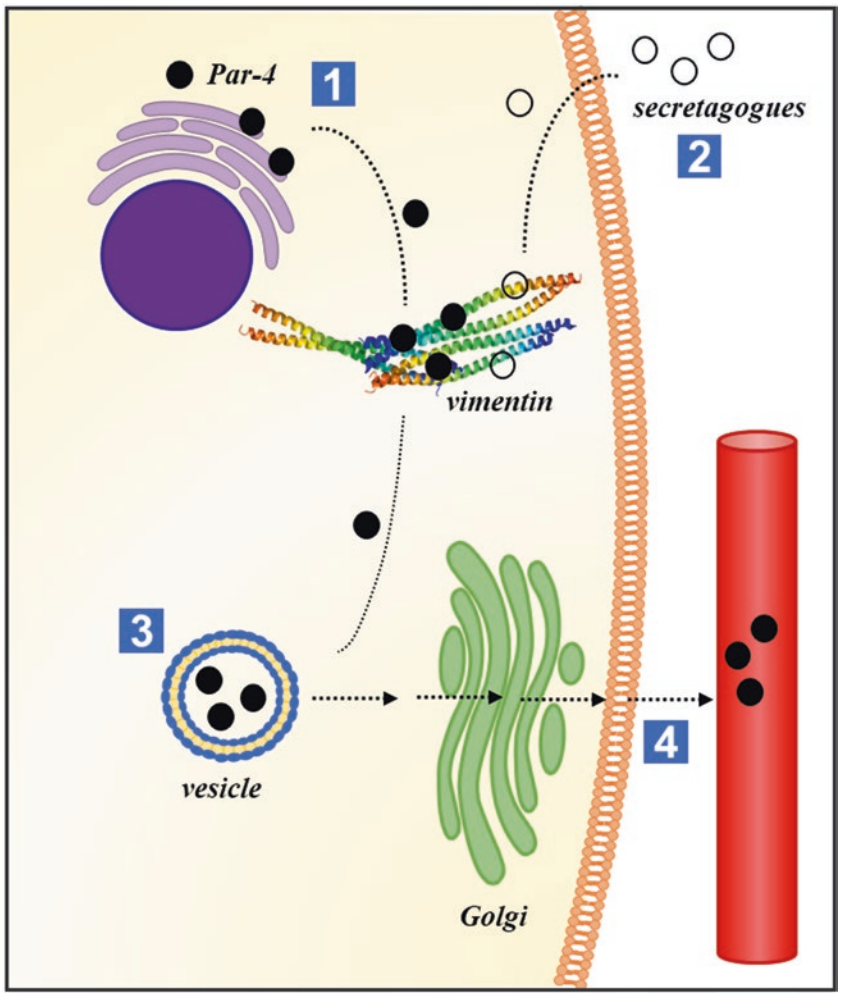

Fig. 1 Schematic representation of secretagogues promoting Par-4 secretion from normal cells. (1) ER biosynthesis of Par-4 and translocation to vimentin; (2) Secretagogues promote release of Par-4 from vimentin; (3) Vesicular capture of Par-4 and translocation to Golgi in conventional secretory pathway onward to the plasma membrane; (4) Translocation of Par-4 across membrane and uptake into circulatory system with delivery to GRP78 on the surface of cancer cells

the levels of Par-4 by various mechanism including inactivation through sequestration, transcriptional down-regulation, or mutation in some cancer cells [77, 93, 94]. In contrast, normal cells expressed Par-4 ubiquitously and typically sequestered Par-4 as a preventative measure.

Both intracellular Par-4 in cancer cells and secreted Par-4 from normal cells played a role in the selective induction of apoptosis in cancer cells by a caspase-dependent [95] mechanism. In the latter case, extracellular Par-4 that appeared during the in vitro secretion into the cell-culture conditioned medium (CM) or during in vivo systemic secretion in mice led to apoptosis. The extracellular Par-4 bound to a multifunctional receptor, a 78 kDa, glucose-regulated protein [96] (GRP78) on the cancer cell-surface and induced apoptosis. For example, Par-4-induced apoptosis in therapy-resistant, prostate cancer cells, such as CWR-R1, 22Rv1, LNCaP-derivative C4-2B, PC-3 and its aggressive analog, PC-3 MM2 [17, 97]. In contrast, normal cells that expressed low to undetectable levels of cell-surface GRP78 resisted apoptosis by extracellular Par-4 [17, 76, 77]. In summary, normal cells produced, sequestered and subsequently exported endogenous Par-4 but only at low levels. Similarly, cancer cells either degraded or sequestered endogenous Par-4 as well as its cell-surface receptor GRP78. If a means were available to augment circulating Par-4 levels from normal cells, evade mechanisms such as its phosphorylation by survival factors including nuclear factor kappa B [98, 99] (NF-κB), protein kinase C-ζ [100] (PKCζ), protein kinase B [101] (Akt) kinase, and casein kinase-2 [102] (CK2) and simultaneously promote translocation of the GRP78 receptor to the cell surface, then the combination of Par-4 selectivity for GRP78 and its capacity of this Par-4-GRP78 binding event to trigger apoptosis would offer a potential application in cancer therapy.

5.2 Synthesis and Evaluation of Potential Secretagogues

The administration of exogenous, recombinant Par-4 [17, 77, 102, 103] or its core, Selective-for-Apoptosis-Induction-Cancer (SAC) domain [104, 105] (*i.e.*, amino acids 137–195) that binds to GRP78 [17], provides a potentially useful alternative to an endogenous source, but Par-4 from mammalian

cells is at least 20 times more potent in inducing cancer cell apoptosis than recombinant Par-4 produced in *E. coli*. The details underlying this difference between exogenous and endogenous Par-4 are unclear but may involve an undetermined, post-translational event. Still other alternatives such as the production of Par-4 analogs with selected mutations that might enhance the wild-type Par-4 activity have not, as yet, come to fruition. Consequently, we focused attention on finding "small-molecule" secretagogues that would disrupt sequestration and promote endogenous Par-4 secretion from normal cells.

Although nutlin-3a (**1**) (Fig. 2a) was originally developed as an MDM2 inhibitor [75], nutlin-3a also stimulated the *in vitro* secretion of Par-4 [97] from mouse embryonic fibroblast (MEF) cells treated with nutlin-3a at a 10 μM concentration. In evaluating the molecular substructure of nutlin-3a and its analogs, such as MI-219 [106] (**2**) (Fig. 2a), we noted the presence of two halogenated, phenyl rings separated by a two-carbon spacer and the presence of a fused, nitrogen-containing heterocycle (*i.e.*, either the *cis*-imidazoline in nutlin-3a or the 4′-phenylspiro[indoline-3,3′-pyrrolidin]-2-one in MI-219), and these features served as a departure point for our efforts to develop new scaffolds that embraced these features. We considered two general scaffolds, designated as α and β (Fig. 2b), that differed in the connectivity of the aromatic rings and the two-carbon bridge between them. Scaffold α incorporated the arrangement of phenyl groups, a two-carbon spacer, and a fused heterocycle as seen in nutlin-3a and its analogs. Scaffold β incorporated, in part, the arrangement seen in MI-219 [106]. We opted to explore scaffold β in which we simplified some of the connectivity seen in MI-219 by inserting a heterocyclic substructure that spanned one of the carbons in the two-carbon spacer and an *ortho*-position in one of the phenyl rings. This approach offered relatively unexplored, yet easily accessible, frameworks for investigation as secretagogues for Par-4. It was unclear, of course, whether these modifications would retain or remove the Par-4 secretory activity seen with nutlin-3a.

Our prior work on an unrelated project identified fluorinated stilbenes [107, 108] bearing *N,N*-dialkylamino sub-

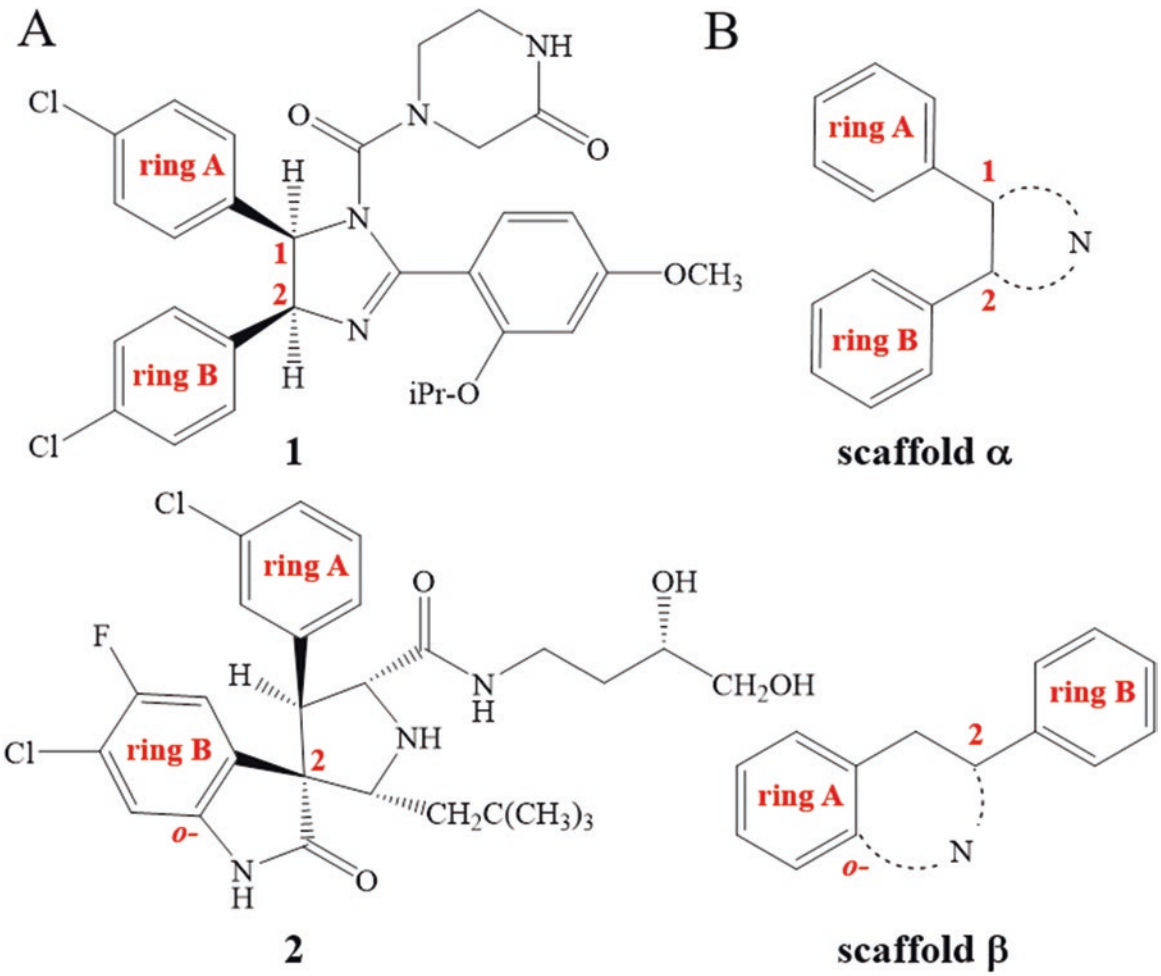

Fig. 2 Nutlins and substructures. Panel A: Nutlin-3a (**1**) and MI-219 (**2**); Panel B: Scaffolds α and β for the development of potential secretagogues

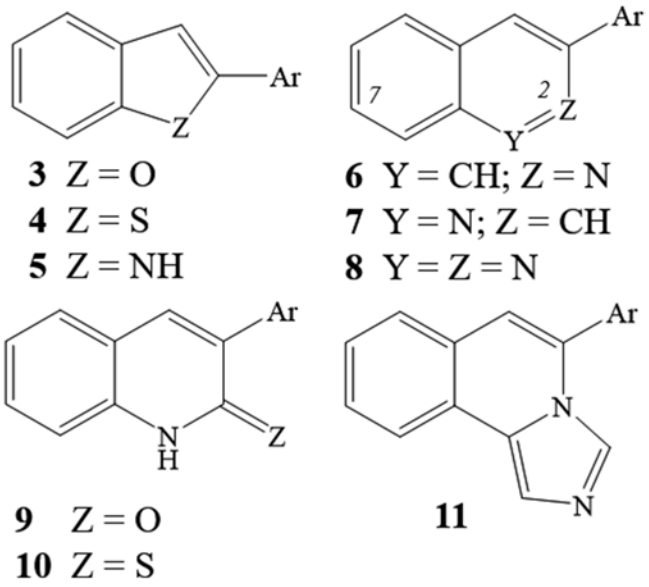

Fig. 3 Exemplars of scaffold β. Legend: Ar = halogenated phenyl group

stituents as potent, antineoplastic agents with activity against primary hepatocellular carcinomas and colorectal cancers in both in vitro cell proliferation studies and in vivo xenograft models. Among these fluorinated stilbenes, (*E*)-4-(2,6-difluorostyryl)-*N,N*-dimethylaniline, for which we coined the acronym "FIDAS," emerged as an initial, leading candidate that inhibited exclusively the catalytic subunit of methionine S-adenosyltransferase-2 (MAT2A) [109]. These analogs possessed several of the features found in nutlin-3a: two phenyl groups, one of which was halogenated phenyl group; and a two-carbon bridge between the two phenyl groups, albeit an unsaturated carbon-carbon double bond. Reduction of the double bond in these FIDAS agents brought them more in line with the substructure of nutlin-3a, but eliminated their activity as MAT2A inhibitors. FIDAS agents possessing the carbon-carbon double bond showed little Par-4 secretory activity (unpublished data).

The potential thermal or photochemical *cis/trans*-isomerization [110] of the carbon-carbon double bond in the FIDAS agents could complicate pharmacokinetic and pharmacodynamic studies in pre-clinical studies and could limit shelf-life stability if these FIDAS agents ever reached Investigational New Drug status. This concern prompted synthetic efforts either to replace the double bond with a triple bond [111] or to introduce an additional, fused heterocyclic ring bridging one of the carbons in the double bond and an *ortho*-position in one of the phenyl rings as a means of precluding *cis/trans*-isomerization. In the former case, the 1,2-diarylacetylenes [111] also selectively inhibited the catalytic subunit of methionine S-adenosyltransferase-2 (MAT2A) [109], possessed nanomolar *in vitro* potency, suppressed *in vivo* human tumor growth in xenograft and patient-derived xenograft (PDX) studies in mice, possessed promising physical properties for pharmaceutical candidates including biologically active, equipotent metabolites, good bioavailability and half-life and minimal toxicity, and induced, as desired, no activation of the human ether-à-go-go-related potassium channels (hERG).

In the latter case, the introduction of an additional, fused heterocyclic ring, as in the β scaffold (Fig. 2b), provided another approach for avoiding *cis/trans*-isomerization that was possible in the stilbenes [107, 108] and as an additional advantage, a nitrogen-containing heterocyclic ring could increase hydrophilicity. Specifically, we synthesized a library of compounds that included 2-arylbenzofurans (**3**), 2-arylbenzothiophenes (**4**), 2-arylindoles (**5**), 3-arylisoquinolines (**6**), 3-arylquinolines (**7**), 3-arylcinnolines (**8**), 3-arylquinolones (**9**), 3-arylthioquinolones (**10**), and 5-phenylimidazo[5,1-*a*]isoquinolines (**11**) (Fig. 3) for evaluation as MAT2A inhibitors. The synthetic routes to these compounds made use of standard, published techniques that proceeded without event from commercial compounds in three or four steps in good yields. In summary, a project geared to provide compounds for testing against colorectal cancers and hepatocellular carcinomas as MAT2A inhibitors led from stilbenes to diarylacetylenes and finally to a collection of heterocyclic compounds (Fig. 3). Concomitant with the synthesis of these compounds, we analyzed their potential activity in cell proliferation assays with various cancer and normal cell lines.

Cell proliferation assays of these heterocyclic compounds (Fig. 3) using colorectal cancer (LS174T), hepatocellular carcinoma (HepG2), and prostate cancer (PC-3) cancer cell lines provided a filter that reduced the number of analogs to a handful of agents with at least 50% inhibitory activity at ≤10 μM concentration. Those with promising activities in these assays, without any foreknowledge as to their biological targets, underwent evaluation as Par-4 secretagogues using normal mouse fibroblasts (MEFs) under conditions that were not toxic to the cells. With a few exceptions, 3-arylquinolines (Fig. 4a) with a fluorinated aryl ring and with additional amino groups at C-2 and/or C-7 proved to be the most active as Par-4 secretagogues (Fig. 4b) among the heterocyclic systems (Fig. 3) that were synthesized.

One particular 3-arylquinoline, that we described as "arylquin-1" (**12**) (Fig. 4a), emerged early in this screening process and produced a six-fold increase in Par-4 in the CM when administered at 500 nM concentration relative to Par-4 secretion induced by vehicle alone. Arylquin-1 became the standard against which later analogs were measured. Other analogs lacking the *ortho*-fluoro substituent (**13**) or possessing the fluoro substituent in the *meta*- or *para*-positions were less active than arylquin-1. Analogs lacking either the C-7 *N,N*-dimethylamino group (**14**) or the C-2 amino group (**15**) retained Par-4 secretory activity and suggested an ave-

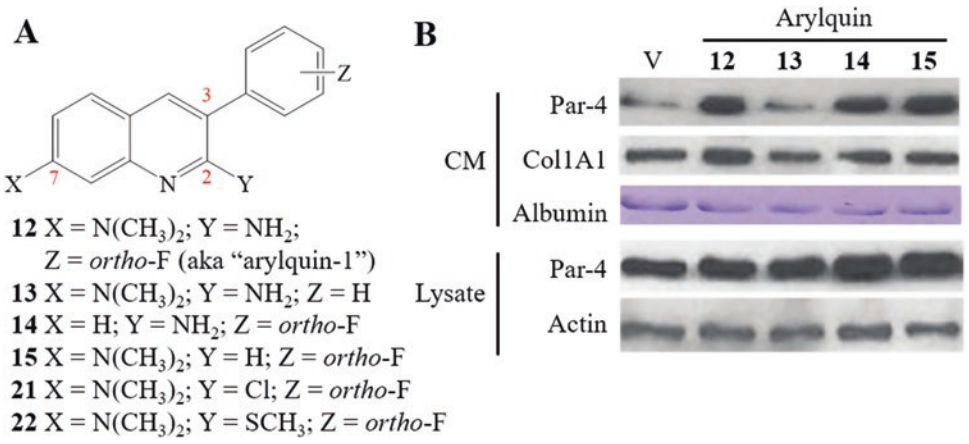

Fig. 4 Initial structure–activity studies leading to the identification of arylquin-1 as a potent Par-4 secretagogue. Panel A: Structures of 3-arylquinolines. Panel B: Mouse embryonic fibroblasts were treated with vehicle (V) or 500 nM of the indicated arylquins and Par-4 secreted in the CM or intracellular Par-4 in whole-cell lysate was quantified by Western blot analysis. Albumin in the serum from CM, collagen 1A1 secreted by the cells into the CM, or intracellular β-actin in the lysate served as a loading control

nue for the development of a biotinylated version of arylquin-1 that ultimately led to target identification (*vide infra*).

The synthesis of arylquin-1 (**12**) made use of the Friedländer condensation [112, 113] of 4-(*N,N*-dimethylamino)-2-aminobenzaldehyde (**16a**) with *ortho*-fluorophenylacetonitrile (Fig. 5). The synthesis of related arylquins lacking either the C-7 *N,N*-dimethylamino group as in (**14**) or the C-2 amino group as in (**15**) utilized similar approaches with either 4-(*N,N*-dimethylamino)-2-aminobenzaldehyde (**16a**) and *ortho*-fluorophenylacetyl chloride or 2-aminobenzaldehyde (**16b**) and *ortho*-fluorophenylacetonitrile, respectively (Fig. 5). These reactions proceeded efficiently to provide various 3-arylquinolines, including arylquin-1 (**12**) that was available as a non-hygroscopic, hydrochloride salt.

5.3 Identification of Target

Although we originally anticipated that the arylquins might function as MDM2 inhibitors like nutlin-3a, initial studies demonstrated that arylquin-1 (**12**) (Fig. 4a) differed dramatically from nutlin-3a in terms of biological response. Arylquin-1 was not merely an MDM2 surrogate, and unlike nutlin-3a, arylquin-1 did not stabilize p53. Treating either mouse embryonic fibroblast MEF cells or human embryonic lung (HEL) fibroblasts with nutlin-3a at a concentration of 10 μM for 24 or 48 h showed a clear stabilization of p53 (Fig. 6). Unlike nutlin-3a, the administration of arylquin-1 at a concentration of 500 nM that was sufficient to induce significant Par-4 secretion over baseline levels had no effect on p53 for these same time periods (Fig. 6). These findings reflected that the structural dissimilarities between the arylquin scaffold and nutlin-3a outweighed the similarities that led to their initial selection for screening. In addition, arylquin-1 produced a dose-dependent secretion of Par-4 in mouse embryonic fibroblast MEF cells (Fig. 7a) and as expected, triggered paracrine apoptosis in prostate cancer PC-3, human non-small cell lung carcinoma H1299 and adenocarcinoma HOP92 cells but not in human embryonic lung (HEL) fibroblasts (Fig. 7b–d).

We synthesized several biotinylated versions of arylquin-1 as a means of identifying a biomolecular target responsible for the observed Par-4 secretory activity [114] (Fig. 8). Among the most obvious positions on arylquin-1 (**12**) (Fig. 4a) on which to station a D-(+)-biotin tag, we initially selected the C-2 amino group. The acylation of this position with a biotin derivative possessing a polyethylene (PEG) spacer between a propionic acid at one terminus and a biotin group at the other led to the biotinylated arylquin (**19**) (Fig. 8). Although this derivative possessed a suitable distance (*i.e.*, 19 Å) between the C-2 carbon in the arylquin and the C-1 carboxy group of biotin, it was inactive as a Par-4 secretagogue (data not shown). It is unclear if the diminished activity reflected either an electronic distortion within the quinoline ring produced by replacing the C-2 amino group in the arylquin with a C-2 amido group or a diminished aqueous solubility of the biotinylated arylquin relative to arylquin-1 despite the presence of the PEG spacer.

Taking a somewhat different tact that would retain the hydrophobic PEG and the distance between the arylquins and the biotin termini, we opted to synthesize a 3-arylthioquinolone analog of the arylquins, namely 7-(dimethylamino)-3-(2-fluorophenyl)quinoline-2(1*H*)-thione, using a two-step procedure involving a Knoevenagel condensation [115] of the adduct between 4-(*N,N*-dimethylamino)-2-aminobenzaldehyde (**16a**) and 2-fluorophenylacetyl chloride

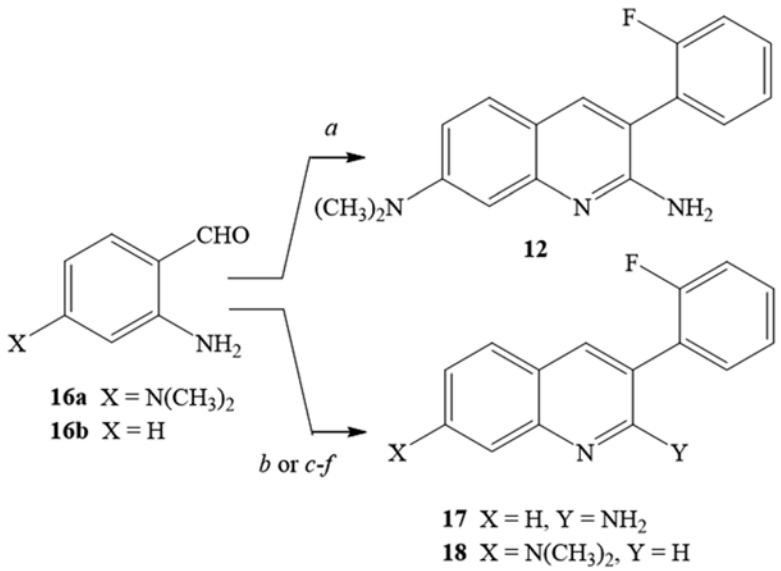

Fig. 5 Synthesis of arylquins with varied C-2 and C-7 substituents. Legend: *a*, **16a**, 2-fluorophenylacetonitrile, tert-BuOK, DMF, 90 °C, 3–4 h; *b*, **16b**, 2-fluorophenylacetonitrile, tert-BuOK, DMF, 90 °C, 3–4 h; *c*, **16a**, 2-(2′-fluorophenyl)acetyl chloride, Et_3N, reflux, 2 h; *d*, K_2CO_3, DMF, 90 °C, 4 h; *e*, $POCl_3$, reflux, 3 h; *f*, Zn, CH_3CO_2H, 75 °C, 1 h

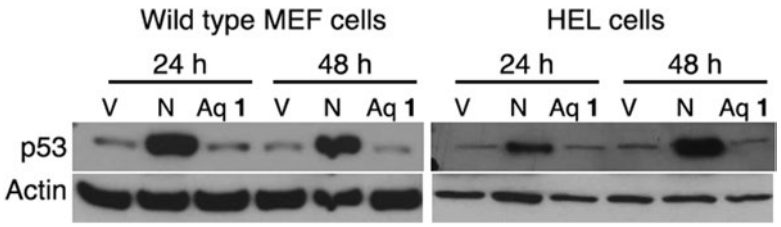

Fig. 6 Contrasting nutlin-3a with arylquin-1. Panels A and B: Arylquin-1 did not stabilize p53. Mouse embryonic fibroblasts (MEFs) and human embryonic lung (HEL) fibroblasts, respectively, were treated with vehicle (V), nutlin-3a (N, 10 μM), or arylquin-1 (Aq-1, 0.5 μM) for 24 h or 48 h, and whole-cell extracts were subjected to Western blot analysis for p53 or β-actin

to acquire a 3-arylquinolone and then treatment with Lawesson's reagent [116] to convert the 3-arylquinolone to a 3-arylthioquinolone. We utilized an S-alkylation of the 3-arylthioquinolone with an α-iodoacetamide derivative of a PEG-modified biotin to acquire the biotinylated arylquin (**20**) (Fig. 8). This biotinylated derivative (**20**) possessed the necessary Par-4 secretory activity (Fig. 9a), albeit at a reduced level from that seen with arylquin-1 itself. Although the distance between the C-2 carbon in the arylquin and the C-1 carboxy group of biotin was somewhat shorter (*i.e.*, 13.6 Å) than in the biotinylated arylquin (**19**), it was sufficient for a successful pull-down experiments using streptavidin-linked, sepharose beads and using either mouse embryonic fibroblasts (MEFs) or human embryonic lung (HEL) fibroblasts. We identified vimentin [117], a cytoskeletal intermediate filament protein, as its principal target (Fig. 9b) using mass spectrometry (Fig. 10). Appropriate controls excluded the adventitious binding of vimentin to the sepharose beads or binding of the PEG spacer to the sepharose beads. The absence of other, significant bands on gels of the pull-down experiment using the biotinylated derivative (**20**) excluded the possible association of arylquins with a secondary protein that in turn bound vimentin.

To confirm experimentally that the binding of arylquin led to Par-4 secretion, we performed co-immunoprecipitation experiments in which a Par-4 antibody co-immunoprecipitated endogenous vimentin, and a vimentin antibody co-immunoprecipitated endogenous Par-4 (Fig. 10). Immunocytochemical analysis confirmed that Par-4 co-localized with vimentin (Fig. 10). On the other hand, cells treated with arylquin-1 showed neither Par-4 co-immunoprecipitation nor co-localization (Fig. 10) with vimentin, indicating that arylquin-1 displaced Par-4 sequestered on vimentin. This action of arylquin-1 was not associated with inhibition of vimentin expression [114] but instead suggested that arylquin-1 either caused conformational changes in vimentin that inhibited its ability to bind and sequester Par-4 or arylquin-1 competed for a hydrophobic binding region on vimentin that was crucial for Par-4 binding. In summary, confocal microscopy established the co-localization of Par-4 and vimentin and the release of Par-4 induced by arylquin-1 treatment. In addition, because the expression levels of vimentin were not altered by arylquins, we excluded the possible inhibition of a transcription factor that could reduce vimentin expression and thereby release Par-4 for secretion. We could not,

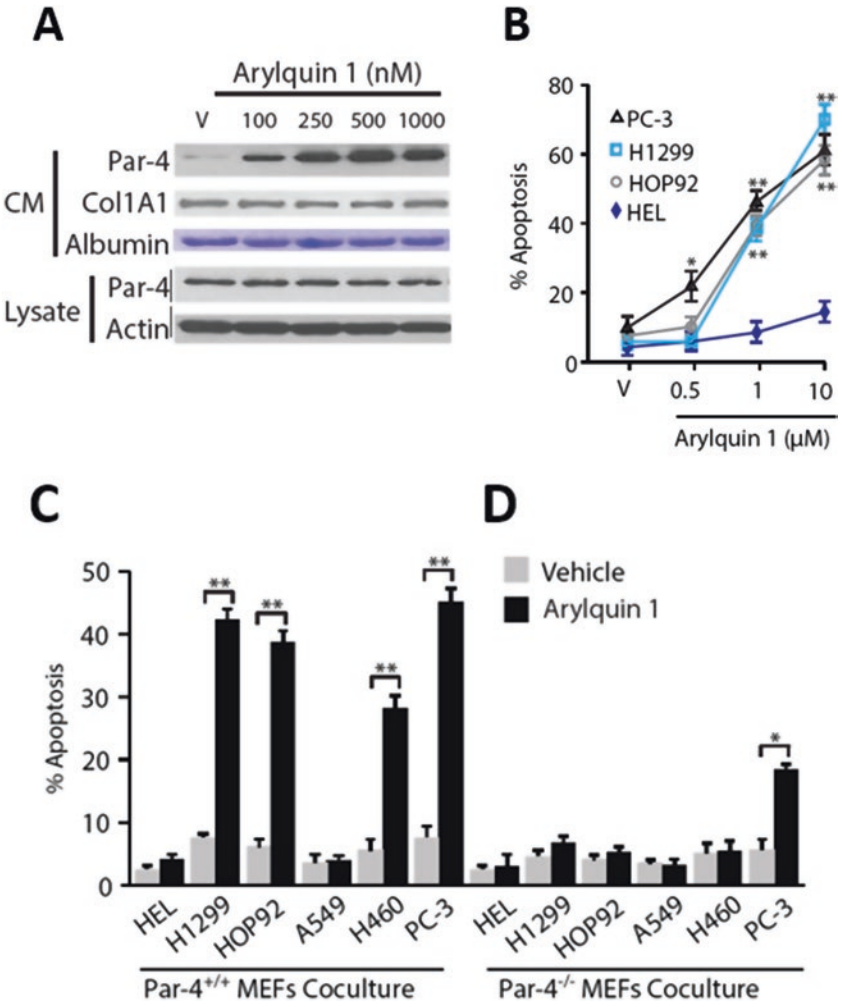

Fig. 7 Arylquin-1 effects on Par-4 secretion and Apoptosis. **Panel A**: Arylquin-1 induced a dose-dependent secretion of Par-4. MEF cells were treated with the indicated concentrations of arylquin-1 or vehicle (V), and Par-4 in the CM or whole-cell lysate was quantified by Western blot analysis. Albumin or collagen 1A1 in the CM, or intracellular β-actin in the lysate served as a loading control. **Panel B**: Arylquin-1 induced a dose-dependent apoptosis of cancer cells. Cancer cells were co-cultured with MEFs and treated with arylquin-1 (500 nM) or vehicle (V) and tested for apoptosis. **Panel C**: Arylquin-1-induced systemic Par-4 pro-apoptotic activity. Serum from mice injected with arylquin-1 (Aq) or corn-oil vehicle (V), was examined by Western blot analysis. **Panel D**: Aliquots of serum from these mice were either directly added to the growth medium of cells in culture, or incubated with the indicated antibody, and then added to the growth medium of PC-3 MM2 cells to test for apoptosis. Panels B-D. Apoptotic cells were scored after 24 h and data shown represent mean values from three independent experiments ± s.d. Asterisks (**) or (*) indicate statistical significance ($P < 0.0001$) or ($P < 0.001$), respectively, by the Student *t* test

at this stage in our studies, determine if arylquins promoted the release of Par-4 though a competitive interaction with the vimentin-Par-4 binding site(s) or through an allosteric effect in which a conformation change promoted the dissociation.

Because of the complexity and oligomeric nature of vimentin, it is not surprising that "small-molecules" other than just arylquins function as Par-4 secretagogues. Several groups noted that ketorolac, a non-steroidal anti-inflammatory agent (NSAID) used for pain management, contributed to increased survival for patients with ovarian [118] and breast cancers [119, 120]. Investigators at Cipla Limited subsequently connected these improved outcomes for ketorolac treatment of renal cell carcinomas [121] with Par-4. Ketorolac, either as a monotherapy or in conjunction with other antineoplastic agents and/or ionizing radiation, led to Par-4 secretion and tumor volume reduction [122], although a direct association of ketorolac with vimentin has not, as yet, been established. The differences in the structures of the arylquins and ketorolac, assuming for the moment that ketorolac interacts directly with vimentin, suggest that other possible small-mole-

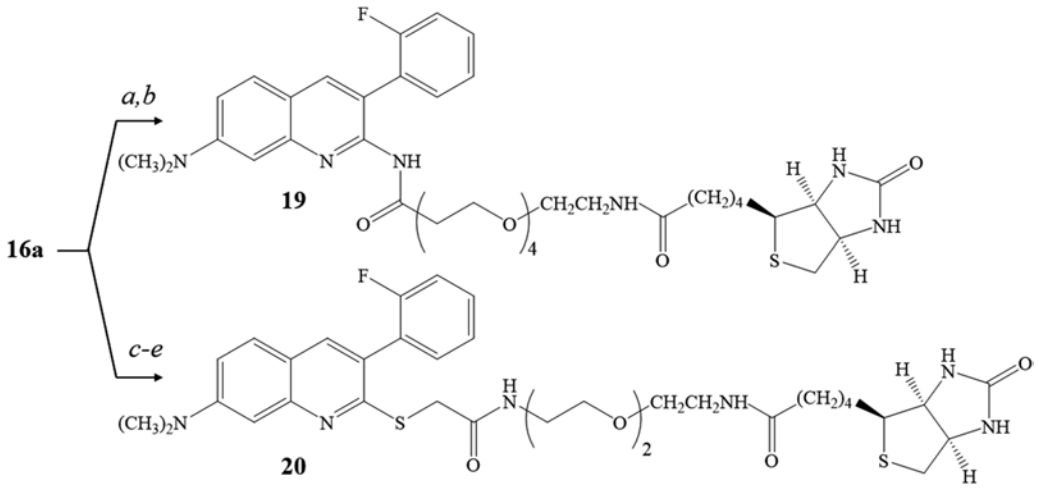

Fig. 8 Synthesis of biotinylated arylquins. Reagents: *a*, 2-(2′-fluorophenyl) acetonitrile, *tert*-BuOK, DMF, 90 °C, 3–4 h; b, 18-oxo-22-((3aS,4S,6aR)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-4,7,10,13-tetraoxa-17-azadocosanoic acid, EDC, HOBt, DMF; *c*, 2-(2′-fluorophenyl)acetyl chloride, Et_3N, reflux 2 h and then K_2CO_3, DMF, 90 °C, 4 h; *d*, 2,4-bis(4-methoxyphenyl)-2,4-dithioxo-1,3,2,4-dithiadiphosphetane (Lawesson's reagent), 1,4-dioxane, reflux 5 h; *e*, (+)-biotinyl-iodoacetamidyl-3,6-dioxa-octanediamine, K_2CO_3, DMF, 12 h

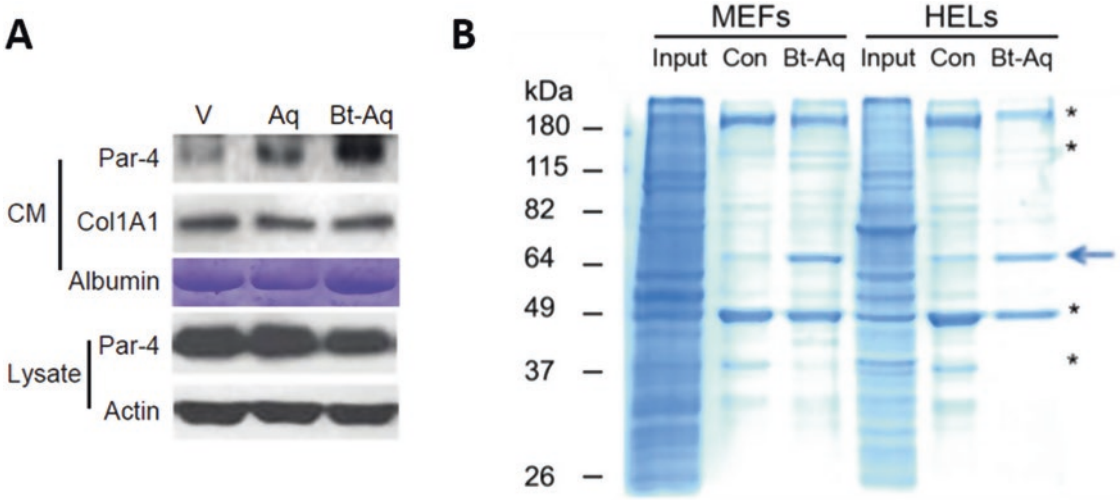

Fig. 9 Validation and application of biotinylated arylquin (**20**) in target identification. Panel A: Biotinylated arylquin (**20**) retains Par-4 secretory potency. HEL cells were treated with either arylquin-1 (Aq, 500 nM), biotinylated arylquin (**20**) (Bt-Aq, 500 nM) or vehicle. Par-4 in the CM or whole-cell lysate was quantified by Western blot analysis. Albumin, collagen 1A1, or actin served as a loading control. Panel B: Biotinylated arylquin (**20**) bound to vimentin. Whole-cell lysates of MEF or HEL cells were subjected to pull-down with biotinylated arylquin (**20**) (Bt-Aq) or with no compound (control, Con), and bound proteins were resolved by SDS-PAGE and stained with Coomassie blue. The indicated band (arrow) was identified as vimentin by mass spectrometry. Asterisk (*) indicates non-specific bands

cules may hold potential value as Par-4 secretagogues that target vimentin.

5.4 Computational Modeling of Secretagogue Binding to Vimentin

Computational modeling using molecular dynamics simulations led to a minimum-energy structure in which arylquin-1 (**12**) (Fig. 4a) bound to tetrameric vimentin in a hydrophobic pocket located between a pair of head-to-tail α-helical dimers (Fig. 11). The spatial arrangement of functional groups within arylquin-1 was ideally suited to stabilize binding to vimentin. Additional modeling revealed that arylquin-1 (**12**) and its analogs **13**, **14**, and **15** (Fig. 4a) bound to vimentin in the same orientation but with binding energies [114] that favored arylquin-1. The relative values of these calculated binding energies were qualitatively consistent with experimental trends for Par-4 secretory activity. These calculations also found that the *ortho*-fluorine group in arylquin-1 was

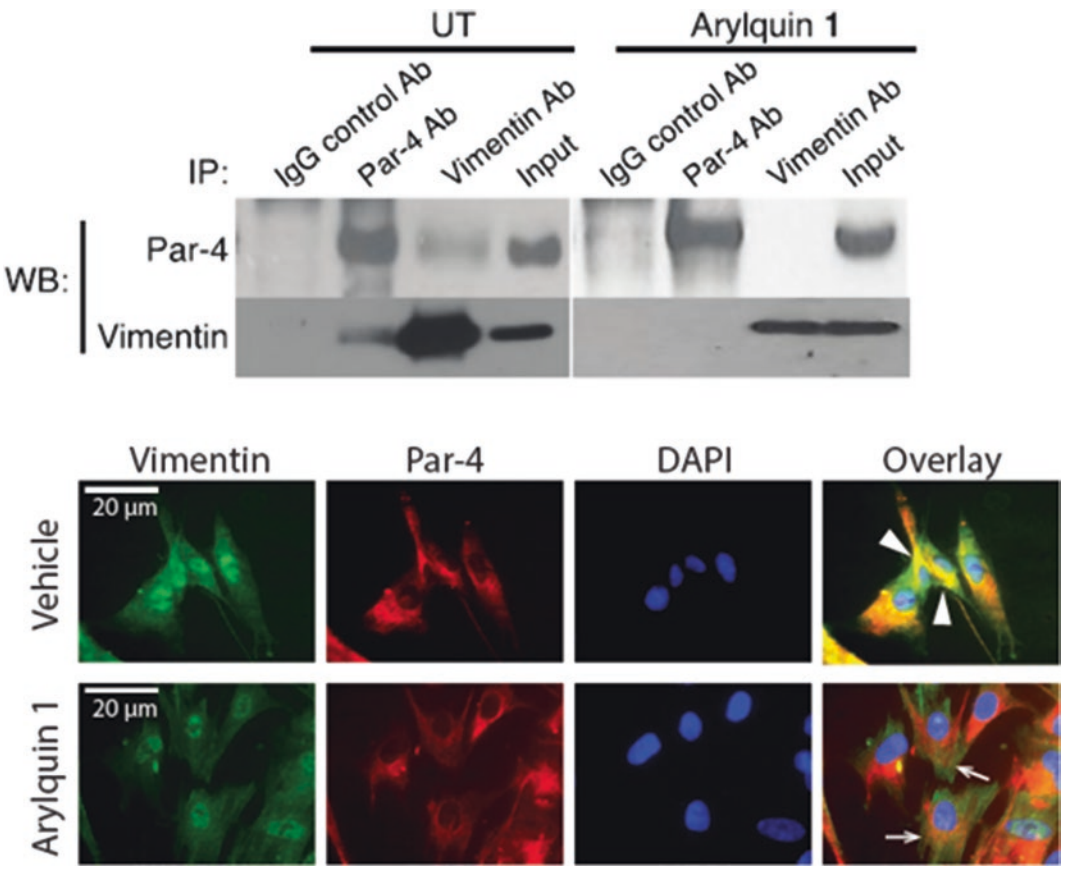

Fig. 10 Arylquin-1 displacement of sequestered Par-4 on vimentin. Top Panel: Arylquin-1 inhibited Par-4 co-immunoprecipitation with vimentin. HEL cells were left untreated (UT) or treated with arylquin-1 (500 nM) for 24 h and were subjected to immunoprecipitation (IP) with the indicated antibody (Ab). The immunoprecipitated complexes and input samples (5%) were subjected to Western blot (WB) analysis. Bottom Panel: Par-4 co-localized with vimentin and was displaced from vimentin by arylquin-1 treatment of cells. HEL cells, treated with vehicle or arylquin-1 (500 nM) for 24 h, were subjected to ICC for Par-4 (red fluorescence) and vimentin (green fluorescence). Cells were stained with DAPI to reveal their nuclei (cyan fluorescence). Co-localization of Par-4 and vimentin in the overlay images shown in vehicle panel is indicated by arrowheads (yellow fluorescence), and dissociation of Par-4 and vimentin (loss of yellow fluorescence, but retention of red and green fluorescence) is indicated by arrows in the arylquin-1 panel

indispensable, and the removal of the fluorine was accompanied by reduced calculated binding energy and was also consistent with the loss of Par-4 secretory activity in analog **13** (Fig. 4a).

5.5 Secretagogue Induction of Apoptosis

Par-4-induced apoptosis in diverse cancer cells but not in normal cells [77]. The ubiquitous expression of Par-4 in normal cells and tissues stood in contrast to its inactivation, down-regulation or mutation in cancers [77, 93, 94]. Both intracellular and secreted Par-4 played a role in apoptosis induction by caspase-dependent mechanisms [77]. Par-4 underwent secretion into the CM or systemically in mice from normal cells, and extracellular Par-4 bound to a specific receptor GRP78 on the cancer cell-surface and induced apoptosis [17, 76]. Normal cells, on the other hand, expressed low to undetectable levels of basal or inducible cell-surface GRP78 and thus resisted apoptosis by extracellular Par-4 [17, 76].

Testing the direct connection between Par-4 secretion and apoptosis, as described in the preceding paragraph, involved studies of truncated Par-4 constructs [14]. As noted earlier in this chapter, deletion of the first 25, N-terminal amino acids (*i.e.*, Δ^{1-25}Par-4) impaired secretion but led to a construct that exhibited two-fold, augmented levels of apoptosis. The processes of secretion and apoptosis are, of course, multistep events, and the unexpected, outcome of augmented apoptosis may simply reflect enhanced binding to GRP78 by the shorter construct than full-length Par-4. Still another feature of the complexity of relationships between secretion and apoptosis emerged in another construct lacking both the N-terminal sequence as well as the nuclear localization signal-2 sequence (*i.e.*, $\Delta^{1-25,\ 137-153}$Par-4). This double mutant underwent secretion but failed to induce apoptosis. The latter

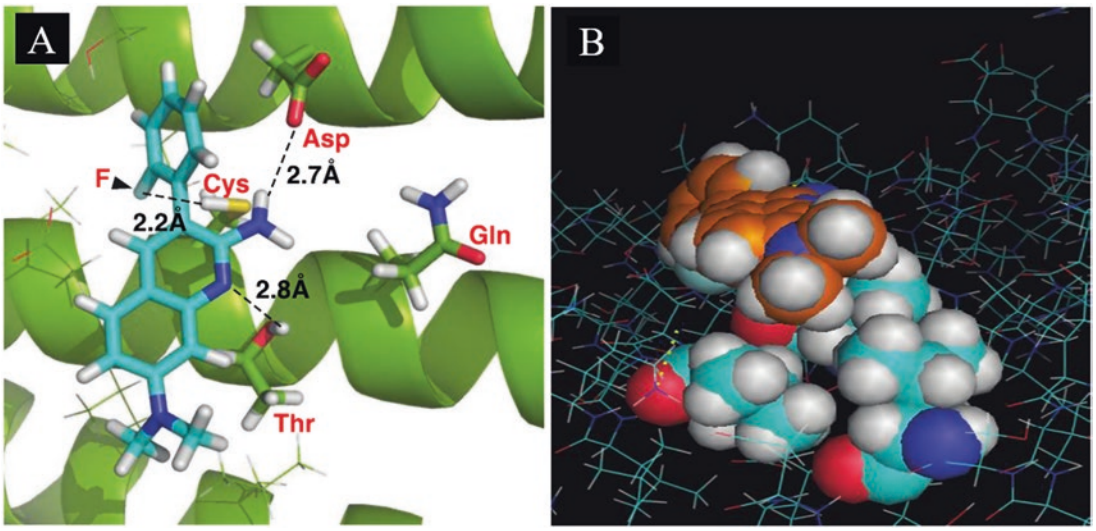

Fig. 11 Computational modeling of arylquin-1 bound to vimentin. Panel A: Binding of arylquin-1 to hydrophobic binding pocket of vimentin. Key stabilizing hydrogen-bonding or charge–charge interactions between 2′-fluorophenyl group and Cys105; C-2 amino group and Asp107; and nitrogen atom in quinoline ring and Thr 15 are shown with dashed lines. Panel B: Interaction of arylquin-1 with vimentin. Van der Waals interactions are depicted between C-7 *N,N*-dimethylamino group of arylquin-1 (in orange) and Leu58 and Val18 (in blue). The fluoro group in the C-3 phenyl group formed a stable, hydrogen bond to a Cys105 residue; the C-7 *N,N*-dimethylamino group interacted with a collection of hydrophobic residues (Leu58, Val18); the nitrogen within the quinoline formed a hydrogen bond to Thr15; and the C-2 amino group interacted with a proximal, acidic residue (Asp107)

outcome may result from the absence of part of the SAC domain (residues 137–195) that is the putative binding element for GRP78, but the restoration of secretion remains as a puzzling outcome.

We tested normal cells and diverse cancer cells for apoptosis driven by the administration of arylquin-1 at a concentration of 500 nM. Arylquin-1 induced a dose-dependent secretion of Par-4 from normal cells (Fig. 7a) with an approximate IC_{50} of 100 nM, and arylquin-1 induced the dose-dependent apoptosis in cancer cells but not in normal cells (Fig. 7b) at concentrations of 500 nM. This concentration of arylquin-1 triggered secretion of Par-4 from normal cells but not lung cancer cells but did not itself directly induce apoptosis in normal or cancer cells. In contrast, 500 nM concentrations of arylquin-1-induced apoptosis of prostate cancer (PC-3) cells and the derived PC-3MM2 cells were sensitive to apoptosis by Par-4. As expected, arylquin-1 had no effect on LNCaP or DU145 cells that were resistant to apoptosis by Par-4 [17, 76].

We tested co-cultures of normal cells with cancer cells for the apoptotic effect of arylquin-1 at a concentration of 500 nM because this concentration induced the secretion of Par-4 from normal cells but did not induce apoptosis in either normal or cancer cells. Arylquin-1 treatment of the co-cultures containing Par-$4^{+/+}$ MEFs and cancer cells resulted in apoptosis of the cancer cells relative to treatment with vehicle alone (Fig. 7c). Only the cancer cells, but not the normal human embryonic lung (HEL) fibroblasts, underwent apoptosis in these co-culture experiments. Again by way of contrast, arylquin-1 treatment of the co-cultures containing Par-4 deficient (*i.e.*, Par-$4^{-/-}$) MEFs and cancer cells did not induce apoptosis (Fig. 7d). Paracrine apoptosis in the cancer cells induced by Par-4 in response to arylquin-1 treatment appeared in Par-$4^{+/+}$ MEFs, but not Par-$4^{-/-}$ MEFs. Vimentin-deficient cells [123, 124] showed robust increase in secretion of pro-apoptotic Par-4 activity in the CM relative to their counterpart wild-type cells, and arylquin-1 did not further induce Par-4 secretion in these cells. In summary, vimentin sequestered Par-4 and prevented its secretion, and arylquin-1 bound to vimentin and thereby altered the vimentin-Par-4 association to facilitate Par-4 secretion. Vimentin represents a particularly important therapeutic target because of its elevation in diverse tumors and its causal role in EMT and metastasis [117]. Arylquin-1 may have additional targets other than vimentin that was identified in pull-down studies. A recent report describes the effects of arylquin-1 on lysosomal membrane permeabilization (LMP) and suggests a non-apoptotic pathway to cancer cell death involving the induction of LMP [125].

Finally, advancing small-molecule secretagogues like arylquin-1 would require more than just potency in promoting Par-4 secretion. In a brief but interesting examination of other, important pharmaceutical parameters, we compared the solubility, the intrinsic clearance by mouse and human microsomes and the inhibition of key cytochrome P450 monooxygenases (CYP) for arylquin-1 and two analogs in

which the C-2 amino group was replaced by either a C-2 chloro [126] (**21**) or thiomethoxy group [126] (**22**) (Fig. 4). The selection of these two analogs was based on our observations that they exhibited no inhibition of the hERG channel (IC_{50} *ca.* 100 μM) as compared to arylquin-1 with an IC_{50} for hERG inhibition of 3.74 ± 1.17 μM. However, only arylquin-1 exhibited modest solubility (3.1 μM) in comparison with either **21** or **22** that were insoluble, and only arylquin-1 possessed intrinsic clearances in mouse and human liver microsomes (205 and 312 μL/min/mg protein, respectively) that compared well with verapamil (282 and 155 μL/min/mg protein, respectively). Among the CYP enzymes that were examined, arylquin-1 at 10 μM concentration inhibited CYP2D6 (85%) but not CYP3A4 and CYP2C9. In summary, arylquin-1 held some promise as a leading candidate but additional SAR work will be required to improve further the pharmaceutical properties found in a clinical candidate.

5.6 Future Perspectives

Among the future directions for our interests in secretagogues lie three lines of investigation. First, we would like to define the precise binding site(s) of arylquins on vimentin as not only a fundamental issue but also as a means of guided SAR development of new arylquins. Secondly, we would like to evaluate structure–activity relationships in arylquins that were not, as yet, explored as a means of promoting Par-4 secretion to levels above that produced by arylquin-1. Thirdly, we seek to define the role of arylquin binding to vimentin on the EMT process and potentially develop agents that impede metastasis.

5.6.1 Arylquins and Vimentin Structural Studies

Monomers of vimentin associate into coiled-coil, dimeric structures that are more rigid than the flexible monomeric counterparts. The dimers form antiparallel tetramers and, eventually, higher order thick, elongated filament assemblies that are 16 dimers thick [127]. Although the exact structure of a fully assembled vimentin filament remains unknown, this assembly possesses a well-featured surface topography. Its overexpression in various cancers, such as prostate cancer, makes it an attractive, albeit difficult, target for drug development [117]. Initial studies suggested that arylquin-1 promoted the release of Par-4 sequestered on vimentin. We subsequently reported structure–activity studies of arylquins and related compounds to identify more potent arylquins than arylquin-1 for these binding studies, and we reported binding experiments between arylquins and a purified, rod domain of vimentin (residues 99–411) that probed the issue of one-site *versus* multiple-binding sites.

Computational modeling suggested that arylquin-1 bound to a single, putative site on a fragment of the vimentin rod domain (residues 328–406) that was competitive with the binding site of withaferin A and presumably with a recently reported 3-azidowithaferin derivative [67, 128, 129]. The "one-site model" suggested that the binding of arylquin **1a** involved three non-covalent interactions: a van der Waals interactions between the C-7 *N,N*-dimethylamino in arylquin-1 and Leu326 and Val330; a hydrogen-bonding interaction between the C-2 amino group in arylquin-1 and Asp331; and another hydrogen-bonding interaction between the *ortho*-fluorophenyl group in arylquin-1 and Cys328. We probed these computational predictions by evaluating the influence of structural modifications on new arylquins. The relative ratio of Par-4 secretion by arylquin-1 at 500 nM to vehicle was 3.5, and the newly synthesized arylquins were compared to this value. In general, modifications of the *ortho*-fluorophenyl group with other halogens at the *ortho*-position, with various halogens at the *meta*- or *para*-positions, and with multiple halogens including analogs with at least one fluoro group at the *ortho*-position results in arylquins with poorer secretory activity than the original arylquin-1 [126]. Secretion is, however, only one factor governing drug development, and replacement of the C-2 amino group in arylquin-1 with a chloro group (*i.e.*, 2-chloro-3-(2-fluorophenyl)-*N,N*-dimethylquinolin-7-amine) led to a secretagogue with potency equal to arylquin-1 but with a significantly increase, as desired, IC_{50} for activation of the hERG channel.

Suspecting that the one-site model for arylquin binding was inaccurate, we turned our attention to measuring directly the stoichiometry of arylquin binding to vimentin. Vimentin is a 53 kDa polypeptide comprised of 466 amino acids, with a highly conserved α-helical "rod" domain flanked by non-α-helical N-terminal head (77 residues) and C-terminal tail (61 residues) regions [130]. Together, vimentin monomers associate in parallel and in-register to form a coiled-coil that forms the basic structural building block for the entire intermediate filament family of proteins [131]. We tested whether arylquins bound the purified rod domain of vimentin (residues 99–411), which is a much larger portion of vimentin than the segment used for the computational modeling. The rod domain consists of helices interrupted by short loops, and it has been demonstrated to form a parallel, coiled-coil dimer splayed at both termini. This dimer is a basic building block of an intermediate filament formed by the full-length vimentin [68]. We titrated arylquin analogs at constant concentrations with the vimentin rod domain and monitored the intrinsic fluorescence of the arylquins. Binding of the vimentin rod

domain to arylquins was typically characterized by 2–three-fold enhancement in arylquin fluorescence. The intrinsic Trp fluorescence contribution to this fluorescence increase was insignificant. For example, arylquin-1 displayed activity in the Par-4 secretion assay bound to the vimentin rod domain with a K_d < 500 nM, but another arylquin, 7-chloro-3-(2-fluorophenyl)quinolin-2-amine, that did not induce Par-4 secretion did not bind the vimentin rod domain to a measurable extent. Beyond computational studies, the challenge before us is to find co-crystallization conditions to confirm some of these understandings and provide the level of structure-guided drug design that we require.

5.6.2 Arylquin SAR Development

Initial studies [114] identified the most active arylquins as those that possessed amino groups at C-2 and/or at C-7 and that possessed an *ortho*-fluorophenyl group at C-3. The synthesis of additional analogs [126] of arylquin-1 made use of either the Friedländer condensation [112, 113] of substituted *ortho*-aminobenzaldehydes and arylacetonitriles to acquire 3-arylquinolines or the Knoevenagel condensation [115] of the adduct between *ortho*-aminobenzaldehydes and α-arylacetyl chlorides to acquire the 3-arylquinolones. Similar condensations of 4-(*N,N*-dimethylamino)-2-aminobenzaldehyde (**16a**) with 2-arylacetonitriles in which the aryl ring possessed either an *ortho*-cyano- or an *ortho*-carboxylate-substituent led to the little-known, tetracyclic dibenzo[*b,f*][1,8]naphthyridine or dibenzo[*b,f*][1,8]naphthyridin-5(6*H*)-one, respectively. The arylquinolones provided also access to the thioquinolones, 2-thiomethoxyquinolines, 2-chloroquinolines, 2-(N-methylpiperazinyl)quinolines, and the 2-(morpholinyl)quinolines in a straightforward series of reactions. Additional SAR studies will explore modifications of other positions within the 3-aryquinolines and will examine the potential for replacing the *ortho*-fluorophenyl group with heterocyclic rings. Apart from testing their potency as Par-4 secretagogues, these efforts will also require pull-down experiments to confirm that vimentin remains as the exclusive target of these agents.

5.6.3 Arylquins and Metastasis

Our studies identified a novel secretagogue, arylquin-1 (**12**) (Fig. 4), that produced a dose-dependent secretion of Par-4 at nanomolar concentrations from normal lung fibroblasts and epithelial cells. We detected vimentin as a single primary target of arylquin-1 in pull-down experiments. Vimentin' importance rests with its elevated levels in tumors and its causal role in EMT and metastasis (Fig. 1). At low concentrations, arylquin-1 by itself does not kill either normal cells or most cancer cells, but arylquin-1 causes robust secretion of Par-4 from normal cells and produced apoptosis in cancer cells only when they were used in co-culture experiments with normal cells. These findings that implicate Par-4 secreted from normal cells in the apoptotic death of cancer cells were corroborated by the observation that arylquin-1-treatment of cancer cell co-cultures with Par-4-null normal cells failed to induce apoptosis of the cancer cells. Thus, arylquin-1 induces paracrine apoptosis in cancer cells *via* Par-4 secreted by normal cells. Because Par-4 produces apoptosis in diverse tumors and because there are no previously reported compounds that act at nanomolar concentrations to produce the levels of Par-4 secretion discovered in this study, our findings potentially have broad translational significance.

The in vitro or in vivo administration of 3-arylquinolines at nanomolar concentrations promoted high levels of Par-4 secretion by inhibiting the sequestering agent, the intermediary filament protein, vimentin (Fig. 1). This finding stimulated our interest in the synthesis and evaluation of additional Par-4 secretagogues because of vimentin's role in EMT during which cancer cells lose their cell–cell adhesion and metastasize. Within three years of either surgery or hormone treatment, a significant number of prostate cancer patients, for example, exhibit castration-resistant tumors that ultimately metastasize to bone, lung, liver, pleura, or adrenal glands, resist further chemotherapeutic treatment leading ultimately to fatal outcomes. Future efforts will further develop the 3-arylquinoline scaffold, will exploit FDA-approved quinolines, such as chloroquine [132] (CQ), and will explore other naturally occurring quinolines as potential Par-4 secretagogues. We will also study other mechanisms that effect Par-4 secretion. The antimalarial drug CQ, for example, induced Par-4 secretion in a p53- and Rab8b-dependent manner [132], and arylquin-1 and nutlin-3a were also dependent on p53 to induce Par-4 secretion. It would be of interest to identify other secretagogues might utilize a vimentin-independent mechanism for Par-4 secretion.

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Competing Financial Interests: David S. Watt and Chunming Liu hold an equity interest in a for-profit corporation, Epionc, Inc., founded for the purpose of advancing translational research involving antineoplastic agents. The co-inventors have complied with the University's policies regarding intellectual property disclosure and conflict of interest issues. The University of Kentucky has filed patent applications for compounds for which these authors are co-inventors.

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Recombinant Production and Characterization of Par-4/SAC

Fang Zheng and Chang-Guo Zhan

Abstract

Recombinant Par-4/SAC protein is capable of inducing apoptosis selectively in cancer cells without affecting the normal cells and, hence, has the potential to serve as a protein drug candidate for cancer treatment. Meanwhile, major challenges exist in the recombinant Par-4/SAC protein drug development, such as the low yield of recombinant protein production and the poor pharmacokinetic (PK) profile (with a short elimination half-life) of the protein. Nevertheless, encouraging progresses have been made during the last few years for improving both the yield of recombinant Par-4/SAC production and the PK profile. Particularly for improvement of the PK profile, the recently developed novel, long-lasting form of Par-4, known as Par-4Ex, is promising, because Par-4Ex has not only fully retained the unique anti-cancer activity of native Par-4, but also significantly prolonged the elimination half-life. As a result, Par-4Ex has a more potent in vivo anti-cancer activity compared to Par-4 itself. So, Par-4Ex may serve as a truly promising protein drug candidate for drug development moving forward. In addition, future effort to further prolong the elimination half-life may also include the structure-based rational design and testing of possible Par-4Ex mutants with improved binding affinity to neonatal Fc receptor (FcRn) in the acidic environment. In this way, the relatively longer elimination half-life of Par-4Ex may be extended further.

Keywords

Recombinant Par-4 · SAC · Anti-cancer activity · Par-4Ex · Pharmacokinetics · Human neonatal Fc receptor (FcRn) · Cancer · Lung tumors

1 Recombinant Par-4 or its SAC Domain as a Promising Therapeutic

Prostate apoptosis response-4 (Par-4) is known as a tumor suppressor protein expressed ubiquitously in a number of tissues. The Par-4 gene was first discovered as an early apoptotic gene in a rat prostate cancer cell line incubated with ionomycin for apoptotic cell death [1, 2]. It was demonstrated that overexpression of Par-4 is sufficient to elicit apoptotic cell death in most cancer cells [3]. In line with this observation, the Par-4 gene has been reported to be mutated in endometrial cancer [4], and significantly down-regulated in many different types of cancer including renal cell carcinoma [5], breast cancer [6, 7], gastric and pancreatic cancer [8], glioblastoma [9], and neuroblastoma [10]. Par-4 down-regulation is associated with tumor recurrence and diminished patient survival [7].

The core domain of Par-4 (including amino-acid residues 145–203 in human Par-4 or amino-acid residues 137–195 in rat Par-4), known as selective for apoptosis induction in cancer cells (SAC domain), serves as the effector domain responsible for its proapoptotic activity [11]. It has been known that the SAC domain is 100% conserved in mouse, rat, and human homologs, implying that Par-4 plays a critical role in the surveillance against tumors [11]. In fact, both mature Par-4 protein and its SAC domain are capable of inducing apoptotic cell death through both an intrinsic pathway (activated by intrinsic stimuli such as biochemical stress or DNA damage, and mainly modulated by Bcl-2 and Bax) [12] and an extrinsic pathway (activated in response to external stimuli such as Fas ligand) [13]. First, it was believed that

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Par-4 protein localizes and acts only in the cytoplasm and the nucleus for apoptosis induction [1, 14]. Subsequent studies revealed that Par-4 protein can be secreted to the extracellular space for action [2]. Extracellular Par-4 protein can induce apoptosis via FADD, caspase-8 and -3 activation following binding to the stress response protein, i.e., glucose-regulated protein 78 (GRP78), expressed on the surface of cancer cells [2]. It has also been demonstrated that exposure to purified recombinant Par-4 protein not only induces apoptosis in multiple types of cancer cells, but also inhibits tumor growth in vivo [3, 15].

Based on the above background, Par-4-related drug discovery may focus on development of either a small-molecule drug that can facilitate Par-4 secretion from normal cells [16] or an efficient method for producing recombinant Par-4/SAC protein as a protein drug [17]. Both the small-molecule drug and protein drug approaches are promising. For the small-molecule drug approach, because Par-4 co-localizes and binds with vimentin (a cytoskeletal intermediate filament protein), a small-molecule drug should be designed to disrupt the vimentin binding with Par-4 so as to facilitate Par-4 secretion from normal cells for Par-4-dependent inhibition of tumor growth [16]. Indeed, arylquin 1 [16], chloroquine (CQ) [18], and hydroxychloroquine (HCQ) [18] were discovered as strong inducers of Par-4 secretion from normal or cancer cells. In a drug repurposing effort, HCQ, an anti-malarial drug, has been tested in Phase 1 clinical trial for cancer treatment [19]. As hoped, eight of the nine patients treated with HCQ showed elevation of Par-4 levels in plasma and had tumors that exhibited TUNEL positivity, indicative of apoptosis. The Par-4 secretion correlated with apoptosis induction in patients' tumors [19].

For the protein drug approach, one will first need to produce the recombinant Par-4 or SAC protein in large scale. There have been efforts for the recombinant Par-4/SAC protein production [17, 21–23]. For another major hurdle of the practical application of the protein drug approach, recombinant wild-type Par-4 protein has a very short elimination half-life in the body because molecular weight (~38 kDa) of wild-type Par-4 protein is below the threshold (~40 kDa) for glomerular filtration so that it undergoes rapid clearance from the blood [17]. Thus, the anti-cancer activity of Par-4 may be diminished due to its limited serum persistence in vivo. It is well recognized that the practical therapeutic efficacy of a protein drug can be greatly increased by improving its pharmacokinetic (PK) profile [22–28]. One may reasonably suggest a possibility that the therapeutic efficacy of the recombinant Par-4 protein for cancer treatment can be improved by prolonging the elimination half-life of the protein. A recently reported study [17] aimed to prolong the elimination half-life, and encouraging progress has been made (see below for the detailed discussion).

This chapter is focused on the protein drug approach, particularly the production and characterization of recombinant Par-4/SAC protein in various forms. Below, we will first briefly discuss the progress of Par-4/SAC protein production research using various expression systems. Then, we will discuss how the elimination half-life of a therapeutic protein can be prolonged and summarize the progress achieved so far.

2 Recombinant Protein Production of Par-4 and SAC

2.1 Protein Expression in *Escherichia coli*

In earlier studies [2, 15] that aimed to understand the detailed molecular mechanisms concerning the anti-cancer activity of Par-4, recombinant Par-4/SAC was expressed in *Escherichia coli* (*E. coli*), usually with a tag on either the N- or C-terminus or with tags on both the N- and C-terminuses, as summarized in Table 1.

The commonly used protein fusion tags for producing the PAR-4/SAC protein to be used in basic research include His_6, glutathione *S*-transferase (GST), green fluorescent protein (GFP), and thioredoxin (TRX). The His-tag is used for

Table 1 Summary of expressed recombinant Par-4/SAC protein forms with a tag

Name	Structure (with references for studies producing the protein)
His-Par-4	A his-tag (usually 6 his residues) is fused to N-terminus of Par-4 [2, 17]
His-SAC	A his-tag (usually 6 his residues) is fused to N-terminus of SAC [2]
TRX-Par-4	A thioredoxin tag is fused to N-terminus of Par-4 [2, 15, 17]
TRX-SAC	A thioredoxin tag is fused to N-terminus of SAC [2, 15]
Par-4-GFP	A green fluorescent protein (GFP) tag is fused to C-terminus of Par-4 [2]
SAC-GFP	A GFP tag is fused to C-terminus of SAC [2, 21]
GST-Par-4	A glutathione *S*-transferase (GST) tag is fused to N-terminus of Par-4 [15]
GST-SAC	A GST tag is fused to N-terminus of SAC [15]
His-Par-4-GFP	A his-tag (usually 6 his residues) is fused to N-terminus of Par-4, and a GFP tag is fused to C-terminus of Par-4 [15]
His-SAC-GFP	A his-tag (usually 6 his residues) is fused to N-terminus of SAC, and a GFP tag is fused to C-terminus of SAC [15]
SUMO-SAC	A small ubiquitin-related modifier (SUMO) is fused to N-terminus of SAC [20]
His-SUMO-SAC	A his-tag (usually 6 his residues) is fused to N-terminus of SUMO-SAC [20]
Fc-Par-4	A fragment crystallizable (fc) region of IgG-1 is fused to N-terminus of Par-4 [17]

convenience of protein purification because a His-tagged protein may be purified most conveniently by using a HisPur™ Cobalt Resin or a HisPur™ Ni-NTA Resin. The GST tag is also used for convenience of protein purification using affinity chromatography because GST binds strongly and specifically to chromatography resins coupled with glutathione. The GFP tag is used for image analysis of the biodistribution.

Like His_6, the TRX tag is also popularly used in protein expression and purification. Particularly, attachment of a TRX tag to a protein of interest can help to improve the solubility of the protein because TRX is highly soluble in water. So, TRX is popularly employed as a fusion tag to avoid inclusion body's formation in recombinant protein production.

It should be noted that all the tags mentioned above are perfectly fine for using the tagged Par-4/SAC to test the protein functions and understand the molecular mechanisms. However, for the protein drug development, it is necessary to cleave the tag after the protein purification, unless the tag can provide some desirable function/property that the unfused protein does not have and it is sure that the tag does not cause any unwanted problems for use in humans.

2.2 Other Potentially Useful Protein Expression Systems

Based on the background discussed above, there is no question that recombinant Par-4 protein or its SAC domain has the desirable anti-cancer activity of a protein drug. However, the promising potential of Par-4 or SAC can be realized only when the recombinant protein can be produced in large scale. Indeed, the desirable large-scale production of Par-4 of its SAC domain is still a matter of concern [21].

Generally speaking, traditional protein expression methods include bacterial and mammalian cell expression. For the cell-based expression systems, the most widely used expression hosts include *E. Coli*, yeast, insect, plant, and mammalian hosts. So, if Par-4 or SAC cannot be expressed in *E. Coli* with a high yield suitable for large-scale protein production, there are many other options available for testing.

Notably, many other protein drug candidates also had the similar problem of large-scale production using a traditional method, and the problem was eventually solved by using an alternative method. For example, recombinant human butyrylcholinesterase (BChE) [29] is considered as a very promising protein drug candidate for treatment of organophosphorus (OP) poisoning associated with OP insecticides and chemical warfare agents, such as tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), and O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate (VX) [30–34]. On the other hand, it has been very challenging to express BChE and its mutants in a commercially feasible expression platform [35]. The low yield of protein expression of BChE in Chinese hamster ovary (CHO) cells would mean the high costs of the protein production for ultimate therapeutic treatment. It was highly desired to identify a low-cost and feasible, sustainable source of BChE production for practical application of BChE-based protein drug. Indeed, higher yields of recombinant BChE protein expression have been achieved with other systems, including transgenic goat [36, 37] and transgenic plant [36, 38, 39]. For example, for BChE expression in transgenic goat, the expressed BChE protein is secreted to milk. A very high yield (1–5 g/L) [36] of BChE protein was produced in the milk of transgenic goat. The transgenic goat-based protein production is scalable.

Similarly, rapid expression of a foreign protein in tobacco plants is also readily scalable for large-scale production with low costs [39, 40]. Interestingly, Mor et al. have demonstrated that tobacco plant can serve as an expression host for wild-type BChE [39, 40] and its mutants [41–44]. It has also been demonstrated that the Fc-fused BChE mutant can be expressed well in plant *Nicotiana benthamiana* leaves [45].

So, the similar transgenic animal/plant methods that have been used to successfully produce recombinant BChE (wild-type or mutant) and its fusion protein may also be proven useful to produce recombinant Par-4 or SAC in large scale.

2.3 Protein Expression in Plant

Indeed, for a proof-of-principle study on large-scale production of the SAC domain of Par-4, Sarkar et al. [21] reported the first successful production of SAC-GFP fusion protein coupled to translational enhancer sequence (5′ AMV) and apoplast signal peptide (aTP) in transgenic *Nicotiana tabacum* cv. Samsun NN plants under the control of a unique recombinant promoter M24. The stability and functionality of their tobacco plant-derived SAC-GFP protein was confirmed by various molecular analyses. Notably, retention to the endoplasmic reticulum (ER) was achieved by adding an ER retrieval signal (SEKDEL) to the C-terminus of the SAC-GFP construct and transiently expressed in the tobacco plant to obtain a glycosylated and proteolytically stable protein. According to their data, the expression of ER-targeted SAC-GFP-SEKDEL in infiltrated leaves reached the highest level at day 3 post-infiltration (dpi), with yields up to 88 mg/kg fresh leaves [21].

It should be noted that the obtained SAC-GFP-SEKDEL or SAC-GFP protein itself might not be practically useful as a protein drug although it can be used as a very valuable tool to further examine the therapeutic potential of SAC. Nevertheless, the successful expression of the SAC-containing fusion protein suggests that it is indeed possible

to pursue large-scale production of recombinant SAC or Par-4 protein in a tobacco plant such as *Nicotiana tabacum.*

2.4 Protein Expression in a SUMO Fusion System

Zhang et al. [20] developed a novel method for producing wild-type SAC in *E. coli* using a small ubiquitin-related modifier (SUMO) fusion construct. As a result, this SUMO fusion system greatly improved the solubility of the protein and enhanced the expression yield of the fusion protein (SUMO-SAC). Technically, a His_6-tag was attached to N-terminus of SUMO-SAC. Hence, they were able to purify the His-SUMO-SAC protein by using the commonly used Ni-NTA affinity resin.

Further, in an attempt to simplify the SAC protein purification process, Zhang et al. [20] purified His-SUMO-SAC and cleaved the His-SUMO tag using a single-step purification strategy. According to their strategy, a SUMO protease was incubated with the His-SUMO-SAC protein bound resin, which allowed the specific and controlled cleavage of the His-SUMO-SAC within the Ni-NTA affinity column to effectively release the untagged SAC into the collected solution. In this way, the SUMO (or actually His-SUMO) tag was removed and untagged SAC was purified in a single step, with the final SAC protein purity reaching 95% and the yield of purified recombinant SAC reaching 25 mg/L in flask fermentation. Their study has demonstrated that SUMO fusion is an effective approach to improve the yield of recombinant SAC production in *E. coli*.

3 Characterization of Recombinant Par-4/SAC

3.1 In Vitro Anti-Cancer Activity

Burikhanov et al. [2] first demonstrated that exogenous recombinant Par-4 and SAC, that were in the forms of TRX-Par-4 and TRX-SAC, respectively, produced in *E. coli*-induced apoptosis in diverse cancer cell lines, including PC-3, H460, and HeLa. This pioneering study confirmed that extracellular Par-4 and SAC can induce apoptosis in cancer cells and that recombinant Par-4 and SAC proteins have the desirable anti-cancer effects [2].

Sarkar et al. [21] demonstrated that the plant-derived SAC-GFP inhibited cancer cell growth and induced apoptosis in vitro. Specifically, they determined the biological activity of plant-derived partially purified SAC-GFP by using MTT assay (mammalian cells proliferation assay) on PC3, MAT-LyLu, and LNCaP cell lines at various concentrations (including 10, 20, 30, 40, 50, and 60μg/ml) for 48 h. According to their data, the SAC-GFP protein dose-dependently reduced the viability of PC3 and MAT-LyLu cells, and maximum cell death was observed up to 63% and 66%, respectively, at 60μg/ml SAC-GFP. It was determined that IC_{50} = 43μg/ml for SAC-GFP against PC5 cells and IC_{50} = 50μg/ml for SAC-GFP against MAT-LyLu cells. However, the SAC-GFP protein was relatively less effective for inhibiting LNCaP cell growth, as it exhibited only a maximum of 10–15% growth inhibition in LNCaP cells. In comparison, they observed that plant-derived SAC-GFP exhibited no cytotoxicity to HEK293 cell line, which is non-cancerous in nature. So, Sarkar et al. [21] confirmed that the plant-derived SAC-GFP is effective only against cancer cells, without cytotoxicity to the normal cell line.

Zhang et al. [20] tested their purified recombinant SAC domain (cleaved from the SUMO-SAC fusion protein) for its inhibitory activity against the growth of ovarian cancer cells SKOV-3, in comparison with HEK-293 cells using an MTT assay. Their data showed that the SKOV-3 cells exhibited typical apoptotic morphology in the nuclei after incubation with 200 nM SAC for 2 days. In comparison, no obvious change was observed on HEK-293 cell nucleus after incubation with 200 nM SAC for 2 days. Further, they demonstrated that the recombinant SAC inhibited the SKOV-3 cell growth in a dose-dependent manner, without cytotoxicity to the normal human cells (HEK-293) [20].

In an effort to test a novel protein entity of Par-4 (see below) in comparison with a wild-type Par-4 form (His-Par-4), Kim et al. [17] incubated E0771 (murine breast cancer cell line) cells with 100 nM recombinant His-Par-4 (100 nM) for 24 h, demonstrating that 100 nM recombinant His-Par-4 significantly induced apoptosis of the E0771 cells. So, all the previously reported recombinant Par-4 and SAC proteins have consistently demonstrated the promising in vitro anti-cancer activities.

3.2 In Vivo Anti-Cancer Activity

Zhao et al. [15] first tested the in vivo anti-cancer activity of a recombinant Par-4 or SAC, demonstrating that both recombinant TRX-Par-4 and TRX-SAC inhibited the growth of metastatic tumors. Specifically, LLC1 cells (1.5×10^5 cells) were injected into the tail vein of B6C3H mice. Five hours later, the mice were injected intravenously (IV via tail vein) with the recombinant protein (500μg/mouse). Four weeks later, the mice were sacrificed, and the lungs were photographed, showing that administration of the recombinant TRX-Par-4 or TRX-SAC protein significantly inhibited lung metastasis of LLC1.

Sarkar et al. [21] examined the in vivo anti-cancer activity of the plant-derived partially purified SAC-GFP protein. In their in vivo tests, MAT-LyLu cells pretreated and co-injected

with SAC-GFP protein or vector control (20μg in 100μl cell suspension) were injected into the flank region of male Copenhagen rats. Ten days later, tumor volumes were measured daily. According to their data, visible tumors were observed only in two of three rats injected with SAC-GFP pretreated MAT-LyLu cells on the 15th day of the cancer cell injection. In comparison, in all the rats injected with the vehicle control (VC) pretreated MAT-LyLu cells had a visible tumor by 6th day of the cancer cell injection.

In a more recently reported in vivo study by Kim et al. [17], the metastatic growth of tumor (E0771) cells (1.5×10^5 cells) were administered (IV via tail vein) in B6C3H mice ($n = 5$ per group). Five hours after administration, 250μg purified recombinant His-Par-4 (or vehicle control) was injected IV via tail vein every alternate day for 12 days (for a total of 1500μg of protein/mouse). Four weeks later, the mice were euthanized, and the lung nodules were then counted. The data revealed that the recombinant His-Par-4 protein (250μg per dose) was indeed very effective for inhibiting lung metastasis of E0771 breast cancer cells in the mice [17].

3.3 Pharmacokinetics

Development of a protein drug must pay close attention to the PK profile of the protein because the actual in vivo activity and therapeutic efficacy of a protein drug are dependent on its PK profile [17, 22–28]. Kim et al. [17] recently reported the PK profile of a recombinant Par-4 form (His-Par-4 or TRX-Par-4) in mice; see Fig. 1 for the PK profile of His-Par-4 (the PK profile of TRX-Par-4 was similar). The in vivo data were based on IV injection (via tail vein) of recombinant His-Par-4 to the mice. Based on the measured time-dependent concentration of His-Par-4 in mouse plasma,

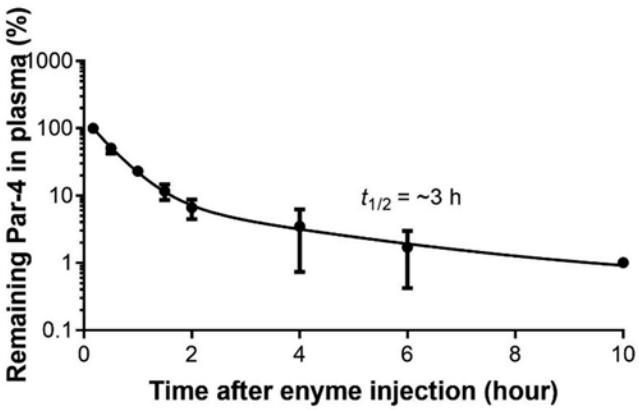

Fig. 1 PK profile of His-Par-4 in mice. His-Par-4 (5 mg/kg) was administered IV (via tail vein), and the serum protein concentrations were determined by ELISA (see original report of the original PK data [17]). Data are shown as mean ± SD

the Par-4 protein has an elimination half-life as short as ~3 h [17]. It would be necessary to improve the elimination half-life of Par-4 before it could be considered as a truly promising therapeutic protein candidate.

4 Fc-Fusion Protein to Improve the Pharmacokinetic Profile

As discussed above, in order for a protein to serve as a therapeutic, the protein must have not only the desirable biological activity and safety, but also a sufficiently long elimination half-life. Various strategies have been developed to improve pharmacological (PK) profiles of protein drugs. A popularly used one is to target human neonatal Fc receptor (FcRn). Below, we will first discuss the general strategy targeting FcRn, and then review the recently reported progress for its application to improvement of the PK profile of recombinant Par-4.

4.1 Human Neonatal Fc Receptor (FcRn) as a Promising Target to Improve Pharmacological Profile of a Protein Drug

Human neonatal Fc receptor (FcRn) for immunoglobulin G (IgG) is a 52-kDa heterodimeric glycoprotein bound on the membrane of endosome. It is composed of a heavy chain and a light chain named as β2-microglobulin (β2m) [46–49]. FcRn is expressed in human placenta and can transfer maternal IgG to the fetus or the newborn, providing humoral immunity for the first weeks of mammalian life [49, 50]. Further studies [51, 52] found that FcRn is expressed in the vascular endothelial cells, epithelial cells, hepatocytes, intestinal macrophages, peripheral blood monocytes, and dendritic cells. FcRn expression was also demonstrated at vascular endothelial cells in brain [53]. The primary function of FcRn is to maintain the long half-life of IgG in the plasma through binding with the Fc portion of IgG [46, 47, 54]. IgG is a class of antibody predominantly present in the normal human serum, and additional amount of IgG could be generated from the secondary response under continuous stimulation of an external pathogen [54, 55]. Without the binding with FcRn, IgG is circulated and degraded quickly through lysosomal degradation pathway, e.g., the half-life of IgG is reduced from 6–8 days to about 1 day in the FcRn-deficient mice [56].

IgG is internalized into FcRn-expressing cells most likely by fluid phase pinocytosis as the steady-state location of FcRn is endosomal [57]. IgG binds with FcRn in the acidic environment of endosome and is later transported to the cell surface where, upon exposure to a neutral pH, IgG is released

back to the main bloodstream. Such pH-dependence of FcRn-IgG binding ensures that IgG binds to FcRn in the intracellular acidic compartments but be released rapidly upon encountering the slightly basic pH 7.4 of the extracellular milieu [54, 56, 57].

Pursuing a longer half-life for a specifically designed therapeutic protein is of great significance and is a grand challenge in the area of protein drug design, discovery, and development [58, 59]. With a growing number of protein drugs being developed, the in vivo half-life extension strategies have attracted more and more attention by the biotech and pharmaceutical industries [60]. Compared to other in vivo half-life prolonging methods, Fc-fusion, i.e., genetically fusing the Fc part of IgG to a protein drug, has become the most clinically and commercially successful strategy with possibly enhanced efficacy, greater safety, and reduced immunogenicity or improved delivery [58, 60]. The Fc part of an Fc-fused protein can bind with FcRn like the Fc part of IgG1 binding with FcRn. So, the Fc-fused protein drug is expected to have a longer elimination half-life compared to the corresponding unfused protein drug. There are a number of marketed and clinical candidate antibodies and Fc-fusion proteins, such as Alefacept and Abatacept, that have successfully taken advantage of the FcRn-Fc binding [61–63]. More recently reported successful stories include long-acting cocaine hydrolases (CocHs) that are human Fc-fused BChE mutants [35, 64–66]. For example, fusion of CocH3 with a rationally designed mutant of IgG-1 Fc has improved the elimination half-life of the CocH3 from ~7 h to 107 h in rats [64].

As reviewed by Huang et al. [67], a number of X-ray crystal structures have been reported in literature, showing how wild-type FcRn and mutants of IgG from various species (human and rat) bind with Fc and various mutants and their pH-dependence. Depicted in Fig. 2 are some representative structures. It has been demonstrated that the overall conformation of FcRn is persistent, indicating that the pH-dependence of FcRn-Fc binding is not mediated by the conformational change of FcRn, but possibly by the electrostatic interactions involving histidine amino acids. Available X-ray crystal structures of rat FcRn bound with rat Fc revealed that the hinge region of Cγ2–Cγ3 domain of Fc binds to the top of α1 and α2 helices of FcRn. The hinge region of Fc was demonstrated to be a consensus site of recognition by a series of proteins associated with Fc [68]. According to available X-ray crystal structures of IgG1 Fc and the M38Y/S40T/T42E mutant [69] under acidic pH condition, the overall shape of Fc is similar to that of a "horseshoe," and most of the internal space is filled with oligosaccharide chains through residue N83 (here we renumbered Fc residues and ignored other portion of IgG1 for convenience). The IgG1 Fc is a homodimer (with two identical subunits) linked by disulfide bridges in the N-terminal region and non-covalent interactions between the C-terminal regions of the two subunits, and each subunit is comprised of two immunoglobulin domains known as Cγ2 and Cγ3. The Cγ2 domain of Fc can take large extent of rigid body motion, leading to a "closed" conformation of Fc when residue N83 is totally unglycosylated and an "open" conformation when residue N83 is fully glycosylated. The distance between the end points of the Cγ2 domains of the two subunits of Fc varies from 10 Å for the "closed" conformation to 14 Å for the "open" conformation.

Interestingly, a large number of Fc mutants have been generated and fused with different effector proteins in order to explore the relationship between the FcRn-Fc binding affinity and the elimination half-life of an Fc-fused protein, as discussed by Huang et al. [67] The reported mutational studies also revealed how the change in the FcRn-Fc binding affinity affects the PK profile of the Fc-fused effector protein, and how to control the safety profile such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDCC). It is also known [67] that there is a dramatic cross-species difference in the affinity of FcRn-Fc binding. At acidic pH, mouse FcRn has a high affinity with either mouse IgG1 or human IgG1, whereas human FcRn has a very low affinity with mouse IgG1. At physiological pH 7.4, mouse FcRn keeps micromolar (μM) range of binding affinity with human IgG1, but no detectable binding with mouse IgG1. These studies established that the Fc mutants with a higher FcRn affinity under acidic pH condition usually have a longer in vivo (elimination) half-life when fused with an effector protein, and a lower cytotoxicity.

The above knowledges about the detailed structures of FcRn and its binding with Fc and various mutants have provided a solid structural and mechanistic base for rational design of Fc-fusion proteins and their mutants with improved binding affinity to FcRn in an acidic environment in order to extend elimination half-life of a therapeutic protein. In fact, in order to prolong the elimination half-life and, thus, decrease the required frequency of the enzyme administration for cocaine use disorder treatment, Zheng et al. [66] carried out a structure-based rational design of a novel CocH-Fc entity by modeling various Fc-fusion CocH (cocaine hydrolase) proteins for its binding with FcRn. The computational modeling studies led to the design and testing of CocH3-Fc(M6), a CocH3-Fc mutant with nearly 100-fold improved binding affinity: from K_d = ~4μM to K_d = 43 nM. As a result, CocH3-Fc(M6) indeed revealed a markedly prolonged elimination half-life ($t_{1/2}$ = 206 ± 7 h or ~9 days) in rats [66], longer than other known Fc-fusion protein drugs such as abatacept and alefacept (for other therapeutic purposes) in the same species (rats). It has been demonstrated that a single dose of 3 mg/kg CocH3-Fc(M6) completely blocked the discriminative stimulus and reinforcing effects of cocaine for

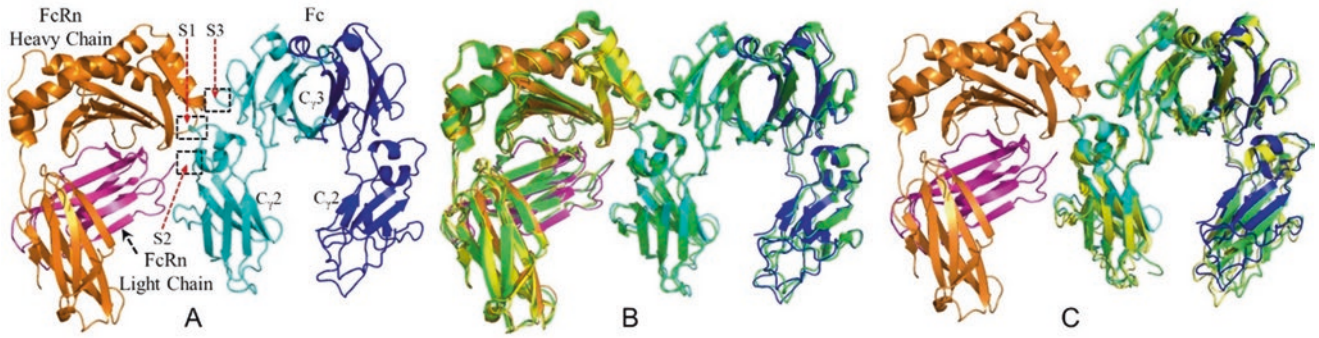

Fig. 2 The energy-minimized structure of human FcRn-Fc binding complex (based on data reported by Huang et al. [67]) in comparison with reported X-ray crystal structures of ligand-free human FcRn and human Fc. (**a**) The heavy chain of human FcRn is represented as orange ribbon, and magenta ribbon for the light chain (β2m) of human FcRn. Human Fc is also represented as ribbon colored in cyan for one subunit and blue for another subunit. The binding interface between human FcRn and human Fc is roughly represented as three subsites (S1, S2, and S3) as labeled. (**b**) Human FcRn-Fc complex superimposed with that of rat FcRn-Fc complex (PDB code 1I1A, green ribbon), ligand-free human FcRn (PDB code 3 M17, yellow ribbon); only Cα atoms were used in the superimposition. The positional RMSD for Cα atoms between human FcRn-Fc structure and the rat FcRn-Fc structure is 0.96 Å, and the RMSD between the human FcRn in human FcRn-Fc complex and the ligand-free human FcRn is 0.79 Å. (**c**) Human FcRn-Fc binding complex superimposed with two typical ligand-free Fc structures. One ligand-free human Fc structure corresponding to PDB code 3AVE (green ribbon) has a positional RMSD value of 1.55 Å for the Cα atoms. Another ligand-free human Fc structure corresponding to PDB code 1H3Y (yellow ribbon) has an RMSD value of 1.85 Å. Obvious differences appeared at the relative position of the Cγ2 domain of each subunit of human Fc dimer

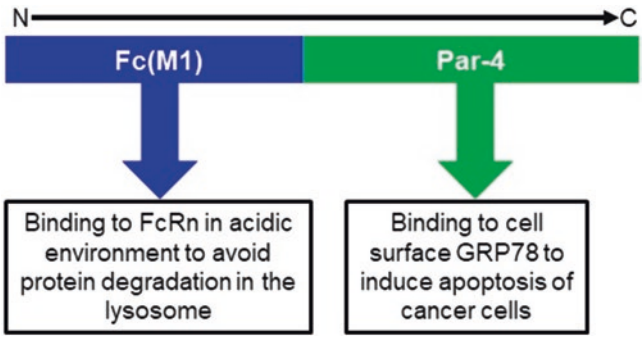

Fig. 3 Par-4Ex protein fusion and functions of its Fc(M1) and Par-4 domains. The Fc(M1) (Fc mutant) was fused to N-terminus of Par-4

24/25 days and continued to significantly attenuate/decrease the cocaine effects for at least 29 days in rats [70]. Reported by Zheng et al. [66] was the first attempt to rationally design a long-acting Fc-fusion enzyme mutant based on combined computational modeling and experimental measurement of the Fc-fusion CocH binding with FcRn. The similar structure-based design strategy may be used to prolong the biological half-lives of other Fc-fusion protein drugs including Par-4 (see below).

4.2 Fc-Fusion of Par-4 to Generate a New Protein Entity Par-4Ex

The first attempt to improve the PK profile of Par-4 through Fc-fusion was reported recently [17]. Specifically, Kim et al. [17] have rationally engineered Par-4 to prolong the elimination half-life and investigated the potential application of an engineered form of Par-4 protein as a novel therapeutic agent for cancer treatment.

As mentioned above, recombinant Par-4 has a very short elimination half-life (see Fig. 1), and the practical therapeutic efficacy of a protein drug can be greatly improved by extending the elimination half-life [22–28]. To address this issue, Kim et al. [17] extended the sequence of wild-type Par-4 and, thus, designed a relatively larger protein (denoted as Par-4Ex which means the Par-4 with extended sequence), with the goal to prolong the elimination half-life of Par-4 without changing its biological function. Par-4Ex is an Fc-fusion protein, with a mutant of Fc, denoted as Fc(M1), of IgG-1 attached to N-terminus of Par-4 (see Fig. 3 for the construct and functions) [17]. The Fc(M1) domain of Par-4Ex was designed to bind with FcRn under acidic environment (pH 6), while the Par-4 domain is expected to bind with GRP78 on cancer cell surface.

Scpecifically, in principle, for rational protein engineering, rational design of a desirable Par-4Ex as a therapeutic candidate must account for several critical issues. First, the molecular weight of a desirable Par-4Ex must be significantly higher than that of Par-4 (~40 kDa). Second, the extra amino-acid residues of the extended protein could prevent it from binding with GRP78 and, hence, make the extended protein (Par-4Ex) inactive against cancer cells. With this issue in mind and considering that the SAC domain is closer to the C-terminus, it would be better to add the extra amino-acid residues (Fc or Fc mutant) to N-terminus of Par-4.

Further, with the goal to prolong the biological half-life of Par-4, one also wants to avoid the possible immunogenicity of the extended protein (Par-4Ex) for human. For this reason,

it would be the best option that the extra amino-acid residues come from a human protein fragment, but without the unnecessary biological function of the protein. The first human protein fragment candidate in mind [17] was the first 233 amino-acid residues, known as the fragment crystallizable (Fc), of human immunoglobulin G1 (IgG1). In fact, as discussed above, protein fusion with the Fc region of human IgG1 (IgG1 Fc) is one of the most popularly used strategies to prolong the elimination half-lives of protein therapeutics [71, 72] although a recently reported study did reveal that the Fc-fusion did not improve the elimination half-life of a protein drug candidate expressed in *E. coli* [64]. However, as well-known, wild-type IgG1 Fc will form a dimer through intermolecular disulfide bonds. The dimerization of Par-4Ex could block the interface of the intermolecular binding between Par-4 and GRP78 and, thus, be risk of losing the binding affinity of Par-4 with GRP78. With this concern, three cystine residues (#6, #12, and #15) of the Fc region are all changed to serine residues to avoid the possible dimerization of Par-4Ex [17]. For convenience, the mutant Fc was denoted as Fc(M1) which refers to the first mutant (mutant 1 or M1) of Fc tested in the reported study [17]. Accordingly, the first (and only) Par-4Ex protein reported was also known as Fc(M1)-Par-4 [17].

The Par-4Ex of Fc(M1)-Par-4 protein was generated and produced using an *E. coli* expression system. This fusion protein can be purified very conveniently by using protein A [17]. So, purification of the *E. coli*-derived soluble Par-4Ex protein was performed using protein A affinity chromatography, followed by an additional ion-exchange chromatographic step to achieve the high purity (see Fig. 4, left panel) [17].

As discussed below, the Par-4Ex protein indeed has the same anti-tumor activity against the cancer cells, but with a significantly prolonged elimination half-life, leading to the improved potency in inhibiting metastatic tumor growth in vivo. Based on the reported in vitro and in vivo data, the newly designed and tested Par-4 entity (Par-4Ex) may be developed as a potentially promising protein therapy for cancer treatment.

4.3 In Vitro Anti-Cancer Activity of Par-4Ex

To test the in vitro activity of Par-4Ex in comparison with Par-4 [17], both Par-4Ex and Par-4 proteins were prepared using the *E. coli* expressing system under the same experimental conditions. Before in vivo testing of the purified recombinant Par-4 and Par-4Ex proteins, one first needed to know whether Par-4Ex retains the unique proapoptotic activity of Par-4 or not, because the Fc(M1) fusion to the N-terminus of Par-4 could block or interfere the protein–protein interactions between Par-4 and GRP78. To address this crucial question, E0771 (murine breast cancer cell line) cells were treated with 100 nM His-Par-4 or Par-4Ex protein, followed by incubation for 24 h. Storage buffer (50 mM Hepes, 20% sorbitol, 1 M glycine, pH 7.4) was used as a vehicle control. It was observed that Par-4Ex and His-Par-4 proteins induced a similar level of apoptosis in E0771 cells in a given treatment condition (Fig. 4, right panel), demonstrating that the anti-tumor activity of Par-4 protein was not lowered by the Fc(M1) fusion to its N-terminus. This observation of the anti-tumor activity of Par-4 protein itself is consistent with the findings of Zhao et al. [15] using TRX-Par-4. The study reported by Kim et al. [17] further demonstrated that Par-4Ex was as active as Par-4 itself in the cell-based anti-tumor activity assays.

4.4 Pharmacokinetics of Par-4Ex

To examine whether Par-4Ex really has a prolonged elimination half-life compared to Par-4, a pharmacokinetic (PK) study was carried out on Par-4Ex in comparison with His-Par-4 in mice. The PK data were based on IV injection of each recombinant protein in the tested mice. The generated PK data are depicted in Fig. 5. As expected, the PK data revealed that Par-4Ex has a much longer elimination half-life (~20.3 h) compared to that (~3 h) of His-Par-4 protein.

4.5 In Vivo Anti-Cancer Activity of Par-4Ex

To examine whether the longer exposure due to the elimination half-life extension can really improve the in vivo anticancer activity, recombinant Par-4Ex was evaluated in comparison with recombinant His-Par-4 for their inhibitory activity against lung metastasis of E0771 breast cancer cells in immunocompetent C57/BL6 mice [17]. The cells (1.5×10^5 cells) were injected IV through tail vein, and then *E. coli*-derived Par-4Ex (250, 150, or 75μg) or His-Par-4 (250μg) was administered IV every other day for 12 days (for a total of 1500 or 900 or 450μg per mouse within 12 days). According to the reported in vivo data, both Par-4Ex and His-Par-4 at the high dose (250μg/injection) significantly suppressed metastatic tumor growth in vivo compared to vehicle-treated control. Notably, Par-4Ex at the lower doses (150 or 75μg/injection) also significantly suppressed metastatic tumor growth in vivo compared to vehicle-treated control. In comparison, earlier studies [15] examined various doses of the unfused Par-4 (TRX-Par-4) for its in vivo activity using the same animal model under the same experimental conditions in the same lab, and found that 250μg/injection was the minimum effective dose; lung metastasis by E0771 breast cancer cells was not significantly reduced by the Par-4 injection at any of the tested doses lower than 250μg/injec-

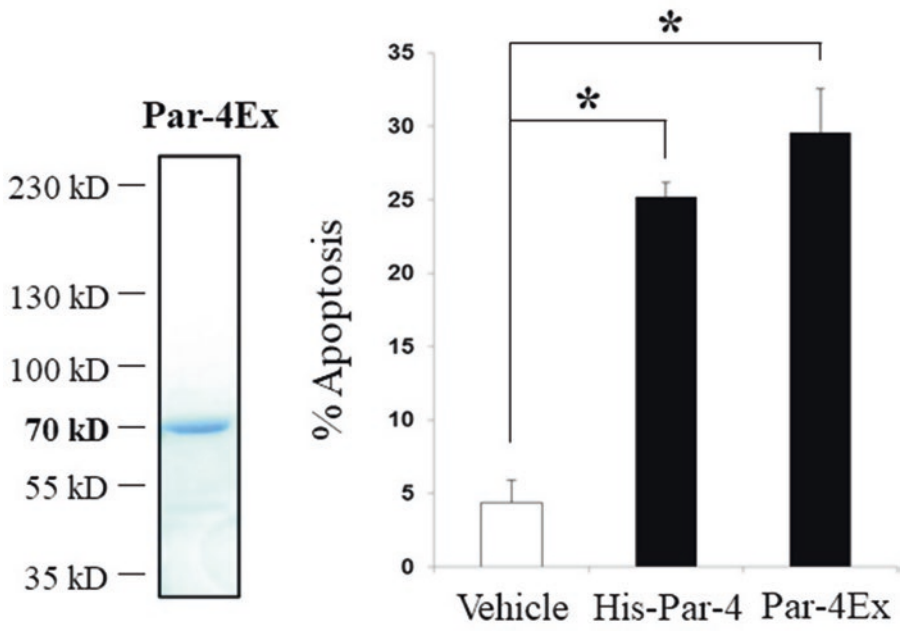

Fig. 4 Recombinant His-Par-4 and Par-4Ex proteins elicit apoptosis in E0771 (murine breast cancer cell line) cells (based on the data reported previously [17]). Left panel: SDS-PAGE of the purified Par-4Ex. Right panel: The cells were treated with purified Par-4 (His-Par-4) or Par-4Ex (100 nM each) or vehicle. 24 h after treatment, the cells were scored for apoptosis by immunocytochemistry (ICC) for caspase 3 activity. Results represent the average of triplicates and the values are expressed as mean ± SD. Asterisk (*) indicates the difference is statistically significant ($p < 0.05$) by Student's *t*-test

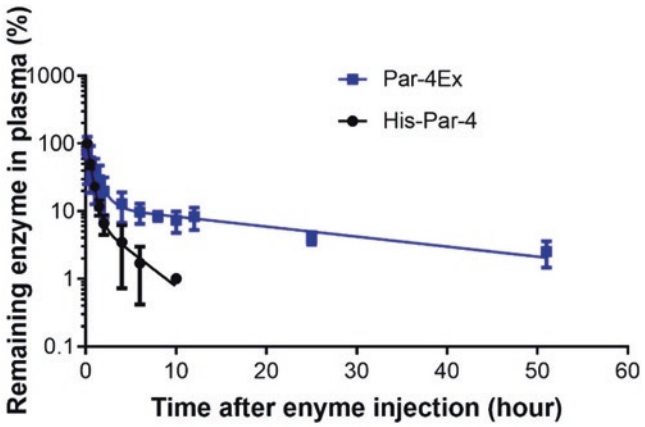

Fig. 5 Serum concentration (%) versus time profiles of recombinant His-Par-4 and Par-4Ex proteins in mice (based on the data reported previously [17]). Par-4Ex or His-Par-4 was administered via IV administration of 5 mg/kg and the serum protein concentrations were determined by ELISA. The data are shown as mean ± SD

tion. Taking all these together, Par-4Ex indeed has an improved in vivo potency than the unfused Par-4 in inhibiting metastatic tumor growth.

5 Concluding Remarks: Summary and Future Perspective

It has been known that recombinant Par-4/SAC is capable of inducing apoptosis selectively in cancer cells without affecting the normal cells. Hence, recombinant Par-4/SAC has the potential to serve as a protein drug candidate for cancer treatment. On the other hand, in general, development of a practical protein drug is generally very challenging, as one must account for multiple other challenging issues beyond the usually considered in vivo potency and toxicity in academic research. For the recombinant Par-4/SAC protein drug development, the specific hurdles that must be overcome include the low yield of recombinant protein production in the widely used *E. coli* expression system and the poor PK profile (with a short elimination half-life) of the protein. Nevertheless, encouraging progresses have been made for improving both the yield of recombinant Par-4/SAC production and the PK profile.

Particularly, for improvement of the PK profile, the recently developed novel, long-lasting form of Par-4, known as Par-4Ex, is promising, because Par-4Ex has not only fully retained the unique anti-cancer activity of native Par-4, but also significantly prolonged the elimination half-life. As a result, Par-4Ex has a more potent in vivo anti-cancer activity compared to Par-4 itself. So, Par-4Ex may serve as a truly promising protein drug candidate for drug development moving forward. The next steps of the Par-4Ex-based protein drug development will include the Par-4Ex protein manufacturing process development including establishment of a master cell bank (MCB) capable of efficiently expressing Par-4Ex, current good manufacture practice (cGMP) protein production, investigational new drug (IND) testing, and, ultimately, clinical trials (Phases I–III). The development of MCB and cGMP protein production should be conducted best through a specialized contract research organization (CRO) capable of routinely manufacturing a therapeutic protein according to pharmaceutical industrial standards.

In addition, future effort to further prolong the elimination half-life, if desirable, may include the structure-based

rational design and testing of possible Par-4Ex mutants with improved binding affinity to FcRn in the acidic environment. In this way, the relatively longer elimination half-life of Par-4Ex may be extended further.

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Role of PAR-4 in Ceramide-Inducible Effects in Neurodegeneration

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Abstract

Recently, the idea that alterations in sphingolipids metabolism contribute to the pathogenesis of various neurodegenerative diseases has been gaining vast acceptance. Especially ceramide, the precursor of all complex sphingolipids, is thought to be a key sphingolipid metabolite and lipid second messenger. For several decades, research on sphingolipids related to neurodegenerative disease focused on myelin constituents and lipid storage diseases. However, recent advances in methods of mass spectrometric lipid analysis (lipidomics) have greatly advanced the knowledge of sphingolipids in neurodegenerative and psychiatric disorder. This was concomitant with the recognition of ceramide as a as key player in lipid cell signaling and regulation of cell death and survival. There is evidence that ceramide is invoked in a myriad of cellular processes related to neurodegeneration such as autophagy, ER stress, mitochondrial dysfunction, and exosome secretion which leads to neurotoxic protein spreading. However, it is still not clear which additional factors may interact with ceramide to determine the specific outcome of a particular ceramide-induced cell signaling pathway. Several studies have shown that interaction with prostate apoptosis 4 (PAR-4) regulates these cell signaling pathways, particularly for the induction of neuronal apoptosis during neurodegenerative disease. We will discuss the function of PAR-4 in the regulation of ceramide neurotoxicity and neuronal apoptosis induced by intrinsic ceramide and PAR-4/ceramide originating in other cells and transported to neurons via extracellular vesicles.

Keywords

Apoptosis · Sphingolipids · Ceramide · Exosomes · Alzheimer's Disease

1 Ceramide

Sphingolipids (SLs) comprise a large group of amphipathic lipids which are abundant in cellular membranes [1–4]. The term "sphingolipids" was coined by J.L.W. Thudichum in 1884 to mark the "sphinx" (mystical)-like nature of the molecules [5–8]. Ceramide is the center component of all complex sphingolipids and its structure consists of a sphingosine long-chain base linked to a fatty acid through an amide linkage [9]. Ceramide can be produced via two distinct pathways; the anabolic pathway and the catabolic pathway, also referred to as de novo and salvage pathways, respectively [10–16] (Fig. 1). The de novo pathway is initiated by the condensation of serine and palmitoyl-CoA to produce 3-ketodihydrosphinganine, catalyzed by serine palmitoyl transferase (SPT). 3-ketodihydrosphinganine is then reduced to sphinganine (Dihydrosphingosine) by 3-ketodihydrosphingosine reductase. Sphinganine is N-acylated to dihydroceramides (DHCer) by ceramide synthases (CerS), a family of acyl-CoA transferases that controls the fatty acyl chain length. There are six established CerSs in mammalian cells, with CerS1 being the most abundant one in neurons, specifically attaching C18 fatty acyl-CoA to the sphingoid base [12, 15, 17–22]. Finally, ceramide formation is completed by dihydroceramide desaturase through desaturation of DHCer, which introduces a 4,5-trans double bond at the sphinganine base of DHCer. On the other hand, the salvage pathway re-utilizes long chain sphingoid bases, mostly derived by hydrolysis from sphingomyelin to produce ceramide.

Sphingomyelin is found in ample amounts in cell membranes, subsequently playing a crucial role in membrane fluidity and homeostasis [23–31]. Sphingomyelin is a substrate

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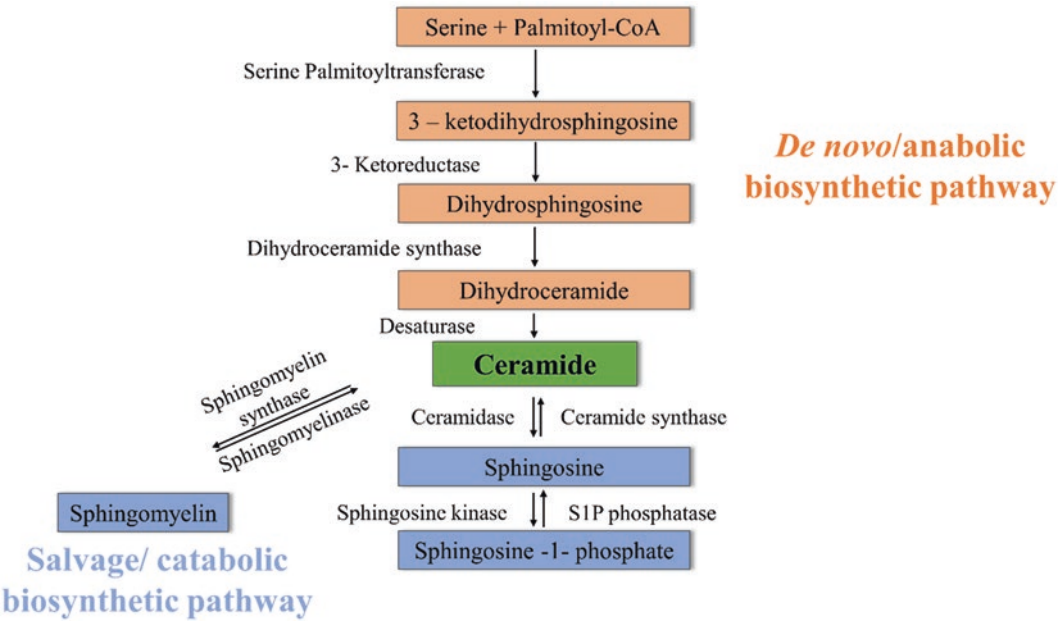

Fig. 1 Ceramide is at the core of sphingolipid metabolism. The anabolic/de novo biosynthetic pathway of ceramide starts in the endoplasmic reticulum by the action of the enzyme serine palmitoyltransferase (SPT), mainly using serine and palmitoyl-CoA as substrates, leading to ceramide generation after sequential enzymatic reactions. The catabolic pathways involve hydrolysis of sphingomyelin, ceramide-1-phosphate and glycosphingolipids, resulting in the formation of ceramide

for a family of enzymes called sphingomyelinases (SMases) which hydrolyze sphingomyelin into ceramide and phosphocholine. There are several SMases, and they can be generally divided into two groups, namely, acid and neutral SMases, depending on their subcellular localization, pH optima, and cation requirements for their enzymatic activity [32–37]. It has been speculated that neutral sphingomyelinase 2 (nSMase2), the predominant SMase in neurons, is present in an inactive form under normal conditions [38]. Upon stimulation with different extracellular factors and intracellular processes, including the proinflammatory cytokines tumor necrosis factor α (TNF-α), interleukin 1β, interleukin 6, and oxidative stress, nSMase2 in the plasma membrane gets activated and facilitates ceramide production and the downstream effects [31, 39–44]. In addition, nSMase2 has been shown to partake in proinflammatory cytokine signaling, associating the enzyme with the progression of several neurodegenerative diseases [34, 42, 45, 46].

Ceramide is a crucial bioactive molecule that has been implicated in mediating several cellular processes. For instance, ceramide has emerged as an important effector in development and stress responses, cell survival and proliferation, autophagy regulation, senescence, and mediating the crosstalk with apoptosis [13, 23, 37, 47–68]. Ceramide levels are tightly regulated through many enzymes and compensatory feedback mechanisms. Alterations in ceramide levels or metabolism have been linked to numerous neurodegenerative diseases including epilepsy, Parkinson's disease, Huntington's disease, Gaucher's disease, Krabbe's disease, and Alzheimer's disease (AD) [21, 50, 54, 56, 69–89]. Being a bioactive lipid, ceramide is known to interact with several proteins to facilitate several aspects of cellular processes and signal transductions, suggesting that ceramide acts as a signaling molecule. However, the precise molecular mechanisms by which ceramide regulates these processes are not well defined yet. One suggested mechanism implicates ceramide in stabilizing lipid rafts, which act as platforms for the concentration of signaling molecules [23, 90–101]. Another mechanism is via direct interaction with target proteins. So far, a few proteins have been reported to directly interact with ceramide including the protein kinase c-Raf and kinase suppressor of Ras (KSR) [102, 103], protein kinase C-ξ [104–113], protein phosphatase 2/inhibitor 2 of protein phosphatase 2A (I2PP2A), protein phosphatase 1 [114–120], and cathepsin D [121]. A third mechanism involves ceramide acting as a "sensitizer" to prostate apoptosis response 4 (PAR4) [110, 122–124].

Several lines of research described roles for ceramide and PAR-4 in neuronal development, differentiation and apoptosis, each participating in the beforementioned processes via distinct mechanisms [122, 123, 125–133]. However, the convergence of both molecules' effects on neurons seems to happen by two—potentially complementary - mechanisms: cis (direct) and trans (indirect) effects. In the cis or direct fashion, e.g. during neural progenitor cells self-renewal and differentiation, upregulation of ceramide serves mainly two

purposes. Firstly, the induction of apoptosis by its interaction with PAR-4, e.g. in excess progeny cells. Secondly, ceramide (in the absence of PAR-4) promotes differentiation and process formation in the surviving daughter cells [124, 134]. It has been speculated that cell fate decisions following neural progenitor cells division depend on the asymmetric distribution of proteins that are either synergistic to or protect from ceramide-induced apoptosis (for detailed discussion please refer to Chap. 8). As for the trans or indirect effect of ceramide and PAR-4 on neurons, it may be mediated through the role of ceramide in the biogenesis, secretion, uptake, and intraneuronal effect of small extracellular vesicles (EVs), particularly exosomes [92, 135–142]. These lipid vesicles facilitate the transfer of many biological molecules from one cell to another [143]. Pertinent to the ceramide/PAR-4 indirect effect on neurons, it has been reported that astrocytes secrete ceramide-enriched and PAR-4-containing exosomes that can be transferred to nearby neurons [122].

2 Ceramide as a Key Player in Neurodegeneration

As mentioned earlier, neurodegeneration is commonly associated with an alteration of sphingolipid metabolism and composition, for both acute and chronic diseases. Among the first responses in many acute injuries such as stroke, concussion, spinal cord injury (SCI), or traumatic brain injury (TBI) is an activation of SMases and glycosidases leading to elevation of ceramide and glycolipid levels [52, 144–149]. In particular, the neuronal ceramides C18:0 and C18:1, which are synthesized by CerS1, are dysregulated within 24 h after the insult. Given the lack of reliable and accurate biomarkers to determine the presence and severity of TBI, this elevation of ceramide levels could serve as a biomarker within a few hours of the blast or impact. The feasibility of accurately measuring ceramide levels via mass spectrometry makes plasma analysis an attractive approach for TBI diagnosis and treatment tracking.

In the context of AD, ceramide has been shown to have a multifaceted role. Due to advances in mass spectrometric methods, several groups were able to show upregulated ceramide levels in the cerebrospinal fluid (CSF), serum, and brains tissues of AD patients [150–153]. In addition, levels of ceramide in plasma have been shown to directly correlate with brain hippocampal volume in late onset AD patients. In a prospective cohort study, Mielke and colleagues followed 99 cognitively normal older women for 9 years as a part of The Women's Health and Aging Study ‖. According to their data, there is a solid relationship between higher baseline serum ceramide levels and the risk of developing dementia and AD, posing serum ceramide as a candidate biomarker for preclinical AD. More specifically, certain ceramide species were proven to be elevated in aging and AD, such as Cer16, Cer18, Cer20, Cer24, and Cer24:1 [151]. On a cellular level, endogenous ceramide within lipid rafts in the plasma membrane, as well as the exogenous ceramide analog C6-ceramide, were shown to directly increase the production of Aβ, the main pathologic protein in AD, by stabilizing the β-site of amyloid precursor protein (APP) cleaving enzymes [154]. Moreover, the levels of both ceramide and Aβ appear to be upregulated through a feed forward mechanism. Exposure to Aβ activates nSMase2, leading to increasing the levels of ceramide and ultimately enhancing the production of Aβ. Concomitantly, Aβ can indirectly increase the production of ceramide through an oxidative stress-mediated mechanism [155, 156].

Another area where the involvement of ceramide gained attention is the biogenesis and formation of exosomes. The work of Trajkovic et al. [149] showed for the first time that the ceramide production pathway is essential for exosomes formation. In general, exosomes can be formed though endosomal sorting complex required for transport machinery (ESCRT)-dependent and ESCRT-independent pathways [157, 158]. In the ESCRT-independent pathway, two enzymes involved in lipid metabolism have been shown to drive the formation of intraluminal vesicles (ILVs) in the lumen of multivesicular bodies (MVBs). Phospholipase D2 (PLD2) hydrolyzes phosphatidylcholine into phosphatidic acid (PA) at the inner leaflet of late endosome membranes, utilizing the negative charge of PA that derives inward budding of the ILVs inside MVBs [159]. The second enzyme involved in ESCRT-independent exosomes biogenesis is neutral nSMase2 [149]. nSMase2 hydrolyze sphingomyelin to produce ceramide [9], a cone-shaped sphingolipid that facilitates spontaneous curvature of membranes leading to invagination and budding of exosomes into the late endosomes (MVBs) [149].

Exosomes formed through the nSMase 2 pathway are packaged with misfolded proteins, which might provide an efficient way for cellular uptake during different neurodegenerative diseases [160]. Earlier reports indicated that exosomes function as a carrier of various pathogenic proteins that are causative of impaired neuronal functions in various neurodegenerative diseases. These proteins include Aβ, p-tau, Parkin, prion proteins, and alpha synuclein [136, 137, 161, 162]. Exosomes are known to cross the blood–brain barrier (BBB) in both directions [163, 164]. Therefore, researchers are able to capture brain-derived exosomes from peripheral circulation and study their content and cargo aiming to find unique exosomal biomarkers related to neurodegenerative diseases, making them a potential diagnostic tool. This is based on the fact that exosomes contain constituents of their cells of origin, essentially making them biomarkers for the secreting cells. Utilizing this concept, efforts have been made to enrich for exosomes from distinct brain cells, especially neurons and

astrocytes. In the context of AD, it is not surprising that the first candidate targets were the major culprits in AD pathophysiology, namely, Aβ, tau, and p-tau-all of which have been identified in serum and plasma exosomes [142, 165, 166]. Neuronal exosomes enriched from the blood of AD patients have significantly higher levels of Aβ, total tau, p-tau181, and p-tau 396 compared to the controls. Exosome screening was able to accurately predict the onset of AD development in preclinical individuals up to 10 years prior to symptoms [165]. Similar results were reported by another group, showing that plasma exosomes from AD patient significantly differed from healthy control based on their morphology, content, and count which might provide a basis for early diagnosis of AD [167].

Similar to AD, peripheral neuronal exosomes have been proposed as a diagnostic tool in mild TBI (mTBI). The recent work of Gill et al. examined the mechanisms underlying mTBI and its chronic symptoms using neuronal-derived exosomes and high-sensitivity detection of neurodegenerative and inflammatory biomarkers [168]. Interestingly, the levels of Aβ42, tau, and IL-10 were significantly higher in military personnel with mTBI when compared to normal individuals. The authors argue that elevated levels of tau were related to chronic post-concussive symptoms. On the other hand, higher IL-10 levels were related to post-traumatic stress disorder (PTSD) symptoms. These exciting findings suggest that exosomes may serve as prognostic and diagnostic biomarkers to identify patients with mTBI at risk for developing chronic symptoms.

Of note, methods have been developed to capture astrocyte-derived exosomes (ADE) in the peripherical circulation, which are reported to be present at lower levels in plasma when compared to neuronal-derived ones (NDE) [166]. Interestingly, ADE have a higher content of APP-derived metabolites (including Aβ42) and APP-processing enzymes like BACE-1, in addition to p-tau when compared to neuronal exosomes [166]. This observation not only leverages ADE to effectively distinguish AD patients from the study controls, it also brings about the idea of trans/indirect effect of ceramide on neurons. The idea to put forward here is that ceramide and PAR-4 have an indirect effect on neurons through exosomes that originate in astrocytes. Knowing that astrocytes contain higher ceramide levels in neurodegenerative diseases, it is reasonable to speculate that this would lead to secretion of exosomes carrying toxic molecules, including ceramide and PAR-4.

3 Trans-Indirect Effect of Astrocyte-Derived Ceramide and PAR-4 on Neurons

Astrocytes are known to be among the most abundant cells in the central nervous system, and they are indispensable for the support of neurons [169]. One characteristic feature of astrocytes is their extended processes, which allows them to make contact with other glial cells, blood vessels, and neuronal synapses. This feature primes astrocytes to participate in a myriad of functions including release and uptake of neurotransmitters, synapse formation and maturation, trophic factor production, and regulation of neuronal survival. Indeed, astrocytes dysfunction has been reported in several neurodegenerative disease such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease [169]. In most of these pathological conditions, astrocytes adopt a reactive phenotype exhibiting cellular process hypertrophy, increased GFAP expression, and elevated release of interleukins and cytokines. Consequently, astrocytes not only fail to support neurons, but also generate a toxic environment that is detrimental to neurons [170]. In addition, several groups reported that activated astrocytes release high numbers of exosomes as a form of response to diverse insults [122, 171–173]. Given the crosstalk, direct and indirect interactions between different cells types in the brain, astrocyte-derived exosomes seemed a good candidate to study their participation in the disease pathology. In the context of Alzheimer's disease, amyloid beta is known to be secreted from neurons to the extracellular milieu where it either aggregates or interacts with glial cells [174]. Wang et al. showed that astrocytes undergo apoptosis upon exposure to Aβ [122]. Interestingly, preincubation with antibodies against PAR-4 and ceramide mitigated the Aβ-induced apoptosis both in vitro and in vivo, suggesting that apoptosis was mediated by exogenous PAR-4 and ceramide. Indeed, analysis of exosomes separated from conditioned media of Aβ treated astrocytes revealed the presence of PAR-4 and ceramide-enriched exosomes, particularly C18:0 ceramide. In the absence of Aβ, these PAR-4/ceramide-enriched exosomes were taken up by astrocytes where they elicited apoptosis [122]. Notably, primary cultured nSMase2-deficient astrocytes did not secrete PAR-4-containing exosomes and showed significantly reduced apoptosis. This clearly indicates that ceramide generated by nSMase2 is crucial for the secretion of PAR-4-harboring exosomes after Aβ exposure. Adding exogenous C18 ceramide restored the secretion of PAR-4-containing exosomes in nSMase2-deficient astrocytes.

These observations suggest that ceramide might have more than one function in exosomes. Ceramide may aid in the formation and secretion of PAR-4-containing exosome as well as facilitating apoptosis in recipient cells by increasing ceramide levels. While this work used astrocytes as recipient cells, it is not excluded that PAR-4/ceramide-containing exosomes target neurons. One earlier study showed that fibrillar Aβ triggers astroglia to release products that were able to kill primary human neurons in transwell experiments [175]. This effect was mediated by nSMase2 as neurons were protected against the toxic effect of Aβ-activated astroglia by antisense

knockdown of nSMase2. However, the ceramide species and exosome production were not assessed in that study. In fact, the specificity of astrocytes-derived exosomes uptake by neurons has been independently shown by separate groups utilizing in vitro models [172]. Recent work by our group corroborated the notion that astrocyte-derived exosomes, termed "astrosomes" selectively target neurons where they induce apoptosis [138, 176]. We reported that exosomes from sera and brains of AD transgenic mice and from AD patients' sera contain this subpopulation of astrosomes that are enriched with ceramide. Astrosomes are specifically taken up by neurons in vivo and in vitro and induce apoptosis through interaction with the mitochondrial protein voltage-dependent anion channel 1(VDAC1) (Fig. 2). Using injection of fluorescently labeled astrosomes, we were able to track astrosomes in vivo and confirm their uptake by neurons [176]. Whether PAR-4 is also carried by these astrosomes, and how this could participate in the deleterious effect of astrosomes on neurons is an active research area in our laboratory. Abnormal induction of PAR-4 in hippocampal neurons of AD transgenic mouse model and in synaptic compartments following toxic insults and its role in subsequent apoptosis has been reported [126, 127, 177]. In addition, long-chain ceramide is elevated in the brains of AD transgenic mouse model leading to apoptosis in astrocytes, essentially due to substantial increased levels of PAR-4 in astrocytes, sensitizing astrocytes to ceramide-induced apoptosis [132]. Knowing that PAR-4 can be carried in exosomes—especially ceramide-enriched astrosomes—shuttling of PAR-4 via these exosomes might present a physiologically relevant mechanism by which ceramide and PAR-4 could contribute to early stages of neuronal apoptosis and degeneration during AD and probably other neurodegenerative disease, too.

4 Conclusions and Future Perspectives

The multifaceted roles of ceramide in neurodegeneration have been well documented. The relatively recent discovery that ceramide induces or mediates the formation of exosomes and the ability of these lipid vesicles to transfer toxic molecules has opened a new avenue in neurodegenerative diseases research. Studies from our group have demonstrated that astrosomes can transfer PAR-4 and Aβ between cells. When taken up by neurons, these Aβ- and/or PAR-4 associated astrosomes interact with proteins at mitochondria, particularly VDAC1 and probably, BCL-2, which leads to caspase activation and ultimately apoptosis (Fig. 2). In future studies, we will explore approaches to block or dissociate the

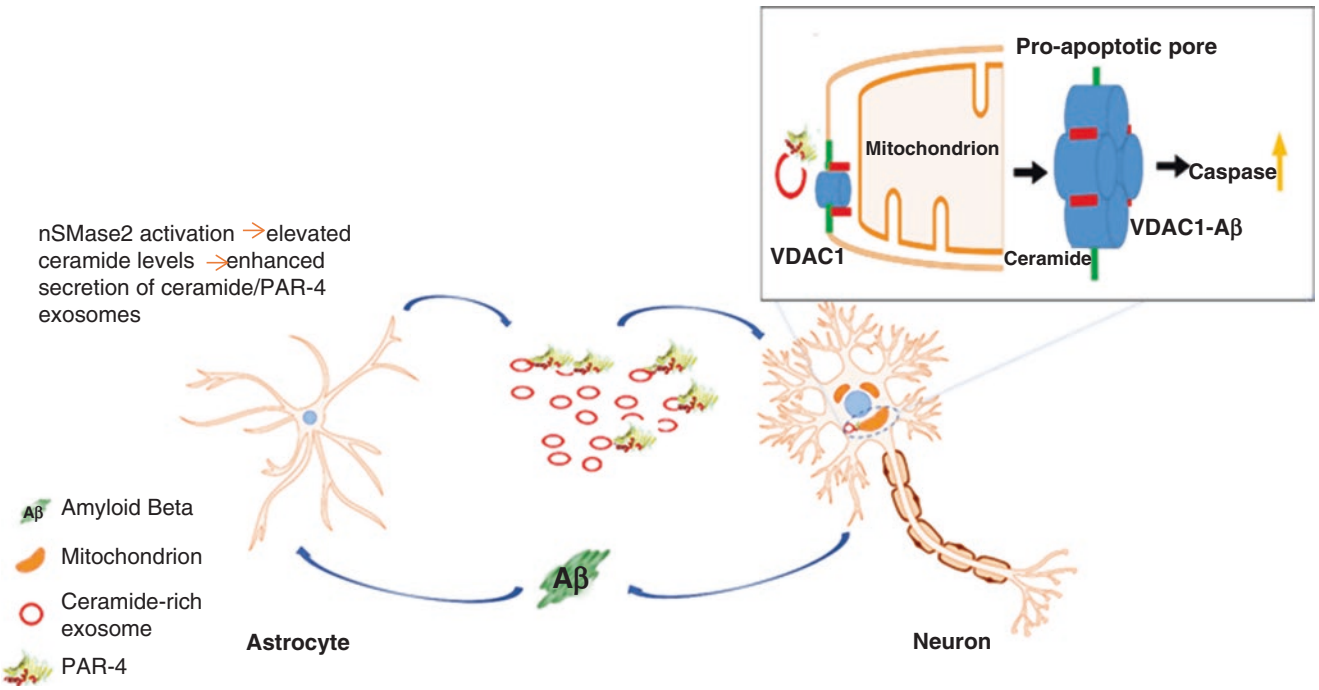

Fig. 2 Indirect effect of Ceramide/Par-4 on neurons: In astrocytes, activation of nSMase2 by several insults or stimuli (e.g. Aβ, cytokines, oxidative stress) leads to generation of ceramide, ultimately inducing formation and secretion of ceramide-rich exosomes, termed astrosomes. Astrosomes associate with Aβ and may contain PAR-4. Upon being taken up by neurons, those astrosomes associate with mitochondria and become mitotoxic. Mitotoxicity is induced by astrosome-mediated effects of ceramide and Aβ (here depicted as interaction with VDAC1 and pro-apoptotic pore formation) or PAR-4 (e.g., by interaction with BCL-2). Release of cytochrome c through the VDAC1 pore and activation of caspase 3 induces apoptosis in neurons

complex formation of astrosomes/PAR-4 and astrosomes/Aβ or inhibit their uptake by neurons.

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Par-4 in Neuronal Death and Survival in Alzheimer's Disease and Other Neurogenerative Diseases

Qing Guo, Jun Xie, and Chelsea J. Guo

Abstract

Alzheimer's disease (AD) is a progressive neurodegeneration in vulnerable regions of the hippocampus and cerebral cortex that leads to loss in thinking abilities and dementia. The main pathological features of AD include loss of neurons and synapses, extracellular amyloid plaques composed primarily of amyloid β peptide 1–42, and intracellular neurofibrillary tangles which are mostly abnormally paired helical filaments of hyperphosphorylated tau protein. The precise mechanisms of neuronal cell deaths in AD are yet to be established. The amyloid hypothesis of AD states that the aberrant processing of the β-amyloid precursor protein (APP) and aggregation of amyloid β-peptide 1-42 (Aβ 1-42) plays a central role in neurodegeneration in AD. Normally, APP is cleaved by α-secretase to release an extracellular fragment known as sAPPα. In contrast, Aβ is produced when APP is cleaved by β-secretase (BACE1) and gamma-secretase. Cleavage of APP by β-secretase generates a soluble NH2-terminal fragment (sAPPβ) and a membrane-bound COOH-terminal fragment C99. Cleavage of C99 by gamma-secretase produces Aβ and an APP intracellular domain (AICD). It is generally believed that Aβ 1–42 is neurotoxic while sAPPα confers neuroprotection. Mutations in familial Alzheimer's disease (FAD) genes, including those in APP and presenilins (which include presenilin-1, PS-1, and presenilin-2, PS-2), are associated with early-onset AD cases. Par-4 (prostate apoptosis response-4) is a cell death-promoting protein that was initially isolated as an apoptosis-associated protein by differential screening for genes upregulated in prostate cancer cells undergoing apoptosis. Par-4 is expressed in neurons, and it is found in both cytoplasmic and nucleus compartments. In this chapter, we provide emerging evidence that Par-4 is involved in neuronal cell death in a variety of neurodegenerative diseases. Specifically, we will focus on the cellular and molecular mechanisms by which Par-4 sensitizes neurons to apoptosis or necroptosis in different experimental models of AD, including the first mouse "knock-in" model of a naturally occurring presenilin-1 mutation responsible for an early-onset form of Alzheimer disease described by our laboratory. Par-4 significantly increases production of the neurotoxic Aβ species while decreases the release of the neuroprotective sAPP by altering cell death signaling, disrupting intracellular calcium homeostasis, and enhancing amyloidogenic processing of APP. Par-4 also interacts with ACID to promote neurodegeneration in AD by regulating AICD-mediated transcriptional activity. Of importance, we identified AATF (apoptosis antagonizing transcription factor), another leucine zipper domain containing protein, to be an endogenous interaction partner and potent inhibitor of Par-4 activity in neurons. AATF confers neuroprotection by interacting with Par-4 via the leucine zipper domain and interfering with binding of Par-4 to AICD. Of importance, AATF is secreted extracellularly by cortical neurons under neurodegenerative conditions, and secreted AATF (sAATF) blocks TLR4-mediated, RIPK3/MLKL-dependent necroptosis of cortical neurons. Surprisingly, a small core peptide from TRL-4 binding region of AATF, termed as SAP-12, provides a much greater neuroprotective potency and broader effective dose range than the full-length sAATF. Participation of Par-4 in other neurodegenerative diseases and neurological disorders as well as future directions is also discussed.

Keywords

Prostate apoptosis response-4 · Amyloid precursor protein · β-secretase BACE1 · Amyloid β peptide · APP intracellular domain · Alzheimer's disease · Apoptosis · Necroptosis · Apoptosis antagonizing transcription factor

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· Presenilin-1 · Amyotrophic lateral sclerosis · Parkinson's disease

Abbreviations

AATF	Apoptosis antagonizing transcription factor
AD	Alzheimer's disease
AICD	APP intracellular domain
ALS	Amyotrophic lateral sclerosis
APP	β-Amyloid precursor protein
Aβ	Amyloid β peptide
BACE1	β-Site APP cleaving enzyme 1
Leu.zip	Leucine zipper domain
Par-4	Prostate apoptosis response-4
PS-1	Presenilin-1

1 Neuronal Cell Death and Survival in Alzheimer's Disease

1.1 Introduction to Alzheimer's Disease

Alzheimer's disease (AD) is an irreversible, progressive neurodegeneration in vulnerable regions of the hippocampus and cerebral cortex [1–8]. The clinical symptoms of AD include memory loss, changes in personality and behavior, decline in thinking abilities, and loss of mental function (dementia). AD affects over five million Americans and 44 million people worldwide. AD primarily attacks people 65 and older, but early-onset familial Alzheimer's disease (FAD) cases between the ages of 30 and 65 are not uncommon [9, 10]. The average life span of patients with AD is 8–10 years, but can be as long as 20 years. In 2020, annual economic toll (health care and lost wages) for all individuals with Alzheimer's disease or other forms of dementia in the USA alone is estimated to be about $305 billion. In 2050, Alzheimer's is projected to cost more than $1.1 trillion unless an effective treatment is found.

1.2 The Amyloid hypothesis of AD

Pathologically, AD is characterized by loss of neurons and synapses, cerebral atrophy, extracellular amyloid plaques (Fig. 1), intracellular neurofibrillary tangles, and microglial activation. Amyloid plaques are composed primarily of amyloid β peptide 1-42 (Aβ 1-42) intermingled with degenerative neurites, while neurofibrillary tangles are mostly abnormally paired helical filaments of hyperphosphorylated tau [1–8].

The cause of neuronal cell death in AD is yet to be established. However, it was generally believed that aberrant processing of APP leading to an increased production and aggregation of Aβ1-42 plays an important role in the pathogenesis of AD [11–41]. Aβ is produced when amyloid precursor protein (APP) is cleaved by β-secretase (BACE1) and γ-secretase [42–52]. Cleavage of APP by β-secretase generates a soluble NH2-terminal fragment (sAPPβ) and a membrane-bound COOH-terminal fragment (known as C99). Cleavage of C99 by γ-secretase produces Aβ and AICD (APP intracellular domain, composed of half of the transmembrane region of 10–12 residues and the cytoplasmic tail of 47 residues) [53–69]. The intramembrane γ-secretase cleavage of APP typically generates two major forms of Aβ that are 40 and 42 amino acids in length [70–98]. It is believed that Aβ1-42 is pathogenic in Alzheimer's disease (AD) because it is the major component of senile plaques and is more aggregable and neurotoxic than Aβ1-40 [11, 13, 23, 24, 34, 40, 99–111]. As a result, Aβ1-42/Aβ1-40 ratio was often used as an indicator of production of the toxic Aβ species [11, 30, 33, 34, 36, 37, 39–41]. Non-amyloid precursor protein (APP) occurs when APP is processed by α-secretase which generates a soluble ectodomain called sAPPα [112–114]. sAPPβ may have different biological properties than does sAPPα (which has an extra 16 amino acids in its C-terminus) although both have been shown to have neuroprotective properties [115, 116]. The biogenesis, subcellular localization, biological activities, and the roles of the AICD in the pathogenesis of AD are not fully established, but available data indicate that AICD may translocate to the nucleus as a transcription factor to predispose to transcription-dependent neuronal cell death [117].

1.3 Genetic Factors in Early-Onset Familial AD

Mutations in familial Alzheimer's disease (FAD) genes, such as β-amyloid precursor protein (APP) and presenilins (which include presenilin-1, PS-1, and presenilin-2, PS-2), have been shown to regulate the processing of APP and result in increased production of the neurotoxic amyloid β-peptide (Aβ1–42) [11, 118–122]. APP is mapped to Ch21, and mutations in APP account for about 3% of early-onset FAD cases. PS-1 and PS-1 are mapped to Ch14 and Ch1, respectively, and they share about 67% of amino acid homology. Mutations in PS-1 account for majority of early-onset FAD cases and have been shown to increase the production of Aβ42 and expose neurons to elevated vulnerability to cell death [11, 118–122]. Of importance, aberrant APP processing and neuronal degeneration in AD may be intricately linked. Abnormal processing of APP and increased production of Aβ could be induced by pro-apoptotic insults [66–71]. Several recent studies showed that APP could be processed in neuronal cells during apoptosis by some cell death proteases, such as caspase-6 and -8, and specific caspase inhibitors can block the apoptotic conversion of APP [102, 103, 123].

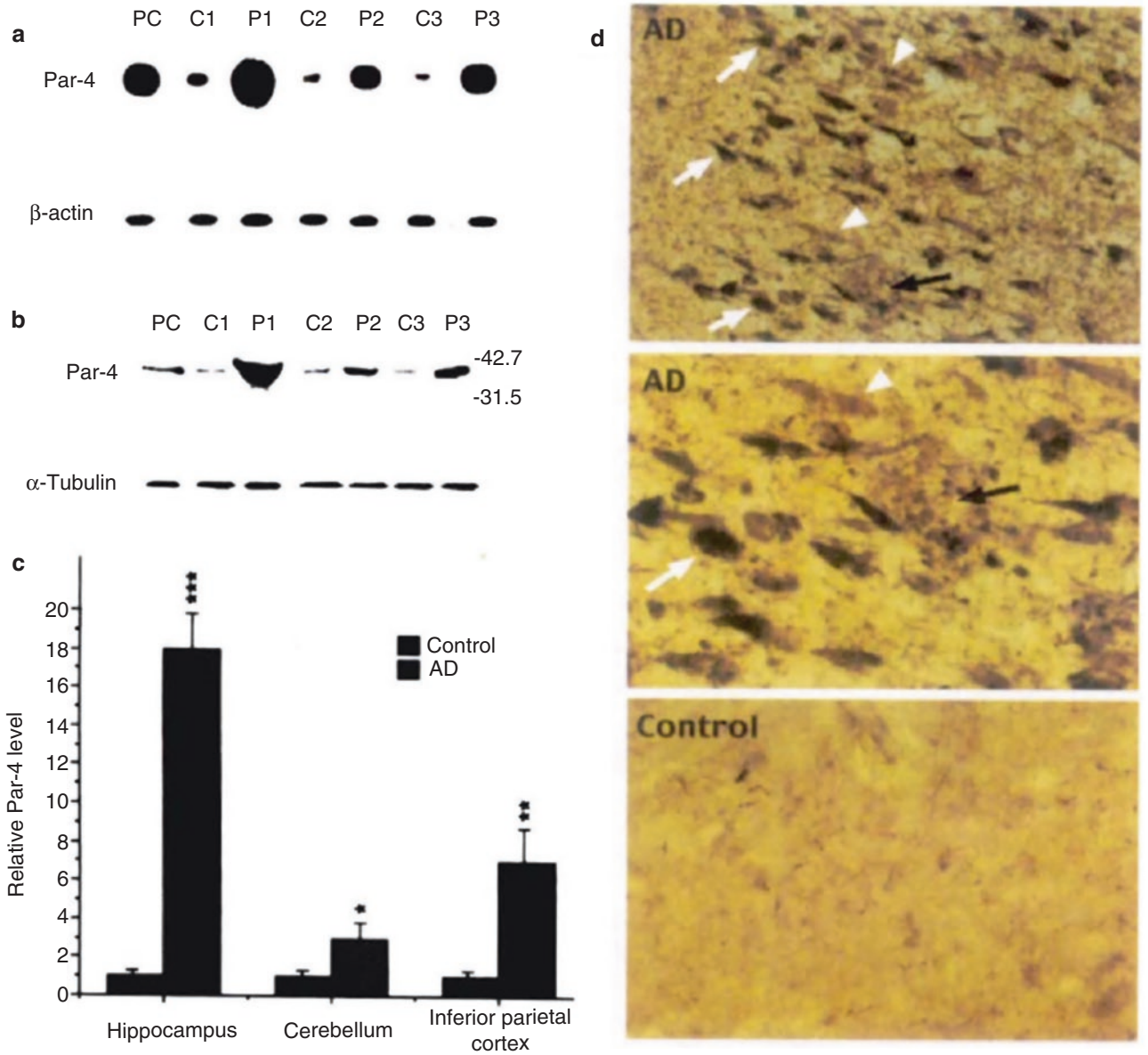

Fig. 1 Levels of Par-4 are significantly increased in the brains of human AD patients. (**a**) Northern blot analysis of Par-4 and β-actin mRNA amounts in hippocampi from three neurologically normal control (C1-C3) and three AD (P1-P3) patients. PC is positive control which represents RNA from fibroblasts overexpressing human Par-4. (**b**) Representative Western blot analysis of Par-4 protein levels in the hippocampus three neurologically normal control (C1-C3) and three AD (P1-P3) patients. PC is positive control which represents authentic Par-4 from cultured 3 T# fibroblasts overexpressing Par-4. (**c**) Densitometric analysis of Par-4 amounts in three brain regions from control and AD patients. Values are the mean and S.D. of determinations made in six control and six AD patients. $***P < 0.001$, $**P < 0.01$, and $*P < 0.05$ compared with corresponding values in the control groups. ANOVA with Scheffe's post-hoc tests. (**d**) Sections of hippocampi from AD patient and an age-matched control patient double-labeled with antibodies against PHF-1 (brown) and Par-4 (black). White arrows, double-labeled neurons; white arrowhead, neurons labeled only with PHF-1; black arrow: neuritic plaque

2 Role of Par-4 in Cell Death and Survival in AD and Other Neurodegenerative Diseases

2.1 Initial Evidence for a Role of Par-4 in Neuronal Degeneration

Par-4 (prostate apoptosis response-4) was initially isolated as an apoptosis-associated protein by differential screening for genes upregulated in prostate cancer cells undergoing apoptosis [124–129]. It is also expressed in neurons and is found in both cytoplasmic and nucleus compartments [11, 33, 124, 130–143]. We performed a series of studies to determine its possible role in neuronal cell death. The first link between aberrant expression of Par-4 and neurodegeneration came

from studies of Alzheimer's disease [11]. Levels of Par-4 mRNA and protein were found to be increased in tissue from vulnerable brain regions of Alzheimer's patients compared to age-matched control patients (Fig. 1). Double-labeling analysis using antibodies against phosphorylated tau (PHF-1 antibody; a marker of neurofibrillary tangle-bearing neurons) revealed that approximately 30–50% of tangle-bearing neurons were also Par-4 positive [11].

Levels of Par-4 also increased in differentiated PC12 cells and primary hippocampal neurons undergoing apoptosis in response to trophic factor withdrawal. Pretreatment of hippocampal neurons with an antisense oligodeoxynucleotide of Par-4 significantly attenuated neuronal apoptosis. Par-4 expression was enhanced by presenilin-1 mutations associated with early-onset inherited AD [11, 33, 105, 137, 142–144]. Analysis of the predicted amino acid sequence of Par-4 indicates that it contains a leucine zipper domain in its C-terminus (within the death domain), indicating that Par-4 may dimerize with itself or other proteins [124–126]. The cell death-enhancing action of Par-4 may involve protein–protein interactions via its leucine zipper domain (Leu.zip) [11, 33, 105, 137, 142–144]. PC12 cell lines stably overexpressing the leucine zipper domain of Par-4, which acts in a dominant negative manner, were resistant to apoptosis induced by trophic factor withdrawal. Overexpression of a deletion mutant of Par-4 lacking the leucine zipper domain does not enhance apoptosis. Additionally, blockade of Par-4 function by par-4 antisense treatment or overexpression of Leu.zip of Par-4 significantly decreases neuronal apoptosis induced by Aβ or overexpression of Alzheimer's mutant presenilin-1 (PS1) proteins [11, 33, 105, 137, 142–144].

2.2 Par-4 Participates in Neuronal Cell death in a Mouse Model of Alzheimer's Disease Expressing a Mutant Form of Presenilin-1: Generation and Characterization of PS-1 M146V Mutant Knock-in Mice

Several animal models have been established to study the pathogenic mechanisms of AD. An ideal AD models requires: (1) behavioral deficit, (2) neuronal and synaptic loss, (3) extracellular amyloid plaques, (4) intracellular neurofibrillary tangles. The APP mutant mice (Tg2576 mice expressing Swedish double mutations) and human Aβ42 transgenic mice showed behavioral deficit and extracellular amyloid plaques, without intracellular neurofibrillary tangles [145–148]. In contrast, mutant Tau (P301L) mice showed behavioral deficit, neuronal and synaptic loss, and intracellular neurofibrillary tangles, without extensive amyloid plaques [145–148].

Mutations in the presenilin-1 gene, located on chromosome 14, account for many cases of familial Alzheimer's disease. We generated PS1 mutant "knock-in" mice in which an exon encoding an AD-linked PS1 M146V mutation was exchanged for the homologous exon in the mouse PS1 gene, resulting in mice that produce (in the homozygous state) only mutant PS1 and no wild-type PS1 (Fig. 2). The pathogenic PS-1 M146V mutation was introduced and the only amino acid polymorphism (between mouse and human PS1) in exon 5 of the murine PS1 gene was "humanized" by introduction of the I145V substitution [120].

In mutant presenilin-1 M146V knock-in (PS1M146VKI) mice, Par-4 expression was enhanced, resulting in increased vulnerability of neurons to apoptosis and increased production of Aβ 1–42 [36, 120, 144, 149]. The mechanism whereby presenilin-1 mutations endanger neurons appears to involve disturbances in calcium regulation in the endoplasmic reticulum which lead to enhanced calcium release when neurons are subjected to various insults [35, 36, 150–153]. The apoptosis-enhancing action of presenilin-1 mutations was significantly inhibited when Par-4 activity is knocked down, indicating that Par-4 participates in the pathogenic mechanism of the presenilin mutations [11, 144, 152, 154]. These data suggest that induction of Par-4 plays a critical role in the abnormal processing of APP and neuronal cell death induced by the Alzheimer's PS-1 mutation (Figs. 3 and 4). Par-4 may exert its cell death-promoting action in the early stages of cell death prior to caspase activation and mitochondrial alterations [11, 144, 152, 154].

2.3 Par-4 Increases Secretion of Aβ 1–42 through a Caspase-Dependent Pathway

In IMR-32 cells, overexpression of Par-4 drastically increased $A\beta_{1\text{-}42}/A\beta_{total}$ ratio in the conditioned media about 6–8 h following trophic factor withdrawal [11, 33, 34]. Consistent with a dominant negative mode of action of Leu.zip involving protein–protein interactions, co-overexpression of Leu.zip abolished the adverse effect of Par-4 on Aβ secretion, indicating that actions of Par-4 in Aβ secretion require its interaction with other protein(s) via the leucine zipper domain. Overexpression of Par-4 drastically increases vulnerability of IMR-32 cells to apoptosis following trophic factor withdrawal. Trophic factor withdrawal-induced caspase-3 activation was exacerbated in cells overexpressing Par-4, and Leu.zip suppressed caspase activation induced by Par-4, indicating an important role for Par-4 in IMR-32 cells in the early period of the apoptotic cascades before caspase activation**.** Par-4 induced an increase in secretion of Aβ 1-42 after trophic factor withdrawal was significantly attenuated by the broad-spectrum caspase inhibitor BD-fmk (Fig. 5). These results suggest that Par-4 may increase secretion of Aβ 1–42 through a caspase-dependent pathway [11, 33, 34].

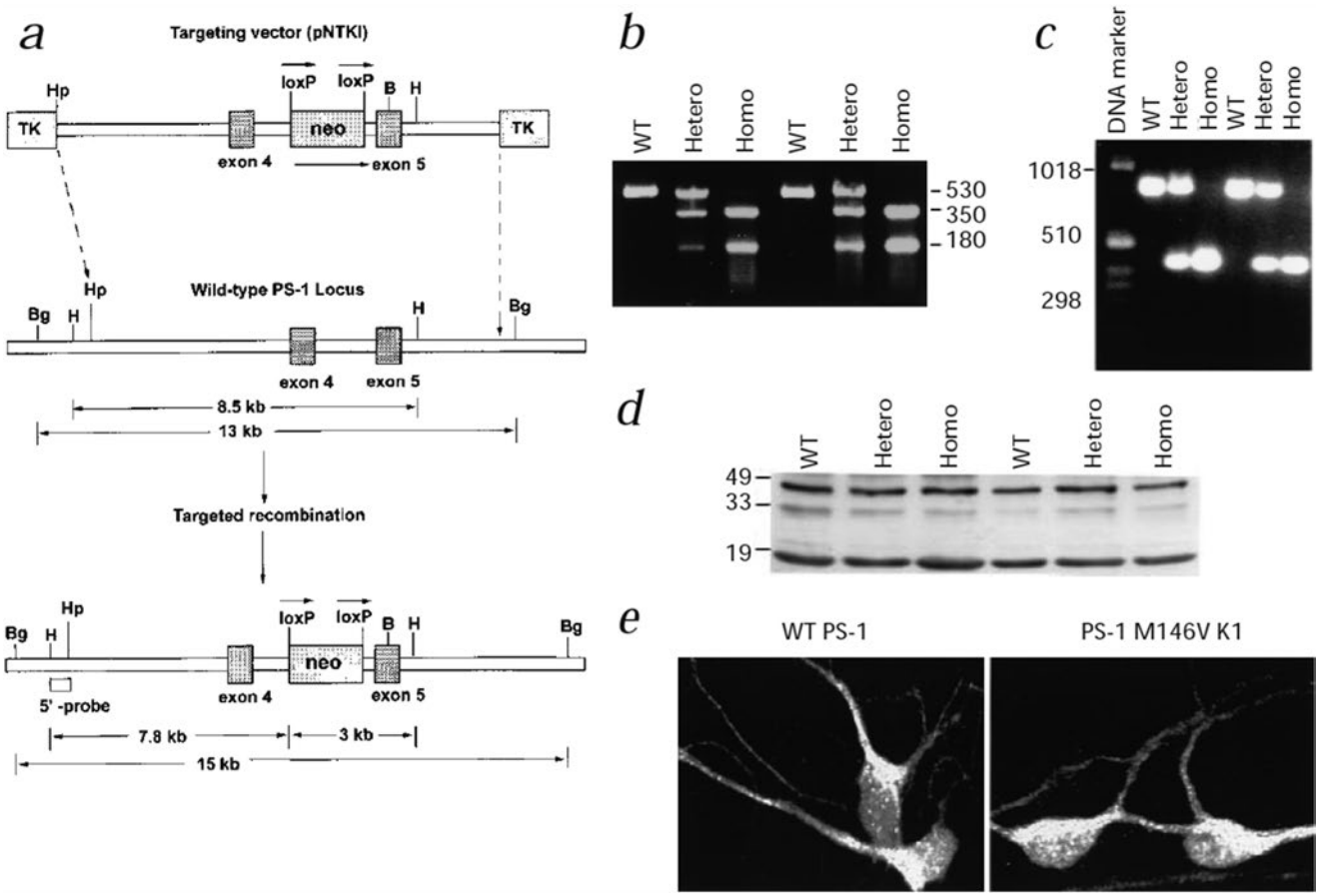

Fig. 2 Generation of PS1M146VKI mice. (**a**) Strategy used to target the M146V FAD mutation to the third coding exon (exon 5) of the murine PS1 gene. The PS1 genomic sequences incorporated into the targeting vector are delimited by the vertical dashed lines. The *neo* selection cassette (flanked by loxP sites) was inserted into an *Fse*I restriction site positioned 375 bp 5′ of exon 5. The 5′ probe was used to screen embryonic stem cell lines for the *Hind*III and *Bgl*I restriction enzyme polymorphisms introduced by the insertion of the *neo* selection cassette. Bg, *Bgl*I; H, *Hin*dIII; Hp, *Hpa*I; B, *Bst*EII. (**b**) Genotypes of F2 pups generated by intercrossing PS1M146V (+/−) mice derived from embryonic stem cell line 106 (lanes 1–3) or 179 (lanes 4–6). Right margin, sizes are indicated in bp. A 530-bp fragment (containing exon 5) was amplified by PCR and the *Bst*EII polymorphism was demonstrated by the cleavage of the PCR product into two diagnostic bands of 350 and 180 bp. (**c**, **d**) Expression from the targeted PS1M146V allele. (**c**) Total brain RNA isolated from the mice in *b* was used to amplify the DNA sequences encoding amino acids 1–298 of PS1 (encoded by exons 3–8). After RT–PCR, the samples were digested with *Bst*EII and separated on a 1.5% TBE gel. The uncut, 868-bp product represents mRNA expressed from the wild-type allele and the digested, 426/442-bp products represent mRNA expressed from the targeted PS1M146V allele. (**d**) Total brain protein was also isolated and separated by SDS-PAGE (100 mg protein/lane). After transfer of the protein, the membrane was probed with PS1 antibody. (**e**) Confocal laser-scanning microscope images of cultured hippocampal neurons from wild-type (left) and PS1M146VKI (right) mice, immunostained with PS1 antibody

2.4 Par-4 Inhibits Secretion of sAPPα Induced by Protein Kinase C Activator PMA

sAPPα has been shown to possess neuroprotective properties [11, 33, 34]. Receptor-coupled protein kinase C (PKC)-dependent mechanisms have been shown to regulate the α-secretase pathway. Indeed, activation of PKC has been shown to favor α-secretase cleavage [155, 156]. For example, treatment of cells with the PKC activator 12-myristate 13-acetate phorbol dibutyrate (PMA) increases secretion of sAPPα, suggesting that stimulation of α-secretase cleavage may be a useful intervention against neuronal cell death in AD [155, 156]. Since Par-4 has been shown to be able to bind to several isoforms of PKC and inhibit their enzyme activity [157], it is possible that Par-4 might also be involved in regulation of secretion of sAPPα. To examine this possibility, transfected IMR-32 cells overexpressing Par-4 and vector-transfected control cells were metabolic labeled with

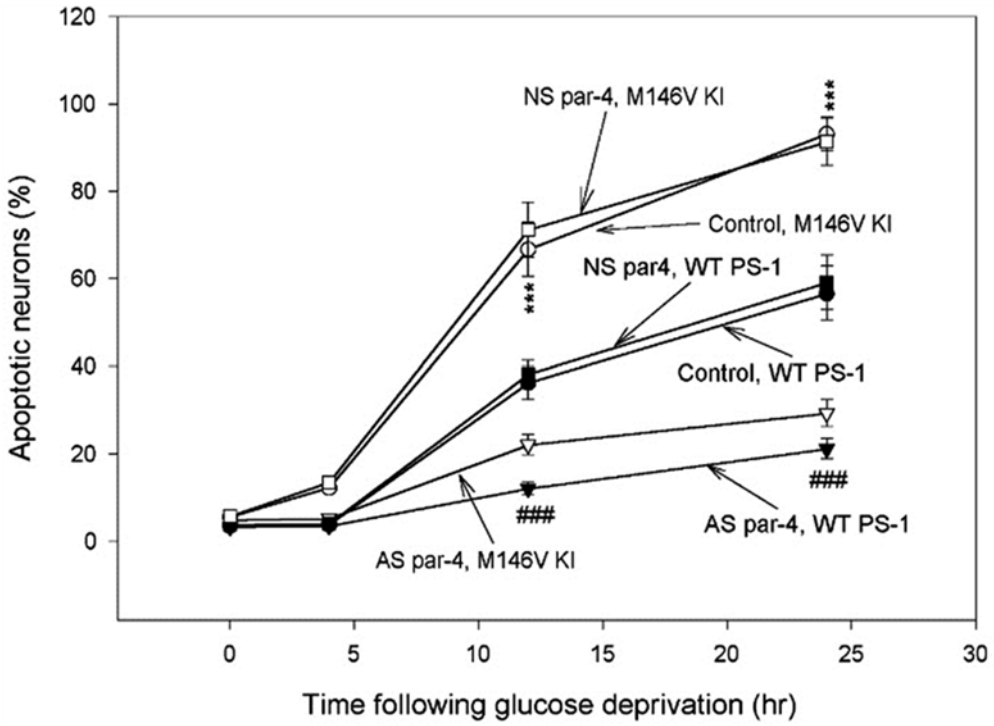

Fig. 3 Aberrant induction of Par-4 is essential for neuronal apoptosis induced by PS-1 M146V mutation following glucose deprivation. Cultures of hippocampal neurons from wild-type (WT PS-1) and PS-1 M146V KI mice were pretreated for 2 h with either par-4 antisense (AS par-4; 25μM), nonsense DNA (NS par-4; 25μM), or no DNA (Control). Cultures were then deprived of glucose for the indicated time periods. Cells with apoptotic nuclei (showing nuclear chromatin condensation and fragmentation) were stained with the fluorescent DNA-binding dye propidium iodide and counted under a confocal laser-scanning microscope. Values are the mean and SE of determinations made in six separate cultures. At least 200 cells per culture were counted. ***$P < 0.001$ compared with corresponding values in control WT PS-1, NS par-4 WT PS-1, AS par-4 M146V KI, and AS par-4 WT PS-1 groups. ###$P < 0.001$ compared with corresponding values in control WT PS-1 and NS par-4 WT PS-1 groups. ANOVA with Scheffe's post-hoc tests

[35S] methionine for 1 h followed by a chase in the presence of absence PMA. As shown in Fig. 6, the monoclonal antibody that recognizes the N-terminus of human APP immunoprecipitated two bands (one strong band and one weak band) of sAPPs. These two proteins are both sAPPα since they also immunoprecipitated with the monoclonal antibody 6E10 (anti-Aβ 1–17), but not with the monoclonal antibody 4G8 (anti-Aβ 17–24). One hour following PMA treatment, secretion of sAPPα in vector-transfected cells was increased by 2.9-fold compared with control cells not treated with PMA. Overexpression of Par-4 largely blocked the PMA-induced secretion of sAPPα. These results demonstrate that Par-4 participates in regulation of secretion of sAPPα.

2.5 Disruption of Intracellular Calcium Homeostasis Contributes to the Aberrant APP Processing Induced by Par-4

While an optimal increase in intracellular calcium levels [Ca2+]c can play a key role in the normal regulation of APP processing, abnormal and sustained elevation of [Ca2+]c may result in aberrant APP processing in favor of increased production of Aβ and/or decreased secretion of sAPP [158–161]. We have found a significant correlation between disruption of intracellular calcium homeostasis and the pathological roles of Par-4 [105]. For example, a sustained increase in intracellular calcium levels has been shown to induce aberrant Par-4 expression in neuronal cells. Indeed, overexpression of Par-4 also results in an enhanced cellular calcium response to apoptotic insults. It was reported that, in a dose-dependent manner, glutamate might induce apoptosis (DNA fragmentation) in IMR-32 cells. Cell death induced by glutamate in IMR-32 cells is associated with an increase in intracellular calcium levels. We found that overexpression of Par-4 sharply increases the calcium response to glutamate, indicating disruption of calcium homeostasis may contribute to the adverse effect of Par-4 (Fig. 7).

An aberrant increase in intracellular calcium levels has been reported to contribute to trophic factor withdrawal-induced apoptosis in several types of neural cells. Overexpression calbindin D28K buffers intracellular free calcium and blocks aberrant elevations in [Ca2+]c in PC12 cells expressing mutant forms of presenilin-1 [110, 162]. To confirm that perturbed calcium homeostasis induced by

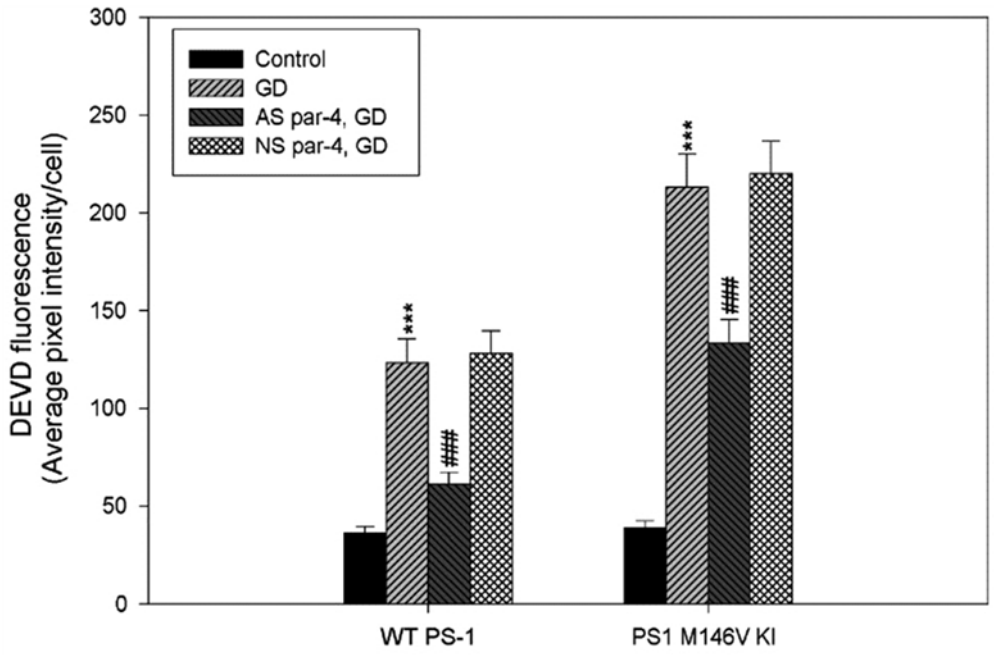

Fig. 4 Induction of Par-4 expression is required for caspase activation in hippocampal neurons expressing PS-1 m146V mutation following glucose deprivation (GD). Cultures of hippocampal neurons from wild-type (WT PS-1) and PS-1 M146V KI mice were pretreated for 2 h with either par-4 antisense (AS par-4; 25μM), nonsense DNA (NS par-4; 25μM), or no DNA (Control). Cultures were then deprived of glucose for 12 h. Levels of cellular DEVD fluorescence, a measure of caspase-3 activity, were quantified. Values are the mean and SE of determinations made in six separate cultures. At least 80 cells were analyzed per culture. ***$P < 0.001$ compared with corresponding values in the control groups. ###$P < 0.001$ compared with corresponding values in GD groups. ANOVA with Scheffe's post-hoc tests

Par-4 is involved in aberrant APP processing, we generated additional IMR-32 cell lines that co-overexpress Par-4 and the calcium binding protein calbindin D28K using similar protocols described in our previous studies [110]. As shown in Fig. 8, inhibiting aberrant calcium response by overexpressing calbindin D28K significantly reduced secretion of Aβ 1-42 induced by Par-4 following trophic factor withdrawal.

2.6 Generation and Characterization of Mice Transgenic for Par-4: Par-4 Increases Production of Aβ 1-42 in Hippocampal Neurons In Vivo

To further study the role of Par-4 in neurodegeneration in vivo, we have generated and characterized Par-4 transgenic mice in which the expression of the par-4 transgene was limited to cells of neuronal lineage by neuron-specific enolase (NSE) promoter [163]. Mice transgenic for Par-4 specifically in neurons have not shown any signs of an overt abnormal phenotype, indicating that neuron-specific expression of Par-4 does not impair the normal development. However, when mice transgenic for Par-4 were crossed with those transgenic for mutant human APPswe, significantly increased production of Aβ1-42 was observed in hippocampal neurons (Fig. 9 and Table 1).

2.7 Par-4 Interacts with AICD and Alters AICD-Mediated Transcriptional Activity and Neurodegeneration in AD

The intramembrane gamma-secretase cleavage of β-amyloid precursor protein (APP) generates two major forms of amyloid β-peptide that are 40 and 42 amino acids in length [11, 13, 23, 24, 34, 40, 99–111]. Emerging evidence suggests that APP might also be a signaling molecule [12–29]. The cleavage of APP in its intramembranous domain by gamma-secretase generates, in addition to Aβ, a free intracellular carboxyl-terminal fragment (CTF), often referred to as APP intracellular domain (AICD) [53–69, 164]. Two major forms of AICD have been reported under normal conditions: CTFγ59 (which represents a major C-terminal product of γ-secretase cleavage in APP) and CTFγ57 (which represent a minor product of γ-cleavage) [164, 165]. Alternatively, it has been shown that a shorter 50 amino acid C-terminal fragment of APP (CTFγ50) could be generated from a second transmembrane cleavage site in APP that is distal to the γ-secretase sites [56, 68, 164]. AICD may function as a tran-

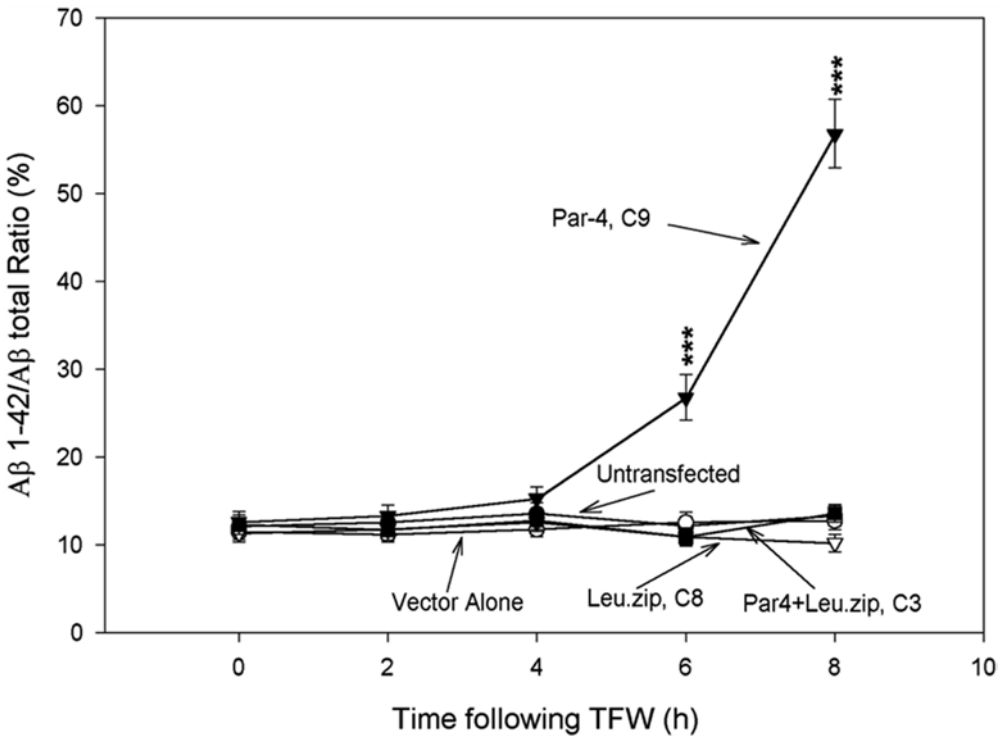

Fig. 5 Par-4 significantly increases secretion of Aβ 1-42 from transfected IMR-32 cells following trophic factor withdrawal: blockade by co-overexpression of Leu.zip. Cultures of the indicated clones of transfected IMR-32 cells were deprived of trophic support for the indicated time periods, and values of $A\beta_{1\text{-}42}/A\beta_{total}$ ratio in the conditioned culture media of transfected IMR-32 cells were measured by sandwich ELISAs. Note that values of $A\beta_{1\text{-}42}/A\beta_{total}$ ratio in untransfected and vector-transfected control cells and in cells overexpressing Leu.zip remained statistically unchanged following trophic factor withdrawal. However, overexpression of Par-4 drastically increased $A\beta_{1\text{-}42}/A\beta_{total}$ ratio in the conditioned media. This effect of Par-4 was not observed until approximately 6 h following trophic factor withdrawal. Co-overexpression of Leu.zip completely abolished the adverse effect of Par-4 on Aβ secretion. Values are the mean and SE. of determinations made in six separate cultures. $^{***}P < 0.001$ compared with corresponding values of $A\beta_{1\text{-}42}/A\beta_{total}$ ratio in untransfected, vector transfected, Leu.zip, and Par4+Leu.zip cell groups. Similar data were obtained from cell lines Par-4 C6 and C3, Leu.zip C10, and Par4+Leu.zip C1 and C12. ANOVA with Scheffe's post-hoc tests

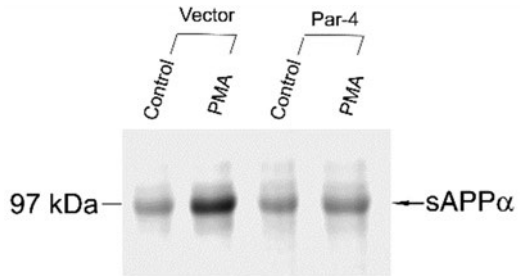

Fig. 6 Par-4 inhibits secretion of sAPPα from transfected IMR-32 cells. IMR-32 cells transfected with vector alone or full-length Par-4 were pulse-labeled for 1 h ad chased with (PMA) or without PMA (control) for 1 h. Secreted APPα was then precipitated from medium, resolved on 7.5% Tris-glycine gels, and quantitated using a Phosphor Imager. Note that the monoclonal antibody that recognizes the N-terminus of human APP immunoprecipitated two bands of sAPPs. These two proteins are both sAPPα since they also immunoprecipitated with the monoclonal antibody 6E10 (anti-Aβ 1-17), but not with the monoclonal antibody 4G8 (anti-Aβ 17-24). PMA significantly increases the release of sAPPα. This effect of PMA was effectively blocked by overexpression of Par-4

scriptional activator by interacting with the phosphotyrosine-binding (PTB) domain of the adaptor protein Fe65 and the histone acetyltransferase Tip60 [54, 166, 167]. The transcriptional activity of AICD in the nucleus was further supported by the observations that CTFγ59 represses retinoic acid-responsive gene expression and causes disappearance of PAT1, a protein that interacts with AICD, from the nucleus [75]. Of importance, ectopic expression of CTFγ58 in H4 human neuroglioma cells leads to apoptosis that was dependent on the interaction of CTFγ58 with Tip60 and nuclear translocation [82]. These results indicate that AICD may be responsible for contributing to signal transduction pathways that predispose to transcription-dependent neuronal cell death in Alzheimer's disease.

Par-4 is found in both cytoplasm and in the nucleus. Structure-function analysis indicated that apoptosis induced by Par-4 is dependent on Par-4 translocation to the nucleus via a bipartite nuclear localization sequence (NLS2) [168]. Thus, both AICD and Par-4 may induce cell death that was

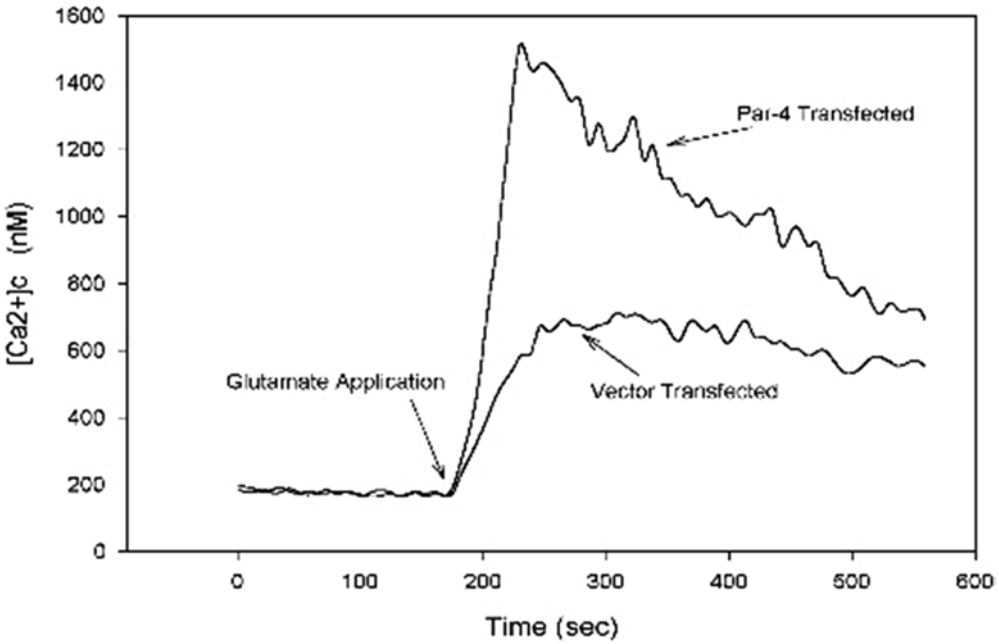

Fig. 7 Overexpression of Par-4 induces aberrant cellular calcium response to glutamate. Vector-transfected control IMR-32 cells and those transfected with Par-4 were exposed 100μM of glutamate and intracellular calcium levels [Ca2+]c was monitored by fura-2 ratio imaging before and after the application of glutamate. Each line represents the mean [Ca2+]c in at least 30 neurons. Note that, following glutamate treatment, IMR-32 cells overexpressing Par-4 showed an early and enhanced increase in intracellular calcium levels compared with vector-transfected control cells levels

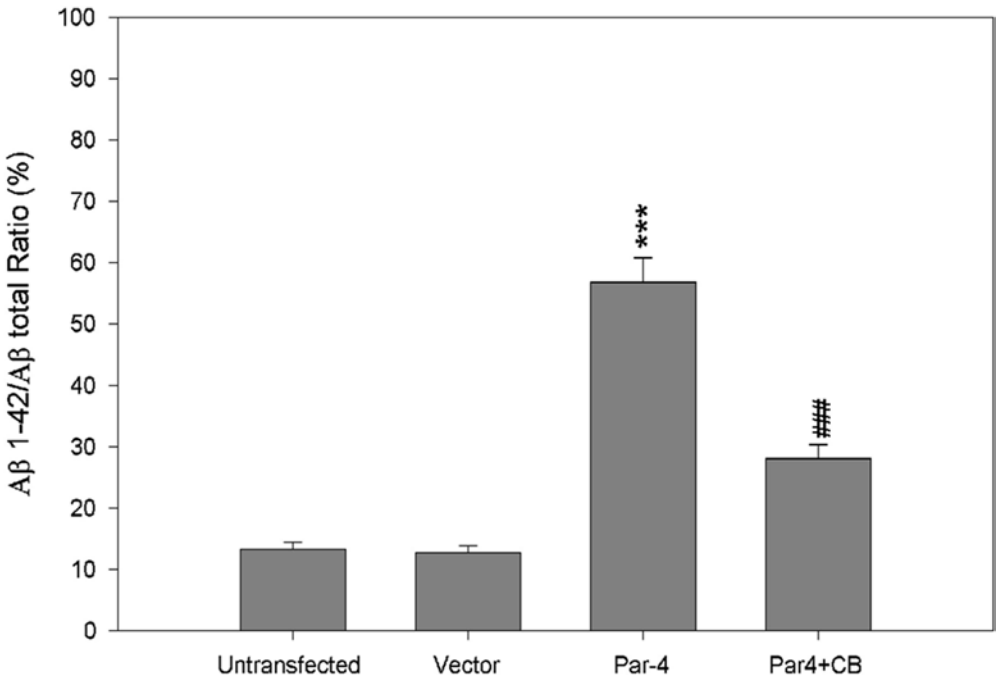

Fig. 8 Calbindin D28K counteracts the adverse effect of Par-4 on secretion of Aβ 1–42. Cultures of the indicated clones of transfected IMR-32 cells were deprived of trophic support for 8 h. Values of $A\beta_{1\text{-}42}/A\beta_{\text{total}}$ ratio in the conditioned culture media of transfected IMR-32 cells were measured by sandwich ELISAs. Values are the mean and SE of determinations made in six separate cultures. ***$P < 0.001$ compared with corresponding values of $A\beta_{1\text{-}42}/A\beta_{\text{total}}$ ratio in untransfected and vector-transfected cells. ###$P < 0.001$ compared with the value of $A\beta_{1\text{-}42}/A\beta_{\text{total}}$ ratio in cells transfected with Par-4 only. Similar data were obtained from at least two clones from each of the transfected cell lines. ANOVA with Scheffe's post-hoc tests

dependent on nuclear translocation. Of importance, we have found that Par-4 specifically interacts ACID. In pull-down assays, Par-4 protein was pulled down by an APP C-terminal fragment peptide (AVTPEERHLSKMQQNGYENPTYKFF) corresponding to the amino acid sequence of CTFγ28–52 immobilized on gel beads. Additional co-immunoprecipitation experiments showed that CTFγ57 binds to C-terminal region (amino acids 181-342) of Par-4. Co-expression of Par-4 significantly exacerbated apoptosis induced by CTFγ57. In addition, RNAi-mediated silencing of Par-4 expression protects against apoptotic cell death induced by CTFγ57. These results suggest that Par-4 may be involved in regulation of AICD activity, possibly by altering nuclear translocation of AICD and/or AICD-dependent pro-apoptotic transcriptional transactivation.

AICD, Fe65, and Tip60 have been reportedly to form a transcriptionally active complex in transiently transfected cells [54, 166, 167]. KAI1 is a specific in vivo gene target of this APP-dependent transcription complex. KAI1 is a tetraspanin, a cell surface molecule acting as a tumor metastasis suppressor that functions in cell adhesion and is capable of interacting with many plasma membrane receptors. In mice transgenic for hAPP, specific binding of AICD/Fe65/Tip60 on the KAI1 promoter was observed. Levels of both KAI1 protein and mRNA expression were found to be significantly increased in mice transgenic for APP [169]. In transfected neural IMR-32 cells, AICD induces KAI1 expression, which is potentiated by co-expression of Par-4 (Figs. 10–12).

Small interference RNA (siRNA) cocktail targeted against Par-4 was generated, and efficiently transferred to primary neurons and cell lines [142]. Suppression of Par-4 by RNAi protects against apoptotic cell death induced by CTFγ57 (Figs. 13 and 14).

2.8 Par-4 Increases BACE1 Cleavage of APP: Effects of RNAi-Mediated Par-4 Gene Silencing

We have found that Par-4 significantly increases BACE1 cleavage of APP and increases neuronal cell death by increasing Aβ42 production [33, 34]. Specific siRNAs used to target endogenous Par-4 mRNA were described previously [33, 142]. For analysis of apoptotic cell death, medium was removed and replaced with Locke's solution without glucose. Par-4 increases β-secretase cleavage of APP (as measured by generation of the β-secretase cleavage products C99/C89 as well as $A\beta_{40}$ and $A\beta_{42}$). RNAi-mediated par-4 gene silencing effectively blocked this adverse effect of Par-4 (Figs. 15 and 16, Table 2).

3 AATF Is a Par-4 Interacting Protein that Functions as an Endogenous Negative Regulator of Par-4 Activity in Neurodegeneration

3.1 Specific Interaction between AATF and Par-4

Because Par-4 plays an important role in aberrant APP processing and signaling of cell death in neurodegeneration, identification of one or more *endogenous* factors that regulate Par-4 activity should have significant therapeutic implications for pharmacological modulation of APP processing and signaling of neuronal death in AD. We have found that AATF (apoptosis antagonizing transcription factor), another leucine zipper domain containing protein, is an endogenous interaction partner and potent inhibitor of Par-4 activity [34, 171–175].

AATF was initially identified as an interaction partner of DAP-like kinase (Dlk), a member of the DAP (Death-Associated Protein) kinase family of pro-apoptotic serine/threonine kinases [171, 173]. Human AATF gene was mapped to chromosome 17q11.2-q12 and encodes a protein that has an open reading frame of 560 amino acids that contain a leucine zipper domain, nuclear localization signals, and potential phosphorylation sites for different kinases. We found that AATF was a novel and potent cytoprotective factor against neuronal cell death. In fact, AATF modulates both apoptotic and necroptotic neuronal cell deaths.

AATF and Par-4 are both localized in the nucleus and cytosol in IMR-32 cells and primary neurons. Importantly, immunoprecipitation/Western blotting analyses showed that Par-4 and APP form a complex in vivo in transfected neural cells. Indeed, AATF and Par-4 interact with each other via the leucine zipper domain, as shown in Fig. 17. A peptide corresponding to the leucine zipper of human AATF (-LKNSHKALKALLRSLVGLQEEL) was shown to bind specifically to Par-4. Co-transfection of AATF with Par-4 in neural cells counteracts the cell-promoting actions of Par-4 [34, 175–177].

3.2 AATF Interferes with Binding of Par-4 to AICD and Protects against Cell Death Induced by CTFγ57

Recent data from our laboratory further suggest that binding of AATF to Par-4 may interfere with Par-4/AICD interaction and thereby inhibit neuronal cell death signaling mediated by CTFγ57. In other words, Par-4/AATF

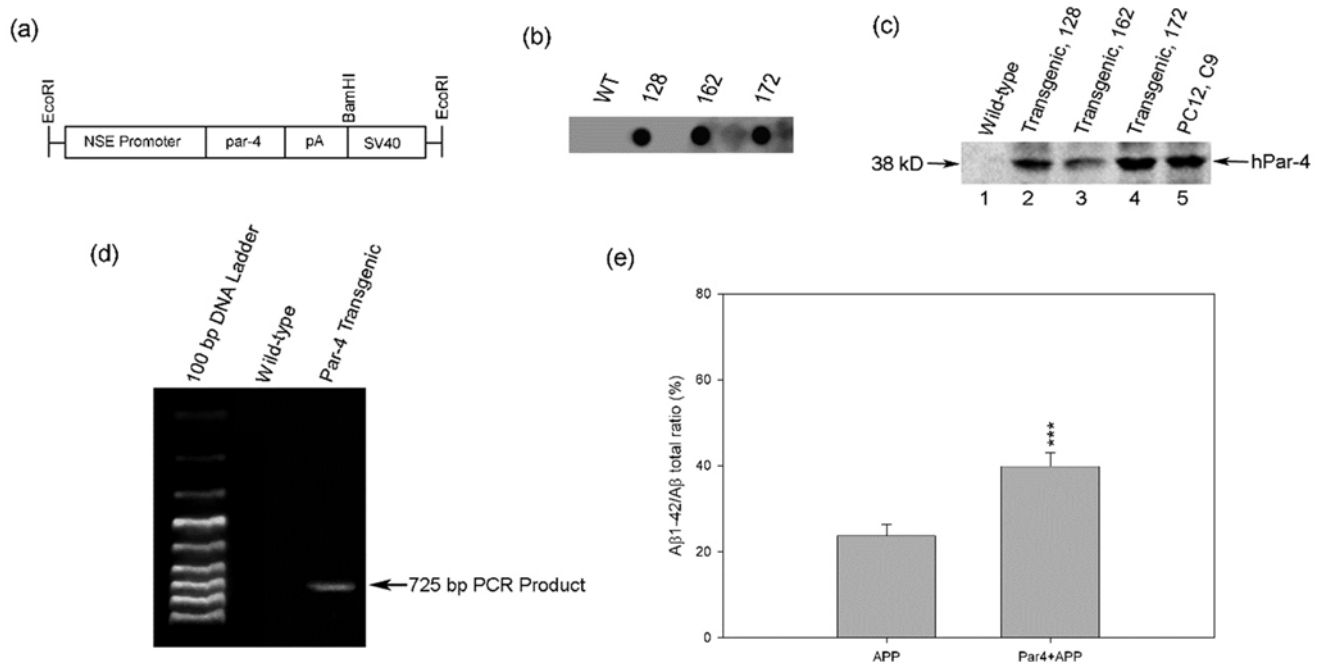

Fig. 9 Generation and Characterization of mice transgenic for Par-4: Effect on Aβ production. (**a**) ***The NSE-par4 construct used to generate Par-4 transgenic mice***. The pNSE-par4 transgenic construct was derived from pNSE-bcl2 by removal of a HindIII and Cla I fragment containing the coding sequence of the human bcl-2 gene, and in frame ligation of a 1.038-kb HindIII/ClaI PCR fragment containing the coding sequence of the human par-4 gene. The pNSE-Par4 plasmid was then digested with EcoR I to recover the approximately 4.0 kb NSE-par4 fragment that contains NSE promoter, par-4 cDNA, pA, and SV40 sequence. The purified NSE-par4 construct was then used for microinjection into zygotes from inbred strain FVB/N. The Oklahoma Medical Research Foundation (OMRF) Microinjection Core has its own FVB/N breeding colony to generate females for zygote donation. Females are superovulated and mated; zygotes are harvested and fertilized zygotes are injected. Injected zygotes which develop further to the two-cell stage are reimplanted into the oviduct of pseudo-pregnant Swiss-Webster recipient females. All resulting pups are subject to characterization for transgenic founder animals and further analysis. pA, simian virus 40 (SV40) polyadenylylation; par-4, coding region of human par-4 cDNA; SV40, SV40 sequence used to genotype the transgenic mice. (**b**) ***Integration of the transgene was detected by dot hybridization of DNA obtained from tail biopsies to the 750-bp EcoRI/BamHI simian virus 40 fragment from pNSE-CAT as a probe***. Three representative transgenic mouse lines (denoted as 128, 162, and 172) are shown. WT indicates DNA samples from a nontransgenic wild-type control mouse. (**c**) ***Representative Western blotting analysis showing high levels of expression of human Par-4 protein in hippocampal neurons from transgenic mice using a specific polyclonal antibody against human Par-4***. Primary cultures of hippocampal neurons were established and Western blot analyses were performed in 7-day-old cultures using an antibody that specifically recognizes a 19 amino acid peptide ($SAMLTRAPPARGPPRSED_{\text{-COOH}}$) corresponding to amino acids 95-112 of human Par-4. Note that high levels of transgenic Par-4 (hPar-4) protein were detected in neurons from Par-4 transgenic mice (lanes 2–4), while little Par-4 was observed in wild-type mice (lane 1). Lane 5 shows high levels of Par-4 from a PC12 cell line stably transfected with pCMV-hPar4 that encodes a full-length human Par-4 cDNA. Little or no NSE-par4 transgene expression in mice was found in other tissues, including liver, lung, kidney, intestine, or thymus (data not shown). (**d**) ***Genotyping of Par-4 transgenic mice using a PCR-based protocol.*** We have developed a quick PCR-based assay of the DNA from tail biopsies to amplify a 725-bp simian virus 40 fragment from pNSE-Par4 vector, which is detectable only in mice transgenic for Par-4 (lane 2), but not in wild-type mice (lane 1). The primers used for the PCR genotyping protocol were: SV40F: 5′-ccaggaagctcctctgtgtc-3′, and SV40R: 5′-gacttaacctgtggaaatattttga-3′. (**e**) ***Par-4 increases production of $A\beta_{1-42}$ in hippocampal neurons***. Mice transgenic for Par-4 (from line 172) were crossed with those transgenic for human APPswe, and cultures of hippocampal neurons were established from the Par4/APPswe double transgenic mice. Values of the $A\beta_{1-42}/A\beta_{total}$ ratio in the conditioned culture media were then measured by sandwich ELISAs. Hippocampal neurons from mice transgenic for human APPswe alone were used as controls. Note that expression of Par-4 significantly increased the $A\beta_{1-42}/A\beta_{total}$ ratio in the conditioned media. Values are the mean and SE of determinations made in six separate experiments. $^{***}P < 0.01$ compared with the value of the $A\beta_{1-42}/A\beta_{total}$ ratio in mice expressing APPswe alone. ANOVA with Scheffe's *post-hoc* tests

Table 1 Par-4 increases production of $A\beta_{40}$ and $A\beta_{42}$ in hippocampal neurons from mice transgenic for APPswe

Transgenic mice	$A\beta_{40}$ (*fmol/ml*)	$A\beta_{42}$ (*fmol/ml*)	Average $A\beta_{42}/A\beta_{total}$ ratio (%)
APPswe	108.7 ± 12.1	32.6 ± 3.3	23.2
Par4 + APPswe	169.1 ± 13.7***	106.3 ± 8.9***	38.6

Mice transgenic for Par-4 were crossed with those transgenic for human APPswe, and cultures of hippocampal neurons were established from the Par4/APPswe double transgenic mice. Values of the $A\beta_{40}$ and $A\beta_{42}$ in the conditioned culture media were then measured by sandwich ELISAs. Hippocampal neurons from mice transgenic for human APPswe alone were used as controls. Co-expression of Par-4 in these mice significantly increased levels of both $A\beta_{40}$ and $A\beta_{42}$ in the conditioned media. Due to expression of the APPswe mutations, the increase in neuronal $A\beta_{42}$ was more pronounced than that in $A\beta_{40}$ (which led to an increase in $A\beta_{42}/A\beta_{total}$ ratio) in Par4/APPswe double transgenic mice. Values are the mean and SE of determinations made in six separate experiments

***$P < 0.01$ compared with corresponding values in mice expressing APPswe alone. ANOVA with Scheffe's *post-hoc* tests

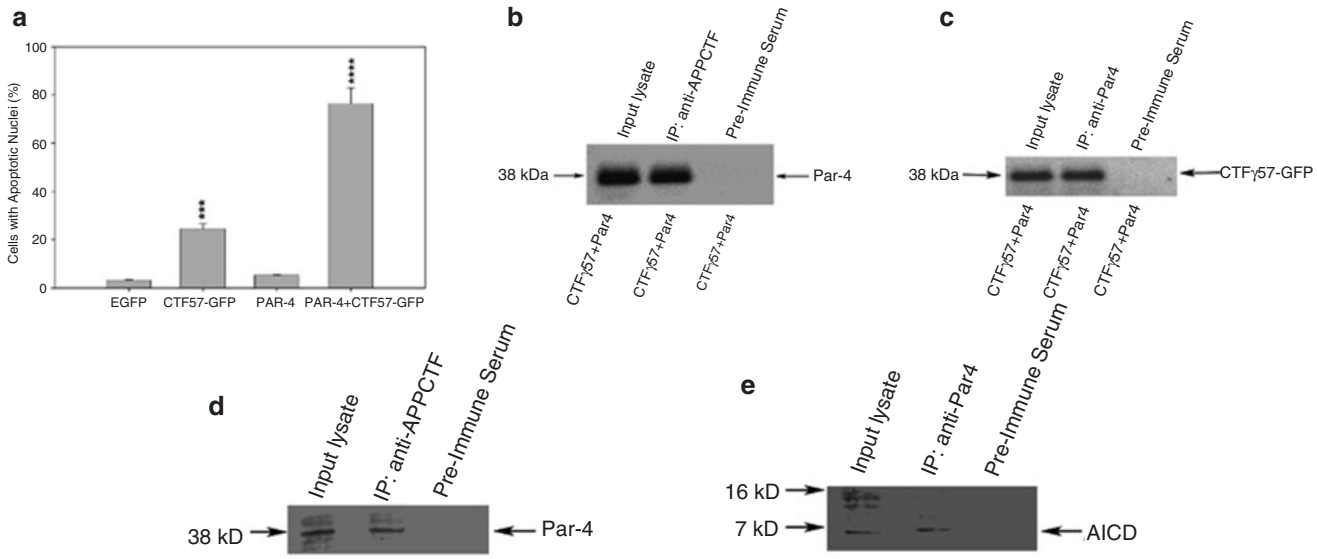

Fig. 10 Par-4 forms a complex with CTFγ57 and promotes the apoptotic activity of CTFγ57. (**a**) *Par-4 exacerbates apoptosis induced by CTFγ57 in IMR-32 cells.* IMR-32 cells were transfected with pEGFP-CTFγ57 (CTF57-GFP) or pREP4-Par4 using the Lipofectamine 2000 transfection reagent. Parallel cultures were co-transfected with pEGFP-CTFγ57 (CTF57-GFP) and pREP4-Par4 (Par4+CTF57-GFP). Cells transfected with vector pEGFP-N1 (EGFP) were used as a control. 48 h following transfection, cells were stained with DNA-binding dye Hoescht 33,342, and the percentage of transfected cells in each culture with apoptotic nuclei (condensed and fragmented DNA) was determined. Note that co-expression of Par-4 significantly increased apoptosis induced by CTFγ57. Values are the mean and SEM of determinations made in at least three cultures. ***$P < 0.01$ compared with the value in EGFP group. ****$P < 0.001$ compared with values in other groups (ANOVA with Scheffe's post-hoc tests). (**b**) *Par-4 is associated with CTFγ57 in transfected IMR-32 cells* in vivo. IMR-32 cells were transfected with pEGFP-CTFγ57 (CTF57-GFP) or pREP4-Par4 using the Lipofectamine 2000 transfection reagent. 24 h following the transfection, cells were lysed and precipitated with the antibody against the carboxyl-terminal 20 amino acid residues of APP770 (anti-APP-CT20), followed by Western blotting with Par-4 antibody. Input lane shows 10% of the total protein used in immunoprecipitation experiments. The pre-immune serum was used as a control. (**c**) *Reverse order of immunoprecipitation/Western blot analysis of the same transfected cells showed similar Par-4/CTFγ57 complex formation (middle lane).* Input lane shows 10% of the total protein used in immunoprecipitation experiments. The anti-APP-CT20 antibody recognized predominantly a band of CTFγ57-GFP fusion protein at about 33 kDa. (**d**) *Physiological relevant interaction between endogenous Par-4 and AICD in primary neurons.* Primary hippocampal neuronal cultures were established from newborn wild-type mouse pups, and interaction between Par-4 and AICD was analyzed in 7-day-old cultures when levels of endogenous AICD accumulated to its highest amounts during differentiation [170]. Cells were lysed and precipitated with the anti-APP-CT20 antibody, followed by Western blotting with Par-4 antibody. Input lane shows 10% of the total protein used in immunoprecipitation experiments. The pre-immune serum was used as a control. (**e**) *Reverse order of immunoprecipitation/Western blot analysis of the same non-transfected hippocampal neurons showed similar endogenous Par-4/AICD complex formation*. The anti-APP-CT20 antibody recognized predominantly a band of AICD at about 6 kDa. The specificity of Par-4/AICD interaction was confirmed by the observation that Par-4 or AICD failed to interact with c-Jun in similar experimental conditions

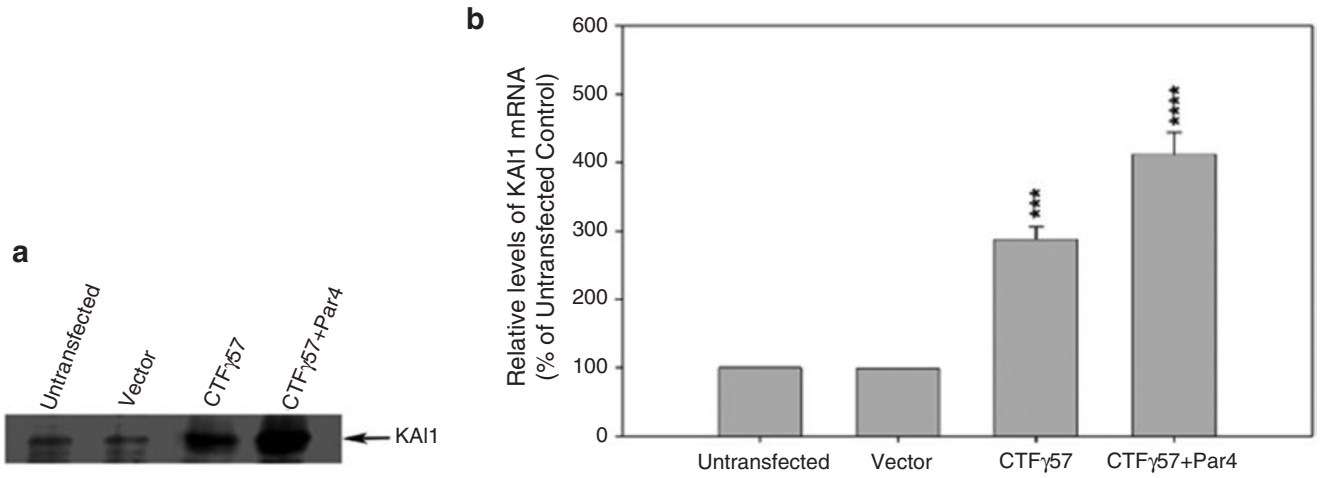

Fig. 11 AICD induces KAI1 expression in IMR-32 cells: potentiation by Par-4. (**a**) *Representative Northern blot analysis showing increased KAI1 gene expression in cells transfected with CTFγ57 and Par-4 in IMR-32 cells.* IMR-32 cells were transfected with pEGFP-CTFγ57 (CTFγ57) alone, or co-transfected with pEGFP-CTFγ57 and pREP4-Par4 (Par4+CTFγ57). Cells transfected with vector pEGFP-N1 (EGFP) were used as a control. 48 h following transfection, total RNA was prepared, and subject to Northern blot analysis using an RT-PCR amplified ^{32}P labeled 268 bp DNA fragment from human KAI1 ORF. (PCR primers used were: forward: ct taggatgggggcctatgt reverse: ttcatgagctcagcgttgtc). Increased KAI1 gene expression was clearly observed in cells transfected with CTFγ57. The increase in KAI1 gene expression was further exacerbated by co-expression of Par-4. (**b**) *Statistical analysis of the Northern blotting data showing effect of AICD and Par-4 on KAI1 gene expression.* Values are the mean and SE (bars) of determinations made in at least six separate experiments. ***$P < 0.01$ and ****$P < 0.001$, respectively, compared to values in untransfected and vector-transfected control groups. ANOVA with Scheffe's post-hoc tests

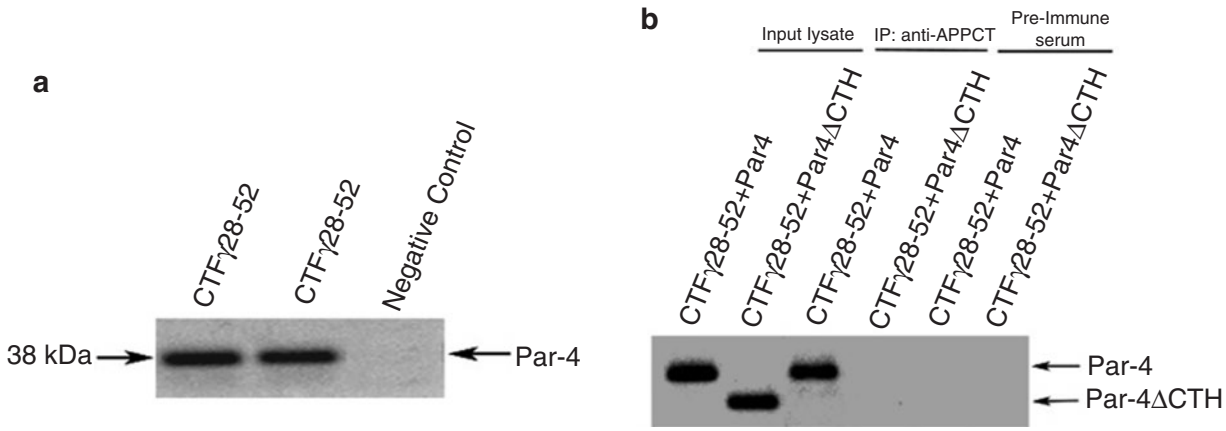

Fig. 12 Mapping of interaction domains in AICD and Par-4. (**a**) *Interaction between Par-4 and AICD: Par-4 binds to an APP C-terminal fragment peptide derived from CTFγ28–52* in vitro. The figure shows SDS-PAGE electrophoresis pattern of the pull-down of Par-4 protein by an APP C-terminal fragment peptide (AVTPEERHLSKMQQNGYENPTYKFF) corresponding to the amino acid sequence of CTFγ28-52 immobilized on gel beads (see methods). The CTFγ28-52 peptide (with an added cysteine residue at the C-terminal end to allow binding to the iodoacetyl groups in Sulfolink Coupling Gel) was covalently linked to Sulfolink Coupling Gel (Pierce). Full-length Par-4 was expressed in IMR-32 cells. The cell lysates were incubated with the gel-linked CTFγ28-52 peptide, and the washed gel samples were then subjected to SDS-PAGE and Western blotting for Par-4. Cysteine-blocked gel was used as negative control. (**b**) *CTFγ57 binds to C-terminal region (amino acids 181–342) of Par-4.* IMR-32 cells were co-transfected with pEGFP-CTFγ57 (CTF57-GFP) and pREP4-Par4 or pREP4- Par-4deltaCTHF (a carboxyl terminus half deletion mutant of Par-4 that lacks nucleotides 541 through 1267 in Par-4 cDNA) using the Lipofectamine 2000 transfection reagent. 24 h following the transfection, cells were lysed and precipitated with the anti-APP-CT20 antibody, followed by Western blotting with Par-4 antibody. Input lane shows 10% of the total protein used in immunoprecipitation experiments. The pre-immune serum was used as a control. Note that CTFγ57 interacted only with the full-length Par-4 but not Par-4 lacking the C-terminal region of the nucleotides 541 through 1267, indicating that the C-terminal domain of Par-4 (amino acids 181–342 of Par-4) is involved in interacting with CTFγ57. Reverse order of immunoprecipitation/Western blot analysis showed similar Par-4/CTFγ57 complex formation (data not shown)

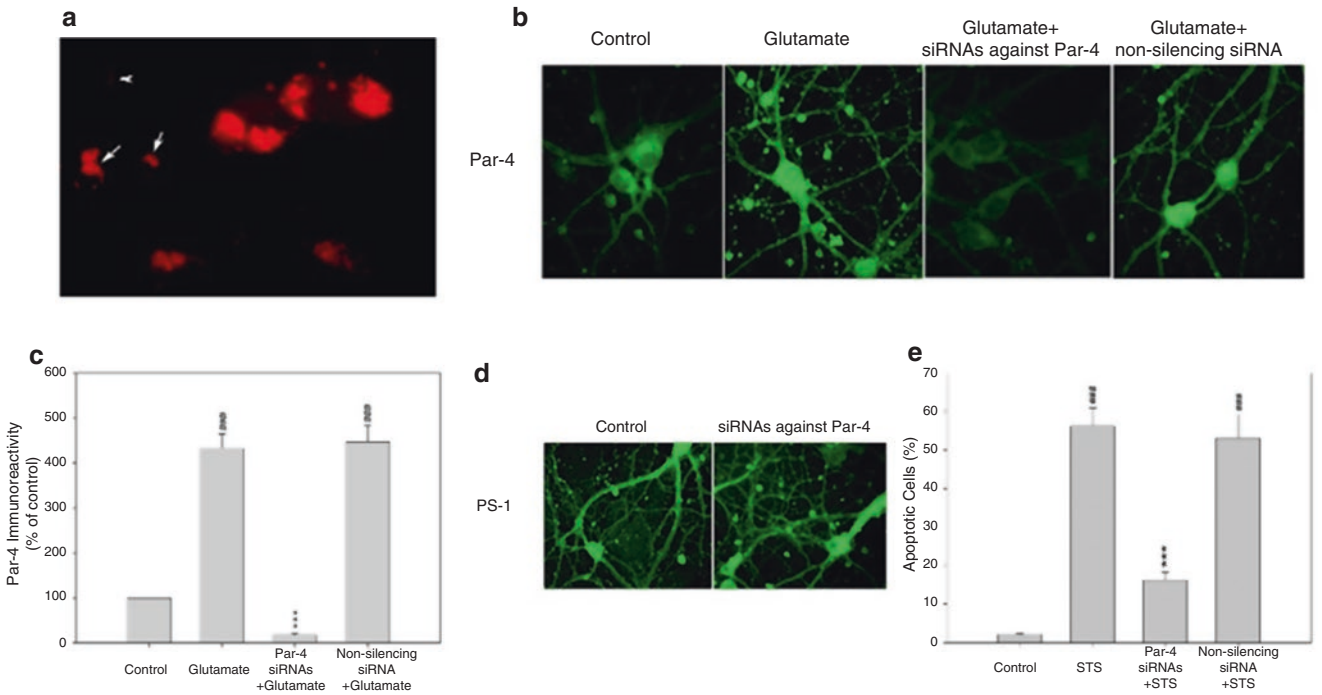

Fig. 13 Knockdown of Par-4 expression by small interference RNA (siRNA) cocktail in primary neurons. (**a**) *Representative microscopic image showing efficient transfection of fluorescently labeled anti-Par4 siRNAs into primary spinal motor neurons*. siRNAs against Par-4 were fluorescently labeled with Cy3 using Ambion's Silencer™ siRNA labeling kit. Labeled siRNAs in transfected cells were then analyzed by fluorescence microscopy. Note that, in this field, strong fluorescent siRNA (red, arrows) signals were observed in the cytoplasmic perinuclear regions in over 80% of neurons, while less than 20% of the neurons (arrowhead) had weak or no siRNA fluorescence. An average of 78.6 ± 6.2% of siRNA transfection efficiency was obtained in six separate experiments. (**b**) *siRNA cocktail targeted against Par-4, but not a non-silencing siRNA, inhibits Par-4 expression induced by glutamate in primary neurons*. Primary spinal motor neurons from mice were either mock-transfected (control), or transfected with siRNAs against Par-4 at a concentration of 100 nM. A non-silencing siRNA (see specific aim 2 for details) was used as a negative control. 48 h after siRNA transfection, cells were treated with either vehicle (control) or 50μM of glutamate for 8 h. The cells were then processed for Par-4 immunoreactivity using fluorescent microscopy. Note that the induction of Par-4 expression was completely knockdown by siRNAs targeted against Par-4, but not by the non-silencing siRNA. (**c**) *Quantitative analysis of Par-4 knockdown by siRNAs in spinal motor neurons as assessed by immunofluorescent microscopy*. Par-4 immunoreactivity obtained with fluorescent microscopy was quantified using LSM 510 image analysis software. Values are the mean and SE (bars) of determinations made in at least six separate experiments. ###$P < 0.001$ compared with the value in control group. ***$P < 0.001$ compared with values in glutamate treated or glutamate plus non-silencing siRNA treated groups. (**d**) *Expression of another protein (presenilin-1) was unaffected by siRNAs targeted against Par-4*. Primary spinal motor neurons from wild-type mice were either mock-transfected (control) or transfected with siRNA cocktail targeted against Par-4. 48 h after transfection, cells were then processed for presenilin-1 (PS-1) immunoreactivity using fluorescent microscopy. (**e**) RNAi knockdown of Par-4 expression protects against staurosporine (STS)-induced apoptosis in primary neurons. Primary cultures of mouse motor neurons were either mock-transfected (control), or transfected with siRNAs against Par-4. A non-silencing (NS) siRNA was used as a negative control. 48 h after siRNA transfection, cells were treated with either vehicle (0.2 dimethyl sulfoxide, control) or 100 nM of STS for 24 hs. Cells were fixed and stained with DNA-binding dye Hoechst 33342, and the percentages of neurons in each culture exhibiting apoptotic nuclei was determined using fluorescent microscopy. ###$P < 0.001$ compared with the value in control group. ***$P < 0.01$ compared with the values in STS alone and non-silencing siRNA+STS treated groups. Data are mean and SE (bars) values of determinations made in six separate cultures. ANOVA with Scheffe's post-hoc tests

interaction inhibits AICD-mediated apoptotic activity by interfering with the recruitment of AICD by Par-4. As shown in Fig. 8, the inhibitory effect of AATF on binding of Par-4 to AICD was analyzed by competition pull-down experiments using a peptide corresponding to the leucine zipper of human AATF (-LKNSHKALKA LLRSLVGLQEEL, which has been shown to bind to Par-4), and a peptide corresponding to CTFγ28–57 (AVTPEERHLSKMQQNGYENPTYKFF, which has also been shown to bind to Par-4). For this procedure, CTFγ28–57 (with an added cysteine residue at the C-terminal end to allow binding to the iodoacetyl groups in Sulfolink Coupling Gel) was covalently linked to Sulfolink Coupling Gel (Pierce). Cell lysates were prepared from IMR-32 cells transfected with Par-4 and mixed with human AATF leucine zipper peptide and incubated with gel bearing immobilized CTFγ28–57 peptide in PBS. The gel beads were pelleted, and the proteins on the gel beads eluted by SDS sample buffer and subjected to SDS-PAGE electrophoresis (Fig. 18).

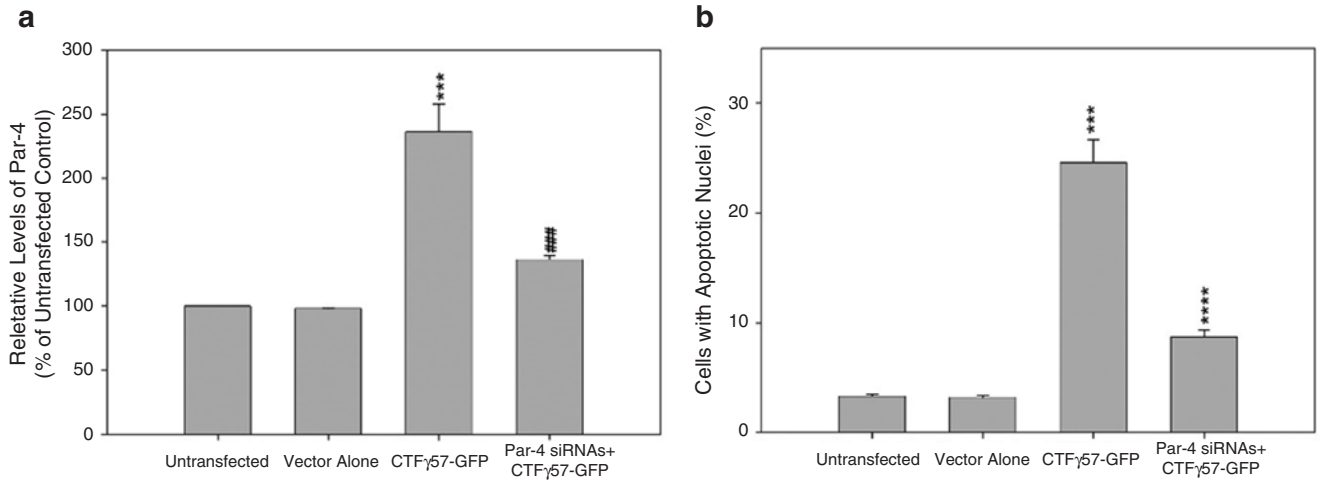

Fig. 14 Suppression of Par-4 by RNAi protects against apoptotic cell death induced by CTFγ57. (**a**) *Transfection of CTFγ57 increases levels of Par-4 in IMR-32 cells: effect if Par-4 siRNAs.* IMR-32 cells were transfected with pEGFP-CTFγ57, or co-transfected with pEGFP-CTFγ57 and siRNA cocktail targeted against Par-4 using the Lipofectamine 2000 transfection reagent. Cells transfected with vector pEGFP-N1 (EGFP) were used as a control. 48 h following transfection, cells were lysed and levels of Par-4 were analyzed by Western blotting. Note that overexpression of CTFγ57 resulted in a significant increase in expression of Par-4, which was significantly inhibited by Par-4 siRNAs. Values are the mean and SEM of determinations made in six separate experiments. ***$P < 0.01$ compared with values in untransfected and vector-transfected control groups. ###$P < 0.01$ compared with the value in cells transfected with pEGFP-CTFγ57 alone. (**b**) *Silencing of Par-4 expression by RNAi significantly inhibited apoptosis induced by CTFγ57.* IMR-32 cells were transfected with pEGFP-CTFγ57, or co-transfected with pEGFP-CTFγ57 and siRNA cocktail targeted against Par-4 using the Lipofectamine 2000 transfection reagent. Cells transfected with vector pEGFP-N1 (EGFP) were used as a control. 48 h following transfection, cells were stained with DNA-binding dye Hoescht 33,342, and the percentage of transfected cells in each culture with apoptotic nuclei (condensed and fragmented DNA) was determined. Values are the mean and SEM of determinations made in six separate experiments. ***$P < 0.01$ compared with the value in untransfected and vector-transfected control groups. ****$P < 0.001$ compared with the value in cells transfected with CTFγ57-GFP. (ANOVA with Scheffe's post-hoc tests)

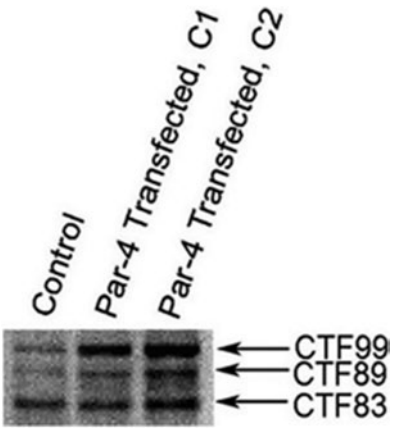

Fig. 15 Par-4 increases β-secretase cleavage of APP. Representative Western blot analysis of a rabbit polyclonal antibody raised against a 22 amino acid synthetic peptide derived from the C-terminus of the human APP. The data illustrate the increased levels of β-secretase cleavage product CTF99/CTF89 in IMR-32 cells transfected with Par-4. The bottom band represents the C-terminal α-secretase cleavage product CTF83, which was not significantly altered by Par-4

4 Neuroprotective Actions of Extracellularly Secreted AATF (sAATF) and SAP-12

4.1 AATF Is Secreted Extracellularly

AATF lacks a classical N-terminal signal peptide and is typically located intracellularly in cytoplasmic and/or nuclear compartments [34, 173, 175, 177]. However, we unexpectedly noted that an unusual amount of intracellular AATF protein was secreted extracellularly by cortical neurons following acute exposure to ischemic insults in vitro and in vivo. As shown in Fig. 19, both endogenous and transduced AATF were secreted extracellularly by cortical neurons. Secretion of AATF was neither dependent on the GFP-tag nor on the death of the cells.

4.2 AATF Is Associated with Cell Surface Receptor TLR-4

Secreted AATF (sAATF) is highly protective against hypoxia-induced neuronal damage. In searching for cellular surface receptors of sAATF, we used antibodies against three potential candidate receptor proteins involved in cell death in initial screening: GRP78 (78-kDa glucose-regulated protein), TLR2 (Toll-like receptor 2), and TLR4 (Toll-like receptor 4). GRP78 was selected based on the fact that it is both an endoplasmic reticulum chaperone and a cell surface

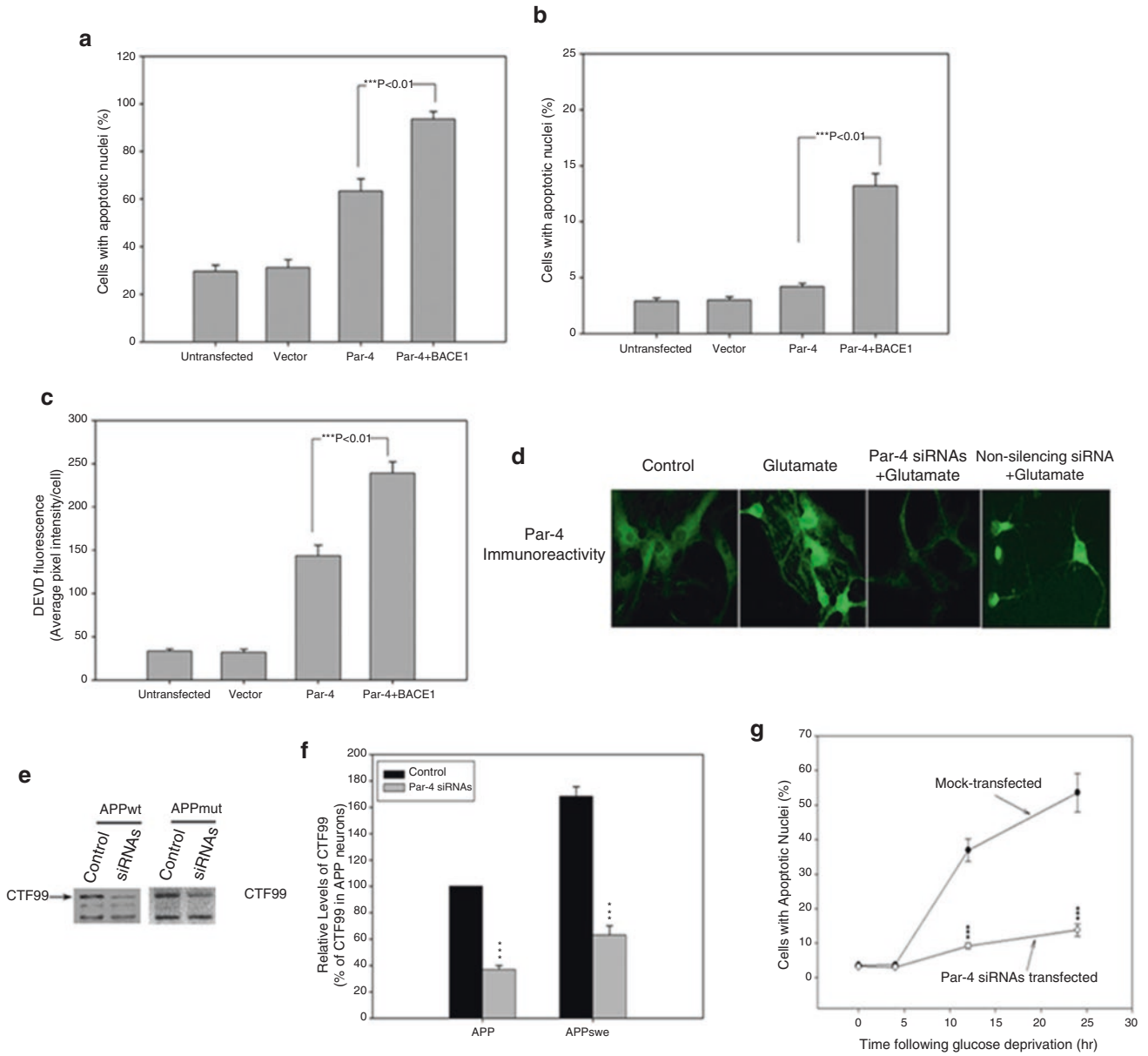

Fig. 16 Par-4/BACE1 double transfection increases cellular susceptibility to apoptosis and increases BACE1 cleavage of APP: blockade by RNAi. (**a**) ***Cells double transfected with Par-4 and BACE1 showed significantly increased vulnerability to trophic factor withdrawal-induced apoptotic cell death compared with those transfected with Par-4 alone***. Cultures of indicated types of transfected IMR-32 cells were deprived of trophic support for 24 h and percentage of cells with apoptotic nuclei was then quantified. Values are the mean and SE of determinations made in six separate cultures. ANOVA was performed with Scheffe's post-hoc tests. (**b**) ***Cells double transfected with Par-4 and BACE1 showed significantly increased basal apoptotic activity under normal culture conditions compared with those transfected with Par-4 alone***. Cultures of indicated types of transfected IMR-32 cells were established by plating equal number of cells (about 2×10^5 cells per 35 mm glass bottom dish) at the same time in the same amount (1 ml) of growth medium. 48 h later, the percentage of cells with apoptotic nuclei (basal apoptotic activity under normal culture conditions) was quantified. Values are the mean and SE of determinations made in six separate cultures. ANOVA was performed with Scheffe's post-hoc tests. (**c**) ***Early and exacerbated activation of caspase activity in cells double transfected with Par-4 and BACE1***. Cultures of indicated types of transfected IMR-32 cells were deprived of trophic support for 2 h, and levels of cellular DEVD fluorescence, a measure of caspase-3 activity, were quantified. Values are the mean and SE of determinations made in six separate cultures. ANOVA was performed with Scheffe's post-hoc tests. (**d**) ***Specific and highly efficient knockdown of Par-4 by RNAi: siRNA cocktail targeted against Par-4, but not a non-silencing siRNA, inhibits Par-4 expression induced by glutamate in primary hippocampal neurons***. Primary hippocampal mouse neurons were either mock-transfected (control), or transfected with siRNAs against Par-4 at a concentration of 100 nM. A non-silencing siRNA (see materials and methods) was used as a negative control. 48 h after siRNA transfection, cells were treated with either vehicle (control) or 50μM of glutamate for 8 h. The cells were then processed for Par-4 immunoreactivity using fluorescent microscopy. Induction of Par-4 expression was completely knocked down by siRNAs targeted against Par-4, but not by the non-silencing siRNA. For more quantitative analysis of the efficacy of the Par-4 RNAi approach, refer to our recent publication: *Xie J.* et al.

receptor for a wide variety of ligands [178]. TLR2 and TLR4 were selected based on their cell surface expression and their involvement in signaling of cell death [179]. Only TLR4 was found to be associated with AATF in neural cells. In addition, *TLR4-mediated signaling of cell death was largely blocked by sAATF* (Fig. 20). A variety of mammalian cell types express toll-like receptors (TLRs) which are transmembrane pattern-recognition receptors that are initially considered to be involved in rapid microbial recognition through detection of pathogen-associated molecular patterns [180]. TLRs may also be activated by host-derived endogenous molecules. Structurally, members of the TLR family share three domains: an extracellular domain with leucine-rich repeats, a transmembrane domain, and an intracellular Toll/IL-1R homologous region. The signaling pathways activated by TLRs are classified into myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways [180]. Of importance, an increased activation of TLR4-mediated pathways has recently been implicated in neurodegeneration [181]. These findings open the possibility that sAATF represents a novel endogenous blocker of cell surface TLR4 receptors. TLR-4 contains the RHIM domain involved in formation of the non-classical necrosome in neuronal necroptosis (programmed necrosis). Necroptosis is a regulated inflammatory mode of cell death that contributes to the pathology of ischemic brain injury and stroke [182–191]. Typically, receptor-interacting protein kinase 3 (RIPK3) recruits and phosphorylates the executioner mixed lineage kinase domain-like protein (MLKL) to signal necroptosis. The assembly of pore-forming oligomers of phosphorylated MLKL (pMLKL) causes membrane rupture in necroptosis [192–200]. sAATF blocks TLR4-mediated, RIPK3/MLKL-dependent necroptosis of cortical neurons. Thus, sAATF represents a novel endogenous blocker of cell surface TLR4 receptors (Figs. 20–22).

4.3 SAP-12 Confers Greater Neuroprotective Potency and Broader Effective Dose Range than the Full-Length AATF

A region corresponding to the amino acid sequence between AATF179 and AATF279 was found to be responsible for interacting with TLR-4. Surprisingly, a small core peptide from this TRL-4 binding region of AATF (SALKNSHKALKA), termed as SAP-12 (secreted AATF peptide of 12 amino acids), provided a much greater neuroprotective potency and broader effective dose range than the full-length sAATF. As shown in Fig. 21, although both SAP-12 and sAATF are highly neuroprotective against hypoxia-induced cell death, SAP-12 has an EC50 of 1.003 fM and confers much greater neuroprotective potency and broader effective dose range than the full-length AATF (AATF has an EC50 of 1.643 pM).

As shown in Fig. 22, confocal laser-scanning microscopy and a flow cytometry method were used to evaluate MLKL immunofluorescence and necroptotic cell death. Levels of MLKL-immunoreactivity and PI^+/Annexin V^+ necroptotic cells were significantly increased in neurons following ischemia-like insults, which was effectively blocked by SAP-12. Western blot analysis showed that levels of RIPK3 and MLKL, as well as phosphorylated MLKL (pMLKL) in the ipsilateral striatum were significantly increased in the ischemic striatum after MCAO, which was effectively blocked by intracerebroventricular injection of SAP-12 in vivo (Fig. 23). Taken together, SAP-12 provides a novel lead in peptide therapeutics for neuronal necroptosis in ischemic stroke, which may set the groundwork for producing new therapeutics in the future, including the optimization of the pharmacokinetic properties and target specificity of SAP-12.

4.4 Extracellular Release of sAATF Is Negatively Regulated by an Intracellular process Involving Binding of AATF by Par-4

We further examined the effect of Par-4 on sAATF in vivo using Par-4 homozygous (Par-$4^{-/-}$) and heterozygous (Par-$4^{-/-}$) null mice (C57BL6 background). Loss of function of Par-4 in these mice was achieved by deletion of exon 2 and the initiating ATG codon. Exon 2 was flanked by loxP sites, and constitutive KO allele was achieved after in vivo Cre-mediated recombination. These mice appear to be normal during embryonic development and they breed most efficiently at age of 2–6 months. PCR-based genotyping of the homozygous Par-$4^{-/-}$ mice was accomplished by identifying a 248 nt DNA fragment after employing a pair of specific

Fig. 16 (continued) J Neurochem 2005;92 (1):59–71. (e, f) RNAi-mediated silencing of Par-4 leads to a decrease in β-secretase cleavage of APP in primary neurons expressing APPwt or APPswe. (e) Representative Western blots showing depletion of Par-4 by RNAi reduces production of CTF99 in hippocampal neurons expressing APPwt or APPswe. (f) Densitometric analysis of Western blots of CTF99 in primary hippocampal neurons expressing APPwt or APPswe. ***$P < 0.001$ compared with corresponding values in control (non-siRNA treated) neurons. Values are the mean and SE of determinations made in six separate experiments. (g) Suppression of Par-4 blocks apoptotic cell death induced by glucose deprivation in basal forebrain neurons. Cultures of control (mock-transfected) neurons and those transfected with Par-4 siRNAs were deprived of glucose for the indicated time periods. Cells with apoptotic nuclei (showing nuclear chromatin condensation and fragmentation) were stained with the fluorescent DNA-binding dye propidium iodide and counted under a confocal microscope. ***$P < 0.001$ compared with corresponding values in mock-transfected control cells. ANOVA with Scheffe's post-hoc tests

Table 2 Co-transfection of Par-4 with BACE1 increases levels of BOTH $A\beta_{40}$ and $A\beta_{42}$

	Without BD-*fmk*			With BD-*fmk*		
Cell lines	$A\beta_{40}$ (*fmol/ml*)	$A\beta_{42}$ (*fmol/ml*)	Average $A\beta_{42}/A\beta_{total}$ ratio (%)	$A\beta_{40}$ (*fmol/ml*)	$A\beta_{42}$ (*fmol/ml*)	Average $A\beta_{42}/A\beta_{total}$ ratio (%)
Untransfected	119.2 ± 8.1	18.3 ± 1.2	13.3	116.2 ± 7.6	16.9 ± 1.3	12.7
Vector alone	115.1 ± 7.2	15.4 ± 0.7	11.8	117.6 ± 8.1	16.2 ± 0.9	12.1
Par-4	121.3 ± 9.7	19.1 ± 1.7	13.6	118.6 ± 6.3	16.9 ± 0.9	12.5
BACE1	163.6 ± 12.2	34.0 ± 2.5	17.2	161.0 ± 11.3	24.1 ± 2.2	13.0
BACE1 ± Par4	177.2 ± 13.7	64.2 ± 4.1	26.6	173.6 ± 11.6	28.4 ± 1.9***	14.1***

Par-4 does not directly interact with major elements of γ-secretase. Co-expression of BACE1 with Par-4 provided increased caspase activation and vulnerability to apoptosis, which may in turn alter APP processing in favor of $A\beta_{42}$ production. Thus, the increase in $A\beta_{42}/A\beta_{total}$ ratio observed under basal culture conditions in cells co-transfected with Par-4 and BACE1 was likely a secondary response to enhanced caspase activation, rather than a direct effect of Par-4 on γ-secretase itself, because inhibition of caspase activation prevents the increase in the secretion of $A\beta_{42}$ in these cells

***$P < 0.01$ compared with corresponding values in BACE1+Par4 cells without BD-*fmk* treatment. The amounts of $A\beta_{40}$ and $A\beta_{42}$ secreted by the indicated types of transfected cells (with or without pretreatment with 100 uM of the broad-spectrum caspase inhibitor BD-*fmk* for 2 h) in the conditioned medium were measured by sandwich ELISAs. The data showed that inhibition of caspase activation by BD-*fmk* largely prevented the Par4-induced increase in the secretion of $A\beta_{42}$ without significantly affecting levels of $A\beta_{40}$ in BACE1/Par-4 double transfected cells, which led to a significant reduction in $A\beta_{42}/A\beta_{total}$ ratio in these cells. Western blot analysis with using a mouse anti-APP antibody (Zymed Laboratories, Inc.) showed that total cellular levels of APP were not significantly altered by transfection of Par-4 (data not shown). Values are the mean and SE of determinations made in six separate cultures from at least three separate clones of transfected cell lines. ANOVA was performed with Scheffe's post-hoc tests

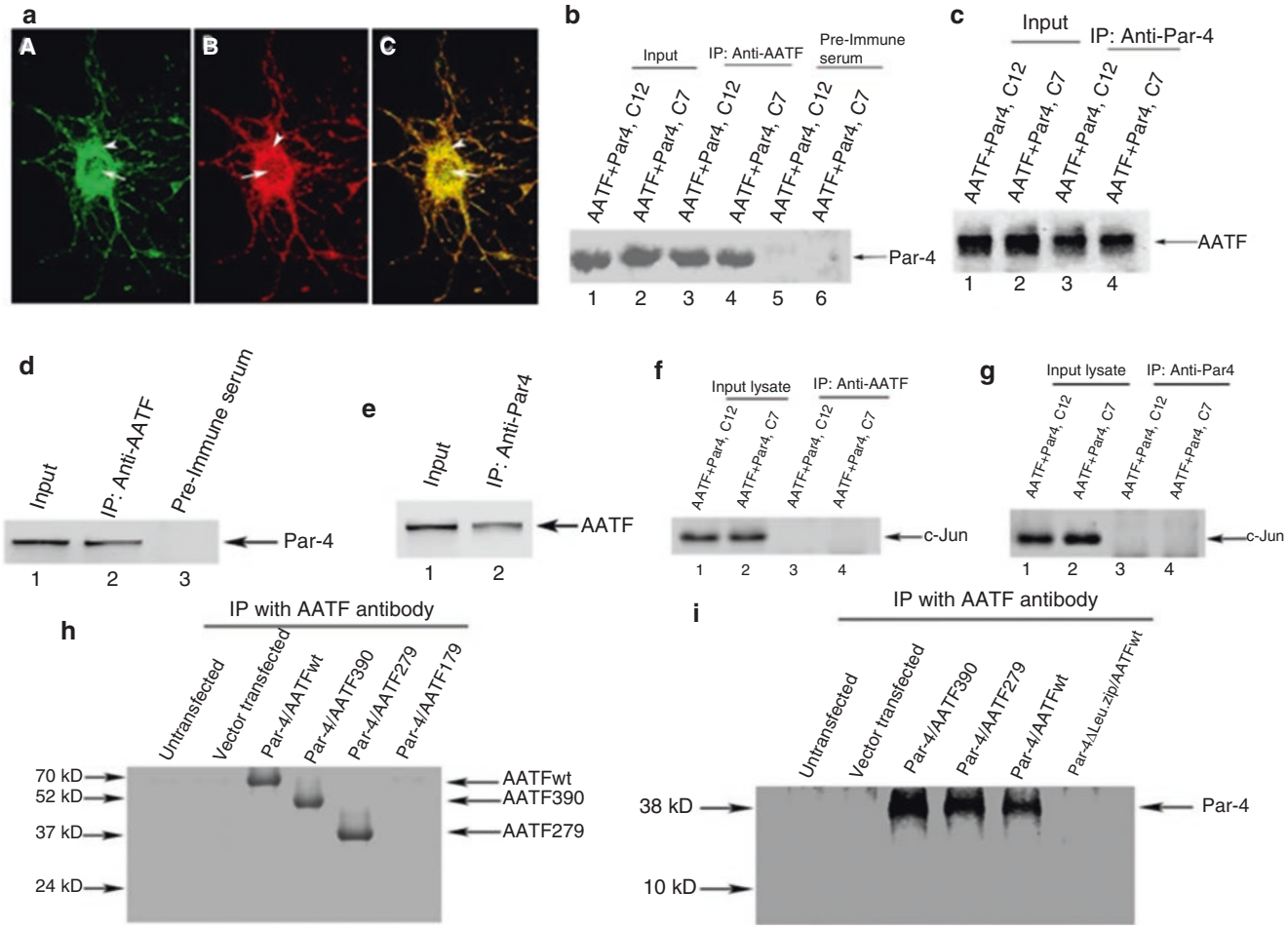

Fig. 17 Specific interaction between AATF and Par-4 via the leucine zipper domain in transfected cells and in primary neurons. (**a**) ***Immunocytochemistry analysis showing colocalization of AATF and Par-4 in primary neurons***. Representative confocal laser-scanning micrographs showing images of primary hippocampal neurons double-labeled with AATF and Par-4 antibodies. (A) Green fluorescence, AATF immunoreactivity; (B) Red fluorescence, Par-4 immunoreactivity; (C) A merged image (anaglyph) of A and B. Note yellow fluorescent areas indicating sites of colocalization of AATF and Par-4 immunoreactivity in both cytoplasmic (arrow head) and nuclear compartments (arrow). (**b**, **c**) ***AATF/Par-4 complex formation in co-immunoprecipitation assays in transfected IMR-32 cells.*** (**b**) Transfected IMR-32 cells co-expressing human AATF and Par-4 were lysed and precipitated with the AATF antibody, followed by Western blotting with Par-4 antibody (lanes 3–4). Input lanes (lanes 1–2) show 10% of the total protein used in immunoprecipitation experiments. The pre-immune serum from rabbits immunized with AATF was used as a control (lanes 5–6). AATF/Par-4 complex was clearly observed in two separate clones (C12 and C7) of transfected cells (lane 3–4). (**c**) Reverse order of immunoprecipitation/Western blot analysis of the same transfected cells showed similar AATF/Par-4 complex formation (lanes 3–4). Input lanes (lanes 1–2) show 10% of the total protein used in immunoprecipitation experiments. (**d**, **e**) ***Interaction between endogenous AATF and Par-4 in primary hippocampal neurons.*** (**d**) Cultures of primary hippocampal neurons were lysed and proteins from total lysates were immunoprecipitated with rabbit anti-AATF antibody (lane 2), followed by Western blotting with anti-Par4 antibody. The pre-immune serum from rabbits immunized with AATF was used as a control (lane 3). Input lane (lane 1) show 10% of the total protein used in immunoprecipitation experiments. (**e**) Reverse order of immunoprecipitation/Western blot analysis of the same hippocampal neurons showed similar endogenous AATF/Par-4 complex formation. The results indicate that AATF/Par-4 complex formation occurs without overexpression of the proteins and is therefore physiologically relevant. (**f**, **g**) ***Absence of interaction between c-Jun and AATF or Par-4.*** (**f**) Transfected IMR-32 cells co-expressing human AATF and Par-4 (clones C12 and C7) were lysed and proteins from total lysates were immunoprecipitated with rabbit anti-AATF antibody (lanes 3–4), followed by Western blotting with anti-c-Jun antibody. Input lanes (lanes 1–2) show 10% of the total protein used in immunoprecipitation experiments. Similar data were obtained in reverse order of immunoprecipitation/Western blot analysis of the same transfected cells (data not shown). (**g**) Transfected IMR-32 cells co-expressing human AATF and Par-4 (clones C12 and C7) were lysed and proteins from total lysates were immunoprecipitated with anti-Par-4 antibody (lane 3–4), followed by Western blotting with anti-c-Jun antibody. Input lanes (lanes 1–2) show 10% of the total protein used in immunoprecipitation experiments. Similar data were obtained in reverse order of immunoprecipitation/Western blot analysis of the same transfected cells (data not shown). Note that, that neither AATF nor Par-4 interacts with c-Jun, indicating AATF/Par-4 interaction was specific. (**h**, **i**) ***Mapping of the binding region of AATF and Par-4 to the leucine zipper domain by immunoprecipitation/ Western blotting analysis in transfected IMR-32 cells.*** (**h**) Representative immunoprecipitation/Western blot analysis showing AATF interacts with Par-4 via the leucine zipper domain of AATF. Double transfected IMR-32 cells used immunoprecipitation studies include: Par-4/AATFwt: cells co-transfected with full-length Par-4 and wild-type AATF; Par-4/AATF390: cells co-transfected with full-length Par-4 and the AATF deletion

primers (1639_28: GAGACTCCAGAACTTAGTTGC and 1640_30: CGTCTCGGAATGGAGG). Wild-type animals of the same genetic background were used as controls. As shown in Fig. 24, secretion of AATF and the neuroprotective actions of sAATF were significantly increased in primary cortical neurons of Par-4 null (Par-$4^{-/-}$) mice. Thus, binding of Par-4 to intracellular AATF decreased extracellular release of sAATF following glucose deprivation and chemical hypoxia in primary cortical neurons.

5 Par-4 in the Pathogenesis of Other Neurodegenerative Diseases and Neurological Dysfunctions

5.1 Par-4 Is a Synaptic Protein Involved in Pathogenesis of Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is characterized by clinically by paralysis and pathologically by specific degeneration and loss of motor neurons predominantly in the spinal cord and brainstem [138, 142, 201, 202]. There are significantly higher Par-4 levels in lumbar spinal cord samples from ALS patients than in lumbar spinal cord samples from neurologically normal patients [138, 142]. Mutations of human Cu/Zn superoxide dismutase (SOD1) are found in many familial amyotrophic lateral sclerosis patients, and expression of high levels of human SOD containing a substitution of glycine to alanine at position 93 caused typical motor neuron disease (ALS) symptoms in transgenic mice, indicating that dominant, gain-of-function mutations in SOD contribute to the pathogenesis of familial ALS [138, 142]. In this transgenic mice model of ALS, the highest Par-4 levels were observed in lumbar spinal cord at a time when they had hind-limb paralysis [138, 142]. Immunohistochemical analyses of human and mouse lumbar spinal cord sections revealed that Par-4 is localized to motor neurons in the ventral horn region. The possible role of Par-4 in motor neuron degeneration in ALS was further supported by evidence from in vitro studies showing that exposure of primary mouse spinal cord motor neurons or NSC-19 motor neuron cells to oxidative insults resulted in a *rapid and large* increase in Par-4 levels that *preceded* neuronal death. Pretreatment of the motor neuron cells with a Par-4 antisense oligonucleotide or expression of a dominant negative regulator of Par-4 (the leucine zipper domain) prevented oxidative stress-induced apoptosis and reversed oxidative stress-induced mitochondrial dysfunction that preceded apoptosis [138, 142]. These data strongly suggest that induction of Par-4 is an important and necessary event in the pathogenic mechanisms of neurodegeneration in ALS.

Signaling and integration events in synapses play important roles in regulating normal neuronal functions in spinal cord. Lower motoneurons in the spinal cord and brainstem receive much synaptic input from motor areas of the cerebral cortex. Dysfunction of synaptic contacts between upper and lower motoneurons may ultimately lead to degeneration of motoneurons. Indeed, synapse loss is a prominent feature of many acute and chronic neurodegenerative disorders, including ALS. Par-4 is enriched in synaptic compartments in spinal cord, and levels of Par-4 are regulated at translational level in synaptic terminals following apoptotic and excitotoxic insults, suggesting that Par-4 might play a role in synaptic function [138, 142]. Indeed, Par-4 was found to mediate the production locally in synaptic compartments of specific cytosolic factor(s) that induces nuclear chromatin condensation and DNA fragmentation (Fig. 25).

Fig. 17 (continued) mutant AATF390; Par-4/AATF 279: cells co-transfected with full-length Par-4 and the AATF deletion mutant AATF279; Par-4/AATF179: cells co-transfected with full-length Par-4 and the AATF deletion mutant AATF179; Par-4deltaLeu.zip/AATFwt: cells co-transfected with Par-4ΔLeu.zip and the full-length wild-type AATF. The indicated double transfected IMR-32 cell lines were lysed and precipitated with the Par-4 antibody, followed by Western blotting with AATF antibody. Note that Par-4 interacted with only wild-type AATF (AATFwt band at about 70 kD) and AATF deletion mutants containing the leucine zipper domain (AATF390 band at about 52 kD, and AATF279 band at about 37 kD), but not the AATF deletion mutant lacking the leucine zipper domain, indicating that the leucine zipper domain of AATF is necessary for interaction with Par-4. In untransfected or vector-transfected control cell lines, AATF immunoreactivity on Western blots was weak, most likely due to the fact that endogenous levels of AATF and Par-4 in IMR-32 cells are relatively low under normal basal conditions. (i) Reverse order of immunoprecipitation/Western blot analysis showing that Par-4 interacts with AATF via its leucine zipper domain. The indicated double transfected IMR-32 cell lines were lysed and precipitated with the AATF antibody, followed by Western blotting with Par-4 antibody. Note that full-length wild-type AATF exhibited a functional interaction only with full-length Par-4, but not with the Par-4 deletion mutant Par-4deltaLeu.zip lacking the leucine zipper domain, indicating that the leucine zipper domain of Par-4 is involved in interaction with AATF. Also, consistent with the results in (h), AATF390 and AATF 279, both of which contain the leucine zipper domain of AATF, also interacted with the full-length Par-4 [34, 175]

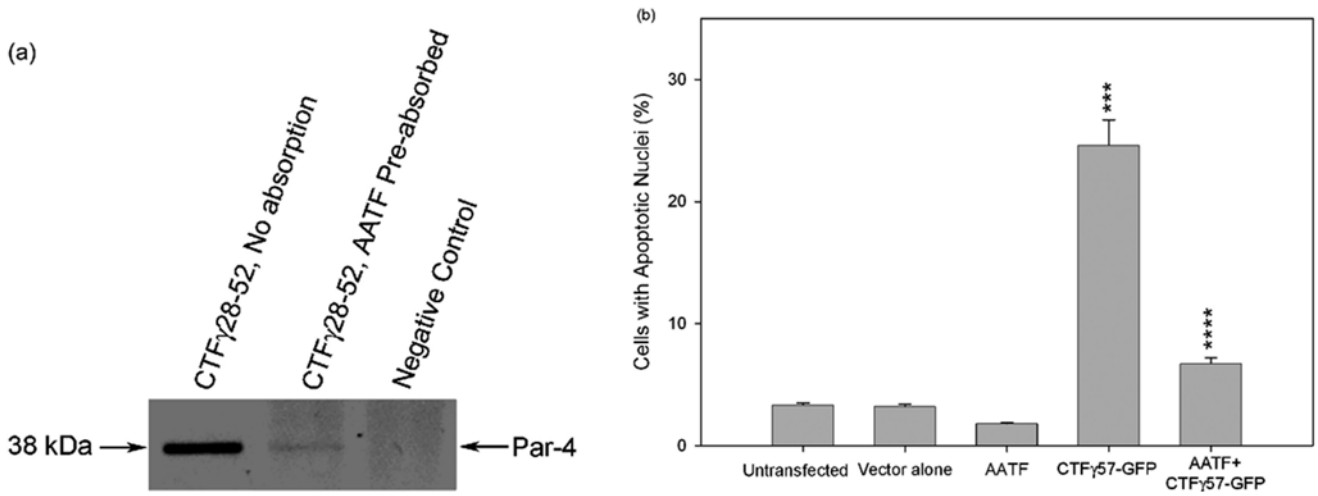

Fig. 18 AATF interferes with binding of Par-4 to AICD and protects against cell death induced by CTFγ57 in IMR-32 cells. (**a**) ***AATF leucine zipper peptide diminishes Par-4/AICD binding in pull-down experiments***. The figure shows SDS-PAGE electrophoresis pattern of the pull-down of Par-4 protein by CTFγ28-57 peptide immobilized on gel beads. The CTFγ28-57 peptide (AVTPEER HLSKMQQNGYENPTYKFF, with an added cysteine residue at the C-terminal end to allow binding to the iodoacetyl groups in Sulfolink Coupling Gel) was covalently linked to Sulfolink Coupling Gel (Pierce). Full-length Par-4 was expressed in IMR-32 cells. The Par-4 protein in the cell lysates was incubated without AATF peptide (left lane) or with 10μg (middle lane) of human AATF leucine zipper peptide (-LKNSHKALKALLRSLVGLQEEL, which has been shown to bind to Par-4) and the gel-linked CTFγ28-57 peptide. The washed gel samples were then subjected to SDS-PAGE and Western blotting for Par-4. Cysteine-blocked gel was used as negative control (right lane). The results provide supporting evidence that binding of Par-4 by AATF diminishes Par-4/AICD interaction. (**b**) ***Co- expression of AATF protect against apoptotic cell death induced by CTFγ57.*** IMR-32 cells were transfected with pEGFP-CTFγ57 (CTF57-GFP) or pREP4-AATF using the Lipofectamine 2000 transfection reagent. Parallel cultures were co-transfected with pEGFP-CTFγ57 (CTFγ57-GFP) and pREP4-AATF (AATF+CTFγ57-GFP). Untransfected cells and cells transfected with vector pEGFP-N1 were used as a control. 48 h following transfection, cells were stained with DNA-binding dye Hoescht 33,342, and the percentage of transfected cells in each culture with apoptotic nuclei (condensed and fragmented DNA) was determined. Note that co-expression of AATF significantly inhibited apoptosis induced by CTFγ57. Values are the mean and SEM of determinations made in six separate experiments. ***$P < 0.01$ compared with the value in untransfected and vector-transfected control groups. ****$P < 0.001$ compared with the value in cells transfected with CTFγ57-GFP. (ANOVA with Scheffe's post-hoc tests)

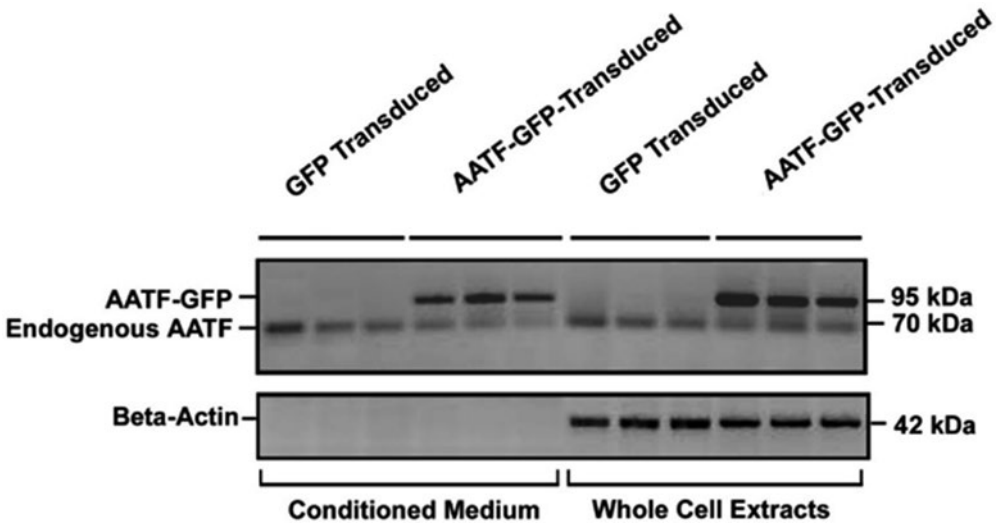

Fig. 19 AATF is secreted extracellularly. *Representative Western blots showing that conditioned medium from the AATF-GFP transduced cortical neurons contained secreted AATF proteins*. The conditioned medium from primary cortical neurons transduced with rAAV particles expressing AATF-GFP or GFP alone was subjected to Western blotting with the anti-AATF- or anti-actin antibody. Protein samples from the whole-cell extracts were used as controls. The conditioned medium from AATF-GFP transduced cells contained both endogenous AATF and AAFT-GFP fusion protein but no detectable levels of actin. As a control, actin was detected in whole-cell extracts. Similar data were obtained in six separate experiments

5.2 Par-4 Is Involved in Degeneration of Dopaminergic Neurons in Models of Parkinson's Disease

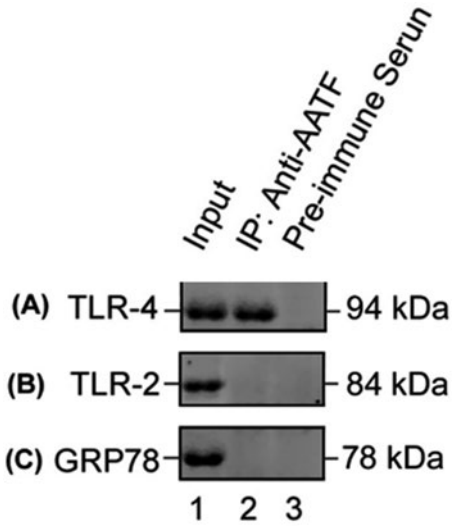

Fig. 20 AATF is associated with TLR4 receptor protein in cortical neurons. (**a**) *Endogenous AATF is associated with TLR4.* Cultures of primary cortical neurons were lysed and whole-cell extracts were precipitated with specific rabbit anti-TLR4 antibody, followed by Western blotting with rabbit anti-AATF antibody (lanes 2). The pre-immune rabbit serum was used as a control (lane 3). Reverse order of immunoprecipitation analysis of the same cells showed similar AATF/TLR4 interaction. (**b**, **c**) *AATF is not associated with TLR2 or GRP78.* Cultured cortical neurons were lysed and proteins from total lysates were immunoprecipitated with rabbit anti-AATF antibody (lanes 2), followed by Western blotting with either anti-TLR2 antibody or anti-GRP78 antibody. Input lanes show 10% of the total protein used in immunoprecipitation

The loss of midbrain dopaminergic neurons is a significant feature of the pathology of Parkinson's disease (PD). Par-4 levels have been reported to increase dramatically in midbrain dopaminergic neurons of monkeys and mice exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, an experimental model of PD) [134, 154, 203, 204]. The increase in Par-4 levels was observed in both neuronal cell bodies in the substantia nigra and their axon terminals in the striatum and precedes loss of tyrosine hydroxylase immunoreactivity and cell deaths. Blockade of Par-4 induction prevented dopaminergic neuronal cell death, demonstrating a critical role for Par-4 in neurodegeneration in PD [134, 154, 203, 204].

5.3 Par-4 as a Molecular Link between Impaired Dopamine Signaling and Depression

There is evidence that Par-4 is a regulatory component in dopamine signaling. Park and colleagues reported that Par-4 directly interacts with the dopamine D2 receptor (D2DR) via the calmodulin binding motif in the third cytoplasmic loop [141]. Calmodulin can effectively compete with Par-4 binding in a Ca2+-dependent manner, providing a route for Ca2+-mediated downregulation of D2DR efficacy. Mice lacking the D2DR interaction domain of Par-4 showed an enhanced dopamine-cAMP-CREB signaling pathway, an impairment in dopamine signaling in neurons, and significantly increased depression-like behaviors [141]. These results suggest that Par-4 functions to link dopamine signaling and depression.

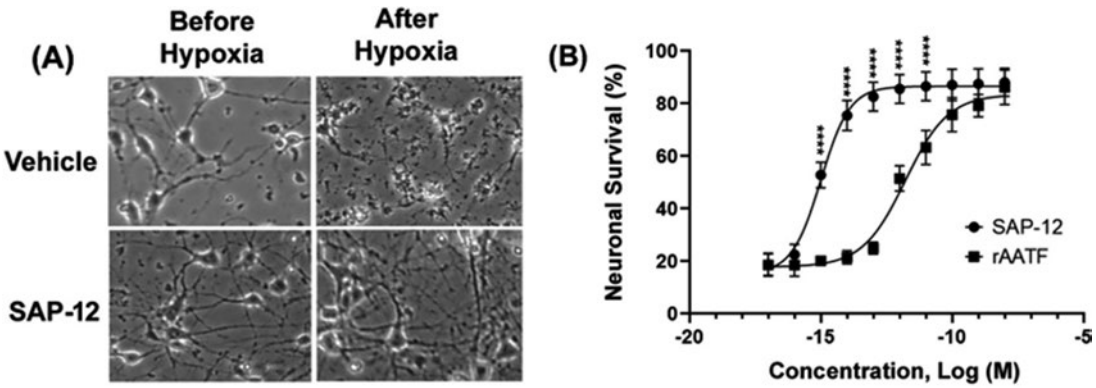

Fig. 21 SAP-12 confers greater neuroprotective potency and broader effective dose range than the full-length AATF. (**a**) *Neuroprotective actions of SAP-12 against neuronal cell death induced by chemical hypoxia.* Primary cultures of cortical neurons were pretreated for 4 h with 0.1 pM of SAP-12, and then subjected to Locke's buffer (vehicle) or chemical hypoxia induced by 5 mM of NaCN for 1 h. Cell death was assessed 24 h after NaCN treatment. Neurons with intact neurites of uniform diameter and soma with a smooth round appearance were considered viable, whereas neurons with fragmented neurites and vacuolated soma were considered nonviable and dead. (**b**) *Dose–response curves showing SAP-12 confers greater neuroprotective potency and broader effective dose range than the full-length AATF*. Primary cortical neurons were pretreated for 4 h with indicated concentrations of SAP-12 or recombinant AATF (rAATF), and then subjected to chemical hypoxia induced by 5 mM of NaCN for 1 h. Cell death was assessed 48 h after NaCN treatment, as described above. Values are the means ± SE of determinations made in six separate experiments. ****$P < 0.001$ compared with corresponding values in rAATF-treated group

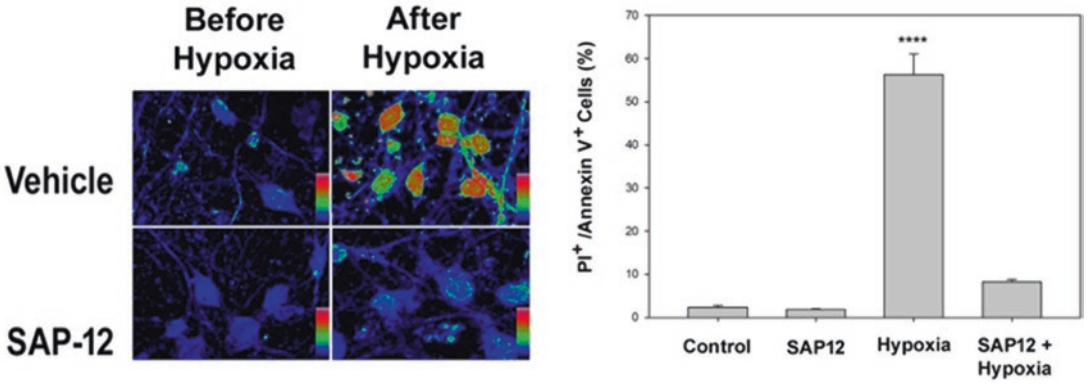

Fig. 22 SAP-12 blocks MLKL-immunoreactivity and neuronal necroptosis induced by chemical hypoxia in vitro. (**a**) *SAP-12 blocks NaCN-induced increase in MLKL expresion in primary cortical neurons*. Cultured neurons were pretreated for 4 h with 100 fM of SAP-12, and then subjected to Locke's buffer (vehicle) or chemical hypoxia induced by 5 mM of NaCN for 1 h. Images of MLKL immunofluorescence were acquired using a confocal laser-scanning microscope, as described in our previous publications. Levels of MLKL were significantly increased following hypoxia, which was effectively blocked by SAP-12. (**b**) *SAP-12 protects against neuronal necroptosis induced by chemical hypoxia*. Primary cortical neurons were pretreated for 4 h with 100 fM of SAP-12 and then exposed to chemical hypoxia induced by 5 mM NaCN for 1 h. A flow cytometry method was used to evaluate neuronal necroptosis, as described previously, and PI^+/Annexin V^+ necroptotic cells were counted 24 h after NaCN treatment. Separate control groups of cells were pretreated SAP-12 (SAP12) or Locke's buffer (Control) alone without NaCN. Values are the means ± SE of determinations made in six separate experiments. ****$P < 0.001$ compared with corresponding values for in Control, SAP-1, and SAP12+hypoxia groups

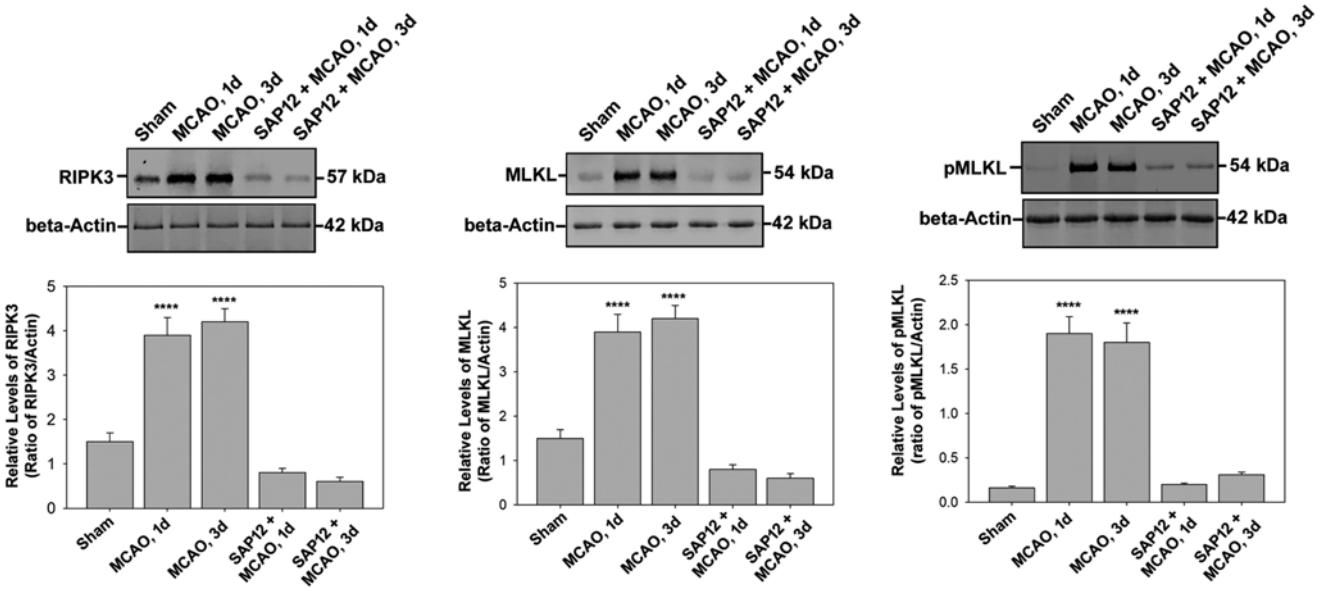

Fig. 23 SAP-12 blocks RIPK3- and MLKL-dependent signaling of neuronal necroptosis following MCAO in vivo. **Top panel**: *Representative images of the Western blot analyses showing the effects of SAP-12 on key markers of neuronal necroptosis (RIPK3, MLKL, and phosphorylated MLKL or pMLKL) in the ipsilateral striatum of sham-operated (Sham) or MCAO mice at 24 h (1 day) and 72 h (3 days) after reperfusion*. 1.5µl of SAP-12 solution (2.5 pM dissolved in Locke's buffer) was delivered intracerebroventricularly by stereotaxic injection into the contralateral cerebral ventricle at a rate of 0.2 ul/min 30 min before MCAO. Sham-operated animals received an equivalent amount of Locke's buffer without MCAO or SAP-12. **Bottom panel**: *Statistical analysis of the effects of SAP-12 on levels of expression of RIPK3, MLKL, and pMLKL in ipsilateral striatum after MCAO*. Values are the means ± SE of determinations made in six separate blots. ****$P < 0.001$ compared with corresponding values in SAP12 + MCAO group

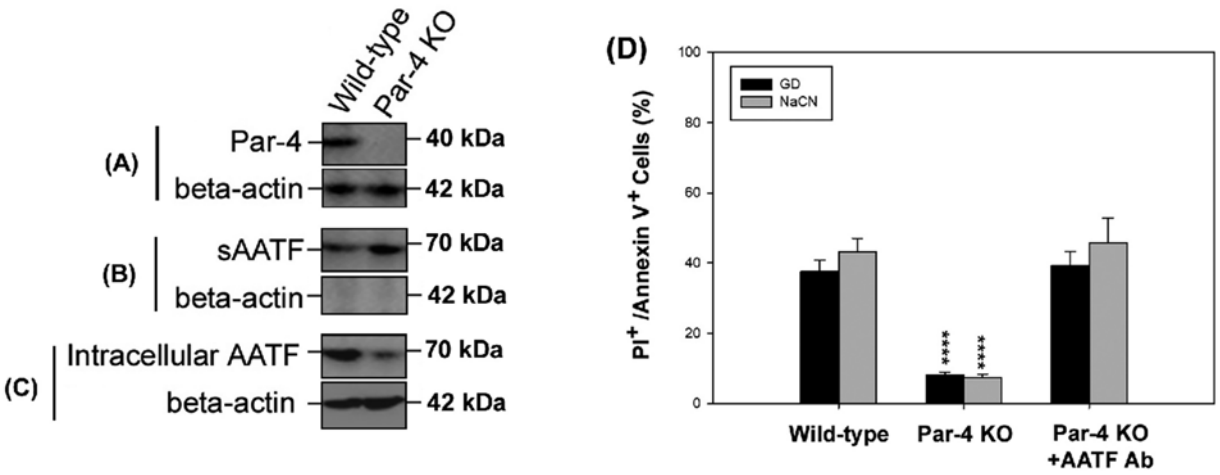

Fig. 24 Par-4 null (Par-4$^{-/-}$) mice show increased extracellular secretion of sAATF and enhanced neuroprotection. (**a**) *Representative Western blotting showing lack of Par-4 protein expression in primary cultures of cortical neurons from Par-4$^{-/-}$ mice*. (**b**, **c**) *Extracellular secretion of sAATF is significantly increased from primary cortical neurons of Par-4$^{-/-}$ mice*. In (**b**), the conditioned medium of Par-4$^{-/-}$ neurons contained significantly elevated levels of sAATF compared with those in wild-type controls. No detectable levels of actin were noted in the conditioned medium. In (**c**), levels of intracellular AATF were significantly decreased in Par-4$^{-/-}$ neurons compared with those in wild-type cells, likely due to increased extracellular release of AATF. (**d**) *Conditioned medium of cultured Par-4$^{-/-}$ cortical neurons protects against necroptosis induced by glucose deprivation and chemical hypoxia*. Cultured cortical neurons were pretreated for 4 h with conditioned medium prepared from Par-4$^{-/-}$ neurons, and then subjected to glucose deprivation (GD) or exposure to 5 mM NaCN for 1 h. The double-labeled (PI$^+$ /Annexin V$^+$) necroptotic cells were then counted using a flow cytometry method described previously. Separate groups of cells were pretreated with conditioned medium from Par-4$^{-/-}$ neurons that was pre-incubated for 30 min with the neutralizing antibody (Ab) for AATF (Par-4 KO+AATF Ab). Values are the means ± SE of determinations made in six separate experiments. ****$P < 0.001$ compared with corresponding values for conditioned medium from wild-type cells and the values for conditioned medium from Par-4$^{-/-}$ cells pre-incubated with the neutralizing antibody for AATF

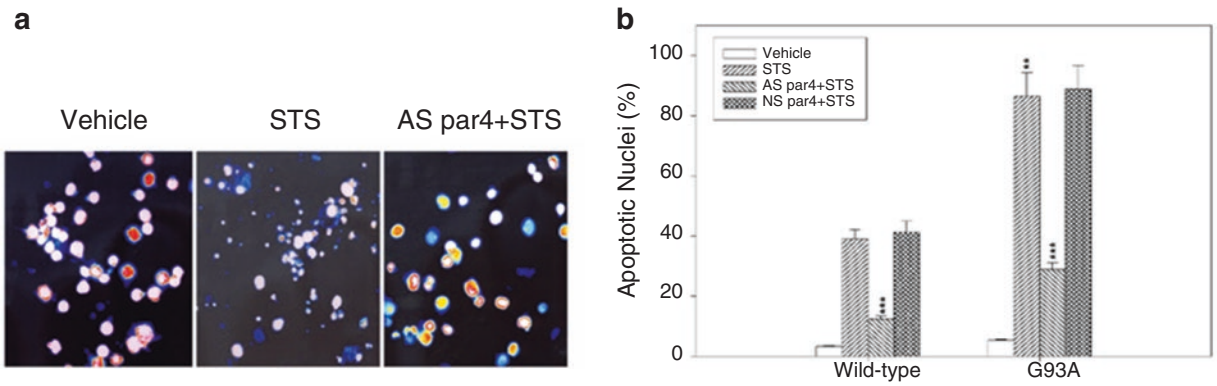

Fig. 25 Evidence that Par-4 mediates the production locally in synaptic compartments of specific cytosolic factor (s) that induces nuclear chromatin condensation and DNA fragmentation. Ventral spinal cord synaptosomes from 2-month-old healthy (with no signs of decline in muscle strength) wild-type mice and those expressing the G93A mutant human SOD1 were left untreated (control) or were treated for 6 h with 25μM par-4 antisense oligonucleotide (AS), or with a nonsense par-4 oligonucleotide (NS). The synaptosomes were then exposed for 4 h to the vehicle (0.2% DMSO) or 100 nM of staurosporine (STS) for 4 h. Cytosolic extracts of the synaptosomes were then prepared, and incubated for 3 h in the presence of isolated nuclei from PC12 cells. The nuclei were then stained with propidium iodide and examined using a confocal laser-scanning microscope. (**a**) Representative confocal images of propidium iodide-stained nuclei following exposure to cytosolic extracts from synaptosomes (of G93A mice) treated with vehicle, STS or Par-4 AS plus STS. (**b**) Statistical analysis of effect of Par-4 antisense treatment on nuclear apoptosis induced by cytosolic extracts from STS-treated synaptosomes of wild-type and G93A mice. Note that nuclear apoptosis (chromatin margination and fragmentation) induced by the extract from STS-treated synaptosomes of G93A mice was much more pronounced than that induced by the extract from STS-treated synaptosomes of wild-type mice. Importantly, pretreatment of synaptosomes with Par-4 antisense before exposure to STS largely prevented the nuclear margination and fragmentation induced by cytosolic extracts of synaptosomes from G93A mice. Direct exposure of isolated nuclei to 100 nM STS, Par-4 AS or Par-4 NS had no effect on nuclear morphology (data not shown), indicating that residual STS, Par-4 AS, or NS in the cytosolic extracts does not by themselves induce nuclear apoptosis. These results suggest that the G93A mutation enhances the production of nuclear apoptosis-inducing factor(s) locally in synaptic compartments in the spinal cord through a Par4-dependent pathway. Values are the mean and SEM from six separate cultures. **$P < 0.01$ compared to corresponding values in WT mice group; ***$P < 0.01$ compared to values in nuclei treated with STS alone in the same group. ANOVA with Scheffe's post-hoc tests

6 Future Directions

Identification of Par-4 and AATF as critical factors involved in the pathogenesis of Alzheimer's disease and other neurodegenerative diseases opens a new area of research in understanding the cellular and molecular mechanisms neuronal cell death and survival. The observation that AATF was secreted extracellularly under neurodegenerative conditions without a classical N-terminal signal peptide was also significant. However, many significant questions remain to be answered. For example, what are the molecular events that regulate the Par-4 activity in APP processing by BACE1 and gamma-secretase? How does Par-4/AICD interaction affect AICD nuclear translocation and APP-dependent transcriptional transactivation involved in neuronal necroptosis? How does Par-4 interact with other pre- and/or post-synaptic proteins to alter neuronal differentiation and synaptic dysfunction under neurodegenerative conditions? Does sAATF interact with other cell surface receptors in addition to TLR-4? Does systemic administration of a recombinant SAP-12 fusion protein (such as SAP12-TAT) that was modified for noninvasive delivery of SAP-12 across the blood–brain barrier (BBB) translates SAP-12 into a viable therapy for neuronal cell death? Of importance, the observations that sAATF counteracts the cell death-promoting actions of Par-4 and that SAP-12 provides improved therapeutic efficacy and better delivery options than the full-length sAATF will help to set the groundwork for producing new therapeutics in the future, including the optimization of the pharmacokinetic properties and target specificity of SAP-12. Genetic and/or pharmacological manipulations of neuronal Par-4 and AATF activities may have broad implications in controlling neuronal cell death and survival in a variety of neurodegenerative diseases.

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Par-4-Dependent Apoptosis of Pancreatic Islet β Cells in Type 2 Diabetes

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Abstract

Pancreatic islet β-cell dysfunction is an underlying cause of type 2 diabetes. Long-term metabolic disorders lead to pancreatic islet β-cell apoptosis, which is one of the main reasons for islet β-cell dysfunction. However, the mechanisms underlying this process are not well understood. The tumor suppressor Par-4, the protein product of the *PAWR* gene, sensitizes a variety of normal tissue cells to apoptosis induced by diverse insults. Our own research demonstrated that Par-4 plays an important role in the apoptosis pathway activated by high glucose/high fat leading to dysfunction of the islet β-cells and type 2 diabetes. Recent investigations also suggest that Par-4 may induce cell death through autophagy dysfunction, which contributes to islet β-cell elimination. We review the role of secreted and intracellular Par-4 in sensitizing islet β-cells to apoptosis via the caspase-8 and caspase-9 pathways. The significance of Par-4 interactions particularly with the NF-κB pathway and telomerase reverse transcriptase (TERT) in regulating autophagy dysfunction and apoptosis of islet β-cells is highlighted.

Keywords

Endoplasmic reticulum stress · Mitochondrial dysfunction · β-Cell · Apoptosis · Autophagy · Type 2 diabetes · Par-4 · TERT · Dopamine receptor DRD2 · Insulin resistance

1 Introduction to Type 1 and Type 2 Diabetes

According to the 2019 global diabetes map released by the International Diabetes Federation, the number of diabetes cases is on a continuous rise in the world. At present, there are 463 million people in the world living with diabetes, and it is estimated that this number will rise up to 700 million people by the year 2045. Most of these patients have type 2 diabetes. China leads the world with a total number of 116 million diabetes patients, India coming in second with 77 million diabetic patients, and the United States next with 31 million diabetic patients (https://www.singlecare.com/blog/news/diabetes-statistics/; IDF Diabetes Atlas, 2019). People rarely die from diabetes directly, and it is more likely that someone with diabetes will die from complications with vital organs. Diabetes is one of the main underlying factors associated with blindness, amputation, heart disease, and renal failure to cause premature death. About 11.3% of all-cause mortality in the world is thus related to diabetes, and about half (46.2%) of diabetes-related deaths occur in individuals who are under 60 years old. Premature death and disability associated with diabetes have a negative impact on economic growth, and is often referred to the "indirect cost" of diabetes [1].

Most of type 1 diabetes cases are autoimmune diseases, and involve genetic and environmental factors that participate in the pathogenesis of the disease. Key features of type 1 diabetes include the destruction of islet β-cells leading to the absolute lack of insulin. The onset of the disease is often acute, with weight loss, polyuria, ketosis tendency that can occur at any age. Most of these patients need lifelong insulin treatment. The role of the pro-apoptotic tumor suppressor protein Par-4 has not been investigated in type 1 diabetes. On the other hand, Par-4 expression and function has been

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examined in type 2 diabetes, which is a heterogeneous, polygenic complex disease.

Type 2 diabetes is caused by genetic factors and lifestyle factors including over nutrition and sedentary lifestyle with lack of physical activity contributing to obesity. Key features of type 2 diabetes include loss of tissue-sensitivity to insulin, the deficiency of β-cell function and insufficiency of insulin secretion, and/or the abnormal function of α-cell leading to the increase of glucocorticoid secretion. Family history is a predisposing factor for type 2 diabetes and manifests mostly in adults. The onset of the disease is slow and initial symptoms are relatively light, with more than half of the people having no symptoms. Most people are diagnosed with the disease due to chronic complications or comorbidities such as obesity, hypertension, or dyslipidemia. Treatment for type 2 diabetes involves lifestyle readjustment with diet and exercise, oral hypoglycemic drug treatment, and insulin treatment [1].

The main difference between type 1 and type 2 diabetes relates to the pancreatic islet cell anatomical differences. Pancreatic islets or islets of Langerhans are composed of α, β, δ, ε-cells, and pancreatic polypeptide (PP) cells. The α-cells produce glucagon, β-cells produce insulin and amylin, δ-cells produce somatostatin, ε-cells produce ghrelin, and the PP cells produce pancreatic polypeptide. In type 1 diabetic patients, two third of the islets have acute insulitis with infiltration of lymphocytes and macrophages, with less than 10% β-cell survival. The local regeneration of islet β-cells is initially seen in patients with a short course of disease, but diminishes with progress of the disease as these cells are destroyed. The exocrine tissue shrinks, β-cells are almost absent, the weight of pancreas decreases, and the weight of islet drops to less than one third of that of normal individuals or type 2 diabetes patients. The islets of Langerhans contain only α cells, ε-cells, and PP cells located at the far end of the head of the pancreas. The number of α and ε-cells in each islet is in the normal range.

Diabetes mellitus and its vascular complications develop as a result of defects caused by oxidative stress. Oxidative stress is accompanied by abnormalities in the telomere-telomerase system and loss of protection of the chromosomal DNA that induces dysfunction in islet β cells, endothelial cells, and insulin resistance [2]. In type 2 diabetes, endoplasmic reticulum stress (ER) and mitochondrial dysfunction is the main mechanism of β-cell apoptosis. Glucose and lipid metabolic disorder may be caused by exposure to high glucose and high fat, amyloid deposition, and inflammatory cytokines that lead to ER stress and mitochondrial dysfunction. These events initiate apoptosis signaling pathways to eliminate damaged cells that cannot be repaired, but the specific process remains unclear [3–7].

2 Par-4 Induces Apoptosis in Islet β Cells that Is Linked to the Pathogenesis of Diabetes

Par-4 is a pro-apoptotic factor with a leucine zipper domain at the carboxyl-terminus and nuclear localization sequence (NLS) within its centrally located SAC domain that are characteristic of the structure of transcription regulators, suggesting that Par-4 may participate in the regulation of cell apoptosis by directing gene transcriptional events [8]. A variety of factors can activate the NLS of endogenous Par-4 and promote its nuclear translocation to induce apoptosis [8, 9]. Endogenous Par-4 activation sensitizes both normal and tumor cells to apoptosis, and this process requires the leucine zipper domain. On the other hand, overexpression of ectopic Par-4 induces apoptosis in cancer cells but not in normal cells, by a mechanism dependent on the centrally situated SAC domain of Par-4. In the nucleus, Par-4 can downregulate the expression of NF-κB and inhibit cell survival [9]. Moreover, Par-4 protein is also secreted, and extracellular Par-4 binds to GRP78 on the cell membrane to initiate and amplify ER stress and induces the extrinsic apoptosis pathway [10]. On the other hand, Par-4 can also be cleaved by caspase-3 activated through the mitochondrial pathway, and the cleaved Par-4 fragment translocates into the nucleus to induce apoptosis [11]. As many diseases result from the pathophysiological changes associated with ER stress and mitochondrial dysfunction, we performed *in vivo* and *in vitro* experiments to test the involvement of Par-4 in apoptosis regulation in islet β-cells [12, 13]. Our studies indicated that in diabetes-induced ER stress, the upregulation of Par-4 is tightly associated with an increase in downstream NF-κB activity, and that Par-4 may participate in the process of ER stress-inducing apoptosis of islet β cells in the pathogenesis of type 2 diabetes [12, 13]. Our key observations are indicated below.

1. Upregulation or down-regulation of Par-4 by itself in islet β cells do not induce apoptosis. However, when associated with high glucose and high palmitate (fat)-induced ER stress, Par-4 upregulation induces apoptosis of islet β cells. Our findings, summarized in Fig. 1, suggest that Par-4 may induce ER stress and lead to apoptosis of islet β cells in type 2 diabetes mellitus. The levels of GRP78 protein are known to be elevated in diabetes and often associated with ER stress. As GRP78 translocates to the plasma membrane in situations of ER stress and serves as a receptor for extracellular Par-4, the downstream pathways induced after extracellular Par-4 engages the GRP78 receptor need further investigation in islet β cells.
2. ER stress induced by high glucose-high fat can promote the secretion of Par-4 and also increase the expression of

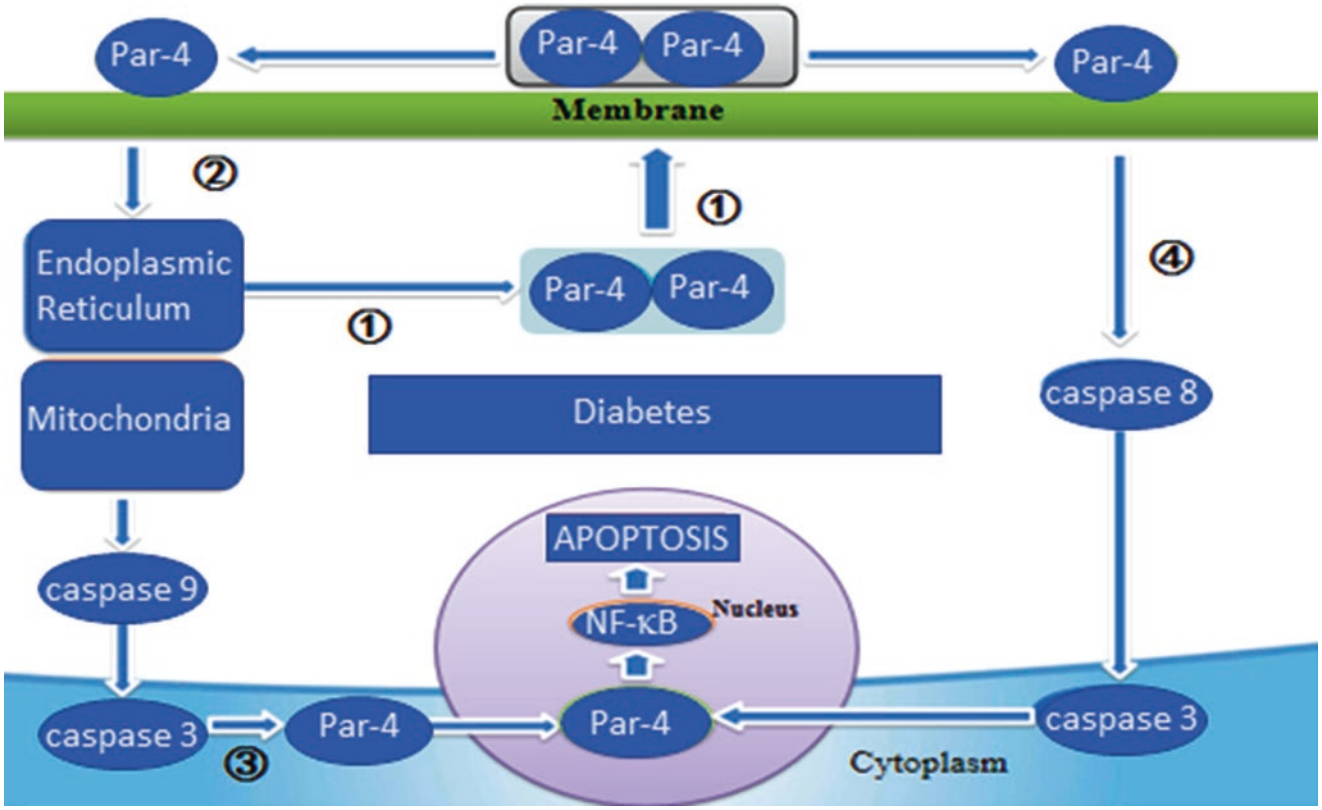

Fig. 1 Mechanisms of apoptosis induced by Par-4 in diabetes. (1) ER stress promotes Par-4 secretion; (2) secreted Par-4 promotes ER stress; (3) mitochondrial dysfunction promotes Par-4 entry into nucleus to induce apoptosis; (4) secreted Par-4 induces apoptosis through the extrinsic/cell membrane pathway

intracellular Par-4 in the nucleus of islet β cells. In contrast to cancer cells where Par-4 inhibits NF-κB activity, we found that Par-4 promotes NF-κB expression and transcription activity for apoptosis of the islet β cells. Moreover, caspase-8 activity that is activated via the cell membrane associated extrinsic apoptotic pathway was significantly increased. We noted that this process is promoted by ectopic overexpression of Par-4 and impeded by inhibition of endogenous Par-4 expression.

3. High glucose-high fat increases the expression of Par-4 in the nucleus of islet β cells, along with a significant increase in the expression of caspase-9, decreased expression of the anti-apoptotic protein Bcl-2, and the release of cytochrome C from the mitochondria. As noted above with the extrinsic apoptotic pathway, overexpression of Par-4 promoted the intrinsic apoptosis process, and inhibition of Par-4 expression impede this process, suggesting that the mitochondrial pathway also plays a key role in pancreatic β-cells apoptosis induced by Par-4/NF-κB. Similar to the effect of diabetes-induced ER stress that causes NF-κB activation and apoptosis of islet β-cells, upregulation of NF-κB produced the same result on its own. NF-κB is a multifunctional transcription factor with a wide range of biological functions. Activated NF-κB exhibits dual regulatory effects involving anti-apoptosis or pro-apoptosis depending on the cellular, environmental, and molecular context, but the precise conditions are not well understood.

3 Par-4/TERT Interaction Regulates the Process of Apoptosis in Islet β Cells

Telomeres are repetitive, non-coding, sequences located at the termini of chromosomes. They act as buffers for coding sequences by capping the end-sequences and exhibit several key biological functions: (1) maintaining the stability of

chromosomes; (2) prevention of chromosome end fusion; (3) protecting chromosome structure; and (4) determining the lifespan of cells. Chronic inflammation, oxidative stress, and insulin resistance in diabetics may induce islet β-cell senescence associated with telomere shortening and subsequent telomerase dysfunction, which can lead to DNA damage, islet β death, and decreased compensatory capacity [14]. All of these mechanisms play an important role in the pathogenesis and progression of diabetes mellitus. An enzyme called telomerase adds repetitive nucleotide sequences to the ends of the DNA to replenishes the telomere cap. Telomerase reverse transcriptase (TERT) is the main catalytic subunit of telomerase protein component that is widely expressed in the cytoplasm. Together with the template component of telomerase RNA, it can reconstruct telomerase activity. PAR-4 is one of six proteins that interact with human telomerase reverse transcriptase [15]. Expression of TERT-shRNA in laryngocarcinoma Hep-2 cells can decrease the expression of TERT, increase the nuclear expression of Par-4, and reduce tumor size in nude mice. Importantly, TERT interacts with Par-4 in the cytoplasm, inhibits the translocation of Par-4 to the nucleus, and reduces cell apoptosis [16][l]. Consistently, the expression of Par-4 increased and TERT decreased in newly diagnosed diabetes patients compared with normal controls. The above two indicators were correlated with the secretary index of islet β-cells (Homa-β), indicating that Par-4 and TERT regulate the function of islet β-cells. Moreover, we noted that high glucose and fat can increase the binding of Par-4 to TERT in the cytoplasm, decrease the expression of TERT, and increase the expression of Par-4 in the nucleus to activate NF-κB [16].

4 Par-4, Autophagy Dysfunction, and Islet β-Cell Apoptosis

Autophagy is generally a cell survival process that can maintain cellular homeostasis through the degradation and recycling of proteins and organelles [17, 18]. This vital process is initiated by starvation, growth factor deprivation, and ER stress [17, 18]. Autophagy eliminates defective proteins and organelles and removes intracellular pathogens. Dysfunctional autophagy is linked to many diseases, such as cancer, inflammatory diseases, and neurodegenerative diseases. As autophagy can reallocate nutrients from unnecessary processes to more pivotal processes required for survival, it plays an important role in the pathogenesis of metabolic disorders including diabetes. Accordingly, loss of autophagy function is noted in diabetic conditions and uncontrolled autophagy leads to non-apoptotic cell death.

In an autopsy report on Japanese patients with type 2 diabetes, it was found that the dysfunction of islet β cells and autophagy dysfunction in type 2 diabetes were closely related to each other [19]. Several laboratories have reported that with the increase in age, the islet β cell itself induces autophagy dysfunction, which leads to the increase of apoptosis and decline of islet β cell [20]. In type 2 diabetic animal and cell culture models, long-term treatment with high glucose and high fat can activate the mTOR signaling pathway, inhibit stearoyl CoA reductase, reduce lysosomal acidity and hydrolase activity, and lead to autophagy corpuscles maturation and fusion with lysosome and degradation capacity deficiency, resulting in autophagy dysfunction [21, 22]. This dysfunctional process is associated with accumulation of a large number of damaged organelles and proteins, and activation of the apoptosis signal pathway in islet β cells [22, 23]. Several laboratories have suggested a role for Par-4 in autophagy [24–27]. We noted that Par-4 induces cell death through autophagy dysfunction as follows: (1) high glucose and high fat increases Par-4 expression, autophagy marker protein accumulation, autophagy obstruction and dysfunction, leading to cell death; (2) upregulation of Par-4 inhibits the binding of Bcl-2 with autophagy gene Becn1, resulting in autophagy cascade obstruction, autophagy dysfunction, and cell death. Moreover, the transcription factors FOXO3a and NF-κB that are regulated by Par-4 are involved in the development of islet β cell autophagy.

Recent studies have shown that TERT is also a key factor in the development of autophagic disorders [28–30]. Overexpression of TERT inhibits the mTOR pathway to activate autophagy. Consistently, the autophagic inducer-rapamycin upregulates the expression of TERT. On the other hand, TERT knockout impairs autophagy in mouse renal tubular epithelial cells with p62 protein accumulation, and rapamycin can only partially restore autophagy and ischemia/reperfusion injury. In addition, TERT expression is regulated by the Akt pathway: Akt activation increases TERT expression and telomerase activity. Consistently, one of the important effects of Par-4 activation is inhibition of Akt activation contributing to autophagy dysfunction. Thus, Par-4 participates in the dysfunction of islet β cells in type 2 diabetes as follows: the expression and secretion of Par-4 is elevated, leading to inhibition Akt phosphorylation and TERT expression in the cytoplasm, and activation of nuclear transcription factors NF-κB and FOXO3a in the nucleus to cause autophagy dysfunction and apoptosis of islet β cells (Fig. 2).

5 Par-4 and Insulin Resistance

A number of studies have revealed that abnormal expression of Par-4 is associated with depression and schizophrenia. For example, Par-4 knockout mice lacking the leucine zipper domain exhibit symptoms of depression [31]. Moreover, an investigation of SNPs of the Par-4 gene in a Taiwanese population led to mutations in exon 2 (P78R) and exon 3(I199M)

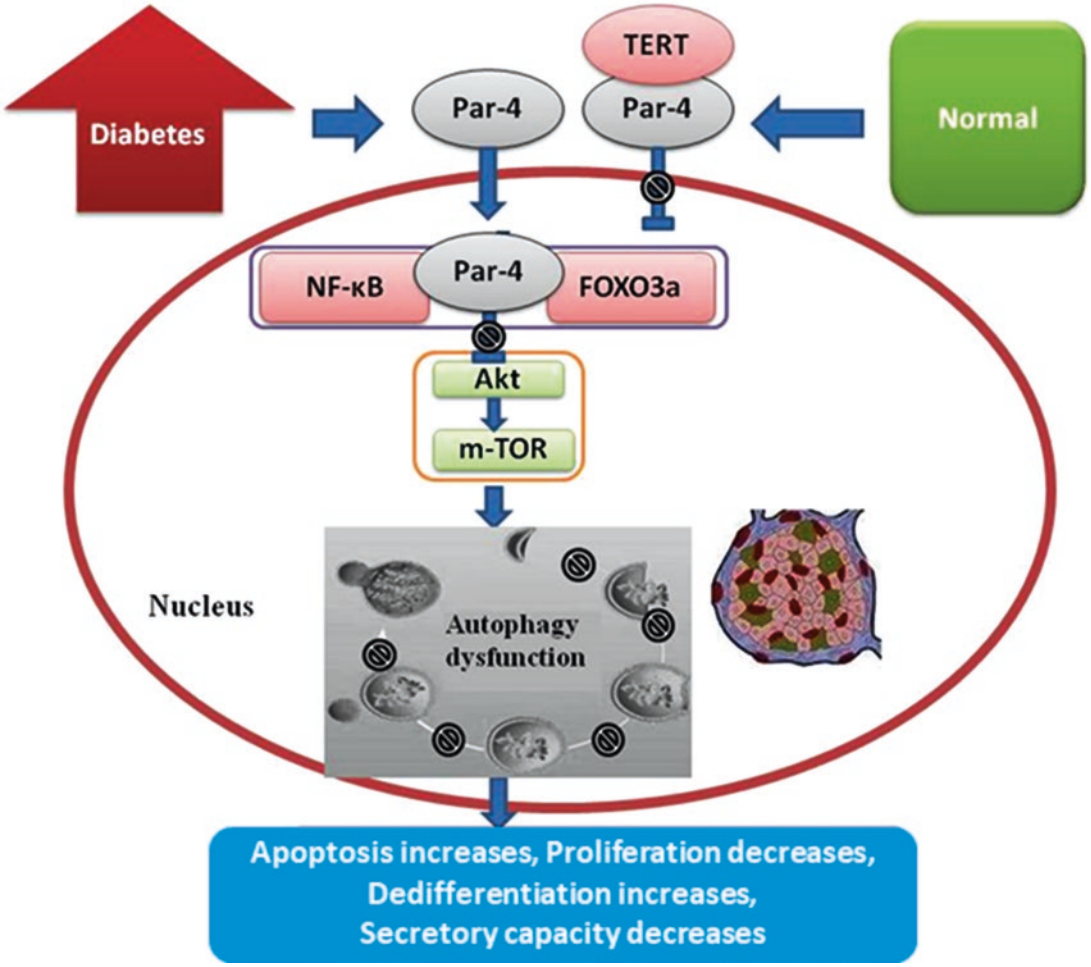

Fig. 2 Par-4-TERT/ Akt and autophagy dysfunction of islet β cells. Under normal conditions, Par-4 is sequestered by TERT in the cytoplasm. On the other hand, in diabetes environment, Par-4 is activated and translocated into the nucleus to activate FOXO3a and NF-κB transcription, inhibit Akt-mTOR signaling pathway, and lead to dysfunction of islet β cells through autophagy dysfunction

that may be associated with schizophrenia. Further studies revealed that these Par-4 mutations interfere with Par-4 interaction with the dopamine D2 receptor DRD2 [32–34]. These observations indicate that Par-4 interaction with DRD2 may be an important mechanism underlying the pathogenesis of schizophrenia and depression. Coincidently, DRD2 is also associated with appetite, obesity, and diabetes [35–40], and the DRD2 agonist bromocriptine that is FDA-approved for the treatment of type 2 diabetes and insulin resistance, also effectively reduces body weight [41]. Moreover, the DRD2 receptor agonist coffee can improve insulin resistance and reduce the risk of diabetes [42]. DRD2 agonists can also inhibit the secretion of insulin, participate in the proliferation and repair of islet β-cells, and high concentration of DRD2 can promote insulin secretion, and regulate apoptosis [43]. As Par-4 regulates the activity of DRD2 [44, 45], we speculate that the interaction between Par-4 and DRD2 is involved in the process of insulin resistance.

6 Future Perspectives

Under physiological conditions, low levels of Par-4 expression may play an important role in the differentiation and metabolism of normal cells. Precisely how Par-4 keeps the balance between ER stress, DRD2, insulin resistance, autophagy dysfunction, and apoptosis is an important question that may shed new light on the role of Par-4 in type 2 diabetes. Also, in view of the potential role of Par-4 in autophagy regulation [46], it would be important to determine how Par-4 activation directs cells to switch between apoptosis and autophagy. Development of Par-4 inhibitors may provide the basis for gene therapy of type 2 diabetes. Moreover, DRD2-based therapy to overcome autophagy dysfunction and apoptosis also may be a promising direction for controlling type 2 diabetes.

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Role of PRKC Apoptosis WT1 Regulator in Ocular Development and Diseases

Tahseen Ahmed, Tuneer Ranjan Mallick, Michael A. Walter, and Moulinath Acharya

Abstract

PRKC apoptosis WT1 regulator (PAWR or prostate apoptosis response-4, Par-4) is a prominent tumor suppressor, reported to be involved in regulating WT1 and selectively induces apoptosis in cancer cells. Role of PAWR is also critical in urogenital development. PAWR acts mostly as a transcriptional repressor thus downregulates many genes including WT1 and the anti-apoptotic protein Bcl2. Similar phenomenon is also observed in eye anterior segment development where PAWR inhibits PITX2 activity, mutations in which cause anterior segment dysgenesis (ASD) and glaucoma. Nonetheless, PAWR interacts differently with FOXC transcription factors, primarily involved in the eye anterior segment development. PAWR-FOXC complex exerts both activation and repression in a target-specific manner. This becomes even more complex when the presence of PAWR along with PITX2 and FOXC proteins upregulates PITX2 activity additively, whereas PAWR alone acts as a repressor of PITX2 transactivation. Genetic interactions of *pitx2*, *foxc1a*, *foxc1b,* and *pawr* have also been observed in zebrafish using morpholino-mediated knockdown experiments. Altogether, PAWR acts as a modulator in this complex regulatory network of transcription factors involved in the development of eye anterior segment. Further functional investigation is certainly needed to decipher the underlying biology of PAWR in ocular development, as the facts mentioned above are mostly obtained from a few published reports that are available so far.

Keywords

Par-4 · PAWR · PRKC apoptosis WT1 regulator · Ocular development · Pituitary homeobox 2 (PITX2) · Forkhead box C1 (FOXC1) · FOXC2 · Anterior segment dysgenesis

1 Introduction

The development of the eye in vertebrates follows a series of interconnected events. This is dependent on the expression of various key molecules including certain transcription factors, growth factors, nuclear proteins, and enzymes that are transcribed during development. The expression of transcription factors is instrumental in determining the fate of organ development because it is not only dependent on the expression level of the transcription factor in a specific tissue but rather also dependent on the particular time (spatio-temporal) when the transcription factor is expressed during development. Therefore, the complex regulatory network comprising of these key molecules are the determinants of not only ocular development but the development of the whole embryo. Here, we will discuss a complex regulatory network consisting of the transcription factors pituitary homeobox 2 (PITX2), forkhead box C1 (FOXC1), and FOXC2 in the ocular development and the interaction of prostate apoptosis response 4 (PAR4), also known as PRKC apoptosis WT1 regulator (PAWR), in determining the activity of these transcription factors. Any mutation or deleterious changes in these genes can cause an imbalance in the regulatory networks and therefore result in developmental defects associated with disorders involving eye anterior segment.

Tahseen Ahmed, Tuneer Ranjan Mallick contributed equally to this work.

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2 Structure of the Human Eye

The human eye is grossly similar to other vertebrates, including fish, amphibians, reptiles, birds, and other mammals. Human eye (Fig. 1) is embedded in the skull in the orbital cavity and cushioned in place by adipose tissue and muscles. The eye is composed of three tunics based on their position. The fibrous tunic is the outermost covering of the eye and it gives the eye its structure. Sclera is an avascular connective tissue that gives the typical white color to the eyes. It is continuous with the transparent cornea that transmits light inside the eye.

The vascular tunic (uvea) is the middle layer of the eye and it supplies blood to the eyeball. The choroid, situated inwards of the sclera, is composed of highly vascular connective tissue. The iris is a muscular ring surrounding the pupil. It is essential for the accommodation of light entering the eyeball and acts as the aperture of a camera. It is pigmented and results in different eye colors.

The internal tunic is the innermost layer and consists of photoreceptors and ganglionic layers.

Perhaps the most important structure of the eye is the transparent lens. It is composed of crystallin protein fibers and focuses the incident light on the fovea centralis. The ciliary body connects the lens to the sclera and encircles the pupil. Zonular fibers form a suspensory ligament that attaches the ciliary body. The lens divides the ocular chamber into two segments—anterior and posterior.

The anterior segment is filled with aqueous humor secreted by the ciliary bodies into the posterior chamber (between iris and lens) and enters the anterior chamber (between cornea and iris) through the pupil. The aqueous humor provides nourishment to the avascular cornea and lens. The aqueous humor drains into the venous blood through the Schlemm's canal. This drainage of aqueous humor is aided by trabecular meshwork that line the base of the ciliary body. Blockage of this canal by closure of the angle between the iris and cornea leads to glaucoma.

The posterior chamber is filled with a jelly-like vitreous humor that supports the eyeball, provides nourishment to the retina, and acts as a refractive medium. The retina is the photosensitive layer and is responsible for generating nerve impulses that give the sensation of vision. The retina is com-

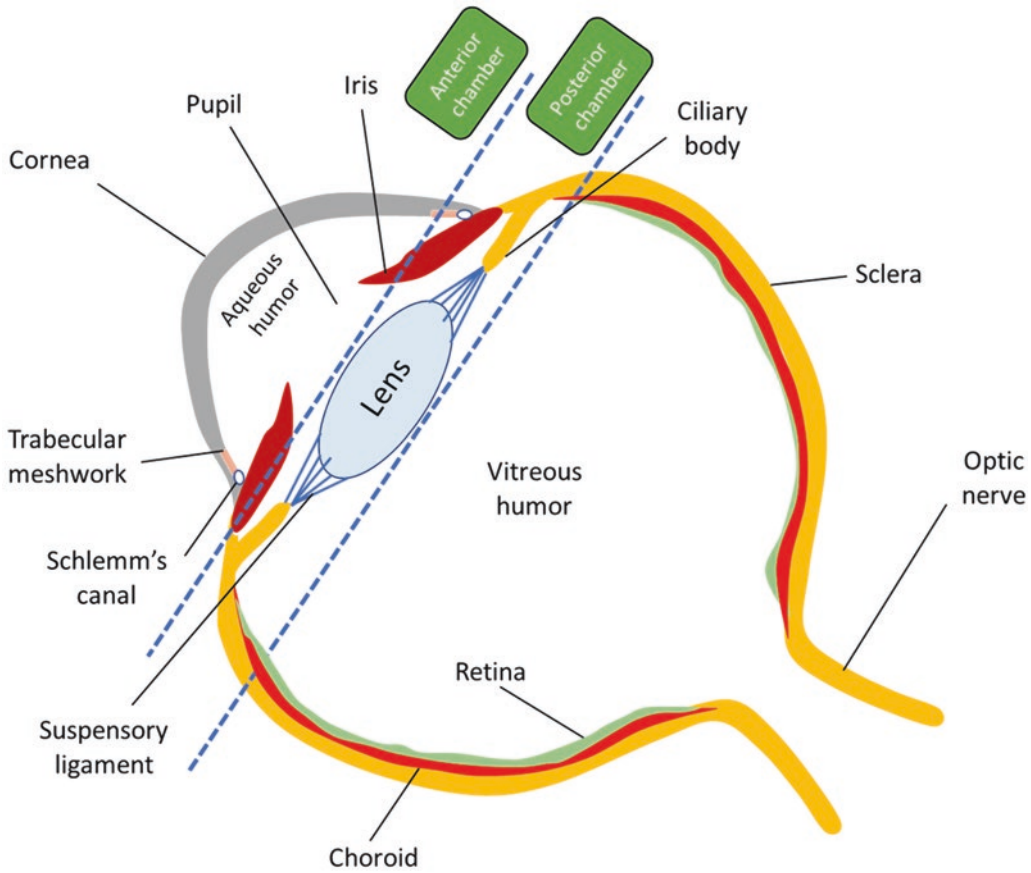

Fig. 1 Structure of a typical human eye

posed of multiple layers of cells that include photoreceptors, ganglionic cells, neural fibers, and pigmented epithelium. Neural fibers gather at the optic disc and give rise to the optic nerve.

3 Development of Eye Anterior Segment

Tissues from different embryonic origin are involved in the development of the vertebrate eye. The retina and the epithelial layers of the iris and ciliary body are from the anterior neural plate whereas the lens and cornea are derived from the surface ectoderm. During gastrulation, a single eye field is formed which then separates into two forming the optic vesicle (Fig. 2a) and the optic cup (Fig. 2b). Various genes including genes encoding cytoskeletal proteins, structural proteins, membrane proteins, and transcription factors become activated during development [1].

Cells from the periocular mesenchyme migrate into the cornea paving way for future corneal stromal development, i.e., sclera, iris stroma, endothelium, trabecular meshwork, ciliary stroma, and ciliary muscle (Fig. 2c, d). Remodeling of vasculature in the corneoscleral transition zone gives rise to the Schlemm's canal and is likely derived from periocular mesenchyme [2]. The iris and the ciliary body arise from the optic cup. The retinal pigmented epithelial cells form from the outer layer of the optic cup, and the main part of the inner layer of the optic cup forms later the neural retina. The retinal ganglion cells grow and form the optic nerve [1].

Mutations in certain transcription factors have been found to cause disease of the anterior segment such as developmental glaucoma, Peters anomaly, Axenfeld–Rieger Syndrome, and anterior segment dysgenesis. Anterior segment development of the eye involves a series of inductive events in a spatio-temporal manner [3]. Malformation of periocular mesenchyme with respect to patterning, migration or differentiation may contribute to glaucoma and ASD [2].

4 Disorders Related to Ocular Development

Anterior segment dysgenesis (ASD) refers to any abnormality caused during the development of the anterior segment of the eye. The tissues of the eye are derived from both embryonic ectoderm (surface and neural) and mesoderm. Hence, a large number of morphogenetic factors are required for proper spatio-temporal organization in ocular development. Mutations in genes coding for these factors lead to ASD.

ASD spectrum disorders can be classified according to their phenotypes in three ways—those with both ocular and systemic phenotypes (Axenfeld–Rieger Syndrome, Peters plus), those with only ocular phenotypes (primary congenital glaucoma), and those primarily having non-ocular systemic phenotype (Alagille syndrome) with a few ASD features (dental abnormalities) [4].

4.1 ASD Disorders Showing both Ocular and Systemic Phenotypes

Axenfeld–Rieger syndrome (ARS): It is characterized by heterogeneous phenotypes with high penetrance. ARS-affected individuals show iris atrophy, corectopia, pseudopolycoria, posterior embryotoxon characterized by an anteriorly displaced Schwalbe's line, and iris strands invading the trabecular meshwork. Iridocorneal angle deformities, iris atrophy, and thickening of meshwork impairs outflow and leads to glaucoma. 50% of the individuals are diagnosed for glaucoma at an earlier period than typical primary congenital glaucoma [5].

Craniofacial abnormalities consisting of tissues of neural crest origin are recognized in making the diagnosis of ARS, particularly in family members with a mild ocular phenotype. Facial deformities include midface hypoplasia, a broad flat nasal root, maxillary and occasionally mandibular hypoplasia, short philtrum, thin upper lip, and larger everted lower lip. Maxillary hypoplasia and maldevelopment of the teeth produce a prognathic profile. Hypertelorism and telecanthus have also been reported. Inspection of the oral cavity often shows various dental deformities [6].

Mutations in the *PITX2* and *FOXC1* [7] genes have been shown to cause ARS. Pedigree analyses showed mutations in 4q25 (PITX2 locus) [8] and 6p25 [9] (FOXC1 locus) in more than half of the probands [10, 11]. Other rare variants identified include 13q14 and 16q24 [11, 12]. Recently, mutations within the coding regions of two additional genes, *COL4A1* [13] and *PRDM5* [14] have been suggested to result in a small fraction of ARS patients (less than 1%).

Peters anomaly and Peters plus syndrome: Like ARS, Peters and Peters plus anomalies also show phenotypic heterogeneity. Both autosomal-dominant and autosomal-recessive forms of Peters anomaly have been described [15, 16]. The most notable hallmarks of the Peters anomaly Type I are central corneal opacity, iridocorneal adhesions, corneolenticular adhesions, and defects in Descemet's membrane. Type II in addition shows lens abnormalities and tends to be bilateral. Like ARS, individuals with Peters anomaly have 50% higher risk of glaucoma [15].

Peters plus syndrome is an extension of Peters anomaly where individuals exhibit systemic phenotypes which includes short stature, cleft lip palate, growth, and mental delay [17, 18].

Peters anomaly is genetically heterogeneous. Mutations in PITX2, PAX6, and CYP1B1[20–22] have been linked to

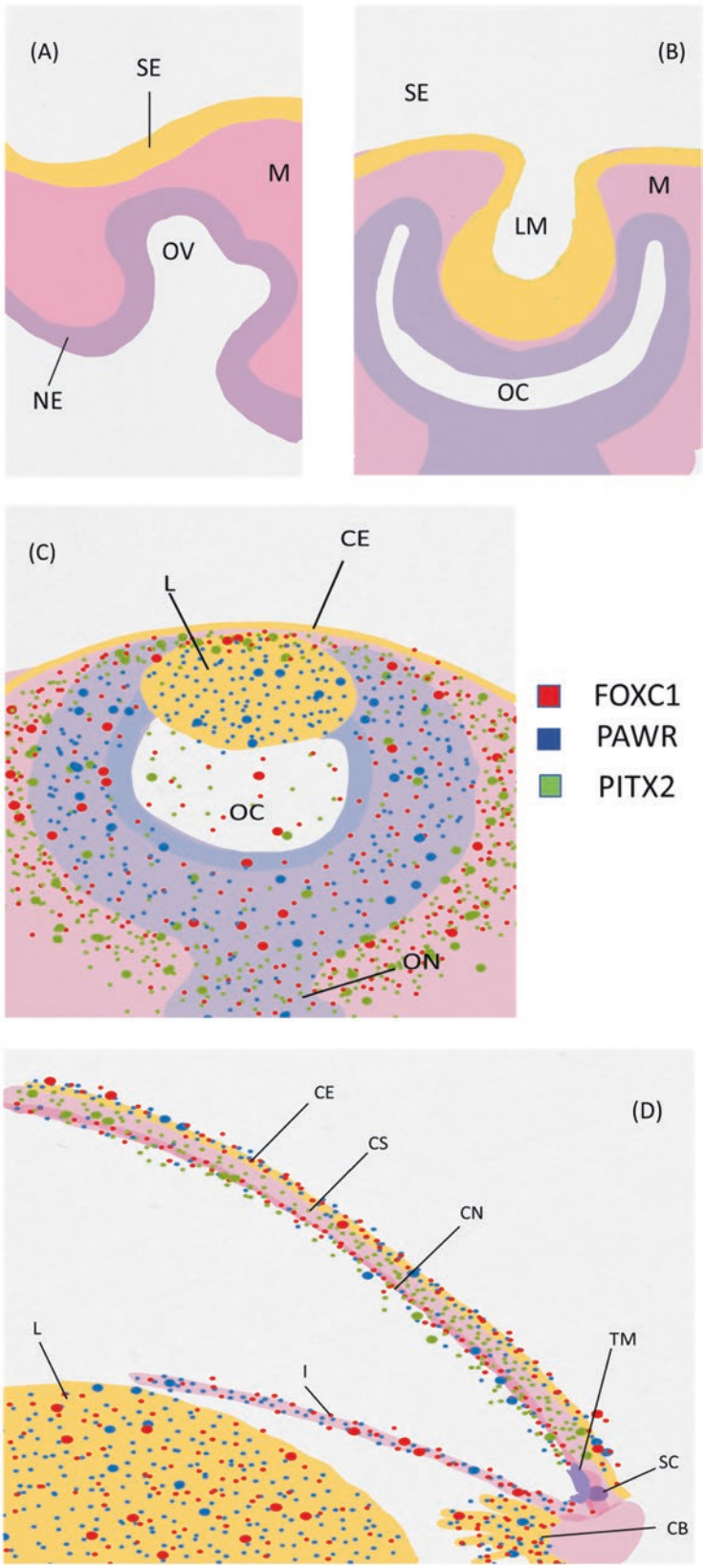

Fig. 2 Development of eye: (**a**) The neural ectoderm (NE), shown in yellow, moves through the periocular mesenchyme (M), shown in pink, and forms a sac-like structure called the optic vesicle (OV) until it touches the surface ectoderm (SE), shown in purple. (**b**) The surface ectoderm invaginates to form the lens primordia (LM) while the optic vesicle forms the optic cup (OC). (**c**) The lens primordia is filled up with fibrous tissue and forms the crystalline lens (L). The lens detaches from the surface ectoderm which gives rise to the corneal epithelium (CE). The optic cup forms the neural retina and optic nerve (ON). (**d**) The SE forms the corneal epithelium while the periocular mesenchyme gives rise to the corneal stroma (CS), corneal endothelium (CN), iris stroma (I), Schlemm's canal (SC), and trabecular meshwork (TM). The optic cup forms the ciliary body(CB) while the mesenchyme forms the muscle of the ciliary body. (The spatio-temporal expression of PAWR (blue), FOXC1 (red), and PITX2 (green) are shown as dots)

disease. Peters plus syndrome is an autosomal-recessive congenital disorder linked to the B3GALTL gene [19].

4.2 ASD Disorders Showing Only Ocular Phenotypes

Primary congenital glaucoma: Glaucoma is a disease related to elevated intraocular pressure (IOP) due to defects in drainage of the aqueous humor. Increased IOP may be caused by blockage of the Schlemm's canal and the trabecular meshwork (open-angle glaucoma) or the closure of the iridocorneal angle (primary angle-closure glaucoma). Glaucoma is typically a late-onset disease with disease phenotype manifesting after 40 years of age. Individuals with ASD have a higher risk of secondary childhood glaucoma. Unlike late-onset glaucoma, PCG develops at birth or within 2 years after birth and is related to maldevelopment of meshwork and Schlemm's canal.

PCG is an autosomal-recessive disorder showing high genetic heterogeneity. Linkage analyses have identified mutations in the cytochrome P4501B1 (CYP1B1) gene in the GLC3A locus as the PCG-causing gene [20, 21]. Strong associations have been found with two additional loci at 1p36 (GLC3B) and 14q24.3 (GLC3C) [4].

Aniridia: The term aniridia means "lack of iris." Aniridia is a rare autosomal-dominant disorder and is associated with iris hypoplasia, ciliary body hypoplasia, lens dislocation, and corneal opacity [22]. Mutations in the PAX6 transcription factor gene is determined as the causative factor, however, one mutation in the FOXC1 gene was found in a patient with aniridia [23].

Corneal hereditary endothelial dystrophy (CHED) caused by mutations in the CHED1 (autosomal-dominant) and CHED2 (autosomal-recessive) genes [24]. Megalocornea is another disorder showing only ocular phenotype and is caused by mutations in the CHRDL1 gene (X-linked) coding for ventroptin, a BMP antagonist [25].

4.3 Non-ocular Systemic Disorders Having ASD Features

Alagille syndrome (AGS)**:** Ocular development is orchestrated by an interplay of various developmental cues and morphogens. Hence, it is not surprising that various systemic disorders often show ocular dysmorphisms. Alagille syndrome (AGS) is an autosomal-dominant disorder caused by mutations in the 20p12 region containing the JAG1 gene [26, 27]. It is characterized by chronic cholestasis resulting from low bile production by hepatocytes or obstruction of bile flow due to maldeveloped hepatic ducts. Other phenotypes associated with AGS are facial dysmorphisms, cardiac anomalies, and butterfly vertebra. However, the preliminary diagnosis for AGS is the presence of posterior embryotoxon (prominent Schwalbe's line between the iris and cornea) [27]. Other systemic disorders showing ocular phenotypes are SHORT and Pierson Syndrome [4].

Occulodentodigital syndrome: This is a condition that affects different parts of the body including eyes, teeth, and fingers. This is inherited in an autosomal-dominant manner. It is caused by mutation in GJA1 gene [28]. People having this condition can have widely spaced small eyes (microphthalmia). The affected individuals might experience vision loss because of glaucoma and strabismus (a condition in which one or both eyes are turned inwards). Tooth deformities are characterized by missing tooth and weak enamel leading to multiple cavities. Syndactyly (webbing of the skin) between fourth and fifth fingers or toes and bony outgrowth in the hands can also happen in the affected individuals [29, 30].

5 PRKC Apoptosis WT1 Regulator (PAWR)/Prostate Apoptotic Response-4 (PAR-4)

PAWR/PAR4 as the name suggests was first identified in the cells of prostate cancer that were undergoing apoptosis [31]. PAR4 has a leucine zipper domain, and it is expressed in a wide variety of cells normal as well as cancer cells [32, 33]. Although endogenous PAR4 might not cause apoptosis, it is essential for apoptosis caused by various exogenous insults [32, 33]. PAR4 has a selective action to promote apoptosis in cancer cells and not in normal cells [34]. It was found that the cancer cells underwent apoptosis if PAR4 was overexpressed in them but this did not happen in normal cells [34]. The region of PAR4 protein responsible for its pro-apoptotic action is the central SAC region which spans from amino acid 137 to 195. Importantly, neither PTEN nor P53 is required for PAR4-mediated apoptosis. Even the overexpression of the anti-apoptotic proteins BCL2 and BCL-XL cannot inhibit the apoptotic action via PAR4 overexpression [34, 35].

Nuclear localization of the SAC domain of PAR4 is critical for the pro-apoptotic activity of PAR4. Cancer cells in which nuclear localization of PAR4 does not happen are resistant to apoptosis; a feature associated with poor prognosis [36]. Although the nuclear translocation of the SAC domain happens in normal as well as cancer cells, PAR4-mediated apoptosis occurs only in cancer cells only [36]. This selective response can be explained by the fact that PAR4 needs to be phosphorylated at the T155 residue via the Protein Kinase A (PKA) activity that is higher in cancer cells than in normal cells [35, 36].

The role of PAR4 as a tumor suppressor has been shown in knockout mice-models where the Par4 knockout mice developed tumors in various tissues, including chemical and hormone-induced tumors [37]. Several mechanisms have been proposed by which the PAR4 protein can become non-functional. There is evidence of the presence of Stop-gain mutations in endometrial tumors [38]. There are other ways by which PAR4 can be rendered non-functional, including post-transcriptional modification and methylation in the case of endometrial tumors [38]. PAR4 can also be inactivated by binding of the cell survival kinase Akt1 which phosphorylates it as in prostate cancer making it a substrate for 14-3-3 chaperone resulting in the sequestration of PAR4 in the cytoplasm [35, 39]. Akt1 binds to PAR4 via its leucine zipper domain. Since the SAC domain of PAR4 lacks the leucine zipper domain, it is not inactivated by Akt. Therefore, the SAC domain of PAR4 can selectively induce apoptosis in cancer cells. The phosphorylated SAC domain of PAR4 translocates to the nucleus and inhibits the prosurvival NF-κB DNA-binding activity [40].

PAWR/PAR4 has been also shown to interact with the Wilm's Tumor 1 (WT1) tumor suppressor gene. While WT1 itself functions as a transcriptional repressor, interactions with PAR4 enhance this activity. PAR4 can bind to WT1 via the leucine zipper domain of PAR4 and the zinc finger DNA-binding domain of WT1. PAR4 was shown to inhibit WT1-mediated transcription and augment WT1-mediated repression. It has been suggested that PAR4-enhanced WT1 repression activity possibly by providing an additional repression domain [3].

6 Regulatory Network of Transcription Factors in Ocular Disease and Development

Up until this point, we have explored the general mechanism of action of the PAWR/PAR4 gene in tumor suppression as well as how PAR4 can act as a transcriptional modulator via its interaction with other proteins/transcription factors. The role of transcription factors is very important if we think about the course of development in an organism because the spatio-temporal expression and interaction of these transcription factors can have a synergistic effect on development. In ocular development, there is a complex regulatory network of transcription factors which function together synergistically, and if there are any deleterious mutations in any of these genes then the complex interaction in this network can be disrupted leading to ocular diseases [41–43].

Pituitary homeobox 2 (PITX2) and forkhead box C 1 (FOXC1) and forkhead box C 2 (FOXC2) are among the transcription factors that are instrumental in ocular development. PITX2 belongs to the paired-bicoid family of homeodomain (HD) transcription factors whereas, FOXC1 protein is a member FOX class of transcription factors characterized by the presence of a conserved domain of 110 amino acids which binds to the DNA (DNA-Binding Domain). PITX2 is involved in the formation of anterior segment of eye, pituitary gland, hind limbs and in brain morphogenesis [44, 45]. FOXC1, on the other hand, regulates the development of various organs and oncogenesis [46]. There are numerous mutations in PITX2 as well as FOXC1 which have been characterized in Axenfeld–Rieger syndrome (ARS). More than 50% of ARS patients present with glaucoma that is often recalcitrant to normally prescribed glaucoma medications [8, 47, 48]. As well, PITX2 mutations have been identified in relation to ocular diseases including iridogoniodysgenesis, iris hypoplasia [49], and rarer cases of a Peters-like anomaly [50]. In case of FOXC1 while frameshift insertions/deletions and nonsense mutations have been found, the majority of FOXC1 mutations are missense mutations occurring in the FHD [44, 51]. Molecular characterizations have shown that mutations within the coding regions of either gene typically result in loss of protein functions which include impaired nuclear localization, DNA binding, protein–protein interactions, and transactivation capacity [52]. These types of mutations give rise to misfolded/truncated proteins that do not function properly and activate the FOXC1 and PITX2 target genes, thus leading to the disease phenotypes [53].

FOXC2 also belongs to the forkhead box family of transcription factors. It shares almost 98% sequence identity in the FHD [54]. The expression pattern of FOXC1 and FOXC2 in embryonic development is largely overlapping [55].

Functional role of PITX2 C-terminal mutations were analyzed in ARS patients. It has been shown that the mutations occurring in the critical DNA-binding homeodomain region of the PITX2 protein result in the significant decrease of function of PITX2 [56], but the mechanism by which the mutations in the C-terminal region of the PITX2 induced pathogenesis gene was unknown. The C-terminal mutations like L105V and N108T lie in the domain of PITX2 shown to be responsible for inhibiting transcriptional activation. Therefore, these mutations did not cause DNA-binding defects in the protein, but instead were responsible for a less stable PITX2 mutant protein with elevated activity. It has been suggested that these C-terminal mutations result in stochastic deregulation, due to elevated activity of PITX2, during ocular development that in turn leads to disease [57].

6.1 Molecular Interaction between PITX2A And FOX Proteins

PITX2 has 4 splice variants (A, B, C, and D) of which PITX2D lacks the HD and thus may be of limited physiological significance. The PITX2A isoform, encoded as a 32 kDa polypeptide, is the best studied isoform in case of ARS. The expression patterns of *Foxc1* and *Pitx2a* during murine development matches with the organs affected in ARS. Immunostaining assays in murine eye primordia showed that at E11.5 (embryonic day) FOXC1 and PITX2 are co-expressed in the periocular mesenchyme cells fated to form the corneal endothelium, choroidal capillaries, and sclera. By E12.5, the co-expression spreads to the posterior of the optic cup. By E16.5, FOXC1 and PITX2 are co-expressed in specific ocular structures like the corneal endothelium, iridocorneal angle, and choroid (Fig. 2). It has been shown that FOXC1 and PITX2 physically interact (Fig. 3) and highly colocalize inside the cell nucleus [57]. Mutations in the NLS region of the proteins interfere with the normal translocation into the nucleus. PITX2A binds to FOXC1's PITX2-binding domain (PITX2BD) located at the C-terminal third (residues 475-553) of FOXC1. The binding domain overlaps with the C-terminal activation domain (AD2; residues 436-553) of FOXC1. The homeobox domain (HD) of PITX2A is extremely crucial for binding to FOXC1, as deletions of the PITX2A HD (residues 39-98) completely ablate the PITX2A-FOXC1 binding (Fig. 3). The degree of co-expression varies across cells suggesting a quantitative difference in relative expression of the two factors in individual cells [3].

PITX2 acts as a negative regulator of FOXC1 transcriptional activity (Fig. 4a). Cells transfected with FOXC1 along with PITX2 showed marked impairment in activation of FOXC1 reporter genes as compared to FOXC1 alone. PITX2 interacts with FOXC2 in a similar way [57].

6.2 Molecular Interaction of PAWR/PAR4 with FOXC Proteins

PAWR/PAR4 interacts with FOXC1 as well as FOXC2 both in vitro and in vivo (discussed in detail in Sect. 8.1). In vitro, full-length 6XHIS-tagged PAWR/PAR4 protein, expressed in bacteria and then bound to Ni^{2+} agarose beads, was able to pull down either V5 epitope-tagged FOXC1 or FOXC2 expressed in human trabecular meshwork cells. This interaction was then also reconfirmed by co-immunoprecipitation. Subsequent investigations revealed that FOXC1 and FOXC2 interact with PAWR through their inhibitory domains. In converse experiments, it was demonstrated that the C-terminal leucine zipper domain of PAWR/PAR4 is sufficient to interact with either FOXC1 or FOXC2 (Fig. 3) [3].

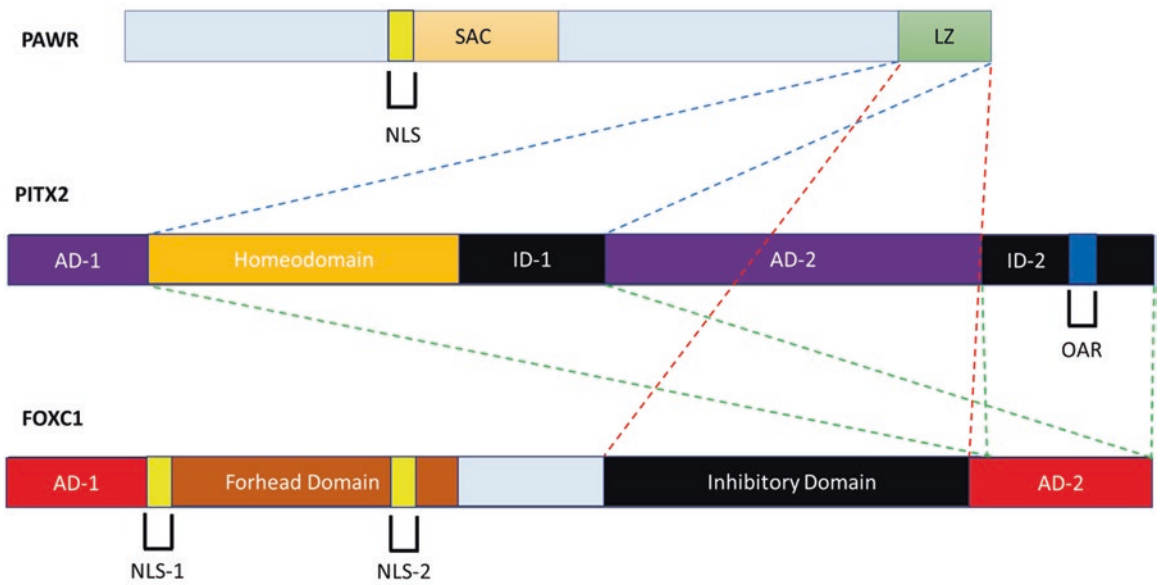

Fig. 3 Domain-map of the interacting regions of PAWR, PITX2, and FOXC1: PAWR interacts with PITX2 (shown in blue) and FOXC1 (shown in red) through its leuzine zipper (LZ) domain and binds to the homeodomain (HD) and inhibitory domain (ID) 1 of PITX2, and inhibitory domain of FOXC1. PITX2 has two binding sites for FOXC1 (shown in green). The activation domain (AD) 2 of FOXC1 binds to both the HD and ID-1, and ID-2 of PITX2

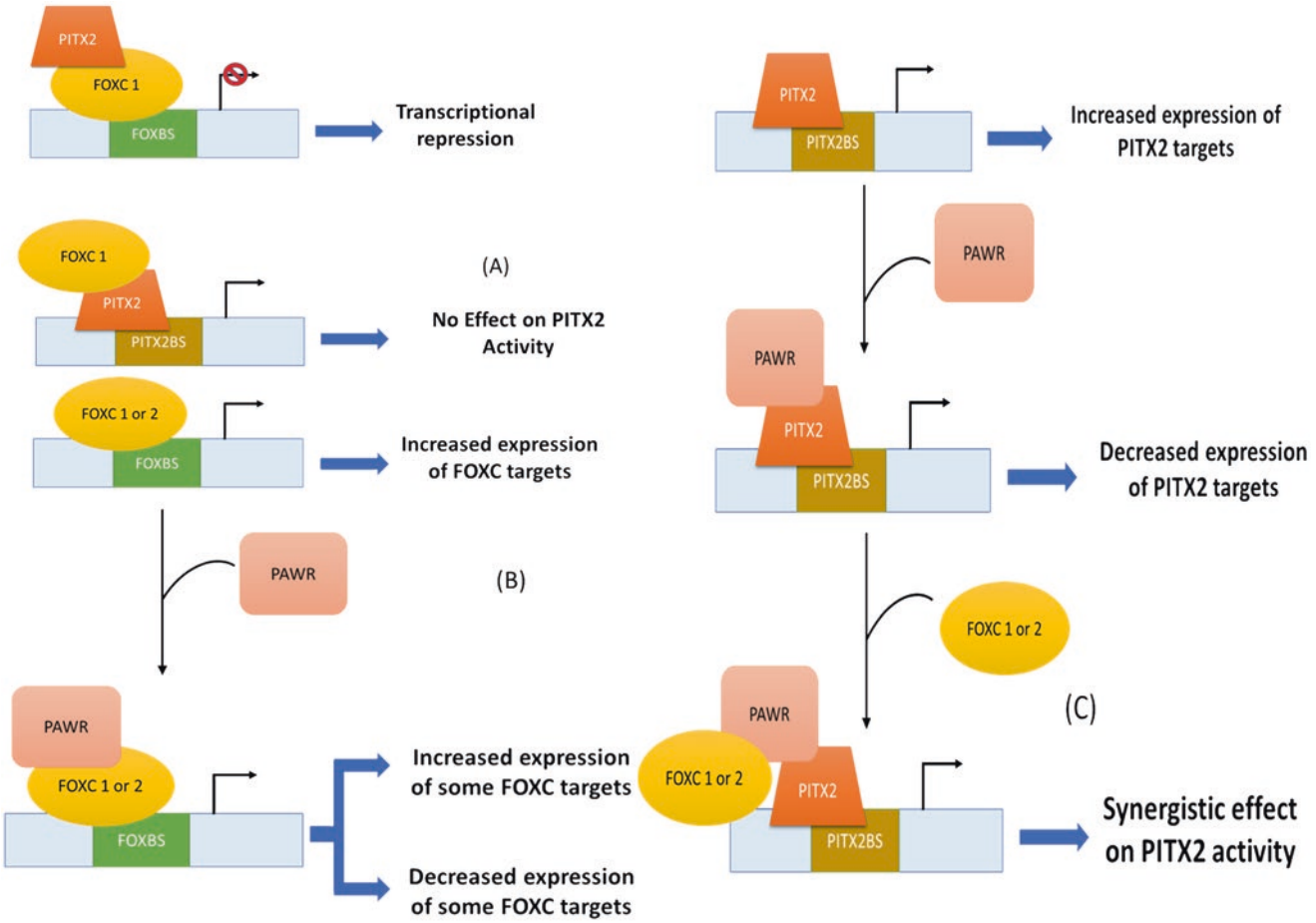

Fig. 4 Molecular Interactions between PAWR, PITX2, and FOXC1: (**a**) PITX2 upon binding to FOXC1 attached to a FOX-binding site (FOXBS) causes transcriptional repression resulting in silencing of the FOX target genes. FOXC1, however, has no effect on the transcriptional activity of PITX2 bound to its binding site (PITXBS) when present alone. (**b**) PAWR influences the activity of FOXC1 in a gene-specific manner. In some cases (FGF19), it causes transcriptional activation, while in some cases (FOXO1A) it causes repression. (**c**) PAWR when present alone suppresses PITX2 activity. However, in the presence of FOXC proteins a synergistic effect on PITX2 activity is observed

6.3 PAWR Regulates PITX2, FOXC1, and FOXC2 Function

The various interactions of PAWR with PITX2 and the FOXC proteins make it a crucial regulator of these transcription factors. Research showed that a complex regulatory mechanism exists among these factors (Fig. 4).

6.4 PAWR Influences FOXC Activity in a Gene-Specific Manner

FOXC1 and FOXC2 binds to the upstream regulatory elements of FOXO1A and FGF19 genes and enhance their expression. FGF19 is responsible for the development of cornea via FGFR4/MAPK pathway as knocking down of FGF19 leads to ASD in zebrafish (*D. rerio*). FOXO1A modulates FOXC1-dependent cell viability and provides resistance to oxidative stress in the eye.

Dual luciferase assays showed that the presence of PAWR significantly enhances transcriptional activity of FOX-binding domains in HTM cells as compared to FOXC1 or FOXC2 alone. However, further analyses showed that PAWR influences FOXC (1 and 2) activity in a target gene-specific manner (Fig. 4b). FOXC and PAWR co-expression positively influenced FGF19 expression. On the contrary, PAWR exerts a negative control on FOXO1A regulatory element activity [3].

6.5 PAWR Influences PITX2 in a Complex Manner

PAWR acts as a repressor of PITX2 activity as HTM cells co-expressing PAWR and PITX2 showed 50% reduced transcriptional activity as compared to PITX2 alone. However, the exact regulation mechanism(s) appears complex. As discussed, PITX2 interacts with FOXC proteins and represses

their activity. Recent findings suggest that FOXC proteins act in synergy with PAWR to upregulate PITX2 activity. PITX2 with FOXC proteins alone did not show any change in transactivational activity. An increase in FOXC2 levels in the presence of PAWR proportionally increased PITX2 activity (Fig. 4c). These findings suggest that PITX2, FOXC2, and PAWR act together to increase PITX2 transactivation. PAWR interacts with FOXC1, FOXC2, and PITX2 through a common domain (LZ) but the changes in activity is not due to competition for binding sites (Fig. 3) [58, 59].

7 PAWR in Ocular Development

FOXC1 and PITX2 are co-expressed in the periocular mesenchyme that gives rise to the tissues of the anterior chamber (Fig. 2). They potentiate migration and condensation of cells forming the cornea and trabecular meshwork. In addition to ocular tissues, PITX2 is expressed in the heart, pituitary, branchial arches, and ventral body wall [60]. In zebrafish, PITX2 is expressed in lateral plate mesoderm and diencephalon early in development; hence, mutations may cause antero-posterior deformities and hydrocephalus. FOXC1 is expressed in the developing vasculature and is crucial for the development of the hyaloid vessel and maintenance of the vascular membrane integrity [3].

In zebrafish, PAWR protein is present in the lens and diffusely throughout the eye during embryonic development [61] (Fig. 2). In mouse, PAWR was expressed in the mesenchyme of the iridocorneal angle of the eye at different developmental stages [8, 59, 62–65].

8 Dysregulation of PAWR Causes ASD

As discussed previously, PAWR acts as a regulator of the transcription factors (FOXC1, FOXC2, and PITX2). Mutations of these transcription factors cause AR phenotypes. Both PITX2 and FOXC1 are required at a strictly enforced level during ocular development as suggested by hypo- and hypermorphic disease-causing mutations, and deletions and duplications of those genes [66–69]. In a cellular context, mutations in PITX2 lead to loss of function of PITX2-target genes as well as dysregulated expression of FOXC1-target genes.

8.1 Phenotypic Effects of Knockdown of PITX2, FOXC1, and PAWR

Morpholino-knockdown experiments on zebrafish effectively showed phenotypic alterations due to PITX2, FOXC1, and PAWR knockdowns. Splice-site blocking morpholinos (MOs) were used against PAWR and PITX2 that produced aberrant products, while translation-blocking morpholinos were produced against FOXC1. Zebrafish embryos at 48 hpf (hours post-fertilization) were injected with morpholinos at two doses: low (2.5 ng/embryo) and high (5 ng/embryo). Embryos injected with PITX2MOs showed several gross deformities, like heart edema, slight hydrocephaly, shorter body length, and a ventrally curved tail. Ocular hemorrhage around the iris was observed at a lower frequency in both low (14%) and high (29%) doses [60] (Fig. 5a).

Translation-blocking *foxc1* MOs were designed for both *foxc1a* and *foxc1b* which are orthologs of FOXC1. *foxc2* MOs could not be used as zebrafish lacks FOXC2. Low doses (1.25 ng/embryo) resulted in ocular hemorrhaging, while high doses (3.25 ng/embryo) resulted in gross abnormalities like hydrocephalus, and reduced body length [3]. Therefore, the phenotypes induced by *pitx2* morpholinos are similar to those induced by *foxc1* morpholinos, indicating a similar role of the two factors in ocular development (Fig. 5a). Splice-site blocking MOs against *pawr* did not show any effects [3].

8.2 Genetic Interactions among PAWR, PITX2, and FOXC1

Concerted knockdown of genes at a sub-effective dosage of MOs is used to determine any genetic interactions between them. Sub-effective dosage is defined as the amount of MO when injected does not produce any phenotype. Sub-effective doses for *pitx2, pawr,* and *foxc1* were determined to be 2, 5, and 1.2 ng/embryo, respectively. Appearance of abnormal phenotype in the presence of MOs for two genes, both at sub-effective dosage signifies a genetic interaction between the genes. As they share a common pathway, knocking down both genes cause amplification of defects resulting in manifestation of abnormal phenotype, which would not be the case if single MOs were injected (Fig. 5b) [3].

Coinjection of *pawr* and *pitx2* MOs at sub-effective doses at 48 hpf resulted in hemorrhaging around the iris, similar to effective doses of *pitx2* MO alone. Hemorrhaging was also observed in the central nervous system (CNS). Coinjection of *pawr* and *foxc1* MOs at sub-effective doses results in hydrocephaly (87.4%), hemorrhaging in the CNS (23.6%). Hemorrhaging was observed in wild type as well as transgenic zebrafish Tg(gata1:dsRed)sd2/+ that express dsRed in erythrocytes (Fig. 5b) [3].

Moreover, additive effects of *pitx2* and *foxc1* MOs resulted in reduced brain size, necrosis, and hydrocephaly. These phenotypes were not observed when the MOs were injected individually, suggesting that they are critical for brain morphogenesis (Fig. 5b) [3].

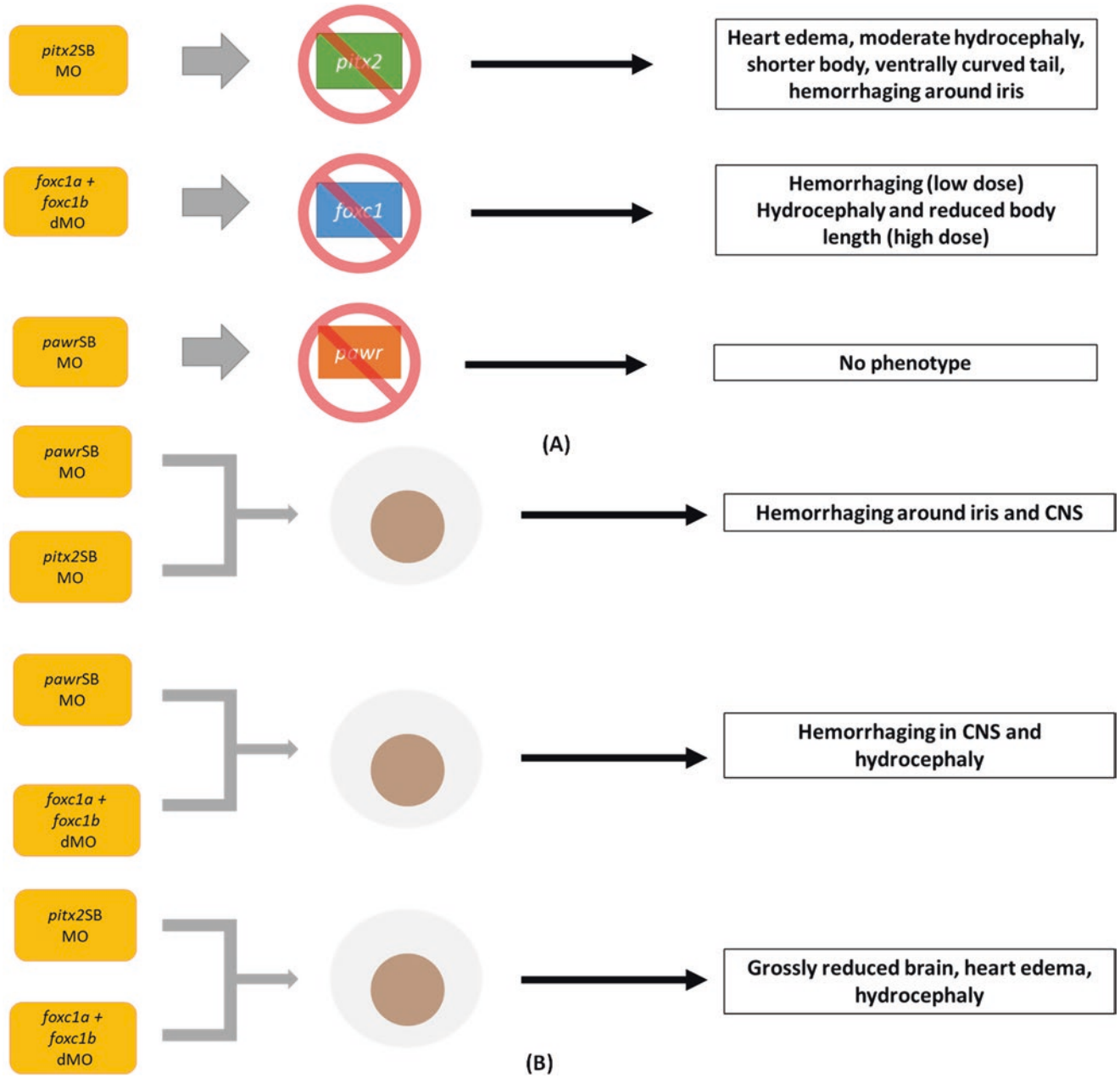

Fig. 5 Phenotypes produced by PAWR, PITX2, and FOXC1 morpholinos in zebrafish: Splice-blocking (SB) MOs were used against PAWR and PITX2, while translation-blocking morpholinos (dMOs) were used against FOXC1A and FOXC1B. (**a**) Effective doses (both low and high) of MOs against FOXC1 and PITX2 produced severe developmental anomalies. However, PAWR MOs did not produce any notable phenotypic alterations. (**b**) Coinjection of MOs in sub-effective doses also produced phenotypes similar to single injection of MOs. This shows a genetic interaction exists between the two factors PITX2 and FOXC1 and PAWR acts as the regulator

Thus, PAWR lies in the same pathway as PITX2 and FOXC1 and affects vasculogenesis and basement membrane formation. PAWR acts as a crucial regulator of both PITX2 and FOXC proteins [3].

9 Future Perspectives

The transcription factors (PITX2, FOXC1, and FOXC2) and the regulator (PAWR/PAR4) are involved in a complex regulatory network as discussed earlier. Mutations in these three

transcription factors are directly involved in ASD and glaucoma pathogenesis. PAWR acts as a crucial regulator of both PITX2 and FOXC proteins in a target-specific manner. It is important to note that while PAWR can repress PITX2 activity, it also works in synergy with FOXC proteins to increase PITX2 activity. PAWR/PAR4 regulates the activity of PITX2 and FOXC transcription factors thus regulating the downstream target gene expression in a complex manner. The morpholino-knockdown experiments in zebrafish also confirmed the biological significance of these genes in ocular development and diseases.

PAWR, being a tumor suppressor has a major role as a regulator and inducer of apoptotic pathways selectively in cancer cells. It has been established as a key regulator, more specifically an inhibitor of WT1 function, which is a transcription factor involved primarily in the development of urogenital system. The fact that PAWR acts as a transcriptional repressor is also established in eye anterior segment development as it inhibits PITX2 activity. However, the interaction between PAWR and FOXC transcription factors becomes complicated where FOXC-PAWR complex exerts target-specific effects of activation and repression. Further it acquires more complexities when PAWR performs a complete role reversal and favors PITX2 transactivation in the presence of FOXC proteins and that too, with an additive effect. While genetic interaction between Pitx2, Pawr and Foxc proteins is established using morpholino-mediated knockdown in zebrafish, the underlying mechanism of this specific additive effect of PITX2 transactivation by PAWR in the presence of FOXC proteins remains elusive. An experimental design from a stoichiometric perspective would have a great potential to resolve this.

So far, the role of PAWR in ocular development, more specifically in eye anterior segment development is somewhat ambiguous and mostly based on a few reports. Apparently, involvement of PAWR in the development of eye anterior segment is not simple and unidirectional but rather convoluted and multifaceted. Thus, complete delineation of PAWR's role in this regulatory network of transcription factors or other similar networks and pathways warrants further in-depth and extensive functional investigation both at cellular and molecular level and in an appropriate animal model where it would be easier to study developmental stages of organogenesis with a special emphasis on ocular development.

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Par-4 in Apoptosis during Human Salivary Gland Development and Tumorigenesis

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Abstract

Human salivary glands (SGs) are complex structures comprising a system of ducts and acini formed in gradual stages termed the prebud, initial bud, pseudoglandular, canalicular, and terminal bud. This process involves growth, proliferation, differentiation, migration, and cell death. Studies in human specimens and in vitro models have demonstrated that apoptosis seems to be important not only during the early developmental stages of the salivary glands, but also contributes to the tumorigenic process and impacts the patient's treatment. Therefore, the screening of proteins associated with apoptosis might contribute to the development of different strategies focusing on cancer diagnosis, prognosis, and target therapies. The prostate apoptosis response-4 (PAR-4) is a 38 kDa protein encoded by the PAWR gene (PKC apoptosis WT1 regulator) that is ubiquitously expressed in different tissues and plays a role in both the intrinsic and extrinsic apoptotic pathways. This chapter explores the current knowledge on the expression of Par-4 during human salivary gland development and in the most frequent salivary gland tumors (benign: pleomorphic adenoma and malignant: adenoid cystic carcinoma and mucoepidermoid carcinoma). In addition to the application of Par-4 as a tumor prognostic marker, the use of targeted therapies against Par-4 is increasingly considered as an important strategy for cancer treatment.

Keywords

Salivary gland development · Salivary gland tumors · Apoptosis · Organogenesis · Par-4 · Differentiation · Morphogenesis · Mucoepidermoid carcinoma · Pleomorphic adenoma · Adenoid cystic carcinoma

1 Aspects of Salivary Gland Development—Brief Morphological and Molecular Considerations

Organogenesis is determined by the coordinated intercellular activity between groups of primitive cells as well as with extracellular matrix (ECM) components. Organogenesis to the final organ architecture depends on cells and tissue rearrangements; these are often accompanied by regulated cell proliferation and/or cell death, which are the primary mechanisms for generating the final shape of a functional mature organ. In this route, the expression of specific cell surface ligands and receptors tightly linked in a complex web of signaling pathways that regulate cell division, migration, and differentiation are essential features of organ development, maturation, and functions (Fig. 1).

Similar to many other glandular organs, the formation of the salivary gland (SG) is driven by the coordination of morphogenetic mechanisms, including regulated cell shape changes, and gene expression; directed cell proliferation, death, and migration lead to a fully developed gland with important secretory functions. Morphologically, all salivary

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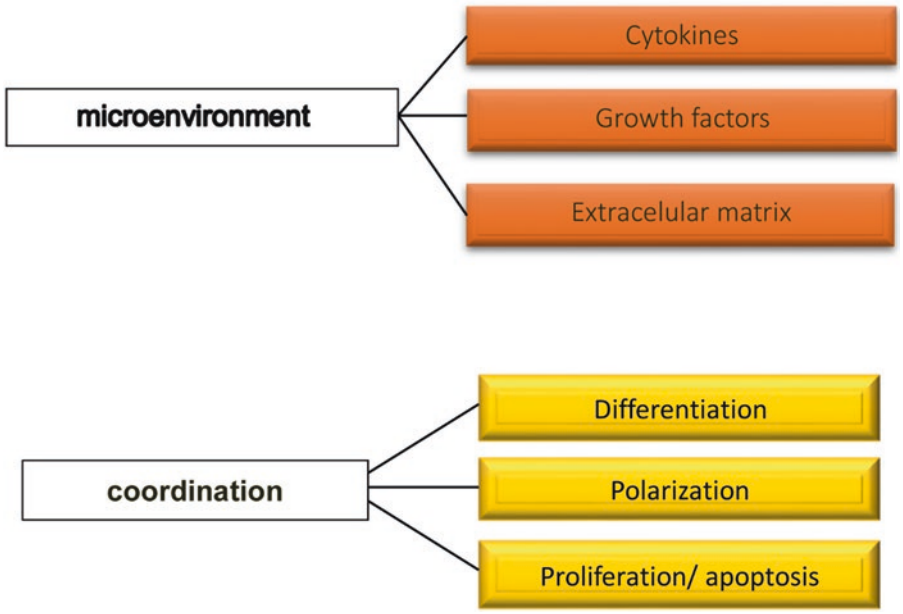

Fig. 1 Mechanisms involved in salivary gland organogenesis

glands develop similarly, with formation starting with the proliferation of a solid cord of cells from the epithelium of the *stomodeum* into the underlying ectomesenchyme. This cord of cells extends deeply into the ectomesenchyme, branches extensively, forming canals with secretory end pieces. This process has been classified into stages of glandular morphogenesis, which is briefly described and illustrated in this text. During the first step denominated *Prebud Stage*, a thickening focus arise from the oral epithelium, which invaginates into the subjacent mesenchyme forming a solid epithelial stalk and a bulb. This solid group of cells undergoes successive proliferations, known as *Initial Bud Stage*. The *Pseudoglandular Stage* presents a presumptive duct formation, and 4–5 end buds are formed by branching morphogenesis. In the *Canalicular Stage*, this branching culminates in an increasing number of buds and the presumptive ductal portion shows initial lumen formation. Finally, at the *Terminal Bud Stage*, ducts and acini are identified showing well-developed structures. It has been accepted that only after birth SG reaches the total differentiated structural maturity, able to perform its major function: to synthesize and to secrete saliva [1, 2].

From early development to mature stages, the SG undergoes branching morphogenesis, which involves epithelial and mesenchymal interactions as well as autocrine and paracrine factors, proliferation, and apoptotic mechanisms [3–6] (Fig. 2).

This complex process involves the interplay of several ECM components (laminin, fibronectin, collagens), growth factors, and their receptors (EGF, FGF, PDGF), and several signaling pathways (WNT and NOTCH pathways) [7, 8]. The orchestration of this process also depends on neuronal and endothelial supply, cellular proliferation and growth, migration, and cell death [9–14], as described in Table 1.

Molecular characterization of the salivary gland development is critical for understanding the basic mechanism controlling morphogenesis and cytodifferentiation, in addition to facilitating the researcher's understanding of which changes can be involved in disease progression and, therefore, be used as indicators of diagnostic and target therapies.

2 Apoptosis and Salivary Gland Development

Since the term apoptosis has been introduced by Kerr et al. [37] to describe the natural cell clearance in mammalian tissues, the importance of this mechanism has progressively increased. The programmed cell death is a key mechanism involved in organizing and sculpting organ shape during development. Through this mechanism, specific cells are eliminated by programmed death. During apoptosis, cells undergo a cascade of well-characterized physical changes. These involve alterations in various cell membranes, including plasma membrane and nuclear breaking up and blebbing, permeabilization of the mitochondrial outer membrane, DNA fragmentation, nucleus disintegration, and cell disintegration into apoptotic bodies, which are finally phagocytosed.

Apoptosis is a genetically programmed cell death mechanism and involves two pathways, the intrinsic and the extrinsic. The intrinsic apoptosis pathway encompasses pro-apoptotic molecules stimuli, which induces mitochondria to release cytochrome-c into the cytoplasm where it associates with Apaf-1. This complex coupled to caspase-9 activates caspase-3 leading to cell death. The extrinsic path-

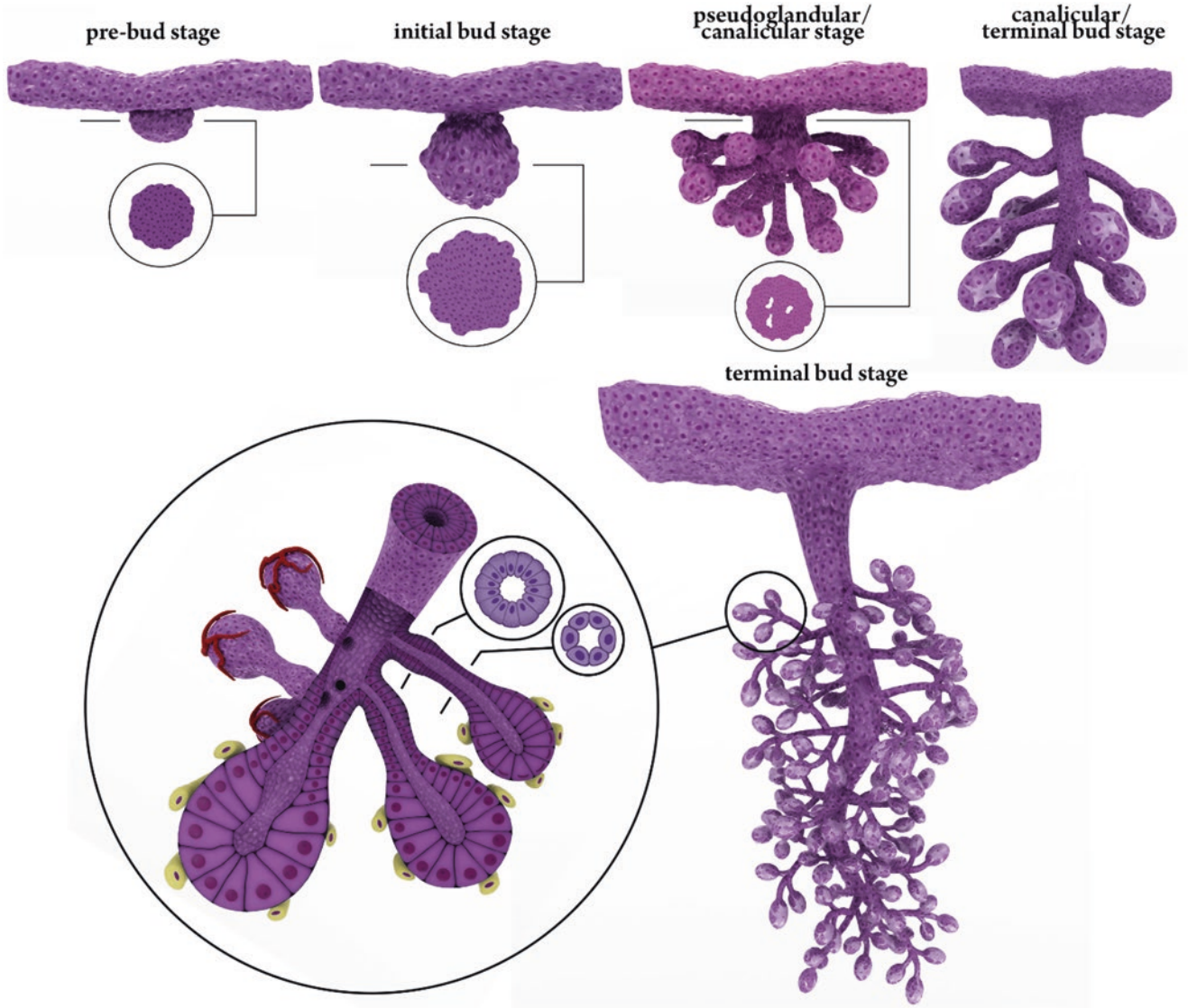

Fig. 2 Schematic representation of salivary gland developmental phases

way is induced by TNF family members, which recognize specific ligands, such as FADD, enabling them to recruit caspase-8 and thereby to activate caspase-3 [38–40]. During the execution of apoptosis, which involves caspases coordination of organelle fragmentation, cellular contents disintegration, and packing, following with efficient clearance of cell remnants, the rearrangement of cytoskeletal elements, as well as significant changes in cell shape, are well documented. This phase also requires alterations and loss in cell-cell and cell-matrix adhesion, which facilitates membrane blebbing.

The morphogenesis of organs involves the coordination of complex mechanisms, which are only partially unraveled to date. These include cell migration, proliferation, apoptosis, and active mobility in cell format and/or polarity, all critical for the final architecture and function of tissues and organs. These mechanisms are yet more intricate in branching morphogenesis due to the final complexity of the organs, such as lungs, mammary glands, pancreas, and salivary glands. The apoptotic cascades and factors involved in salivary gland branching morphogenesis are only partially determined, and there are many questions to be answered in this field to fully understand the process, and mechanisms of controlling the programmed cell death in salivary gland development, physiology, and disease.

In human salivary gland development, the intrinsic apoptosis pathway has been described in the early stages of development [9, 13]. In a series of work, [13, 14] demonstrated that in human specimens and in vitro, salivary glands' initial lumen formation in the SG main stalk seems to occur by an apoptosis-driven mechanism (intrinsic pathway). Subsequently, the maintenance of the luminal space seems to

Table 1 Factors involved in salivary gland differentiation and function

Cytoskeleton	Acinar and ductal cells	K5, K14: Associated with basal cells and regenerative compartments CKs 7, 8, 18, and 19 label luminal duct cells with some degree of morphological differentiation	[15]
	Myoepithelial cells	Actin, calponin, caldesmon: Highlight myoepithelial cell maturation from early development	[16–18]
Intercellular and Cell-matrix adhesion	Cell/cell	E-cadherin: Regulates ductal lumen formation during branching morphogenesis of the salivary gland	[19]
	Cell/matrix	Integrins: Mediate cell and ECM interactions performing adhesion as well as "inside out and outside in signaling." integrin beta-1 appears during bud stage in a few cells (considered pluripotent cells) and its distribution increases as salivary gland morphogenesis progresses. Claudins: Involved in controlling tight junctions and increase their expression from early morphogenesis to fully mature salivary gland.	[4, 5, 20]
Water channels	Aquaporins	Expressed from early stages of SG morphogenesis and exhibit complimentary expression patterns that may contribute to the morphogenesis of salivary glands; AQP-1 is important for myoepithelial cells interactions at the salivary gland boundaries.	[10, 21]
Growth factors and signaling	NGF	Regulation of sympathetic innervation	[3, 22–27]
	TGF-beta	TGF-beta 1: Appears during canalization stage in the surrounding mesenchyme and, in the more differentiated stages, it is detected the cytoplasm of acinar cells; TGF-beta 2: Occurs since the bud stage of the salivary gland in ductal cells; TGF-beta 3: Present from the canalization stage of the salivary gland. It is the only factor detected on myoepithelial cells.	[28]
	EGF	EGF supports branching morphogenesis of SMG rudiments, especially cleft formation	[29, 30]
	Fgf10	Fgf10 is detected in the mesenchyme surrounding the developing salivary glands; essential for both salivary bud outgrowth	[31]
	WNT	Mesenchymal Wnt signaling regulates salivary gland growth indirectly by inducing the expression of paracrine factors such as Eda	[32]
Blood supply	External carotid artery and branches	During branching morphogenesis remains unclear And whether the vascular glandular supply is critical for glandular maintenance	[12, 33]
Innervation	Parasympathetic	Early developmental stages	[3, 34, 35]
	Sympathetic	Late development stages/ mature salivary glands	[3, 34, 35]
	Muscarinic receptors M1 and M3	Interact with acetylcholine in the parasympathetic Nerves to stimulate fluid secretion	[3, 34, 35]
	Noradrenaline And beta-1 adrenoceptors	Promote protein secretion	[3, 34, 35]
Extracellular matrix	Laminin, collagen, tenascin	Collagens: Epithelial branching morphogenesis; laminin and LAMA5 receptor interactions with integrin α 3 β 1, necessary for basement membrane formation and epithelial organization	[36], for review

be associated with other mechanisms, as there is a decreasing expression of apoptotic cells through SG development either in ducts or acinar structures.

Additional evidence has added new data on the participation of the extrinsic apoptotic pathway in salivary gland morphogenesis, with the participation of distinct players in the mechanism, and Par-4 seems to stand as a key factor in this process. The prostate apoptosis response-4 (Par-4) is a pro-apoptotic and suppressor protein ubiquitously expressed in various tissues [41], including the salivary gland [9, 42]. Par-4 is localized in both the cytoplasm and the nucleus, and the translocation to the nucleus occurs upon apoptosis induction [41, 43, 44]. Par-4 promotes the down-regulation of the anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) [44]. This stimulus triggers pro-apoptotic molecules to induce mitochondria to release cytochrome-c into the cytoplasm and

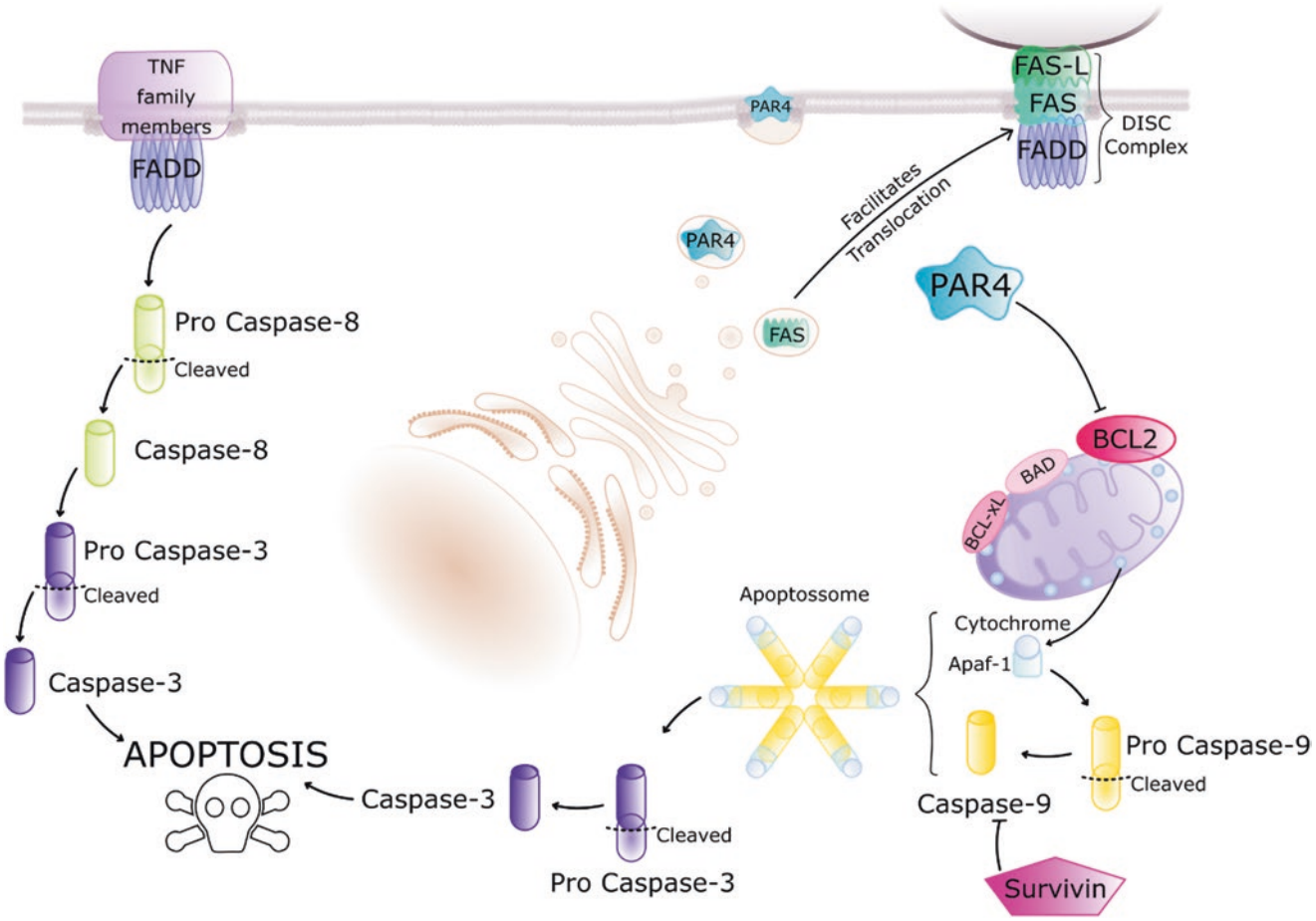

Fig. 3 Schematic representation of the intrinsic and extrinsic apoptotic mechanisms involving cell machinery

trigger the cascade of caspases, leading to cell death [45] (Fig. 3).

The extrinsic pathway of apoptosis triggered via Fas cell surface death receptor (Fas) and Fas ligand (FasL) was found to be associated with Par-4, which has the ability to inhibit the nuclear factor kappa B (NF-κB) activity. This interaction enabling the Fas-FasL complex to be trafficked to the plasma membrane, which is transduced by the activation of the Fas-associated death domain (FADD), causes activation of the Caspase-8, leading to apoptosis [46].

de Mello Gomes et al. [9] described the participation of Par-4 during salivary gland morphogenesis: at the initial bud stage—at the beginning of the rudimentary luminal structure—this protein helps to promote the removal of the central cells of the cylindrical cords, allowing the organization of the future ductal system. At the early development stages, strong nuclear Par-4 expression and weak cytoplasmic expression was observed, and intense activity can be noted at the branched regions a nuclear pattern during the luminal opening. On the other hand, at the later phases of the morphogenesis, nuclear Par-4 expression is observed in intercalated ducts near the secretory lobules, and cytoplasmic staining occurs in the excretory ducts. Fully developed human salivary glands present only focal expression of Par-4 (Fig. 4).

Par-4 protein seems to play an important role during salivary gland development, and understanding this mechanism is important to comprehend tumorigenesis since both processes share similarities [47]. In line with this information, the screening of Par-4 and other proteins associated with apoptosis might contribute to the development of different strategies focusing on cancer diagnosis, prognosis, and target therapies [9].

3 Roles of Par-4 in Salivary Gland Tumorigenesis

Salivary gland tumors (SGT) comprise a heterogeneous group of lesions with distinct biological behavior, accounting for 3–6% of the head and neck tumors. These tumors are divided into benign and malignant [48, 49]. The most common SGT is pleomorphic adenoma (PA). It is a benign tumor accounting for 40–70% of SGTs [50]. Malignant SGT types

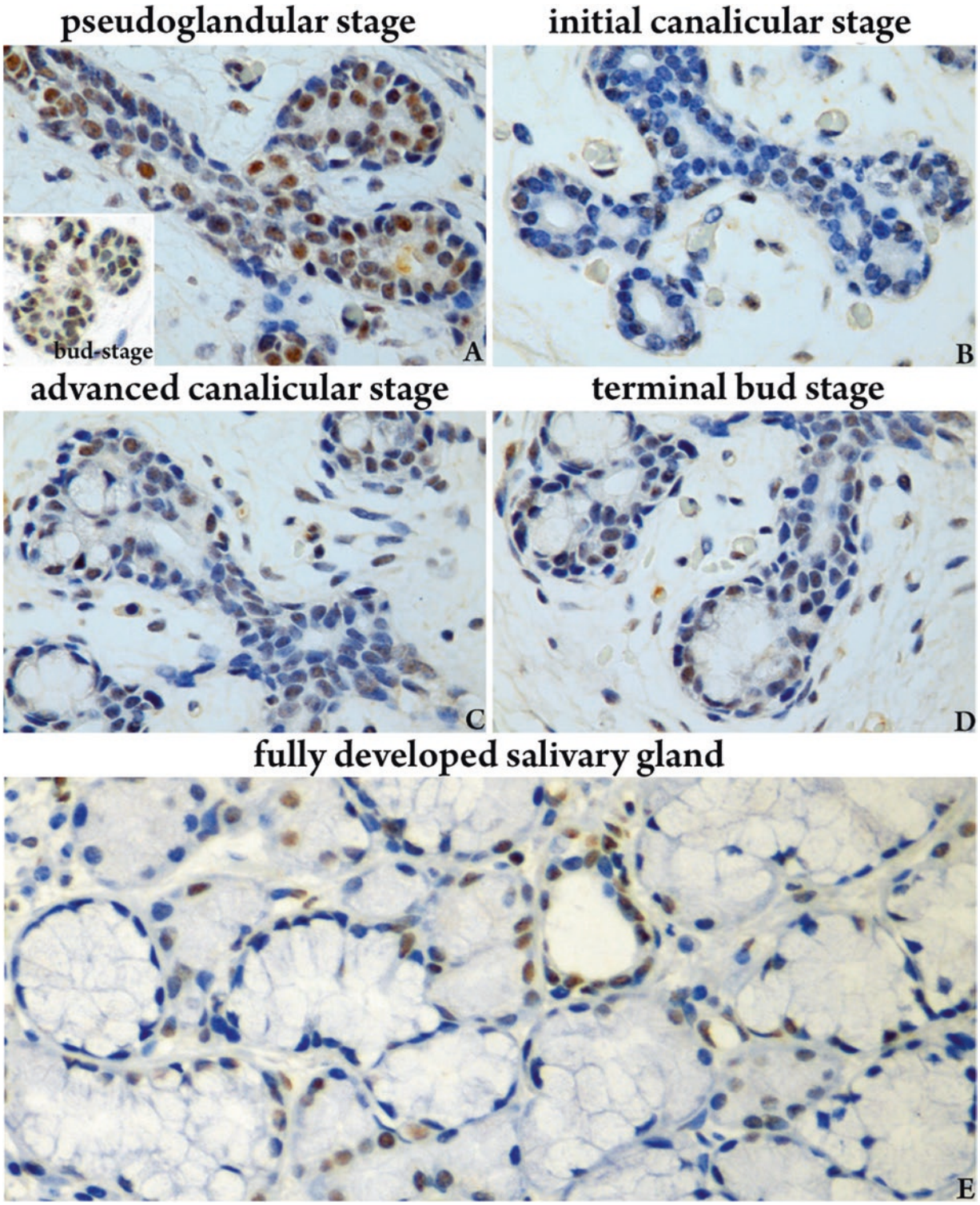

Fig. 4 Par-4 immunoexpression in developing and fully developed human salivary gland

include, most commonly, mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (ACC) [51–53]. Although there is growing knowledge on the molecular pathogenesis of salivary gland tumors [54, 55], in clinical practice the current treatment for salivary gland cancer is surgical resection and postoperative radiotherapy.

As discussed above, apoptosis plays an important role in the organogenesis of glandular structures, including salivary glands, and alterations of the apoptotic mechanisms have been described in different tumors, including SGTs [56–62].

Different authors have reported altered Par-4 expression in endometrial, breast, gastric, pancreatic cancer, and others. Par-4 down-regulation has also been associated with recurrence and poor survival rates in cancer patients [63].

Da Silva et al. [42] evaluated Par-4 expression in mucoepidermoid carcinoma and observed prominent cytoplasmic/nuclear expression in intermediate cells (nuclear staining), mucous (cytoplasmic staining), and epidermoid cells (cytoplasmic staining). In pleomorphic adenoma, Par-4 presented cytoplasmic/nuclear expression and was detected in epithelial/myoepithelial cells (unpublished results). In adenoid cystic carcinoma, Par-4 presented prominent nuclear expression in tubular structures whereas in cribriform structures focal expression was observed (unpublished results) (Fig. 5).

Considering the subcellular localization of Par-4 expression in mucoepidermoid carcinoma, Da Silva et al. [42] observed that cancer-specific survival rates of patients with nuclear or nuclear and cytoplasmic Par-4 expression were different although without statistical significance (survival probability in 5 years was 59.3% for patients with nuclear Par-4 expression and 40.0% for patients with nuclear and cytoplasmic Par-4 expression, as described in Fig. 6). This difference in survival probabilities might be due to the fact that, in tumor cells, induction of apoptosis is associated with Par-4 nuclear translocation.

Since the description of Par-4 down-regulation in different types of cancers, targeted therapies against Par-4 are increasingly considered as an important strategy for cancer treatment [63]. Recent studies reported a role of Par-4 restraining epithelial-mesenchymal transition (EMT) properties and unveil Par-4 as a key therapeutic target and the use of pharmacological modulator as potential tools to suppress EMT and associated chemoresistance, which could be exploited clinically for the treatment of aggressive cancers [64, 65]. The use of molecules that increase Par-4 expression could enhance the susceptibility of cancer cells to apoptosis [66]. Considering that studies revealed Par-4 protein secretion to the extracellular space [67], drug discovery efforts have focused on the development of small-molecule drugs that can facilitate Par-4 secretion [68].

4 Conclusions and Future Perspectives

The participation of apoptosis pathway in human salivary gland development and tumorigenesis has been described and Par-4 seems to stand as a key factor in this process [9, 13, 42].

During the salivary gland development, apoptosis is important for initial lumen formation whereas in the later phases, other cellular processes seem to be fundamental. In line with this, Par-4 expression is more prominent during the initial developmental phases. The understanding of the mechanisms involved in salivary gland development is important to comprehend tumorigenesis since both processes share similarities [47].

Regarding SGTs, the expression of Par-4 has been evaluated in the three most frequent types. However, SGTs comprise a heterogeneous group of lesions with distinct histological characteristics and diverse clinical behavior. Therefore, for better understanding the role of Par-4 during salivary gland tumorigenesis, other different histological subtypes should also be evaluated.

The main treatment for salivary gland neoplasms is surgery. Postoperative radiation therapy has been used to improve local disease control and to increase survival rates for patients with high-grade tumors, positive surgical margins, or perineural infiltration, and the role of chemotherapy in the management of these tumors is still controversial [69–72]. Considering the options of personalized therapy and target therapies, the identification of molecular alterations and the elucidation of their role in these tumors is fundamental, since it allows the design of more appropriate therapies for each patient [55, 73, 74].

The low incidence of SGTs impairs the development of a robust statistical analysis comprising larger cohorts. Da Silva et al. [42] observed a difference in the survival rates of mucoepidermoid carcinoma patients regarding Par-4 subcellular localization. However, due to the small number of samples, the result did not reach statistical significance. In order to overcome the difficulties in studying salivary gland tumors, in vivo and in vitro models are being developed and will allow a better understanding of the molecular pathways involved, drug tests, evaluation of the toxicity/safety of potential targets [75–77].

In summary, the expression of Par-4 has been described not only during the development of salivary gland tumors but also in salivary gland tumors. Par-4 seems to be a promising candidate to be employed as a tumor prognostic factor in surgical pathology evaluation of salivary gland tumors. It may also be further explored as a potential molecule target in cancer individualized therapy. Frontier laboratory models for testing these possibilities may aid to address these important possibilities.

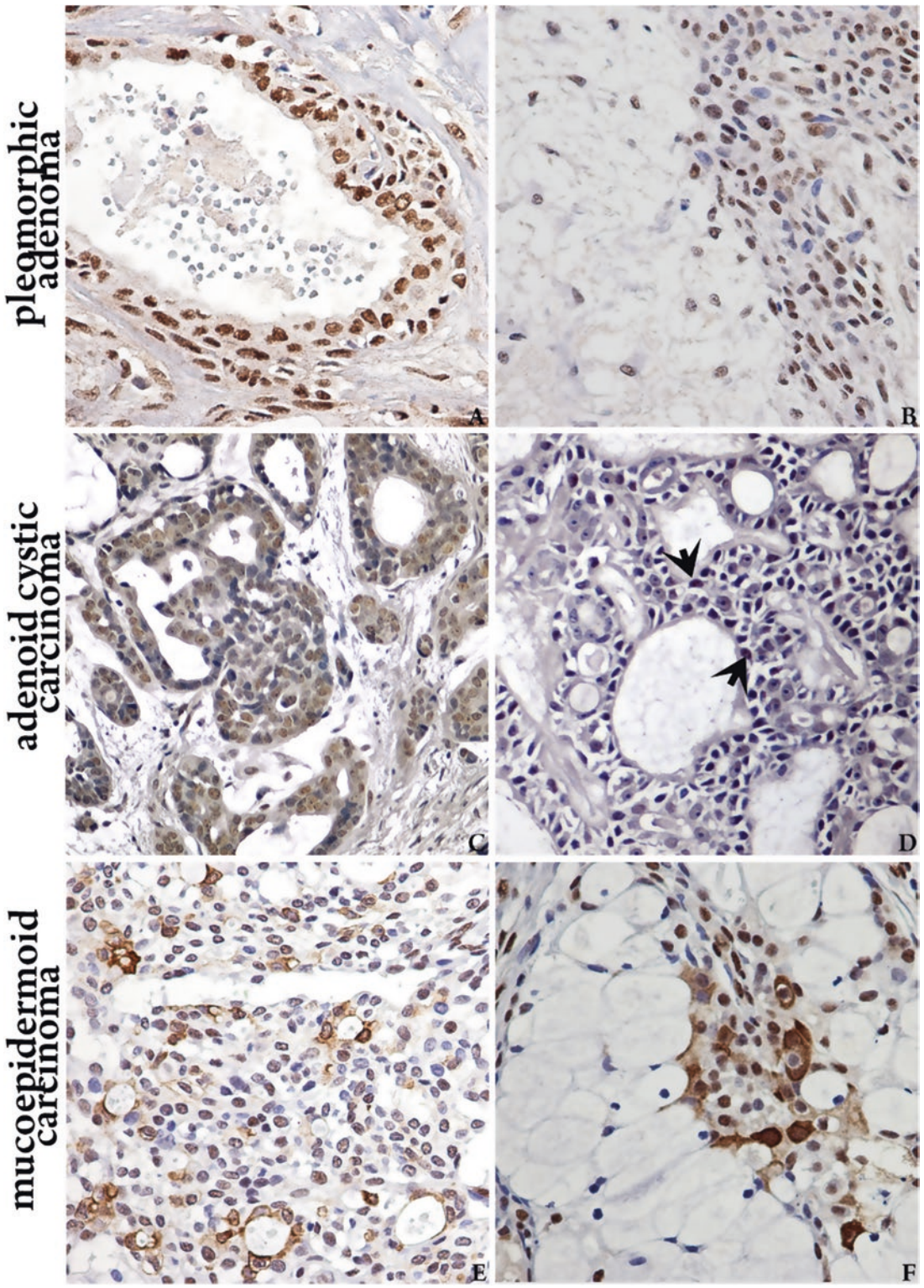

Fig. 5 Par-4 immunoexpression in salivary gland tumors. (**a**, **b**) Pleomorphic adenoma: numerous cells with Par-4-positive nuclei in distinct structures (original magnification x400); (**c**, **d**) adenoid cystic carcinoma: in (**c**) numerous positive cells in tubular structures; in D cribriform structures present only scattered cells positive for Par-4 (arrows) (original magnification X400); (**e**, **f**) mucoepidermoid carcinoma: Par-4 positivity concentrated in intermediate cells that compose the neoplasm (original magnification X400)

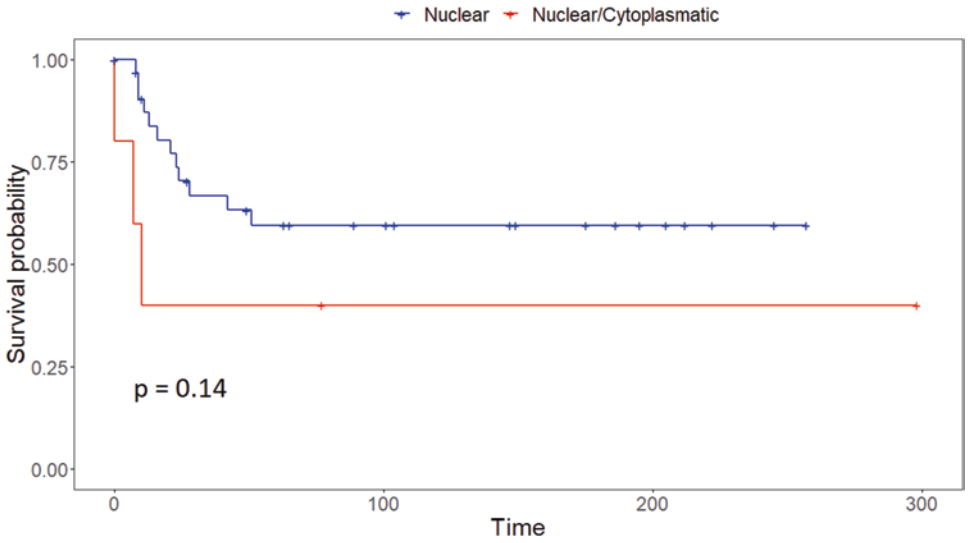

Fig. 6 Survival curves of patients with mucoepidermoid carcinoma considering nuclear or nuclear and cytoplasmic Par-4 expression

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Roles for Par-4 in Kidney Pathophysiology

Lu Ren and Shaolin Shi

Abstract

Kidney acts to filter blood to remove metabolic wastes, balance fluid and maintain electrolyte levels in the body. Kidney disease affects many people, e.g., chronic kidney disease has a prevalence of ~10% worldwide. The pathomechanisms underlying various kidney diseases have not been well characterized. Nevertheless, apoptosis is known to play a role in the development of kidney diseases, and many molecules and signaling pathways have been implicated in the apoptosis of kidney cells. This chapter describes a potential role for Par-4 in kidney disease development, focusing on its pro-apoptotic effect.

Keywords

Prostate apoptosis response-4 · Par-4 · Pawr · Kidney disease · Tubular cells · Glomerulus · Podocyte · Apoptosis · Injury · WT1 · Cancer · Therapeutic target

1 Kidney and Kidney Diseases

1.1 Kidney Anatomy and Physiology

Kidneys are paired and situated in the posterior part of the abdomen on each side of the vertebral column in the body. A kidney is composed of nephrons, renal interstitium, and microvasculature. Kidneys function to filter blood to remove wastes, balance electrolytes and fluid, and maintain blood pressure in the body. The nephron is the basic structural and functional unit of kidney, consisting of a glomerulus, a renal tubule, and a collecting duct (Fig. 1). One kidney of human contains roughly one million nephrons.

The glomerulus is composed of a capillary network lined by endothelial cells, a central region of mesangial cells with matrix material, podocytes covering the capillaries with glomerular basement membrane (GBM) in between, and the parietal cells in the Bowman's capsule (Fig. 1). The endothelial cells, GBM, and podocytes form a filtration barrier that prevents blood cells and macromolecular proteins, but allows small blood components (e.g., metabolic wastes), to pass through to get to the urinary space. The podocytes are terminally differentiated cells and are unable to proliferate normally. Podocytes are specialized to produce long primary processes that are further branched to form an extensive array of foot processes. The foot processes of neighboring podocytes interdigitate with each other and form the slit diaphragm between them. The slit diaphragm is the last barrier of glomerular filtration. Mesangial cells are equivalent to vascular smooth muscle cells and can undergo contraction and relaxation to regulate the glomerular hemodynamics and glomerular filtration rate. With extracellular matrix, the mesangium provides mechanical support for the glomerulus. The parietal epithelium forms the outer wall of Bowman's capsule and is capable of transdifferentiating into podocytes in disease [1].

The renal tubule is subdivided into several distinct segments: the proximal convoluted tubule (PCT), the loop of Henle, the distal convoluted tubule (DCT), and the collecting tubule. The PCT helps maintain the electrolyte and acid-base balance in the body and reabsorbs glucose, proteins, amino acids, and water in the filtrate from the glomerulus. It also secretes ions of hydrogen, ammonia, creatinine, and potassium into the filtrate. The Loop of Henle consists of a descending limb and ascending limb, and the former functions to concentrate the fluid of filtrate while the latter pumps sodium out of the fluid. The DCT secretes hydrogen, potas-

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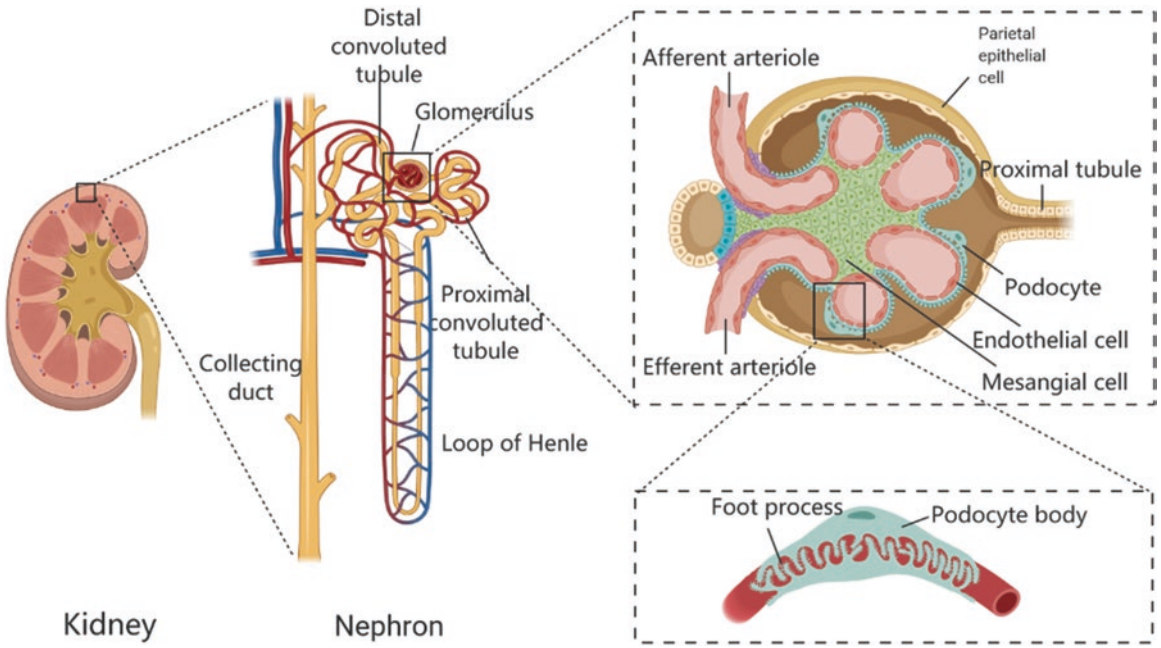

Fig. 1 The anatomy of kidney

sium, NH3, etc. into the fluid while reabsorbs HCO3-, sodium, and water, thereby maintaining the pH and sodium and potassium levels in the blood. The collecting tubule reabsorbs the water and transports the fluid to the collecting duct, where the water is further reabsorbed and the urine is finally formed.

The renal interstitium is a connective tissue between nephrons or collecting ducts. It is composed of fibroblasts, macrophages, and extracellular matrix material (e.g., proteoglycans). The matrix material is convenient for the diffusion and penetration of molecules. It also acts as support for renal structure.

1.2 Kidney Diseases and the Pathogenesis

Kidney diseases can be classified into different types according to different criteria. According to kidney structure and compartments, they can be divided into glomerular disease, renal tubular disease, renal interstitial disease, renal vascular disease, etc. By pathological causes, they are categorized into primary and secondary kidney diseases. The primary kidney diseases refer to the conditions that start with renal cells themselves; while the secondary kidney diseases are caused by other non-renal diseases, such as diabetes, obesity, and hypertension. Kidney diseases can also be divided into acute kidney injury (AKI) and chronic kidney disease (CKD) according to the disease progression.

AKI is a condition with a sudden decrease in kidney function. It is not a single disease entity but is designated for a group of conditions that share common diagnostic features, e.g., increases of the blood urea nitrogen (BUN) and serum creatinine (SCr), and a reduction in urine excretion. AKI severity ranges from asymptomatic and transient changes in renal function as determined by the estimated glomerular filtration rate (eGFR) to rapid and fatal derangements in circulating volume regulation and in electrolyte and acid-base composition in the plasma of patients.

CKD is defined as kidney damage with GFR below 60 ml/min per 1.73 m^2 for 3 months or more. Its prevalence is ~10% [2]. CKD can progress to an end stage, resulting in a condition called end-stage renal disease (ESRD) in which the kidney is no long able to function to meet the need of body and the patients require renal replacement therapies, e.g., kidney transplantation or blood purification by dialysis. The most common causes of CKD leading to ESRD are diabetes, hypertension, glomerulonephritis, and cystic kidney disease (https://health.usnews.com/conditions/kidney-disease/).

CKD is characterized by kidney fibrosis. In the presence of injurious stimuli, kidney responds by adaptive change that leads to either healing thus functional recovery or scarring with loss of kidney function [3]. Healing occurs primarily in AKI, in which injured cells recover and renal cells are replenished by cell proliferation or transdifferentiation, resulting in restoration of renal integrity and function. On the other hand, in most forms of CKD, kidneys take the fate to progressive scarring/fibrosis with loss of function. Inflammatory cells infiltration contributes greatly to the scarring process [4]. Scarring affects both the glomeruli (glomerulosclerosis) and tubule and interstitium (tubulointerstitial fibrosis).

Glomerulosclerosis can be triggered by inflammation or metabolic insults that first attack glomerular endothelium, leading to the accumulation of inflammatory factors. The inflammatory factors can stimulate the mesangial cells to undergo transformation, proliferation, ECM synthesis and deposition, and death. Due to the inability of proliferation, podocytes cope with their loss by stretching along the GBM. However, podocyte stretching may still be not adequate to cover the GBM fully, resulting in the denuded areas of GBM. The parietal epithelial cells can attach to the denuded spots of GBM, leading to the formation of capsular adhesions and subsequent glomerulosclerosis. In AKI, the tubular cells can undergo necrosis or apoptosis, followed by an attempt to regenerate the cells for repair [5]. An insult on kidney may induce epithelial mesenchymal transformation (EMT) of kidney cells, which facilitates fibrosis by producing and depositing more ECM [6, 7].

1.3 Genes and Signaling Pathways Commonly Involved in Various Kidney Diseases

As mentioned earlier, there are many kinds of kidney diseases and each has a distinct underlying pathomechanism, especially at the initial stage of disease development. Thereafter, these diseases undergo similar processes, including inflammation, cell death, and fibrosis, eventually leading to loss of kidney function.

Some growth factors and cytokines are known to promote renal cell death and fibrosis. TGF-β is considered one of the most important players in the disease development, which induces tubular epithelial to mesenchymal transition and cell injury that lead to tubule damage and fibrosis [6–9]. TGF-β has also been shown to induce podocyte apoptosis (a hallmark of various glomerular diseases) [10] and to stimulate mesangial cells to produce ECM in glomeruli [11]. Importantly, TGF-β expression can be upregulated and activated by oxidative stress and hyperglycemia, and signaling molecules, e.g., angiotensin II and complement molecules in kidney. TGF-β has also been proposed to play an important role in the transition from AKI to CKD [12].

Tumor necrosis factor (TNF) signaling has been implicated in renal necrosis and apoptosis in kidney diseases as shown by that the abrogation of TNFR2, a TNF receptor, alleviates experimental kidney injury [13]. TRAIL, a TNF ligand, can activate NF-kappaB and promote apoptosis in tubular cells and podocytes. Another ligand TWEAK induces inflammation and tubular cell death or proliferation. TWEAK is potent in promoting the production of inflammatory factors, including MCP-1, RANTES, CCl21, and CCL19 [14].

Although WNT-β-catenin signaling is required for kidney development and renal tissue regeneration after injury, uncontrolled overactivation of WNT signaling promotes renal cell injury and fibrosis [15]. Other factors, including Angiotensin II [16], CTGF, PDGF, and EGF, are also involved in kidney diseases [17].

Particularly, Par-4 has been shown to interact with many of these signaling pathways, e.g., TGF-β [18], Wnt [19], and TNF-alpha (TRAIL) [20], suggesting that Par-4 may be involved in kidney disease development by interacting with these molecules in similar manner in the non-renal cell types.

2 Par-4 Expression in Kidney

Par-4 was first identified in a screening for apoptosis-associated genes that are induced in response to apoptotic insults in prostate cancer cells [21]. The Par-4 protein comprises of 340 amino acid residues in human, which shares a high homology with its orthologs in mouse and rat [22]. Two nuclear localization sequences (NLS) are identified at the N-terminal region [23]. There is an SAC domain containing a threonine residue that can be phosphorylated by PKA [24]. The SAC domain confers the ability to selectively kill cancer but not normal cells [23]. At the C-terminal region is the leucine zipper domain that mediates protein–protein interactions to form Par-4 homodimer or its heterodimer with binding partners. Par-4 binding partners include WT1, DLK, FOXC2, and aPKC [25–28].

Par-4 transcripts are present in many organs, including kidney, according to the Human Protein Atlas (www.proteinatlas.org). Since kidney is made up of many cell types, it is important to localize Par-4 expression to distinct kidney cell types or compartments, and the single-cell RNA-seq (scRNA-seq) has made this possible.

Recently, scRNA-seq has been performed on human kidney [29] and the results are available in the KIT database (Kidney Interactive Transcriptomics; http://humphreyslab.com/SingleCell/). We found that PAR-4 transcript levels differ in distinct cell types with high levels in podocytes, mesangial cells, and the limb of Loop of Henle of tubules, respectively. In contrast, it is absent or at low levels in endothelial and other cell types (Fig. 2a).

Consistently, the Human Protein Atlas (www.proteinatlas.org) shows abundant Par-4 protein in kidney by immunohistochemical staining (Fig. 2b). In glomeruli, Par-4 exhibits staining pattern of podocyte and mesangial cells (Fig. 2c). The parietal epithelial cells are also stained positive (Fig. 2c). In the renal tubules, PAR-4 staining is intense in proximal convoluted tubule segments whose identity can be discerned by the presence of border brushes in the lumen side of the cells. Par-4 is stained in other tubules with reduced intensities (Fig. 2b). In addition to the cytoplasm, Par-4 protein is also localized in the nucleus of cells, particularly, the glomerular cells. This is consistent with previous studies that

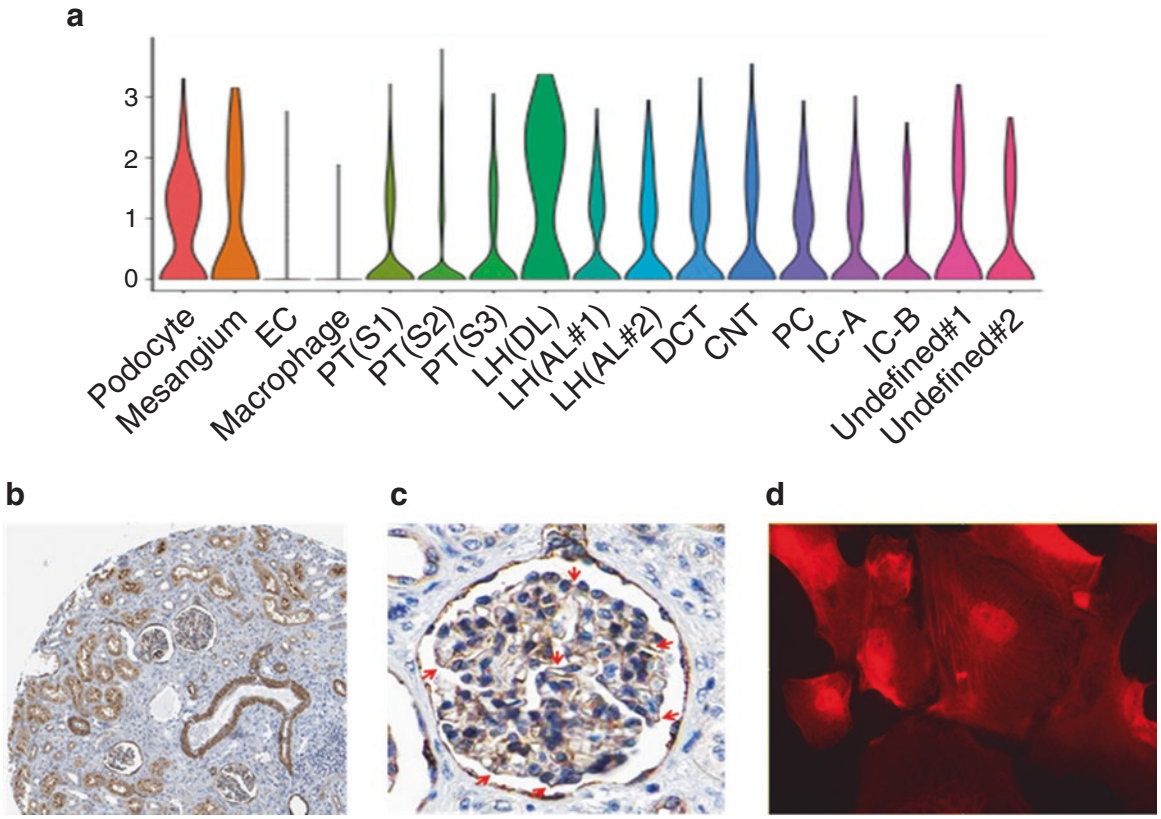

Fig. 2 Par-4 expression in kidney. (**a**) The single-cell RNA-seq of human kidney show heterogeneous expression of Par-4 across differenct renal cell type (KIT database). (**b**, **c**) Par-4 protein is detected by IHC in human kidney, the both tubules and glomeruli (Human Protein Atlas database). (**d**) Immunofluorescence staining of Par-4 in cultured podocytes, showing both cytoplasmic and nuclear localizations of Par-4

have shown the nuclear localization of Par-4 in cancer cells [23]. Interestingly, the nuclear localization of Par-4 is required for its pro-apoptotic activity [23]. We examined the subcellular localization of PAR-4 in cultured podocytes and found its presence in both cytoplasmic and nuclear compartments of the cells (Fig. 2d).

There have been very few reports concerning Par-4 expression regulation in kidney diseases; and, in fact, the Nephroseq, a kidney disease transcriptome database, shows no Par-4 transcript level changes in various kidney diseases essentially. It is not known whether Par-4 expression would be changed at the protein level by post-transcriptional regulation in the kidney diseases.

3 Involvement of Par-4 in Kidney Diseases

3.1 Apoptosis in Kidney

Massive apoptosis can be observed in kidney disease development, especially in AKI; and different renal compartments or cell types may exhibit differential apoptotic activity dependent on the nature of the diseases [30]. It is believed that apoptosis may be beneficial to kidney structure and function by removing injured and abnormal cells at the initial stage of disease, but aggravates renal deterioration later.

Apoptosis occurs massively in the tubules but not glomeruli in ureteral obstruction [31]. In 5/6 nephrectomy CKD model, tubular cells undergo apoptosis, resulting in tubular cell depletion, tubular atrophy, and fibrosis [32]. Apoptosis has also been documented in the diseased glomeruli [33, 34], for example, podocyte apoptosis has been reported in diabetic nephropathy, which results in podocyte depletion, glomerular filtration barrier disruption and proteinuria [35].

A large number of cellular factors have been identified to regulate the apoptosis. Depending on different stimuli, apoptosis occurs via extrinsic or intrinsic pathway. The extrinsic pathway involves binding of extracellular ligands, such as Fas/FasL and TNF ligands, to their corresponding receptors. The intrinsic pathway is activated by intracellular stimuli such as oxidative stress, endoplasmic reticulum (ER) stress,

hypoxia, and DNA damage. Both extrinsic and intrinsic pathways have been described in renal cell apoptosis [36].

In cancer studies, intracellular Par-4 has been shown to play a role in the trafficking of the Fas/FasL to the plasma membrane of prostate cancer cells, resulting in activation of Fas and FasL and induction of apoptosis [37]. Under an injurious stress, intracellular Par-4 binds to GRP78 to facilitate GRP78 translocation from the ER to cell surface, where it acts as a receptor for extracellular Par-4. The binding of Par-4 to cell surface GFR78 enables the adaptor protein FADD to recruit caspase-8 to the membrane, and the activated caspase-8 then triggers the basic apoptotic machinery involving caspase 3 and other downstream effector proteins [38]. It will be important to test whether this mechanism that involves Par-4 exists in renal cell apoptosis.

3.2 Actin Cytoskeletal Injury

A common pathological cause of proteinuric glomerular disease is the effacement of foot processes (FP) and the loss of slit diaphragm (SD) integrity of podocytes, which involve the rearrangement of the podocyte actin cytoskeleton. It is known that Myosin II motor protein is complexed with actin filaments, forming actomyosin that controls podocyte contractility and motility for normal structure and function of podocytes. Abnormal contractility and motility are involved in podocyte injury [39, 40].

Phosphorylation of myosin light chain (MLC) is the primary event in the regulation of actomyosin contractility, and the MLC phosphorylation level is determined by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) in many cell types [41]. In smooth muscle cells, Par-4 was found to regulate MLCP activity through a "padlock" model in which Par-4 binds to MYPT1 (the targeting subunit 1 of MLCP) to sequester the inhibitory phosphorylation sites of MYPT1, resulting in MLCP activation. Once Par-4 is phosphorylated, it becomes unlocked and displaced from the MLCP complex, resulting in exposure of MYPT1 to ZIPK for phosphorylation and thus inactivation of MLCP [42]. It is possible that Par-4 can act by similar mechanism in podocytes because we found that all the genes mentioned above are expressed in podocytes according to mouse podocyte RNA-seq data (GSE17142, GEO).

3.3 Par-4 Interacts with the Molecules Implicated in Kidney Pathophysiology

Although the exact physiological and pathological roles of Par-4 in kidney are largely unknown, they may be inferred based on the pathways and molecules that are present in renal cells and known to interact with Par-4. All of the interacting partners of Par-4 in kidney are associated with cell survival and apoptosis. We describe several of them that are expressed in podocytes as example.

3.3.1 Wilms' Tumor Suppressor 1 (WT1)

WT1 is a transcription factor and acts as the master regulator for the development and homeostasis of podocytes by promoting the expression of many genes essential for podocyte structure and function [43]. WT1 mutations are found in some patients of focal segmental glomerulosclerosis (FSGS) and the patients with Denys-Drash syndrome characteristic of infantile diffuse mesangial sclerosis, gonadal dysgenesis, and Wilms' tumor [44]. These studies support a crucial role for WT1 in podocytes. Indeed, mouse model of WT1 deficiency exhibits podocyte injury, proteinuria, and glomerulosclerosis [45]. It has been shown that Par-4 can bind to the zinc finger of WT1 via its C-terminal leucine zipper domain to inhibit WT1-mediated transcription activation or augment WT1-mediated transcriptional repression, thus acting as a transcriptional repressor [25]. Based on above studies, we speculate that Par-4, at least excessive amount of Par-4, would be harmful to podocytes by inhibiting the function of WT1.

3.3.2 BCL-2

BCL-2 is an important inhibitory factor for apoptosis and it suppresses apoptosis in a variety of cell types by preventing the release of cytochrome c from the mitochondria and other actions. BCL-2 also functions similarly in renal cells [46]. Par-4 has been shown to bind to the promoter of Bcl-2 gene and repress the transcription of Bcl-2 [47, 48]. Therefore, Par-4 may promote apoptosis in renal cells by inhibiting protective BCL-2 expression. This speculation needs to be tested for better understanding of the mechanism underlying podocyte apoptosis.

3.3.3 aPKC

The atypical protein kinase C (aPKC) members, aPKCλ/ι and aPKCζ, which localizes to the tight junctions and interacts with slit diaphragm proteins, play a crucial role in maintaining podocyte foot processes and glomerular filtration barrier as defective aPKC signaling (e.g., aPKC knockout in mice) results in disruption of glomerular architecture and causes severe proteinuria [49, 50]. The leucine zipper domain of Par-4 was found to bind to the zinc finger region of the aPKCs [28]. The interaction of Par-4 with aPKCs may be involved in podocyte injury, an issue deserving investigation.

3.4 Role of Par-4 in Kidney Diseases

At present, there have been very few studies investigating the role of Par-4 in kidney regardless of its expression in kidney. Par-4 protein was shown markedly reduced in human renal cell carcinoma specimens compared with normal tubular cells, and replenishment of Par-4 conferred sensitivity to apoptosis [51]. This finding also suggests that Par-4 might promote apoptosis in normal renal tubular cells.

Ischemia reperfusion-induced renal injury (IRI) is the most common cause of acute kidney injury (AKI) and it manifests with damage of renal tubular cell in addition to glomerular cells. Xie et al. showed that Par-4 was expressed in human renal proximal tubules and it was upregulated by three- to four-folds in the cortex tubular cells in the experimental model of renal IRI. In human kidney proximal tubule HK2 cell line and primary proximal tubule cells, Par-4 levels were elevated predominantly in response to chemical ischemia. Moreover, Par-4 was able to sensitize primary proximal cells and HK2 cells to apoptosis induced by chemical ischemia [52]. Conversely, prevention of Par-4 upregulation in the ischemia cell model alleviated apoptosis. These processes involve mitochondrial dysfunction and caspase activation, and Par-4's action is upstream of apoptotic cascade in IRI [52].

Oxidative stress is well known to play an important role in the development of various kidney diseases, including IRI, and can cause apoptosis. It was found that Par-4 inhibition prevented oxidative stress-induced apoptosis in renal tubular cells, partially via PI3K/AKT signaling that sustained expression of VEGF [53]. These studies have identified Par-4 as a novel and early mediator of renal tubule cell injury, which may be a potential therapeutic target for renal IRI and AKI.

At present, there has not been any study addressing the role for Par-4 in glomerular pathophysiology. This issue certainly deserves exploration given that Par-4 expression in glomerular podocytes and mesangial cells at high levels (Fig. 2) and that many molecules and signaling pathways involved in glomerular injury can interact with Par-4 functionally as described in many other cell types.

4 Future Directions

Although the role of Par-4 in tumor cells has been well investigated, its role in physiology and pathology of kidney is largely elusive. Considering that the kidney consists of several tens of cell types and subtypes and that Par-4 is expressed in many of them, Par-4 may have common and distinct functions in the distinct cell types. Elucidation of the roles of Par-4 in the different cell types under physiological and pathological conditions will provide better understanding of kidney pathophysiology. To achieve the goal, a large amount of work is required in the future.

The expression and regulation of Par-4 in kidney cell types in physiological and various pathological conditions require further detailing in both human and model organisms. Since the kidney is an organ with complex structure and function and consists of many cell types, single-cell analysis of Par-4 expression is particularly advantageous over the other conventional approaches. Several studies have provided single-cell RNA-seq data for both human and mouse kidney [29, 54–56], with which the researchers can determine Par-4 expression in distinct renal cell types under different conditions, particularly, of various kidney diseases. At present, however, the available scRNA-seq data of kidney have limited depth of sequencing and do not provide quality data of some types of cells because they tend to be injured and lost due to their vulnerability to the process of single-cell preparation. Therefore, the accuracy of Par-4 expression profile in kidney is an issue but will be improved with the technical advancement of single-cell RNA-seq in the future.

Animal models are essential for investigating the role and the underlying mechanism of Par-4 in kidney. The kidney of Par-4 knockout mice appears normal but this needs to be examined more carefully, e.g., prolonged observation with age, different genetic background, and molecular changes in kidney cell types deficient in Par-4. More importantly, the Par-4 knockout mice should be stressed by various stimuli and compared with wild-type control mice. Furthermore, kidney cell type-specific Par-4 knockouts should also be generated to determine its roles in distinct kidney cell types. At present, cell type-specific gene knockout has been achieved for podocytes, endothelial cells, proximal tubular and collecting duct cells in mouse, thanks to availability of the Cre transgenes expressing specifically in the cell types. It is expected that more kidney cell type-specific Cre transgenes will be available in the future.

Given that the roles of Par-4 in distinct kidney cell types can be revealed by various experimental approaches, the challenge followed is the elucidation of mechanisms underlying its roles. One convenient approach is to test the mechanisms that have been well established in other cell types, particularly cancer cells. Our preliminary studies have indeed shown certain mechanistic similarity between cancer and podocyte injury concerning Par-4. On the other hand, the behavior of a gene is often cell-type dependent, and thus Par-4 may function via some unique mechanisms in kidney cell types. To explore novel mechanisms in kidney cell types, various databases that have been continuously expanding can be helpful in finding the mechanistic clues. For example, gene expression profile of a cell type under both normal and disease conditions can be acquired and used to construct interaction network involving Par-4 using bioinformatics tools, followed by experimental validations.

Par-4 may be a potential target for kidney diseases due to its pro-apoptotic effect. There are several considerations concerning the use of Par-4 as therapeutic targets. First of all, it is necessary to screen for small molecules that can inhibit Par-4. Currently, there have been no studies reporting any small molecules capable of inhibiting the pro-apoptotic activity of Par-4. Finding a Par-4 inhibitor may not be interesting to cancer researchers and clinicians, but it could be important for kidney disease treatment. Discovery of Par-4 small molecule inhibitors and successful confirmation of their effectiveness and safety in treating kidney diseases will take long but it is anyway an essential direction to explore. Alternatively, small molecule inhibitors that block the upstream regulators of Par-4 expression can be considered. This approach requires detailed mechanistic understanding of Par-4 expression at levels of both transcription and post-transcription. Of course, blocking the pathway downstream Par-4 that leads to apoptosis of kidney cells can be an additional choice. With the accumulating data from Par-4 molecular and cellular studies, these approaches would become possible.

Finally, Par-4 is a promising target for cancer treatment; however, a cancer regimen that activates Par-4 might have adverse effect on kidney because of the pro-apoptotic effect of Par-4 in kidney cells. This concern is particularly important for patients with unaware kidney disease. The prevalence of chronic kidney diseases is ~10% of the population and most of them are unaware of the diseases. This situation holds true for cancer patients.

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Lessons from Mouse Models

Nathalia Araujo, Nikhil Hebbar, and Vivek M. Rangnekar

Abstract

Prostate apoptosis response-4 (Par-4) is a tumor suppressor protein known to be mutated in endometrial cancer, inactivated in prostate cancer, and downregulated in several other cancers such as renal cell carcinoma, neuroblastoma, and pancreatic cancer. Par-4 downregulation is also associated with poor prognosis and recurrence in breast cancer. Expressed in all tissues and present in multiple cellular compartments, Par-4 protein shares homology of its key domains across human, mouse, and rat species. The most well-defined domains of Par-4 consist of two nuclear localization sequences in the N-terminal region (NLS1 and NLS2), a leucine zipper (LZ) domain at the C-terminus, and the effector SAC domain (Selective for Apoptosis in Cancer) naturally placed in the center of the molecule. Additionally, Par-4 and its effector domain can be secreted and found in the plasma of mice, normal individuals, and cancer patients. To better understand the function of Par-4 as a tumor suppressor, several groups have generated mouse models and studied the implications of deletion or overexpression of Par-4 in vivo. Par-4 loss in mice results in increased susceptibility to spontaneous tumors, as well as inducible tumors in various tissues. On the other hand, overexpression of Par-4 leads to longer cancer-free lifespan. Par-4 is also relevant outside the context of cancer as it promotes differentiation of T-cells toward the Th2 lineage in detriment of Th1, and Par-4 interacts with the dopamine receptor to ensure proper dopamine signaling. This chapter will discuss the most relevant studies utilizing mouse models that provide deeper insights into Par-4 function.

Keywords

Prostate apoptosis response-4 (Par-4) · Knockout mice · Tumor growth · Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) · Apoptosis · Selective for apoptosis in cancer cells (SAC) · Leucine zipper domain · Depression · Atypical protein kinase Cζ (ζPKC) · c-Myc · Ras · Inflammation · PKA · Chloroquine · Radiation

1 Par-4 Deletion in 129Sv Background

Multiple research groups have utilized mouse models to study the function of Par-4 in vivo. In 2003, preceding the in vivo studies, mouse embryonic fibroblasts (MEFs) lacking Par-4 were generated via disruption of the Par-4 gene by homologous recombination. A linearized construct eliminating exons 1 and 2 from the Par-4 sequence was electroporated into embryonic stem (ES) cells in the 129Sv background. A chimeric male was produced from one ES clone. The chimeric male was then crossed with CD1 females to generate F1 Par-$4^{+/-}$ mice. Embryonic fibroblasts were isolated at 13.5 days from the mouse embryos resulting from crosses between Par-$4^{+/-}$ mice (F1 intercrosses) to generate Par-$4^{-/-}$ MEFs [1]. These Par-$4^{-/-}$ MEFs were used to elucidate several signaling pathways. Western blotting for phospho-Thr410 of active atypical protein kinase C (aPKC) demonstrated that

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basal activity of aPKC was increased in Par-4$^{-/-}$ MEFs compared to wild-type controls. Further increase in aPKC activity was obtained in Par-4$^{-/-}$ MEFs relative to Par-4$^{+/+}$ MEFs when tumor necrosis factor alpha (TNF-α) was added to the medium, revealing Par-4 as a negative regulator of aPKC. Additionally, when either Par-4$^{-/-}$ or Par-4$^{+/+}$ or wild-type (WT) MEFs were incubated with TNF-α in the presence of cycloheximide, and apoptosis was measured using TUNEL (TdT-mediated dUTP nick end-labeling) analysis, Par-4$^{-/-}$ MEFs showed significantly reduced apoptosis when compared to WT MEFs. The absence of Par-4 also enhanced NF-κB activation. When Par-4$^{-/-}$ and WT MEFs were transfected with an NF-κB luciferase reporter and stimulated with TNF-α or interleukin-1, NF-κB transcription was 2–3 times higher in the Par-4$^{-/-}$ MEFs when compared to WT MEFs. Re-expression of Par-4 in the Par-4$^{-/-}$ MEFs abolished the increase in NF-κB activity, demonstrating that Par-4 was as negative regulator of NF-κB activity [1]. Downstream of TNF-α, Par-4 affected both c-Jun amino-terminal kinase (JNK) and p38 kinase pathways. Inhibition of sustained activation of c-Jun and p38 was obvious in Par-4$^{-/-}$ MEFs when compared to WT MEFs when cells were treated with TNF-α. However, the absence of Par-4 did not affect early TNF-α stimulation of either JNK or p38 kinase. Because X-chromosome-linked inhibitor of apoptosis (XIAP) is decreased when NF-κB is not active, and XIAP can alter sustained JNK activation, the authors showed that TNF-α-induced XIAP was much more robust in Par-4$^{-/-}$ MEFs, explaining the transient activation of JNK when Par-4 was deleted. Moreover, basal level expression of XIAP was higher in Par-4$^{-/-}$ MEFs relative to WT MEFs [1].

Having demonstrated that Par-4 is a negative regulator of aPKC, and in view of the fact that ζPKC (an isoform of aPKC) promotes immune system alterations, a follow-up study characterized the immunological phenotype of Par-4 knockout mice [2]. Par-4$^{-/-}$ mice were born at the expected Mendelian ratio. Although moderate splenomegaly was observed in Par-4 knockout mice, neither the overall cell number nor differentiation of T- and B-cell populations and subpopulations were affected. When B cells were activated through the B-cell receptor (BCR), however, the proliferative response was significantly increased in the population where Par-4 was absent. Enhanced proliferation was not observed in Par-4 knockout cells when B cells were activated by LPS or CD40, which was consistent with the role of ζPKC in BCR signaling. Proliferation of peripheral T-cells was increased in Par-4$^{-/-}$ cells after stimulation with anti-CD3 monoclonal antibody, especially in the absence of the co-stimulant CD28. This was rather unexpected and suggested that Par-4 interfered with T-cell proliferation independently of ζPKC since ζPKC is not known to play a role in T-cell receptor (TCR) signaling. Enhanced proliferation of Par-4$^{-/-}$ cells was observed in both CD4^{+} and CD8^{+} populations, and this was not a result of differences in the percentage of memory/effector or regulatory T-cells. Production of interleukin-2 (IL-2), a marker of T-cell activation, was also augmented by Par-4 loss; however, expression of CD25 (IL-2Rα) was comparable in WT and Par-4$^{-/-}$ cells upon anti-CD3 stimulation, with or without anti-CD28. Moreover, there was no difference in proliferation between WT and Par-4$^{-/-}$ T-cells when they were stimulated with IL-2.

The effect of Par-4 loss in T-cell apoptosis was also assessed. Following a 24h stimulation by anti-CD3, Par-4$^{-/-}$ T-cells showed reduced Annexin V staining compared to WT T-cells. These results were further corroborated by decreased sensitivity of Par-4$^{-/-}$ T-cells to apoptosis when a protocol to recapitulate the activation-induced cell death (AICD) phenomenon was used. The study by Lafuente et al. also investigated the effect of Par-4 deficiency in the T-cell signaling cascade. CD3-stimulated Par-4$^{-/-}$ T-cells displayed enhanced activation of aPKC as demonstrated by two-fold increase in phosphorylation of the kinase T-loop site when compared to aPKC phosphorylation in WT T-cells. Total amount of aPKC did not differ between WT and Par-4-null T-cells and co-stimulation with anti-CD28 did not further augment phosphorylation. Par-4 loss also significantly increased the amount of NF-κB in the nucleus after anti-CD3 stimulation with and without anti-CD28 co-stimulation. Because ζPKC does not affect T-cell proliferation [3] and because both ζPKC and λ/ιPKC can activate NF-κB [4], these data suggested that Par-4 function in T-cells is mediated by λ/ιPKC [2].

Similar to the effect of Par-4 loss in MEFs [1], JNK, but not p38, activation was dramatically decreased in the Par-4$^{-/-}$ T-cells even though the total amount of c-Jun protein was indistinguishable between Par-4$^{-/-}$ cells and WT T-cells. Moreover, stimulation of T-cells that lacked Par-4 promoted robust activation of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), corroborating previous findings [5] where JNK activation inhibited NFATc1 phosphorylation. Since NFAT plays an important role in IL-2 synthesis, these results implied that Par-4 loss lead to JNK inhibition. JNK inhibition, therefore, promoted NFATc1 activation and increased IL-2 production, leading to enhanced T-cell proliferation. In WT cells, stimulation with anti-CD3 (or anti-CD3 with anti-CD28) resulted in increased Par-4 levels, suggesting that Par-4 accumulation in T-cells limited the activation of aPKC. These kinases were relatively more active in Par-4$^{-/-}$ T-cells [2]. Also noteworthy was the effect of Par-4 loss on JNK signaling and cytokine production: JNK2 was necessary for synthesis of interferon-gamma (IFNγ), a Th1 cytokine; and JNK1, through NFATc1 inhibition, repressed synthesis of IL-4, a Th2 cytokine. When Par-4$^{-/-}$ T-cells were stimulated with anti-CD3 (with or without anti-CD28), although IFNγ production was comparable to that of WT T-cells, there was a significant increase in the production of

IL-4 in Par-4$^{-/-}$ CD4^{+} T-cells. In summary, Par-4 deletion led to hyperactivation of aPKCs that led to inhibition of JNK, enhanced IL-4 production, and Th2 differentiation [2].

Next, Jorge Moscat and colleagues investigated the tumorigenesis potential of Par-4$^{+/+}$, Par-4$^{+/-}$, and Par-4$^{-/-}$ mice in the CD1/129Sv 3:1 background [6]. The average lifespan of Par-4$^{-/-}$ (19 months) mice was decreased when compared to that of either Par-4$^{+/-}$ or Par-4$^{+/+}$ mice, which had an average lifespan of 24 or 25 months, respectively. Histopathological analyses were performed after euthanasia of mice that were moribund and typical age-related alterations (i.e., glomerulonephritis and cysts) were observed with comparable frequencies in all three genotypes. The incidence of tumors, however, was remarkably higher in the Par-4$^{-/-}$ mice (87%) when compared to Par-4$^{+/+}$ mice (55%); Par-4$^{+/-}$ mice displayed intermediate tumor incidence (71%). Moreover, 43% of Par-4-null mice and 33% of Par-4 heterozygous mice carried more than one tumor at the time of the death (versus 5% of Par-4 WT mice). It was noted that 57% of the tumors in the Par-4$^{-/-}$ mice were carcinomas, versus 27% and 15% of the tumors in Par-4$^{+/-}$ and Par-4$^{+/+}$ mice, respectively. The tissues most affected by tumor development were bladder, uterus, and prostate [6]. Of note, maximal Par-4 mRNA levels are observed in the urinary bladder of WT mice, and this is relevant because Par-4$^{-/-}$ mice developed spontaneous tumors of the bladder, a tumor type that very rarely affects aged WT mice. When Par-4$^{-/-}$ mice and their littermate controls Par-4$^{+/+}$ mice were treated with the bladder carcinogen BBN ((N-butyl-N-(4-hydroxybutyl) nitrosamine), WT mice survived up to 55 weeks after initiation of the treatment, whereas Par-4$^{-/-}$ mice perished before 40 weeks. These results further corroborated the susceptibility of Par-4 knockout mice to urinary tumors and strengthened the relevance of Par-4 in suppressing tumors [6].

The most prominent carcinoma in Par-4$^{-/-}$ mice was endometrial adenocarcinoma, which affected 36% of the female mice. At 3 months, there were no noticeable lesions in the uteri of Par-4-null or Par-4 WT female mice. At 9 months, 80% of Par-4$^{-/-}$ female mice displayed endometrial hyperplasia. Mechanistically, XIAP levels were significantly elevated in the uteri of Par-4$^{-/-}$ female mice when compared with Par-4$^{+/+}$ littermates. These observations corroborated the function of Par-4 as an inhibitor of ζPKC–NF-κB–XIAP pathway described by Garcia-Cao et al. [1], explaining the endometrial cancer susceptibility in Par-4 knockout female mice. Moreover, Par-4 protein was significantly reduced in the uterus of WT female mice following treatment with estradiol, a strong inducer of endometrial cell proliferation, further suggesting that Par-4 is a negative regulator of proliferation [6]. The prostate was also markedly affected by Par-4 deletion. Compared to 8% of WT and 15% of heterozygous males, 77% of Par-4 knockout males presented lesions in the prostate. Carcinogenic treatment with testosterone and estradiol further corroborated the increased sensitivity of Par-4$^{-/-}$ to the development of prostatic lesions as all Par-4-null mice displayed prostate hyperplasia, and no lesions were observed in the prostate of WT mice. Analogous to what was observed in the endometrium, XIAP levels were elevated in the prostate of Par-4$^{-/-}$ mice. In summary, loss of Par-4 predisposed both males and females to spontaneous and inducible tumors of the prostate and the endometrium, which could be explained by upregulation of the ζPKC–NF-κB–XIAP pathway [6].

The study by Joshi et al. in 2008 expanded the role of Par-4 as a suppressor of tumor growth to other tissues in addition to the bladder, endometrium, and prostate [7]. The authors reasoned that Par-4 would most likely have an important tumor suppressor function in tissues that expressed the highest levels of Par-4. The levels of Par-4 protein in the lungs were comparable to those of the prostate, as determined by immunoblot analysis. Moreover, Par-4 mRNA levels were the highest in the prostate, and also high in the liver, lungs, and kidneys. Immunocytochemistry (IHC) of mouse lungs indicated that Par-4 was mostly expressed in epithelial cells of the airways and in the alveoli. A similar trend was observed in human lung tissue. In fact, Par-4 was absent in 47% of tissue microarrays (TMAs) of human non-small cell lung carcinoma (NSCLC). Lack of Par-4 also correlated with the type of lung tumor: 41% of adenocarcinomas were negative for Par-4, whereas only 6% of squamous cells did not show Par-4 expression. Because adenocarcinomas highly correlate with oncogenic Ras, this study tested whether oncogenic Ras in Par-4$^{-/-}$ mice would increase tumor burden. Par-4$^{-/-}$ mice were crossed with WT mice-expressing oncogenic Ras in the lungs. Not only did the tumor burden jump from 18% in Ras-expressing WT lungs to 75% in Ras-expressing Par-4-null lungs, but also tumor progression was accelerated in Par-4-null/Ras adenocarcinomas. Additionally, the lifespan markedly decreased in the Par-4 knockout-expressing oncogenic Ras, suggesting that Par-4 is a negative regulator of lung tumorigenesis [7].

As demonstrated for the endometrium and prostate, XIAP was elevated in the lungs of Par-4 knockout mice when compared to WT mice. Moreover, phospho-ζPKC was increased in the alveoli and in the airways of Par-4-null mice, and enhanced ζPKC activity was noted in Par-4$^{-/-}$ whole-lung extracts. Nuclear levels of p65/RelA were also increased in the lungs of Par-4$^{-/-}$ mice, and this was consistent with the fact that ζPKC is required for nuclear translocation of NF-κB in the lungs. In addition to enhanced XIAP and NF-κB cell survival and proliferation signaling, Par-4 loss was correlated with increased protein kinase B activation (Akt, phosphorylated at serine 473) in lung extracts. p-Akt-Ser473 was elevated in Ras-expressing Par-4$^{-/-}$ tumors although nuclear RelA was not detected in either Par-4$^{-/-}$ or WT Ras-expressing tumors. These data suggested that elevated

tumorigenesis in Par-4-null/Ras-expressing tumors correlated with enhanced Akt activity, but not with NF-κB activation [7]. Par-4$^{-/-}$ MEFs showed increased Akt activation when compared to WT MEFs; however, re-expression of Par-4 in Par-4$^{-/-}$ MEFs drastically impaired Akt activation. Negative regulation of Akt by Par-4 translated from mouse lungs and MEFs to human cancer cells as well. When A549 and HEK293 cells were treated with Par-4 small interference RNA (siRNA), p-Akt-Ser473 levels were significantly increased. Interestingly, Akt activation by phosphorylation at threonine 308 was also augmented in the context of Par-4 loss. In Par-4$^{-/-}$ cells, the phosphorylation of both activation sites of Akt was independent of PI 3-kinase levels, as PIP3 levels were reduced in Par-4 knockout cells [7]. When HEK293 cells were transfected with ectopic Par-4, Akt phosphorylation at Ser473 was decreased; however, co-transfection of Par-4 and ζPKC impaired the inhibitory effect of Par-4 on Akt activation. These observations suggested the existence of a Par-4/ζPKC/Akt signaling cascade. A kinase assay using [γ-^{32}P] ATP, in which recombinant ζPKC was incubated with purified His-Akt, demonstrated that ζPKC was able to induce Akt phosphorylation at both Ser473 and Thr308. This effect was not due to Akt autophosphorylation. Interestingly, phosphopeptide analyses of Akt phosphorylation by ζPKC uncovered another Akt phosphorylation site at the Ser124 residue. Akt-Ser124 was in fact the most abundantly detected site for phosphorylation upon ζPKC treatment and was observed even without ζPKC, whereas Akt-Ser473 and Akt-Thr308 were only detected after incubation with ζPKC. By phosphorylating Ser124 in vivo, ζPKC impacted total Ser473 phosphorylation through the TORC2 complex. These findings were corroborated by severe impairment in Akt-Ser473 and Akt-Thr308 phosphorylation in ζPKC-null MEFs when compared to WT ζPKC MEFs. Moreover, in lung extracts from Par-4/ζPKC double-knockout mice, Akt phosphorylation was abrogated at both residues. In summary, the Par-4/ζPKC interaction was a relevant regulator of Akt activation, likely due to ζPKC's ability to phosphorylate Akt-Ser124, which promoted the phosphorylation of the key sites Ser473 and Thr308 [7].

In 2009, more insights were gained on the relevance of Par-4 in tumor development. Following the studies describing benign lesions of the prostate in Par-4$^{-/-}$ mice [6] and the importance of Par-4 regulation of Akt through ζPKC in Ras lung tumors [7], Fernandez-Marcos et al. investigated the contribution of ζPKC for the development of tumors in Par-4-null mice using the ζPKC/Par-4 double-knockout mouse model [8]. The prostate phenotype of Par-4$^{-/-}$ mice was reversed in ζPKC/Par-4 double-knockout mice, and nuclear p-Akt levels were increased in the prostate of Par-4$^{-/-}$ mice, but not in WT or ζPKC/Par-4 double-knockout mice. Additionally, NF-κB activation (marked by p65/RelA nuclear localization) was enhanced in the Par-4$^{-/-}$ mouse prostate, but not ζPKC$^{-/-}$/Par-4$^{-/-}$ double-knockout mouse prostate. The authors evaluated human prostate carcinomas to establish the relevance of Par-4 as a tumor suppressor and noted that 41% of the tumors were Par-4-positive and 59% were Par-4-negative or Par-4-low. Low Par-4 levels were associated with Par-4 promoter methylation. When PTEN expression was assessed in these prostate carcinomas, a significant correlation emerged: PTEN negative/low tumors were also negative/low for Par-4, whereas PTEN-positive tumors were Par-4 positive. Moreover, similar to what was observed with PTEN loss, Par-4 deficiency was associated with higher Gleason scores [8]. To address the implications of PTEN deficiency for the development of prostate neoplasia in Par-4-null mice, Par-4$^{-/-}$ mice were crossed with PTEN$^{+/-}$ mice. Assessment of multicentric proliferative disease to determine overall survival of generated cohorts demonstrated that neither Par-4$^{-/-}$/PTEN$^{+/-}$ nor Par-4$^{+/-}$/PTEN$^{+/-}$ cohorts were affected suggesting that Par-4 dosage was not relevant for PTEN-driven lymphoproliferative disease. Par-4 gene dosage, however, was important for cancer initiation and increased incidence of PIN in PTEN$^{+/-}$ background. Percentage of PIN I (low-grade prostatic intra neoplasia) was two-fold higher in Par-4$^{+/-}$/PTEN$^{+/-}$ mouse prostates, whereas Par-4$^{-/-}$/PTEN$^{+/-}$ mouse prostates displayed four times more PIN when compared to WT Par-4 PTEN$^{+/-}$ mouse prostates. Progression of PIN was also impacted by the Par-4 status. While PTEN heterozygous prostates did not show any high-grade PIN (i.e., PIN III and PIN IV), Par-4$^{+/-}$/PTEN$^{+/-}$ mice developed high-grade PIN with 100% penetrance that correlated with enhanced proliferation assessed by Ki67 staining. Moreover, apoptosis was reduced with Par-4 loss, demonstrating that cooperation between Par-4 and PTEN deficiency enhanced both proliferation and survival of neoplastic prostate cells. On the same note, PIN progressed to micro-invasive carcinoma in Par-4$^{+/-}$/PTEN$^{+/-}$ mice, whereas Par-4$^{-/-}$/PTEN$^{+/-}$ mice displayed highly penetrant and fully invasive carcinomas at 6 months of age. The severity of the lesions observed in the mouse prostate recapitulated the aggressive characteristics of human prostate cancer. Together, these findings illustrated the relevance of Par-4 loss in the context of PTEN deficiency and demonstrated the translational importance of the Par-4/PTEN model in recapitulating human prostate cancer. Intriguingly, nuclear p-Akt in Par-4$^{-/-}$/PTEN$^{+/-}$ mouse prostates was not dramatically increased when compared to Par-4$^{-/-}$ or PTEN$^{+/-}$ mouse prostates suggesting that the p-Akt status was not adequate to explain the striking effect on tumor onset and progression observed in Par-4$^{-/-}$/PTEN$^{+/-}$ mice. A synergistic effect of the two mutations, however, was observed in the activation of NF-κB. While only moderate p65 nuclear translocation was observed in Par-4$^{-/-}$ or PTEN$^{+/-}$ mouse prostates, dramatic activation of NF-κB was observed in the prostates of double-knockout mice.

Corroborating this finding, transcripts of NF-κB target genes IL-6 and TNF-α were also synergistically elevated in the prostates of Par-4$^{-/-}$/PTEN$^{+/-}$ mice. Furthermore, activation of the NF-κB pathway was demonstrated in the human prostate cancers where both Par-4 and PTEN were inactivated. This strengthened the importance of NF-κB activation in the context of Par-4 and PTEN-deficient prostate cancers. Fernandez-Marcos et al. also demonstrated that the synergistic effect of Par-4 and PTEN could be extrapolated to other cell lines. When Par-4$^{-/-}$ MEFs were knockdown for PTEN using a lentiviral shRNA (PTENi), cell proliferation was 2.5 times higher when compared to proliferation of Par-4$^{-/-}$ or WT/PTENi MEFs. Additionally, Par-4$^{-/-}$/PTENi MEFs formed colonies in soft agar, whereas PTENi alone did not. As observed in the double-knockout prostates, double-knockout MEFs showed only additive increase in p-Akt staining when compared to Par-4 or PTEN single knockout MEFs, whereas NF-κB activation displayed synergistic increase in double-knockout MEFs. Modest Akt activation paired with robust p65 nuclear translocation was also observed in PTEN-null CaP2 cells when Par-4 was knocked down using a lentiviral shRNA (Par-4i). As expected, IL-6 mRNA levels were elevated in the CaP2-Par-4i cells. The increase in IL-6 expression correlated with enhanced Stat3 phosphorylation that suggested IL-6 was active in these samples. Altogether, these results indicated that there was cooperation between Par-4 and PTEN in a cell autonomous manner and that Akt and NF-κB activation were important for their function. Knockdown of Akt or IKKβ using siRNAs in CaP2-Par-4i cells blocked enhanced proliferation observed with Par-4 knockdown alone, further corroborating the value of Akt and NF-κB pathways for the phenotype of Par-4$^{-/-}$/PTEN$^{+/-}$ mouse prostates [8].

2 Deletion of C-Terminal Region of Par-4

A different model of Par-4 deletion was studied by Affar et al. to understand the role of the C-terminus portion of the Par-4 protein [9]. Rat Par-4 cDNA was used to screen a DNA library from 129Sv mouse. Clones containing exons corresponding to the C-terminus regions were isolated and mapped using restriction digestion. A targeting vector was constructed where LoxP sites flanked the exons 4 and 5 of the *par-4* gene. The conditional knockout mouse was then crossed with the EIIA strain, which expressed Cre recombinase in both somatic and germinal cells, to generate a mutant mouse with whole-body deletion of the Par-4 carboxyl-terminal, i.e., lacking the leucine zipper domain. Par-4 mutant mice were normal compared to wild-type littermates: no differences were observed in body weight or overall appearance, animals were fertile and born at the expected Mendelian ratio, and no morphologic abnormalities were observed upon histological analysis. In addition, overall survival and spontaneous tumorigenesis were comparable to control mice. These findings suggested that the C-terminal domain of Par-4 was not involved in embryonic development. When MEFs isolated from mutant and WT mice were treated with agents known to induce cell death via the mitochondria pathway and cell viability was measured by the MTT assay, no significant differences were observed between WT and Par-4 mutant MEFs upon treatment with calcium ionophores. Similarly, sensitivity of Par-4 mutant MEFs was comparable to wild-type MEFs when cells were treated with DNA-damaging agents, etoposide and adriamycin, or the PKC inhibitor staurosporine. More specifically, no distinction in apoptotic cell death was observed between mutant and WT MEFs when cells were treated with UVC or staurosporine and subjected to fluorescence-activated cell sorting analysis of the sub-G0 population. These findings were further corroborated by analyzing the cleavage products of Lamin A/C (marker of caspase activation), where both WT and mutant MEFs displayed similar extent of Lamin A/C in response to etoposide and UVC treatment [9]. Sensitivity to receptor-mediated apoptosis was similar in Par-4 mutant MEFs and WT MEFs. The extent of cell death in WT and mutant cells was comparable using trypan blue exclusion assay to determine cell viability upon treatment with either Fas ligand or TNF-α in the presence of cycloheximide. Additionally, no differences were observed in caspase activation after Fas or TNF treatments. These observations suggested that deletion of the C-terminus of the Par-4 protein was not sufficient to abrogate its apoptotic function. In hindsight, these findings were obvious in view of the fact that the mutants retained the SAC core domain for apoptosis [10]. When RNAi was used to knockdown endogenous levels of Par-4 mutant MEFs, and viability was measured in response to various cell death-inducing agents, viability was comparable in both control- and Par-4 RNAi-transfected cells. However, Par-4 depletion in HeLa cells using RNAi led to resistance to most apoptosis-inducing agents when compared to HeLa cells that received control RNAi. These results suggested that full-length Par-4 or its SAC domain but not the LZ domain were essential for sensitivity to apoptosis-inducing agents [9].

Deletion of the LZ domain of Par-4 in the knockout mice shed light on a novel role of Par-4 in the context of dopamine signaling and depression [11]. To better understand the intracellular signaling of Dopamine D2 receptor (D2DR), the predominant D2-like receptor subtype, Park et al. [11] searched for proteins that interacted with D2DR. Using a yeast two-hybrid screen containing the intracellular loop of the human D2DR as bait and the human fetal embryonic brain library. These studies identified Par-4 as a D2DR-binding protein. More specifically, the clone recovered from

the hybrid screen contained the amino-acids residues 245–342, which encompasses the LZ domain of the Par-4 protein. The interaction between the LZ domain of Par-4 and the D2DR was confirmed both in vitro, in a binding assay using Par-4 and D2i3 (intracellular loop of the long isoform of human D2 receptor) purified proteins, and in vivo, where endogenous D2DR co-immunoprecipitated with Par-4 in mouse brain lysates. The specificity of this interaction was further confirmed through yeast-two hybrid assays using other G-couple receptors, such as the dopamine D3 receptor, in which no interactions were identified. Additionally, 97% of Par-4-positive cells in the striatum, which is the site of most dopaminergic inputs, were also positive for D2DR. These results suggested a role for Par-4 in dopaminergic signaling in the striatum, likely due to Par-4 interaction with D2i3 [11]. The physiological relevance of this interaction was tested in vivo using the Par-4 mouse model mentioned previously [9] in which the C-terminal region, containing the leucine zipper, was deleted. Authors assessed the importance of the Par-4/D2DR binding for the dopamine-cAMP signaling by comparing cAMP accumulation in striatal neurons from WT vs LZ-domain deleted embryos. Although no morphological differences were observed between WT and ΔLZ cultured neurons, there was significant enhancement of the cAMP levels upon treatment with dopamine in Par-4-ΔLZ neurons when compared to its WT counterpart. These results indicated reduced inhibitory tone on dopamine-dependent cAMP signaling. Of note, the range of dopamine concentration necessary to produce this effect was within the physiological concentration of phasic dopamine in the striatum. When Par-4-ΔLZ neurons were co-treated with the D1DR inhibitor SCH23390 and dopamine, no increase in cAMP response was observed, which suggested that cAMP response was not controlled by D1 receptors. However, co-treatment with the D2DR inhibitor sulpiride and dopamine elevated cAMP response in WT neurons, but produced no effect in Par-4-ΔLZ neurons. The data indicated that the D2DR activity promoted inhibitory tone on the cAMP system and further supported that D2DR function was impaired in ΔLZ neurons. It was likely that reduced inhibitory tone in ΔLZ neurons contributed to the dose-dependent dopamine upregulation of cAMP response. Furthermore, in WT neurons, treatment with dopamine decreased serine 133 phosphorylation of CREB (cAMP-responsive element-binding protein), a site that is phosphorylated by cAMP-dependent protein kinase PKA, in a dose-dependent fashion. In ΔLZ neurons, on the other hand, dopamine treatment led to elevated upregulation of the p-CREB at the serine-133 residue, indicating that the lack of Par-4/D2DR interaction affected cAMP downstream signaling. Examination of the physiological effects of the disruption of such interaction demonstrated that Par-4-ΔLZ mice displayed increased depression-like behaviors. When both WT and ΔLZ mice were subjected to the Porsolt's force swim test (FST), Par-4 mutant mice showed higher immobility with no attempt to escape when compared to what was observed in WT mice. These findings were reflective of an enhanced depressive behavior. Additional evaluation, such as tail suspension test (TST) and novelty-suppressed feeding (NSF), further verified the depression-like behavior in ΔLZ mice by demonstrating increased immobility and increased latency to contract food, respectively. Corroborating the findings of depression-like behavior, the performance of ΔLZ mice to the challenges were not due to increased anxiety levels or impaired motor coordination. Ambulatory pattern center activities were similar in ΔLZ and WT mice, demonstrating that anxiety levels were not altered in the Par-4 mutant mice. Likewise, performance in the rotarod test was not different between WT and ΔLZ mice. In summary, the study by Parker et al. [11] revealed a role for Par-4 in central nervous system and indicated that deletion of the C-terminal of Par-4 altered dopamine signaling and led to depression-like behavior in ΔLZ mice.

3 Par-4 Overexpression and Tumor Resistance

In 2007, Zhao et al. generated a transgenic mouse overexpressing the SAC effective domain for apoptosis by Par-4. Fertilized B6-C3(F1) mouse embryos were injected with either a CMV enhancer-chicken β-actin promoter-SAC-eGFP followed by an intron (pCA/SAC-GFP) or CMV enhancer-chicken β-actin promoter-eGFP followed by an intron (pCA/GFP) construct. Offspring was screened for the presence of the transgene, and SAC-GFP or GFP only was detected in all tissues. However, expression of SAC-GFP or GFP transgene was variable in different tissues, implying either differential tissue-specific protein stability or differential regulation of expression [12]. No differences in fertility as judged by litter size, development, and body weight were observed among SAC-GFP, GFP and non-transgenic littermate control mice. Interestingly, SAC-GFP mice lived longer than either set of control mice although those differences were not sex-based. To test the function of the SAC transgene, MEFs from either GFP, SAC-GFP or littermate controls were transfected with oncogenic Ras (or co-transfected with oncogenic Ras and c-Myc) and assayed for apoptosis. As expected according to previous publication on the function of the SAC domain in the presence of oncogenic Ras and/or c-Myc [13], spontaneous apoptosis was elevated in the MEFs from SAC-GFP in comparison to either control MEFs. SAC-GFP MEFs were also resistant to cellular transformation after adenoviral constructs for c-Myc, oncogenic Ras or GFP-control were transduced into MEFs. These results indicated that the SAC transgene was functional in

the MEFs. Additionally, since the SAC domain was known to promote apoptosis through inhibition of NF-κB activity, reporter assays were conducted to determine basal as well as Ras and c-Myc-inducible NF-κB activity in GFP, SAC-GFP and non-transgenic littermate MEFs. Although basal NF-κB activity was comparable among the three groups of MEFs, the introduction of the oncogenes enhanced NF-κB activity in GFP MEFs and littermate control MEFs, but not in SAC-GFP MEFs. Furthermore, when littermate control MEFs were transduced with either an IκB super-repressor (IκB-SR) or a GFP adenovirus to test whether the inhibition of NF-κB activity was sufficient to promote apoptosis in MEFs expressing Ras and c-Myc oncogenes, induction of apoptosis was observed in MEFs transduced with IκB-SR, but not in the ones transduced with GFP [12]. Thus, the SAC domain induces apoptosis by inhibition of NF-κB activity in cells expressing oncogenic Ras and c-Myc.

The SAC-GFP transgenic mice were monitored for the development of spontaneous growth of hepatocarcinomas and lymphomas, which are common in the B6C3F1 background. Hepatocarcinomas and lymphomas were found in about 50% of littermate control mice and GFP-controls, but the SAC mice failed to develop liver or spleen tumors, implying that the SAC transgene inhibited the development of spontaneous tumors. To further test the ability of the SAC transgene to suppress tumor growth, SAC-GFP mice were crossed with tumor-prone TRAMP mice, known to produce prostate adenocarcinoma. PCR genotyping was used to identify $SAC^{+/-}/TRAMP^{+/-}$ or $GFP^{+/-}/TRAMP^{+/-}$ mice; $SAC^{-/-}/TRAMP^{+/-}$ and $SAC^{+/-}/TRAMP^{-/-}$ mice were used as the littermate controls. By 3 months of age, 62–83% of $SAC^{-/-}/TRAMP^{+/-}$ and $GFP^{+/-}/TRAMP^{+/-}$ mice had developed high-grade PIN and 12–16% displayed prostate adenocarcinoma whereas only 50% of $SAC^{+/-}/TRAMP^{+/-}$ showed PIN and 0% displayed adenocarcinoma. By 6 months, 100% of controls exhibited adenocarcinoma of the prostate whereas only 21% of $SAC^{+/-}/TRAMP^{+/-}$ animals showed progression from PIN to adenocarcinoma. The results indicated that the SAC transgene inhibits progression of aggressive TRAMP tumors. More interestingly, GFP expression in $GFP^{+/-}/TRAMP^{+/-}$ mice was observed both in PIN and in adenocarcinoma of the prostate, but $SAC^{+/-}/TRAMP^{+/-}$ mice exhibited loss of SAC within both the PIN and the adenocarcinoma sections. Moreover, by 12 months, loss of SAC expression was observed in all tumors of $SAC^{+/-}/TRAMP^{+/-}$ mice. Taken together, the data strongly implied that downmodulation of SAC was required for the development of the adenocarcinoma in the prostate. TUNEL staining of prostates from $SAC^{+/-}/TRAMP^{+/-}$ mice showed larger number of cells undergoing apoptosis in all the PIN lesions when compared to $GFP^{+/-}/TRAMP^{+/-}$ and $SAC^{-/-}/TRAMP^{+/-}$ PIN lesions, suggesting that SAC domain induced apoptosis in the PIN lesions to halt progression to adenocarcinoma. These findings were confirmed in vitro using TRAMP-C1 cells (derived from TRAMP tumors) and immortalized prostate epithelial BPH-1 cells as control, where transfection of either SAC domain or full-length Par-4-induced apoptosis in TRAMP-C1 cells, but not in BPH-1 cells. Additionally, in TRAMP-C1 cells, inhibition of NF-κB activity using either SAC or IκB-SR constructs was sufficient to trigger apoptosis, suggesting that SAC domain was able to induce apoptosis in TRAMP-C1 cells through NF-κB inhibition. In summary, overexpression of the SAC domain in mice suppressed the development of both spontaneous and aggressive transgenic tumors, and these effects were likely due to induction apoptosis by inhibition of NF-κB activity [12]. It is noteworthy that the role of Par-4 in the mouse prostate corroborated the findings of Boghaert et al. in the rat prostate where Par-4 levels increased after castration when ductal cells undergo apoptosis [14]. The studies suggested that Par-4 induction is necessary for prostate epithelial cells to undergo apoptosis.

Similar results were observed in mice overexpressing full-length Par-4, generated using the same strategy for generation of SAC transgenic mice, where the actin promoter and the CMV enhancer were used to drive expression of the Par-4-GFP. The spontaneous tumor rate in the liver and in the spleen of transgenic mice was remarkably low relative to the rate of tumors observed in both littermate and GFP-controls [15]. Serum of Par-4 transgenic mice contained detectable levels of Par-4-GFP protein. Additionally, serum of Par-4 transgenic mice induced ex vivo apoptosis of prostate cancer PC-3 cells, whereas no effect was observed when these cells were treated with serum from either GFP or littermate control mice. These data indicated that Par-4 protein is secreted in mice and is functional in promoting apoptosis of cancer cells [15].

4 Secreted Par-4 and Inhibition of Tumor Growth

The discovery of the secreted version of Par-4 expanded the possible mechanisms of tumor suppression by Par-4. Zhao et al., in a study published in 2011, investigated whether secreted Par-4 could inhibit non-autochthonous tumor growth [16]. When MEFs from GFP-control and GFP-SAC mice were co-cultured with either immortalized or transformed cells, apoptosis was observed only in the transformed cells co-cultured with SAC-GFP MEFs. Additionally, the conditioned-media (CM) of GFP-SAC MEFs, but not the CM from GFP-control MEFs, was able to induce apoptosis in the transformed cells. The data suggested that a secreted protein was responsible for inducing apoptosis. When serum from either SAC or

GFP-control transgenic mice was used treat several cancer/transformed cell lines, apoptosis was observed only in cells treated with serum from SAC mice. Apoptosis effect of serum from SAC-GFP mice was neutralized by GFP antibody, but not by the TRAIL antibody. Strikingly, when Lewis lung carcinoma (LLC-1) cells were injected into the flanks of littermate controls, GFP-controls or GFP-SAC mice, tumor positivity was only 15% in GFP-SAC mice compared to 100% for both littermates and GFP-controls (n = 20). Furthermore, tumor volume in SAC-GFP mice was roughly one third of the volume of tumors in the control group. Together the data indicated that secreted SAC-GFP is responsible for inducing apoptosis in cancer/transformed cells and robust inhibition of non-autochthonous tumor growth. The function of secreted SAC was further demonstrated by transferring the bone marrow from SAC-GFP, littermate or GFP-control mice to irradiated control mice. GFP expression was confirmed in recipient mice after transplantation. Serum from SAC-GFP recipient mice induced apoptosis in LLC-1 cells whereas no apoptosis was observed in LLC-1 cells treated with serum from mice that received bone marrow from either littermate or GFP-control. The data indicated that control mice successfully secreted active SAC-GFP and that SAC apoptotic activity could be transferred between mice through the bone marrow. Furthermore, recombinant TRX-Par-4 and TRX-SAC proteins, known to induce apoptosis of LLC-1 and PC3 cells in cell culture, were injected into immunocompetent C57BL/6 mice bearing LLC-1 cells. Both TRX-Par-4 and TXR-SAC significantly inhibited LLC-1 lung metastasis. The findings demonstrated that systemic Par-4 was able to inhibit not only primary tumor growth, but also metastases [16].

5 Par-4 Deletion in C57BL/6 Background

A different Par-4 whole-body knockout mouse was generated by our laboratory to investigate the role of Par-4 in mediating apoptosis. In the C57BL/6 background mice, a fragment containing exon 2 of the Par-4 gene flanked by loxP sites, and the neomycin selection flanked by FRT site was introduced into intron 2. Whole-body constitutive knockout was generated upon crossing with a Rosa26-Cre mouse, resulting in the deletion of Par-4 exon 2. Since exon 2 contains the start codon for initiating protein translation, Par-4 protein was absent in these mice. Corroborating the results by García-Cao et al. [6], Par-4-null mice in the C57BL/6 background also developed spontaneous tumor in various tissues, including liver, endometrium, and intestine.

These Par-4-null mice were also crossed with EμTcl1 mice to determine the effect of Par-4 in the development of chronic lymphocytic leukemia (CLL). Interestingly, it was found that Par-4 deficiency delayed the onset of CLL and prolonged survival of EμTcl1 mice compared to EμTcl1 mice that expressed Par-4. The authors hypothesized that Par-4 loss activated p21, which promoted growth arrest at G1/S and slowed down CLL progression [17].

The relevance of Par-4 in the context of breast cancer [18, 19] raised the possibility of increased growth of mammary orthotopic tumors in Par-4-null females. To address this question, EO771 mammary cancer cell line was transfected with pGL4.50 luciferase construct and luciferase positive cells were selected using hygromycin. EO771-luc cells were injected into the mammary fat pad of WT and Par-4-null females and tumor growth was monitored by in vivo luciferase IVIS imaging. As shown in Fig. 1, 3 weeks after injec-

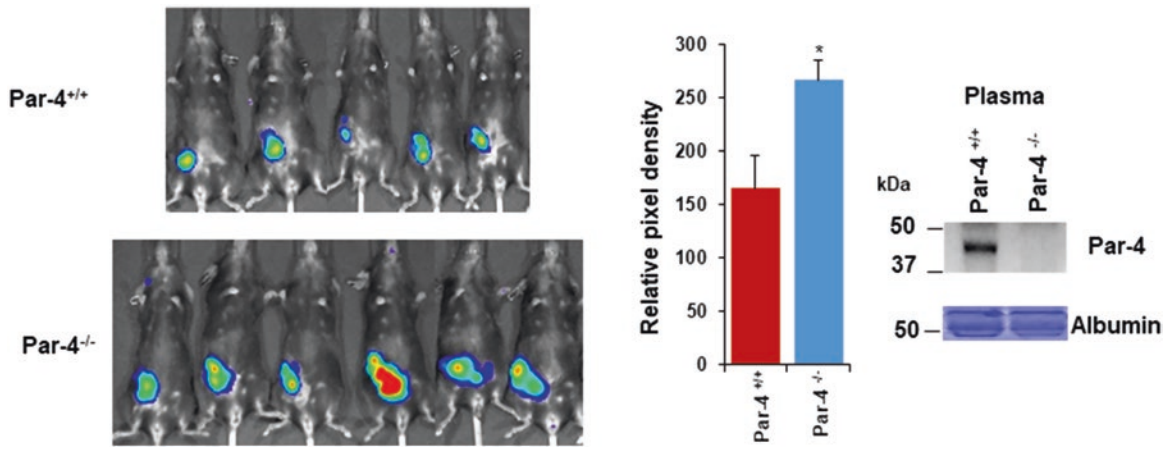

Fig. 1 EO771-luc cells grow larger mammary gland tumors in Par-4$^{-/-}$ mice. Mouse mammary EO771-luc cells (1×10^6) were injected into the mammary fat pad of Par-4$^{+/+}$ and Par-4$^{-/-}$ C57BL/6 female mice and tumor growth was monitored using luciferase imaging. Three weeks after mammary fat pad injection, tumors were significantly larger ($P < 0.05$ by the Student's t-test) in the Par-4$^{-/-}$ mice (left panel) as judged by increased relative pixel density (middle panel). Western blot analysis confirmed the absence of Par-4 in the plasma of Par-4-null mice (right panel)

tion, tumors grew larger in Par-4$^{-/-}$ mice compared to WT mice. Similarly, intravenous injection of mouse LLC-1 lung cancer cells into these Par-4-null mice and WT control mice produced far greater number of lung nodules in Par-4-null mice relative to the control mice. Moreover, the FDA-approved drug chloroquine strongly inhibited the growth of the LLC-1 derived lung nodules in WT mice relative to the Par-4-null mice [20]. These studies indicate that solid tumors resulting from breast cancer cells or lung cancer cells show accelerated growth in the absence of Par-4 in the micro- and macroenvironment.

6 Effect of Extracellular and Intracellular Par-4 on Tumor Growth

The relevance of systemic Par-4 in inhibiting tumor growth and metastases propelled the field to look for molecules that could enhance the secretion of Par-4 (such molecules called Par-4 secretagogues) as a strategy to treat cancer. In 2014, the arylquin family of small molecules was discovered to induce Par-4 secretion from normal cells. Arylquin-1 was found to bind vimentin, which released Par-4 for secretion; then secreted Par-4 induced paracrine apoptosis in cancer cells [21]. Growing interest in the repurposing of FDA-approved drugs and in the discovery of other Par-4 secretagogues led to the identification of chloroquine (CQ) as another robust inducer of Par-4, via the classical pathway involving p53 and Rab8b activation. Chloroquine was shown to inhibit metastases in a Par-4-dependent manner in both immunocompetent and immunocompromised mice [20]. Additionally, chloroquine has been used in combination with radiation for the treatment of glioblastomas and brain metastases [22, 23]. In the light of these findings, the Rangnekar Lab investigated the use of chloroquine and radiation as a radiosensitizer for lung cancer and melanoma cell lines. A549 lung cancer cells with either normal (A549-shControl) or knockdown levels of Par-4 (A549-shPar-4) were treated with vehicle, 10 μM or 20 μM of CQ, and subjected to ionizing radiation (0, 2, or 4 Gray). As shown in Fig. 2, CQ radiosensitized A549 lung cancer cells in a Par-4-dependent manner. Similar radiosensitization by CQ was observed in the melanoma cell lines Mel 1617, which is sensitive to the BRAF-inhibitor PLX4032, and Mel 1617BR, which are resistant to PLX4032 [24], when these melanoma lines were treated with either vehicle, 1 μM or 5 μM CQ and subjected to either 0 Gy or 4 Gy radiation (Fig. 3). In vitro results with melanoma cell lines were verified in vivo by injecting either Mel 1617 (Fig. 4) or Mel 1617BR (Fig. 5) into the flanks of nude mice. Mice were subjected to a single dose of radiation (0 or 3 Gy) and treated with either vehicle (saline) or chloroquine at 25 mg/kg body weight, once daily

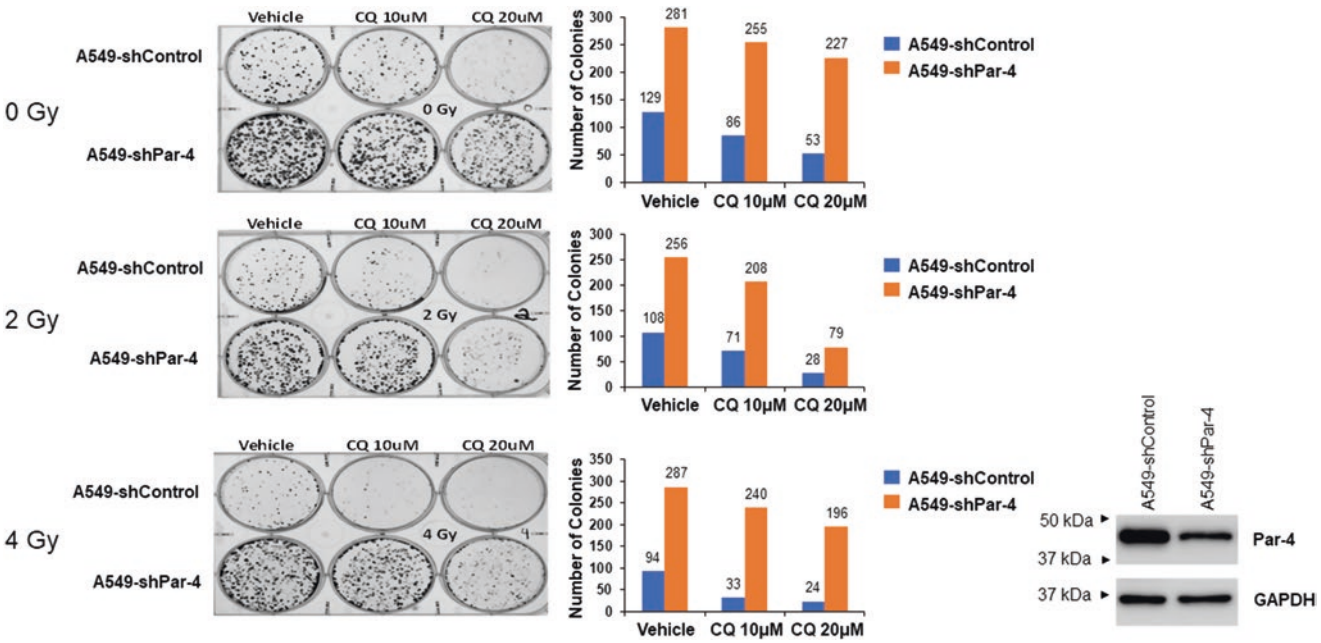

Fig. 2 Chloroquine radiosensitizes A549 lung cancer cells in a Par-4-dependent manner. A549-shControl and A549-shPar-4 were subjected to either 0, 2, or 4 Gy ionizing radiation and immediately treated with either vehicle, chloroquine (CQ) 10 or 20 μM. After 10 days, plates were stained with 0.5% Gentian Violet and colonies were counted. Note that CQ radiosensitized A549-shControl cells, but not A549-shPar-4 cells, at both 2 and 4 Gy doses. Western blot analysis confirmed Par-4 knockdown in A549-shPar-4 cells (bottom right panel)

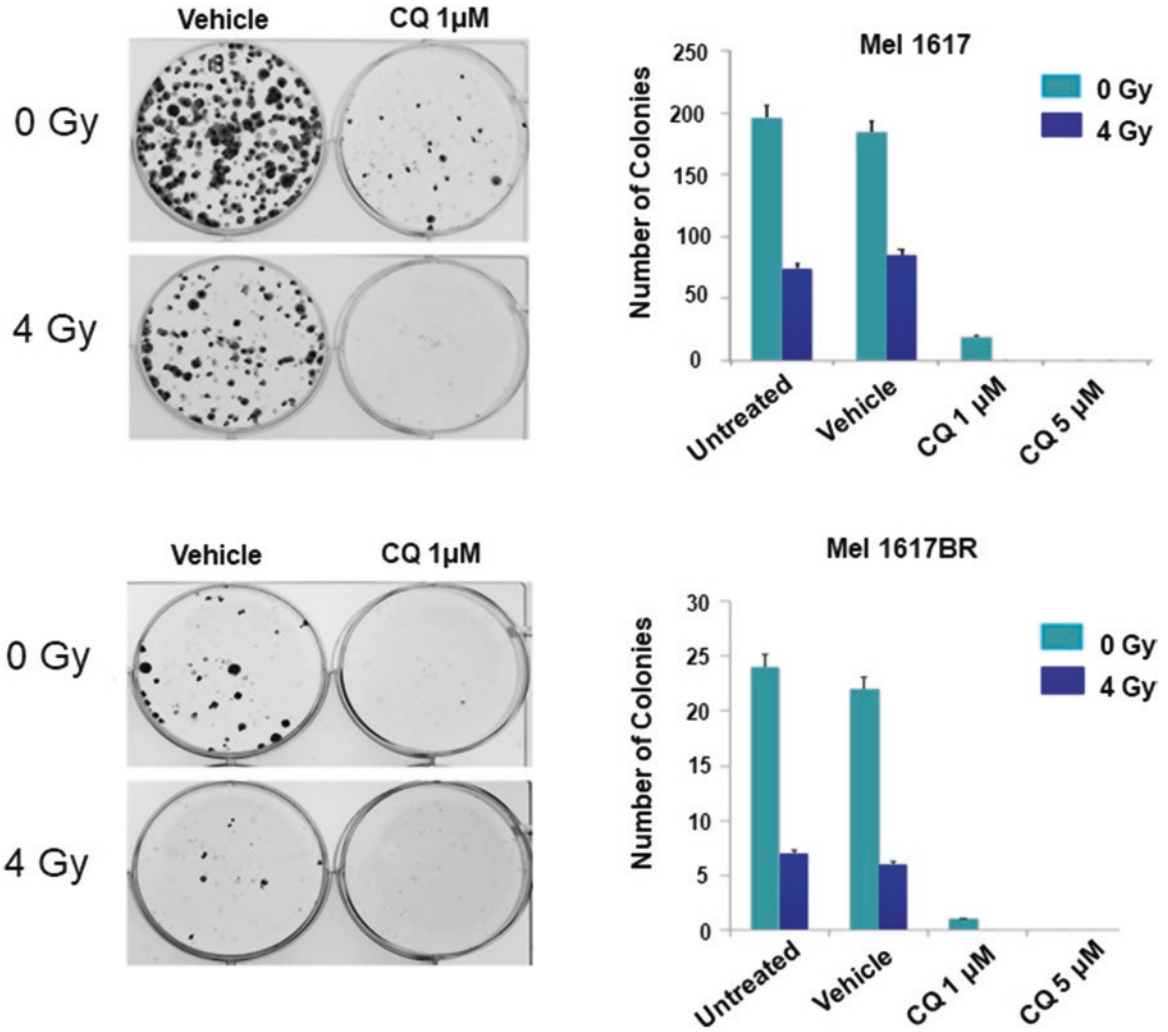

Fig. 3 Chloroquine radiosensitizes melanoma cells. Melanoma cells Mel 1617 that are sensitive to chemotherapy (top panel) and Mel 1617BR that show BRAF mutation and are resistant to therapy (bottom panel) were subjected to either 0 or 4 Gy radiation and immediately treated with vehicle or CQ at 1 or 2 μM concentrations. Plates were stained with Gentian Violet and colonies were counted. Both cell lines showed increased sensitivity to radiation upon CQ treatment

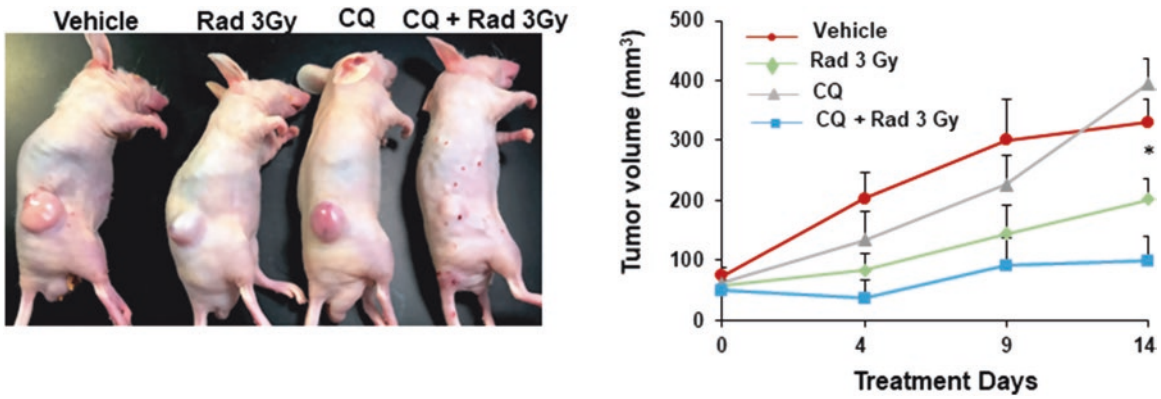

Fig. 4 Chloroquine and radiation combination inhibits growth of tumors derived from BRAF-inhibitor sensitive Mel 1617 melanoma cells. Mel 1617 cells (1.5×10^6 cells in saline) were injected into the flank of nude mice. When tumor volume reached appropriate size, mice received either 0 or 3 Gy radiation (day 0). Treatment with either vehicle or CQ (25 mg/kg body weight) was then started (day 0 after radiation). Mice were treated once every day for 2 weeks, tumor size was measured and tumor volume was calculated. In combination with CQ, radiation showed greater inhibition of tumor growth when compared to the effect of radiation alone. (*$P < 0.05$ by the Student's *t*-test)

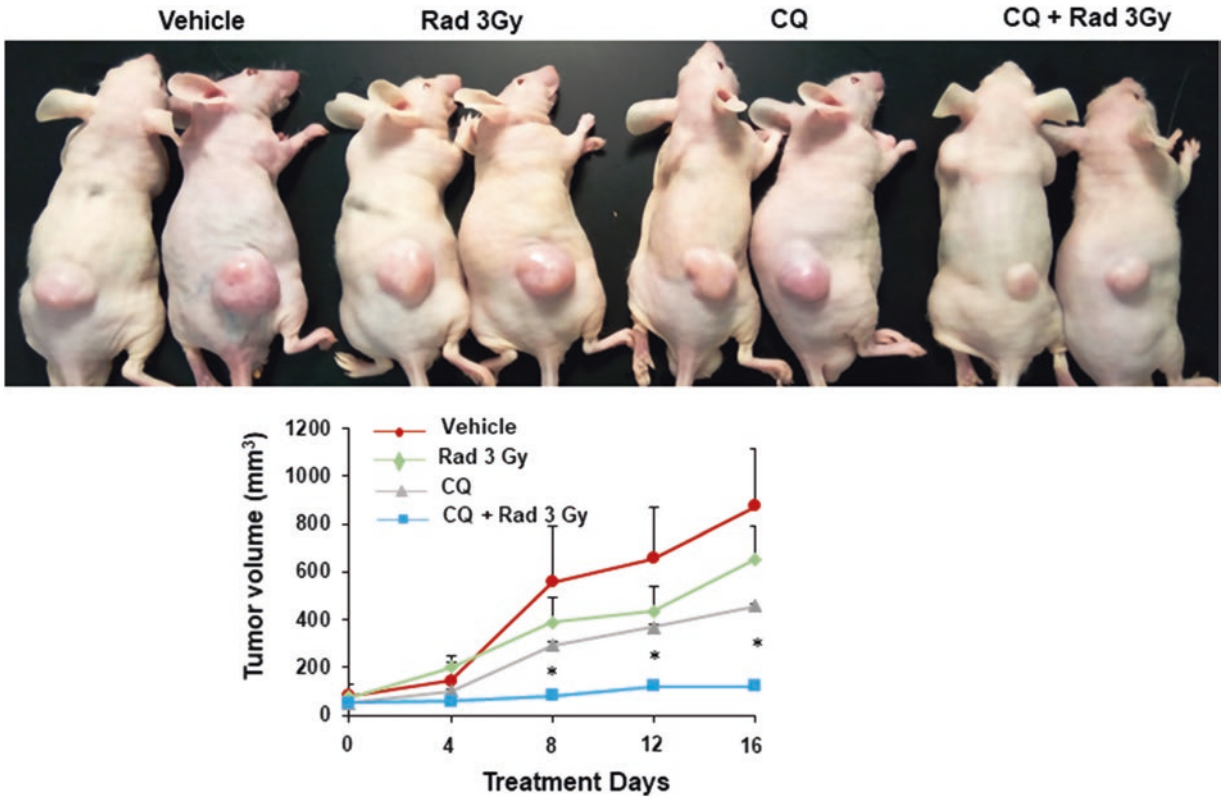

Fig. 5 Chloroquine radiosensitizes tumors derived from BRAF-mutant inhibitor PLX4720-resistant Mel 1617BR melanoma cells. Mel 1617BR cells (1.5×10^6 cells in saline) were injected into the flank of nude mice. One week after injection, when tumors reached appropriate size, mice were subjected to either 0 or 3 Gy dose of radiation (day 0). Treatment with either vehicle or CQ (25 mg/kg body weight) started on day 0 and was performed once every day for 16 days. Combination of CQ and radiation showed greater inhibition of tumors relative to the effect of either CQ or radiation alone. (*$P < 0.05$ by the Student's *t*-test)

for 2 weeks. Decreased rate of tumor growth in the group treated with CQ plus radiation indicated that combination treatment was most effective in inhibiting tumors derived from the melanoma cell lines.

The role of intracellular Par-4 in the ability of cancer cells to form tumors was also investigated in the context of lung cancer. A549-shControl and A549-shPar-4 were cells were injected in the left and the right flank, respectively, of nude mice. As shown in Fig. 6, tumor growth, monitored for the span of 3 weeks, demonstrated that A549 cells with shRNA knockdown of Par-4 grow tumors at a faster rate that A549 cells that contain normal levels of Par-4. Thus, reduced expression of intracellular Par-4 by shRNA knockdown in cancer cells induces rapid tumor growth.

7 Future Perspectives

Par-4 inhibits both primary and metastatic tumor growth. Circulating levels of Par-4 can be altered to restrict tumor progression. Combination of treatments that reduce tumor size and follow-up with Par-4 secretagogues to induce apoptosis of remaining cells offers an exciting and effective strategy, as indicated by the study combining chloroquine and radiation. Concerning the diverse mouse models available, understanding the role of Par-4 in specific tissues by generating tissue-specific deletions or overexpression would be important to determine which cancers are more susceptible (or resistant) to Par-4. Some of the unanswered questions

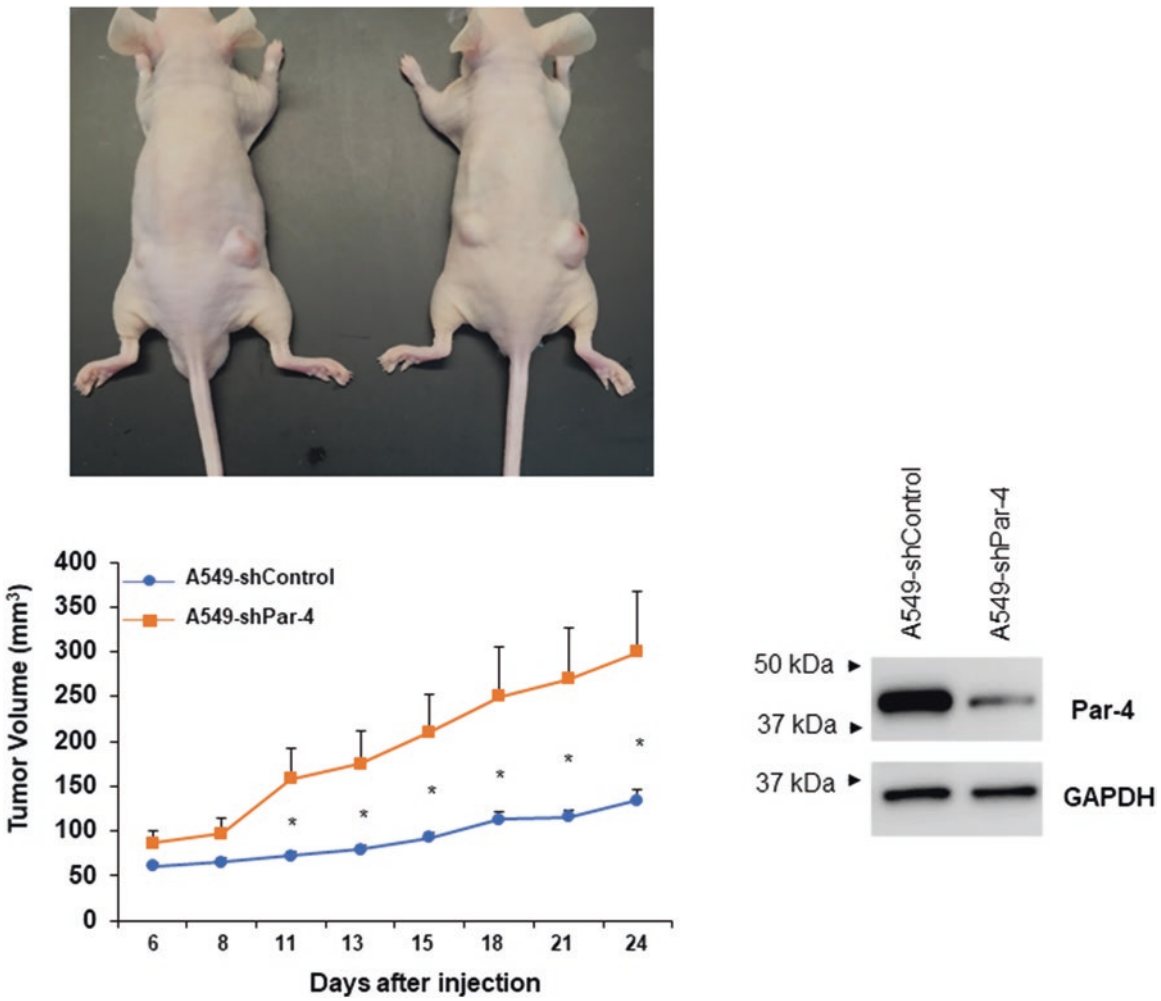

Fig. 6 Par-4 knockdown A549 lung cancer cells grow tumors at a faster rate than A549 control cells. A549-shControl cells (1.5×10^6 in saline) were injected into the left flank and A549-shPar-4 cells (1.5×10^6 in saline) were injected into the right flank of nude mice (n=3). Tumor growth was monitored over a period of 24 days. Tumor size was measured 3 times a week and tumor volume calculated. Note that Par-4 knockdown with shRNA resulted in significantly greater growth of tumors. Western blot analysis confirmed Par-4 knockdown in A549-shPar-4 cells relative to control shRNA containing A549 cells. $^*P < 0.05$ by the Student's *t*-test

include a) which tissues and cells secrete Par-4 and b) can systemic Par-4 compensate for tissue-specific deletion of Par-4? Future studies should help determine the appropriate scenarios to introduce Par-4-based treatments.

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Par-4 Secretagogues in Clinical Trials

Peng Wang and Zhonglin Hao

Abstract

Originally approved by the FDA to treat malaria, chloroquine (CQ) and hydroxychloroquine (HCQ) were later identified as potent secretagogues of the tumor suppressor protein, prostate apoptosis response-4 (Par-4). When released from normal cells via the classical secretory pathway requiring activation of p53, Par-4 triggers paracrine apoptosis in cancer cells. A phase I study demonstrated the relationship between elevation of Par-4 plasma levels and tumor response to HCQ, providing a new approach for cancer treatment.

Keywords

Chloroquine · Hydroxychloroquine · Par-4 · Apoptosis · p53 · GRP78 · Classical secretory pathway · Radiation · Autophagy · Clinical trial

Abbreviations

ADT	Androgen deprivation therapy
AEs	Adverse events
AMPK	AMP-activated kinase
AR	Androgen receptor
ATG	Autophagy-related genes
BCR	Biochemical recurrence
BFA	Brefeldin
CM	Cell-culture conditioned medium
CQ	Chloroquine
CYP	Cytochrome P450
DLT	Dose limiting toxicity
GBM	Glioblastoma multiforme
GRP78	Glucose-regulated protein-78
HBECs	Human bronchial epithelial cells
HCQ	Hydroxychloroquine
HEL	Normal human lung fibroblasts
MEFs	Mouse embryonic fibroblasts
MTD	Maximum-tolerated dose
mTOR	Mammalian target of rapamycin
mTORC1	mTOR Complex 1
NSCLC	Non-small cell lung cancer
Par-4	Prostate Apoptosis Response-4 Protein
PCM	Precision cancer medicine
PI3K	Phosphoinositide 3-kinase
PLK1	Polo-like kinase 1
PrECs	Normal human epithelial cells
PrSCs	Normal human prostate stromal cells
RP	Radical prostatectomy
RP2D	The recommended phase II dose
UPR	Unfolded protein response

1 Introduction

Prostate apoptosis response-4 (Par-4, also called PAWR), a tumor suppressor protein, induces apoptosis exclusively in diverse cancer cells [1]. Par-4 functions through caspase-dependent mechanisms by binding to its receptor, GRP78, on the cancer cell surface [1–4]. Par-4 is ubiquitously expressed in normal cells and tissues [1] and is located in various cellular compartments. Baseline levels of Par-4 are inadequate to induce significant apoptosis in malignant cells [5]. Chloroquine (CQ) and hydroxychloroquine (HCQ) have been identified as Par-4 inducers [5]. CQ and HCQ are also well-known for inhibition of autophagy primarily by interfering with lysosome activities [6–8]. Focusing on its anti-autophagy function, clinical efficacy of CQ or HCQ in combination with other treatment modalities has been assessed in multiple clinical trials [9]. Progressively more clinical attention is being drawn to paracrine apoptosis in cancer cells induced by Par-4 secretagogues such as CQ or

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HCQ. Multiple ongoing early phase clinical trials are attempting to address the biological response of Par-4 and clinical outcomes.

2 Par-4 as a Secreted Tumor Suppressor

CQ-induced Par-4 from normal cells may generate paracrine apoptosis in cancer cells. In a study by Burikhanov et al. [5], regardless of p53 status, p53-deficient prostate cancer cells (PC-3), p53-deficient lung cancer cells (H1299, Hop62, and KP7B), and wild-type p53 lung cancer cells (H460) became sensitive to apoptosis after co-cultured with CQ-treated Par-$4^{+/+}$ MEFs (mouse embryonic fibroblasts) (Fig. 1a). The same phenomenon of apoptotic activity was observed when co-cultured with conditioned media (CM) from CQ-treated Par-$4^{+/+}$ MEFs. Both the Par-4 antibody, by neutralizing Par-4 in CM, and the GRP78 antibody, by inhibiting the binding of Par-4 to its receptor GRP78 on the cancer cell surface, inhibited Par-4-induced apoptosis (Fig. 1b). As a GRP78 inducer, PS-1145 (the nuclear factor κB inhibitor) sensitized cancer cells to apoptosis by CQ-treated CM. Mouse models with metastatic lung cancer were established by injecting LLC1 (lung cancer) cells intravenously into Par-$4^{+/+}$ (wild-type) or Par-$4^{-/-}$ mice. After 5 days of CQ injection (i.p., 25 mg/kg body weight), only plasma from CQ-treated Par-$4^{+/+}$ mice showed significant induction of Par-4 compared to the plasma from vehicle-treated mice (Fig. 2a). Par-$4^{-/-}$ mice had significantly higher tumor burden in lung compared to Par-$4^{+/+}$ mice. After CQ treatment, the tumor volume in the lungs showed far greater inhibition in Par-$4^{+/+}$ mice than in Par-$4^{-/-}$ mice. Plasma from CQ-treated Par-$4^{+/+}$ mice showed the ability to induce apoptosis ex vivo in LLC1 cells (Fig. 2b). Par-4 and GRP78 antibodies blocked this induction of apoptosis. These results indicate that basal levels of Par-4 in wild-type mice slowed down cancer genesis, but not adequately enough to inhibit cancer progression unless Par-4 levels were induced by secretagogues such as CQ or HCQ.

More importantly, induction of plasma Par-4 was observed in renal cancer patients following HCQ treatment in a clinical study. Different cancer cell lines underwent apoptosis with the plasma from these patients. Subsequently, apoptotic activity was inhibited by adding Par-4 or GRP78 antibody. In conclusion, Par-4 tumor suppressor protein secretion is induced in response to CQ or HCQ and triggers apoptosis of cancer cells [5].

3 CQ and HCQ

Both CQ and HCQ were produced for prevention and treatment of malarial infection. CQ penetrates into most human tissues with concentrations several hundred-fold above plasma concentrations and therefore has a large volume of distribution. As a result, serum drug levels may be maintained in humans for up to 2 months [10]. Cytochrome P450 3A isozymes (CYP3As) and CYP2D6 are two main enzymes involved in CQ metabolism [11].

Both CQ and HCQ are 4-aminoquinoline derivatives. They are structurally similar, differing only by replacement of an ethyl group in chloroquine with a hydroxyethyl group in HCQ (Fig. 3). HCQ demonstrates similar PK profile to CQ. Multiple cytochrome P450 enzymes including CYP2D6, 2C8, 3A4, and 3A5 are involved in HCQ metabolism [12]. HCQ comprises 95 percent of all rheumatic disease anti-

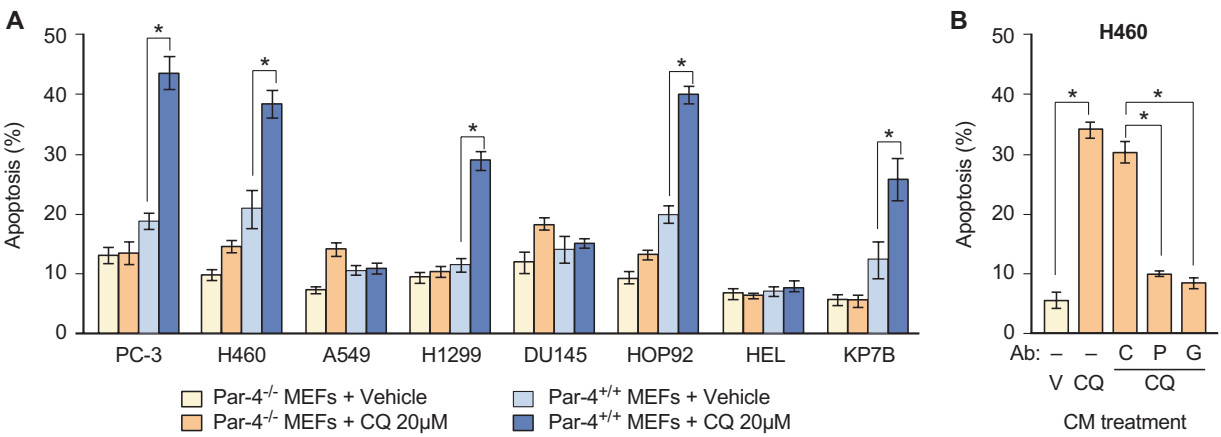

Fig. 1 CQ-induced paracrine apoptosis of cancer cells through induction of Par-4 secretion. (**a**) Par-$4^{+/+}$ or Par-$4^{-/-}$ MEFs were co-cultured with various cancer cells or normal cells and treated with CQ or vehicle. After 24 h, the cells were scored for apoptosis. (**b**) CQ-induced paracrine apoptosis in cancer cells by a Par-4-dependent mechanism. Aliquots of conditioned media from wild-type MEFs treated with CQ (20 μM) were incubated with control (C) antibody (Ab), Par-4 (P) Ab, or GRP78 (G) Ab and then transferred to H460 cells. After 24 h, the cells were scored for apoptosis. *V* vehicle. *(Adapted from Cell Rep. 2017; 18(2): 508–519. doi:*https://doi.org/10.1016/j.celrep.2016.12.051*)*

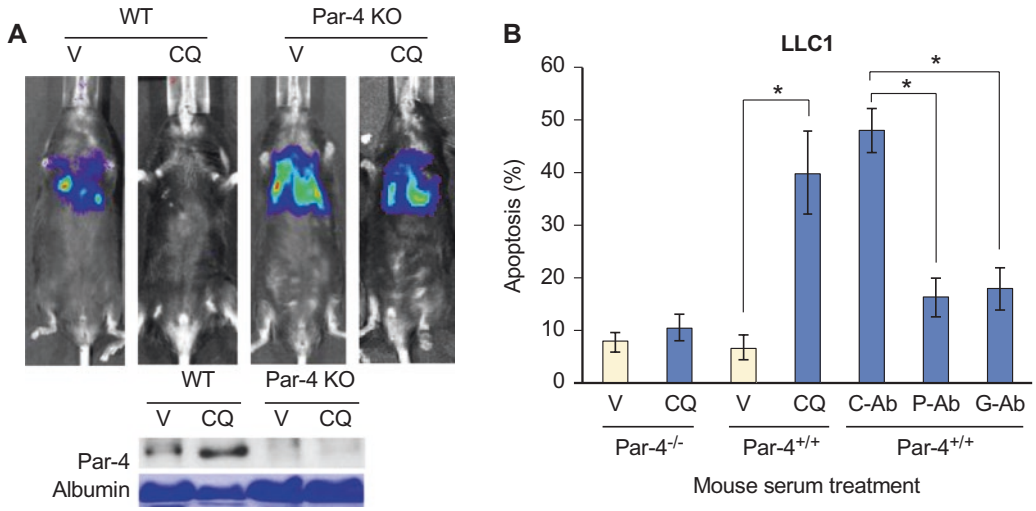

Fig. 2 CQ-induced tumor growth inhibition by a Par-4-dependent mechanism. (**a**) CQ-induced Par-4 secretion and tumor growth inhibition. Wild-type (WT or Par-$4^{+/+}$) or Par-4 knockout (Par-4 KO or Par-$4^{-/-}$) C57BL/6 mice were injected i.v. with LLC1 cells (expressing luciferase) and, 24 h later, injected i.p. with CQ (25 mg/kg body weight) or vehicle (V) once every day for 5 consecutive days. Plasma from mice was collected 24 h after the last injection and subjected to western blot (WB) analysis for Par-4 or Coomassie blue staining for albumin (lower panel). Tumor growth in the mice was followed by fluorescent imaging for luciferase expression using an IVIS imager, and representative images are shown (upper panel). (**b**) Par-4 in plasma from CQ-treated mice induced ex vivo apoptosis in LLC1 cells. Aliquots of plasma from Par-$4^{+/+}$ or Par-$4^{-/-}$ mice treated with CQ or vehicle (V), tested by western blot analysis in (A), were incubated with LLC1 cells for 24 h, and the cells were scored for apoptosis. Moreover, aliquots of plasma from Par-$4^{+/+}$ mice treated with CQ were incubated with control antibody (C-Ab), Par-4 antibody (P-Ab), or GRP78 antibody (G-Ab) and then transferred to LLC1 cells. After 24 h, the cells were scored for apoptosis. *$p < 0.0001$, by Student's *t*-test. *(Adapted from Cell Rep. 2017; 18(2): 508–519. doi:*https://doi.org/10.1016/j.celrep.2016.12.051*)*

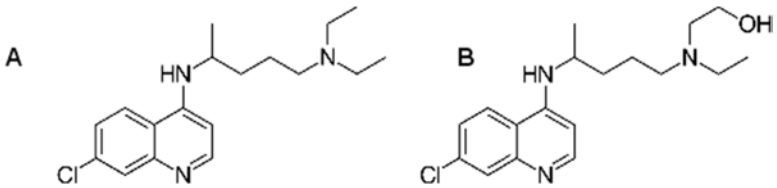

Fig. 3 Biochemical structure of CQ and HCQ. CQ and HCQ are structurally similar, differing only by replacement of an ethyl group in CQ (**a**) with a hydroxyethyl group in HCQ (**b**)

malarial prescriptions and is primarily eliminated through kidneys.

In both CQ and HCQ, severe adverse reactions are extremely rare. Mild gastrointestinal discomfort with nausea, vomiting, diarrhea, and stomach pain are the most commonly described side effects. These side effects are more likely to occur at higher doses [13]. Rash and itching may also occur due to the long half-life of the compounds. Retinal toxicity becomes a concern if chronic and cumulative doses exceed 1000 g [14, 15].

4 Rationale for Using Par-4 Secretagogues and Identification of CQ and HCQ as Secretagogues

Par-4 is ubiquitously expressed in normal cells and tissues [1]. Baseline levels of Par-4 are inadequate to induce significant apoptosis in malignant cells [5]. Thus, a search was undertaken to identify a drug that would boost Par-4.

Screening a mini-library of 17 FDA-approved generic drugs including either quinolone or quinolone pharmacophores (Table 1) revealed that CQ and HCQ had robust ability for induction of Par-4. CQ-induced Par-4 was dose-dependent following co-culture with various concentrations of either CQ or vehicle for 24 h with various normal cells including wild-type p53 MEFs, normal human prostate stromal cells (PrSCs), and epithelial cells (PrECs) and from normal human lung fibroblast (HEL) cells and epithelial cells (HBECs). The process of inducing Par-4 by CQ does not affect autophagy since there was no difference in p62/SQSTM1 levels between the Par-$4^{+/+}$ and Par-$4^{-/-}$ cells regardless CQ treatment [5]. In contrast to Par-4 induction in normal cells by CQ, cancer cells including prostate cancer cells (LNCaP, C4-2B, DU145, and PC-3) and lung cancer cells (H460 and A549) failed to secrete Par-4 after CQ treatment. To confirm these findings, following 24-h treatment with either CQ or control vehicle, plasma samples from immunocompetent mice were collected and tested for systemic levels of Par-4. The levels were significantly elevated in CQ-treated mice compared to vehicle treatment. Importantly, CQ induction of

Table 1 FDA-approved compounds screened for induction of Par-4 secretion

Compound	Drug class	Target
Ciprofloxacin	Fluroquinolone	Anti-bacterial
Difloxacin	Fluroquinolone	Anti-bacterial
Enrofloxacin	Fluroquinolone	Anti-bacterial
Flumequine	Fluroquinolone	Anti-bacterial
Lemofloxacin	Fluroquinolone	Anti-bacterial
Levofloxacin	Fluroquinolone	Anti-bacterial
Norfloxacin	Fluroquinolone	Anti-bacterial
Ofloxacin	Fluroquinolone	Anti-bacterial
Pefloxacin	Fluroquinolone	Anti-bacterial
Sparfloxacin	Fluroquinolone	Anti-bacterial
Nalidixic acid	Quinolone	Anti-bacterial
Primaquine	8-aminoquinoline	Anti-malarial
Tafenoquine	8-aminoquinoline	Anti-malarial
Amodiaquine	4-aminoquinoline	Anti-malarial
Chloroquine	4-aminoquinoline	Anti-malarial
Hydroxychloroquine	4-aminoquinoline	Anti-malarial

Par-4 required the classical secretory pathway. When activated by wild-type p53, binding of p53 with its transcriptional target, Rab8b, led to secretion of Par-4 from normal cells (Fig. 4). Loss of Rab8a did not block CQ-induced Par-4 secretion, indicating that Par-4 secretion was independent of the non-conventional autophagic pathway.

These findings were further confirmed by a clinical trial. In this clinical trial, eight out of nine patients with various malignancies showed elevation of plasma Par-4 from baseline following 2 weeks of HCQ treatment (Fig. 5).

5 Autophagy Induction by Anti-Malarial Drugs

In addition to being powerful Par-4 secretagogues, CQ and HCQ can be effective inhibitors of autophagy. This is important since many preclinical studies have shown that suppression of autophagy is implicated as a promising approach in cancer therapy [16–18].

5.1 What Is Autophagy

The term "autophagy" was first coined by Christian de Duve, the scientist who discovered the self-eating phenomena (autophagy) [19] in 1963, following his discovery of the lysosome in 1955 for which he was honored by a Nobel Prize in Physiology or Medicine in 1974. Yoshinori Ohsumi was subsequently recognized by a Noble Prize in 2016 for his contribution to the mechanism of autophagy using yeast as the model organism [20]. Autophagy is an evolutionarily conserved catabolic cellular process during which dysregulated cellular organelles or macromolecules are degraded by enzymes in the lysosomes to maintain cellular hemostasis. Three different types of autophagy are recognizable in cells. Macroautophagy (hereafter referred to as autophagy), microautophagy and chaperone-mediated autophagy all have different mechanisms and functions [21]. The autophagy process involves fusion of double membraned vesicles called autophagosomes with lysosomes to form autolysosomes. Eventually autolysosome contents are degraded into amino acids which cellular organelles or macromolecules can recycle and use for energy or building blocks for subsequent synthesis of new macromolecules [22, 23]. The autophagy process is typically divided into distinct stages including initiation; nucleation of the autophagosome; expansion and elongation of the autophagosome membrane; fusion with the lysosome to form autophagolysosome and degradation of intravesicular contents. The size of the protein targets that can be degraded is virtually limitless. Energy is generated during the autophagy process via the mitochondria. Since endoplasmic reticulum is part of the autophagy machinery, the autophagy process maintains endoplasmic reticulum stress.

Autophagy is thought to happen at a basal level in all cells and is also induced by diverse cellular stresses such as nutrient starvation, aggregation of toxic proteins, mitochondria depolarization, and pathogen infection [24]. Autophagy-related genes (ATG) include core proteins belonging to the autophagy machinery and a set of signaling proteins that regulate autophagy. ATG1/ULK1 participates in the initiation process in coordination with ULK2, ATG13, ATG101, and FIP200 which activates a class III phosphoinositide 3-kinase (PI3K) complex formed by proteins including VPS34, VPS15, and Beclin 1, a putative tumor suppressor; whereas ATG5-ATG12 and LC3 complexes conjugate to expand the autophagosome. Multiple genes upstream of autophagy are involved in regulation of the autophagy process. Among them is the mammalian target of rapamycin (mTOR) known to play a role in controlling cellular homeostasis. mTOR senses multiple environmental cues such as nutrients, energy levels, stresses, and the presence of growth factor. It integrates signaling from many upstream pathways such as the PI3K pathway and the AMP-activated kinase (AMPK) pathway. During cell starvation, when either amino acid levels or growth factor concentration drop, the mTORC1 (mTOR Complex 1) is inhibited. This in turn takes away the inhibition of mTORC1 on ATG1/ULK1 resulting in activation of autophagy.

5.2 Autophagy and Cancer

Autophagy is a survival mechanism conserved from yeast to human cells. It is also an important survival mechanism across different types of cancer cells. Cancer cells tend to

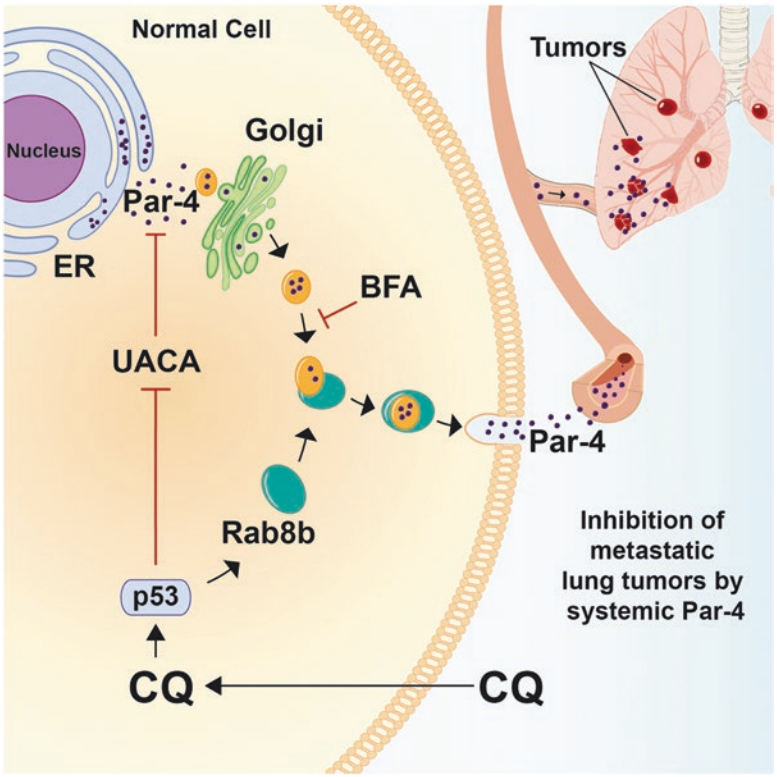

Fig. 4 Schematic representation of Par-4 secretion from normal cells is p53 dependent. CQ-induced Par-4 secretion from normal cells by a mechanism that was dependent on tumor suppressor p53 and its transcriptional target, Rab8b. Par-4 was essential for paracrine apoptosis of p53-deficient cancer cells and tumor growth inhibition by CQ. Moreover, CQ-induced secretion of Par-4 was prevented by brefeldin A (BFA), which blocked the conventional pathway but not the non-conventional pathways. This finding indicates that Par-4 secretion occurs independently of the non-conventional autophagic pathway. *(Cell Rep. 2017; 18(2): 508–519. doi:*https://doi.org/10.1016/j.celrep.2016.12.051*)*

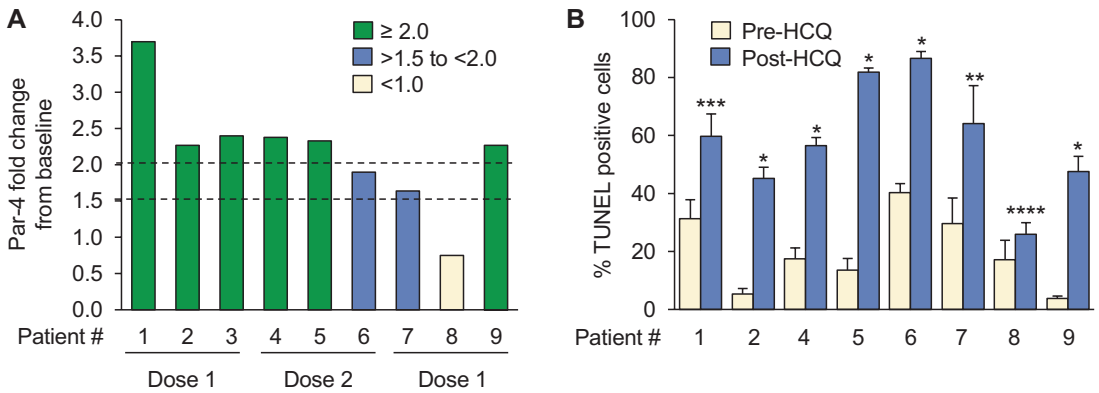

Fig. 5 HCQ-induced apoptosis in cancer patients via induction of plasma Par-4. (**a**) Plasma samples from patients were collected pre-HCQ (Day 0), and Day 14 post-HCQ treatment and analyzed by western blot for Par-4 level. Fold increase in level at Day 14 relative to Day 0 is shown. (**b**) HCQ induces apoptosis in patient tumors. Diagnostic biopsies and paired resected tumor specimen were analyzed by TUNEL assay. TUNEL-positive cells were scored relative to total number of cells and percentages are presented. Mean of three separate tumor sections + SD are shown. $*P < 0.0001$, $**P = 0.0012$, $***P = 0.0002$, $****P = 0.0343$ by the mixed linear model (for Patient 8, $P = 0.077$ by the paired t-test). *(Adapted from Genes Cancer 2018; 9(5–6):190–197. doi:* https://doi.org/10.18632/genesandcancer.181*)*

activate autophagy constitutively through metabolic reprogramming in order to maintain cellular homeostasis given that growth signal autonomy and insensitivity to growth inhibitory signals render them deficient in growth arrest [25–27]. Constitutive activation of autophagy has been linked to treatment resistance [28, 29]. Autophagy can also protect cells from undergoing apoptosis or programmed cell death. Furthermore, certain mutations render the cancer cell dependent on autophagy. For example, mutations in the RAS pathway are often associated with high levels of autophagy inside the tumor cells to maintain metabolism in pancreatic cancer, non-small cell lung cancer and melanoma that has high frequency of BRAF V600E mutation [30, 31]. In colon cancer, functional JUN N-terminal kinase was required for hypoxia-induced autophagy [32]. Autophagy supports breast cancer stem cell growth by regulating IL-6 secretion and signal transduction and activator of transcription 3 (STAT3) as a marker for cell dependence on autophagy for growth [33].

Targeting autophagy in cancer has been the basis of numerous clinical trials since Amaravadi et al. [16] reported that the anti-tumor effects of anti-cancer drugs improved when used in combination with either genetic or pharmacologically inhibited autophagy activity employing genetically engineered mouse model or patient-derived xenograft models.

5.3 Autophagy Inhibition by Anti-Malaria Drugs

Autophagy can be targeted at different stages through different mechanisms. Anti-malarial drugs chloroquine and hydroxychloroquine are well-known for their anti-autophagy effects. They function primarily by interfering with lysosome activities by acidifying them [34–36]. At neutral pH, both CQ and HCQ remain unchanged and are able to diffuse across the cell membrane freely. However, in acidic environments like inside a tumor, CQ and HCQ become protonated and get trapped inside the lysosome causing them to acidify. Acidified lysosomes are not optimal environment for its degradative enzymes. Therefore, autophagy stalls at the last step before protein degradation. Cells treated with CQ or HCQ exhibit vesicular organelle accumulation and blocked autophagy process.

6 Early Phase Clinical Trials in Oncology

Early phase or first-in-human trials are key steps in drug development, representing the first administration of a new drug, a new indication (i.e., repurposing), or drug combination in humans [37]. In oncology, these trials are either performed in patients with advanced cancer who have no other therapeutic options available or in patients with early stage cancer who do not need subsequent treatment as standard-of-care. Early phase clinical trials, especially phase I clinical trials, mainly focus on safety parameters in determination of the maximum-tolerated dose (MTD) and the recommended phase II dose (RP2D). Various biomarkers (i.e., plasma Par-4 levels) as exploratory objectives are analyzed as well [38]. The design of early phase clinical trials must be supported by a well-documented scientific rationale. Given obvious limitations (small number of participants) of phase I studies, clinical efficacy cannot be properly analyzed. Use of expansion cohorts in phase I trials is increasing in an attempt to detect potential signals of clinical efficacy. The traditional framework of drug development involving inclusion of safety evaluation in phase 1, early efficacy assessment in phase 2, and final evaluation compared to standard-of-care in phase 3, has gradually faded out [39]. In the era of precision cancer medicine (PCM), rapid phase 1 dose-escalation studies followed by large expansion cohorts are replacing traditional phase 1 and phase 2 studies. In parallel, to optimize the process of biomarker-driven drug development, new trials are emerging, for example, adaptive studies with basket and umbrella designs [40].

7 HCQ Used as a Par-4 Secretagogue and Blocker of Autophagy in Clinical Trials

Autophagy is an important mechanism of survival and treatment resistance in various types of cancer. CQ has displayed pleiotropic mechanisms of action including inhibition of autophagy and normalization of tumor vasculature [6–8]. CQ induces cytotoxic effects in tumors by blocking autophagy, but in mouse pancreatic tumors containing oncogenic K-Ras and lacking functional p53, loss of autophagy accelerates tumor progression [41]. The presence of a hydroxyl group at the N-ethyl substituent makes HCQ different from CQ. Despite different biological structure, HCQ has pharmacokinetics similar to CQ, but with quick gastrointestinal absorption [42, 43]. More importantly, HCQ elimination is mainly from the kidney. Side effects on short-term usage are minimal [44]. These biological features make HCQ an ideal choice for repurposing in clinical trials. Several recent clinical trials were performed using HCQ in combination with standard-of-care anti-cancer modalities [9]. These clinical trials focused on inhibition of autophagy by HCQ. None of them determined the relationship between HCQ-induced Par-4 levels in patient plasma or serum and apoptotic activity in response to treatment.

To test the hypothesis that HCQ is a safe and potent Par-4 secretagogue in humans and that secreted Par-4 will subsequently lead to apoptosis in diverse cancers, we reported pharmacodynamic results from a phase I, single-institution clinical trial using 14-day oral HCQ (200 or 400 mg twice daily) prior to planned surgery for patients with surgically removable early stage solid tumors. This was the first-in-human study to assess biological response of HCQ as a Par-4 secretagogue. In this single-institute clinical trial, an adaptive, nonparametric, isotonic regression model with two dose levels of HCQ was employed. Dose escalation of HCQ was based on isotonic regression to model safety and biological effect based on plasma Par-4 analysis. A total of nine patients with early stage solid malignancies were enrolled and allocated to 200 mg twice daily (dose level 1) or 400 mg twice daily (does level 2) cohorts. Four patients had prostate adenocarcinoma, two had non-small cell lung cancer (NSCLC), and the other patients had diverse malignancies, including papillary thyroid carcinoma, squamous cell carcinoma of larynx, and carcinoid tumor of lung. Six patients were in the dose level 1 cohort; three patients were in the dose level 2 cohort. No HCQ-related dose limiting toxicities or severe

Table 2 Summary of toxicities and Par-4 response. Six patients were enrolled in dose level 1 (200 mg oral twice a day) and 3 patients were enrolled in dose level 2 (400 mg oral twice a day). *(Genes Cancer 2018; 9(5–6):190–197. doi:* https://doi.org/10.18632/genesandcancer.181*)*

Dose level	Pt#	Type of cancer	Adverse events (Grade)	DLT	Par-4 (fold) Response >2	Par-4 (fold) Response >1.5–<2
1 *200 mg oral twice a day (n = 6)*	1	Papillary thyroid cancer	None	No	Yes	No
	2	Prostate adenocarcinoma	None	No	Yes	No
	3	NSCLC, squamous cell	Diarrhea (G1) Abdominal pain (G3)	No	Yes	No
2 *400 mg oral twice a day (n = 3)*	4	Squamous cell carcinoma of larynx	Floaters (G1) Anorexia (G1)	No	Yes	No
	5	Prostate adenocarcinoma	None	No	Yes	No
	6	Prostate adenocarcinoma	Blurred vision (G1) Chest pain, cardiac (G3) Thromboembolic event (G2)	No	No	Yes
1 *200 mg oral twice a day (n = 6)*	7	Prostate adenocarcinoma	None	No	No	Yes
	8	NSCLC, squamous cell	None	No	No	Yes
	9	Carcinoid cancer of lung	Nausea (G1) Urinary tract infection (G2)	No	Yes	No

DLT dose limiting toxicity, *NSCLC* non-small cell lung cancer

adverse events were observed (Table 2). Eight of the nine patients showed increased plasma Par-4 levels over basal levels after 14 days of HCQ treatment (Fig. 5a) More interestingly, a significant increase in apoptosis in resected tumors from these eight patients were observed by TUNEL assay compared to pre-HCQ treatment (Fig. 5b). Induction of p62/sequestosome-1 was revealed in resected tumors from all nine patients, which indicated autophagy inhibition by HCQ as well as HCQ dose compliance. Increase of apoptosis was not noted in one patient who failed to show any increase in plasma Par-4. However, resected tumor from this patient showed induction of p62, which is indicative of HCQ compliance. Thus, this clinical study not only confirmed HCQ as a potent inducer of Par-4, but it also showed biological effects on induction of apoptosis in tumor cells likely by HCQ-induced Par-4 secretion, but not by autophagy inhibition alone.

8 Future Clinical Trials with Par-4 Secretagogues

Given the well-documented biological effects of Par-4 secretagogues such as HCQ, a clinical study using adjuvant HCQ for cancer patients following definitive treatments for curative intent is ongoing at the University of Kentucky. Combined with biological features as autophagy inhibitors, CQ and HCQ had shown effects on delaying or overcoming resistance to enzalutamide, a second-generation androgen receptor (AR) inhibitor in a xenograft model [45]. Future clinical trials combining CQ or HCQ with enzalutamide or other AR inhibitors to delay or overcome resistance in metastatic prostate cancer are warranted. The benign side effect profile of HCQ and CQ makes this approach feasible.

HCQ, as a Par-4 secretagogue, may also function as a radiosensitizer. In glioblastoma multiforme (GBM) cell lines, Par-4 up-regulation increased apoptosis when used in combination with radiation, chemotherapy, or both [46]. Animal experiments performed using melanoma cell lines (Mel1617 and Mel1617BR) showed that Par-4 secretagogue CQ synergized with radiation therapy (unpublished data). Par-4 also shows potential inhibition of radiation-induced NF-kB and Bcl-2 expression in human prostate cancer cells [47]. These preclinical data provide a strong rationale for design of clinical studies. Localized prostate cancers are most often treated with radical prostatectomy (RP). However, 20–40% of patients undergoing RP experience biochemical recurrence (BCR) within 10 years [48, 49]. Salvage radiation alone is commonly used in these settings, but more than 50% of patients develop disease progression within 10 years [50, 51]. The role of concurrent short-term androgen deprivation adjuvant therapy or AR inhibitor with radiotherapy is still unclear [51, 52]. The concept of concurrent salvage radiation with HCQ, as both radiosensitizer and Par-4 inducer, is gaining attention as a possible clinical study at the University of Kentucky. This same concept may be applied to other malignancies when radiation is indicated (i.e., GBM, head and neck cancer, lung cancer). As an example, a clinical trial with radiation to oligo metastatic prostate cancer in combination with HCQ with the goal of delaying systemic androgen deprivation therapy (ADT) is ongoing at the University of Kentucky.

Par-4 functions via binding to its receptor GRP78 on the cell surface. GRP78 is considered a crucial regulator of endoplasmic reticulum (ER) homeostasis [53], and a master regulator of unfolded protein response (UPR). UPR is needed to facilitate peptide folding, prevent misfolding, aggregation and target misfolded protein for degradation. UPR has been

linked to cancer initiation, tumor aggressiveness, angiogenesis, and metabolic processes [54]. Cancer cells are known to have heightened UPR and high level of ER stress and UPR is critical in conferring chemotherapy resistance [55–57]. Therefore, drugs that are known to increase ER stress could be combined with a Par-4 secretagogue to curb cancer treatment resistance. (S)-crizotinib induces endoplasmic response in lung cancer cells by increasing intracellular levels of reactive oxygen species thus increasing the apoptotic response [58]. Therefore, combination therapy using HCQ and the (S)-enantiomer of crizotinib will be of interest especially in the minimal residual disease settings outlined above.

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Par-4 as a Therapeutic Target in Cancer and Other Diseases

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Abstract

The tumor suppressor Par-4 is a multi-faceted protein with intracellular cytoplasmic and nuclear functions, as well as extracellular paracrine functions. Intracellular Par-4 acts by interacting, via its Par-4 amino-terminal fragment (PAF) domain, centrally placed selective for apoptosis in cancer (SAC) domain, or carboxyl-terminal leucine zipper (LZ) domain, with other proteins to regulate downstream gene transcription or posttranslational events. These activities are integral to developmental processes in specific tissues, inhibition of cellular transformation, tumor growth and metastasis and Par-4 implements them by apoptosis-dependent or -independent mechanisms. Extracellular Par-4 functions via its cell surface GRP78 (csGRP78) receptor that allows Par-4 to trigger signaling pathways to endoplasmic reticulum (ER) stress induction and apoptosis. Moreover, both intracellular and extracellular Par-4 regulate inflammation and EMT that are associated with cancer, metastasis, and a broad range of morbidities. A number of strategies are being tested to reinstate the Par-4 regulated pathways in order to overcome diseases associated with loss of Par-4 such as cancer or schizophrenia and depression, or to impede the active involvement of Par-4 in Alzheimer's, Parkinson's, cardiac aging, diabetes, and other diseases in which Par-4 acts, in consort with other incriminating pathways, to sensitize the cells to apoptosis. This chapter addresses some of the strategies that utilize the key biochemical features of Par-4 such as transcription regulation by its LZ domain, binding or sequestration of functionally significant proteins by its LZ or PAF domain, or receptor mediated signaling by its SAC domain for effectively targeting various diseases. Moreover, as Par-4 loss is associated with therapy resistance and tumor relapse, secreted or intracellular Par-4 levels may guide prognosis and treatment decisions.

Keywords

Prostate apoptosis response-4 (Par-4) · PAWR · Arylquin-1 · Chloroquine · Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) · Apoptosis · Secretagogues · Selective for apoptosis in cancer cells (SAC) · Par-4 amino-terminal fragment (PAF) · Leucine zipper (LZ) domain · Nuclear localization signal (NLS) · Tumor suppressor · Cell surface glucose-regulated protein 78 (csGRP78) · Cancer · Alzheimer's · Stem cells · Neurodegeneration · Ocular diseases · Cardiac aging · Diabetes

1 Salient Features of Par-4

Par-4 protein is ubiquitously expressed in various normal tissues and cell types [1]. Par-4 has been shown to play an integral role in the process of metamorphosis of the tadpole tale [2], development of the salivary gland [3], or the eye [4, 5], and involution of the prostate [1] in conjunction with other co-parallel cues. This is exemplified, for example, by the upregulation of Par-4 in the tadpole tail that coincides with elevation in thyroid hormone levels to cause apoptosis of the myoblasts and resorption of the tail [2]. Similarly, Par-4 is induced in the secretory epithelial compartment of the prostate, and Par-4 sensitizes these luminal cells to apoptosis fol-

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lowing testosterone depletion in castrated rats or mice. It is important to note that this process of apoptosis is blocked in Par-4-null mice, implying a functional role for Par-4 in apoptosis and involution of the prostate after testosterone depletion [1, 6, 7]. This observation is particularly relevant as androgen-deprivation is the mainstay for prostate cancer treatment. Moreover, intracellular Par-4 is phosphorylated by chemotherapeutic agents that induce phosphorylation of its T163 (T155 in rat Par-4) residue in the SAC domain leading to Par-4 activation and its translocation into the nucleus to induce apoptosis [8].

Although the Par-4 gene is induced at the RNA level by apoptotic insults [9], full-length Par-4 protein is often transiently induced and short-lived, and this may in part be due to downstream activation of caspase-8 and caspase-3 by Par-4 that subsequently results in cleavage of Par-4 at D131 residue. The carboxyl-terminal 132–340 amino acid fragment of cleaved Par-4 translocates into the nucleus and induces apoptosis [10–14]. The amino-terminal fragment (designated PAF, 1–131 amino acids) is primarily secreted and induces paracrine caspase-8/caspase-3 activation in cancer cells that are resistant to therapy, as well as those that are resistant to full-length Par-4 [12].

As Akt1 phosphorylates human as well as rat Par-4 protein and subsequently the chaperone 14–3-3 sequesters Par-4 in the cytoplasm [15], and Par-4 indirectly inhibits Akt1 through inhibition of ζPKC [16], Akt inhibitors may have applications in nuclear translocation of Par-4. In the nucleus, activated Par-4 interacts directly with a number of proteins such as topoisomerase-1 to inhibit DNA relaxation [17], THAP1 to regulate gene splicing events [18, 19], or WT1 to inhibit transcription of the anti-apoptotic gene Bcl-2 [20–25]. There is evidence that Par-4 may interact with other transcription factors such as E2F1 [26] or with chromatin modulators such as SMARCA4 [2], and such interactions may potentially modulate global changes in gene expression events.

The heterodimeric transcription factor NF-κB, which is involved in inflammation as well as anti-apoptosis in cancer cells, is another important target of Par-4 [27, 28]. Inhibition of NF-κB occurs in a context dependent manner either in the cytoplasm following Par-4 interaction with ζPKC that prevents NF-κB activation and it's translocation into the nucleus, or inhibition of NF-κB-dependent transcription of cell survival IAP genes such as XIAP and cIAP [27–30]. In addition, other targets signify the role of Par-4 in regulation of EMT [31–33], metastasis [34, 35], as well as its apoptotic and autophagic regulatory function [2, 35]. As Par-4 contains a nuclear export sequence embedded in its LZ domain, retention of Par-4 in the nucleus using selective inhibitors of nuclear export is a promising strategy to block pancreatic cancer cell proliferation and reduce tumor growth [36]. This strategy utilizing drugs such as KPT-185 that inhibit the binding of exportin 1 or chromosomal region maintenance 1 (CRM1) to Par-4 and prevent Par-4 nuclear export is expected to have broad applications in view of the nuclear apoptotic function of Par-4 in diverse cancers [37]. It is particularly noteworthy that while, on the one hand, Par-4 serves as a tumor suppressor protein dependent on its ability to induce apoptosis, it also plays a role as an inhibitor of cellular transformation or metastasis, and these latter functions are executed by either apoptosis-dependent or independent mechanisms [23, 24, 34, 38, 39].

In addition to its intracellular role, Par-4 protein is also secreted [40]. Although the ability to secrete Par-4 appears to be attenuated in cancer cells, chronic lymphocytic leukemia (CLL) cells show deregulated expression and upregulated secretion of Par-4 [41]. As Par-4 secreted from these cancer cells is functional in inducing paracrine apoptosis of other epithelial tumor cells, it is likely that the csGRP78 receptor or its downstream signaling pathway is blocked to prevent apoptosis of the cells. On the other hand, the intracellular pathway for NF-κB inhibition, apoptosis and growth inhibition following overexpression of Par-4 is intact in CLL [42]. In fact, although most cancer cells show upregulation of csGRP78 owing to sustained genetic aberrations and ER-stress [40, 43], treatment with Par-4 results in apoptosis of only a segment of the cancer population. This selective action may be attributed to downmodulation of csGRP78 as a mechanism activated by cancer cells for survival. Reinstating csGRP78 levels is therefore an important strategy to sensitize cancer cells to apoptosis by extracellular Par-4. Most, albeit not all, normal cells express low levels of cell surface GRP78 that are below the threshold to induce apoptosis, thereby rendering them refractory to apoptosis by extracellular Par-4 [29, 40]. Collectively, these observations imply that Par-4 upregulation may allow selective inhibition of inflammation, transformation, tumor growth and metastasis. As upregulation of Par-4 on its own does not induce apoptosis in normal cells [8, 44, 45], Par-4 therapy may be an ideal approach against cancer. However, as upregulation of Par-4 sensitizes normal cells to the action of other apoptotic agents [8, 46], it is important to take a holistic approach and monitor the effects of Par-4 in other normal tissues, especially neuronal or pancreatic islet β-cells that are prone to erosion associated with Par-4 in conjunction with beta-amyloid protein or high glucose/fat diet, respectively [46–48].

2 Par-4 Gene Therapy in Cancer

The first set of experiments indicating that Par-4 upregulation can inhibit the growth of tumors were performed in PC-3 prostate cancer cell-derived xenografts [38]. A biosafe "gut-less" ψ5 adenovirus that produced full-length Par-4 or the empty adenovirus vector as control was introduced into

tumors in the flanks of nude mice as a first step toward testing whether Par-4 is suitable for tumor growth inhibition. These experiments produced over 80% inhibition of tumor volume with a single intra-tumoral injection of the Par-4-producing adenovirus relative to the control adenovirus. Elevated levels of intracellular Par-4 not only activated the FasL/Fas extrinsic apoptotic pathway, but also inhibited the cell survival NF-κB induced pathway leading to reduction in tumor growth. This unexpected level of tumor reduction was especially remarkable because a parallel series of experiments using a green fluorescent protein (GFP)-producing ψ5 adenovirus indicated that the tumor transduction frequency never exceeded 40% of the tumor cells. Similarly, a single injection of the Par-4-producing ψ5 adenovirus induced more than 50% tumor growth inhibition in orthotopic tumors derived from aggressively growing RM1 cells in the C57BL/6 mouse prostate [2]. As the adenovirus used was replication- and integration-deficient and its expression was expected to diminish as the tumor cells replicated, these observations indicated a bystander effect associated with Par-4 upregulation in tumors [38]. Almost a decade later, these findings were explained by the discovery that Par-4 protein is secreted and that secreted Par-4 inhibits the growth of tumors [40, 49]. Thus, in retrospection, both intracellular and extracellular Par-4 produced by the Par-4-producing adenovirus must have cooperated to produce massive apoptosis and reduction of tumor growth noted by Chakraborthy and colleagues [38].

The safety of Par-4 overexpression was apparent in our transgenic mice that overexpressed Par-4 or its SAC domain ubiquitously in all tissues [40, 45, 49, 50]. These mice survived longer than control mice, and especially the SAC domain transgenic mice failed to develop spontaneous or oncogene-inducible tumors (33). Moreover, these mice resisted the growth of non-autochthonous mouse tumors in their flanks, confirming the tumor growth inhibitory role of Par-4 secreted by normal cells [49].

Based on the above findings, and the fact that Par-4 is a generic tumor suppressor that induces apoptosis in diverse cancer cells, adenoviruses (AVs) or adeno-associated viruses (AAVs) that produce full-length human Par-4, or its functionally important domains, such as PAF, SAC, or including both SAC + LZ may be effective in inducing apoptosis and growth inhibitory effects in primary and metastatic tumors. Moreover, the ΔNLS1 mutant of Par-4 may readily translocate into the nucleus to induce robust apoptosis. Full-length Par-4, the ΔNLS1 mutant, SAC domain, and 132–340 amino acids fragment of Par-4 are expected to regulate gene transcription events. Full-length Par-4 and PAF are primarily secreted and trigger paracrine apoptosis. High-capacity AVs and particularly those that can be regulated appear to exhibit superior effects owing to evasion of degradation by the immune system, thereby allowing viral longevity [51]. Alternately, bioengineered AAV vectors have experienced clinical success due to their superior safety profile and high gene delivery efficacy [52]. Regardless of the delivery system, intra-tumoral overexpression of Par-4 may be most efficient as the tumor will be subject to the effects of both intracellular and extracellular Par-4. As intra-tumoral delivery may not always be feasible, another approach involves injection of the AVs or AAVs into normal tissue to cause secretion of Par-4 that may target metastasis via systemic circulation. In either case, the effect may be augmented by co-parallel treatment of the tumor with radiation or a drug that might selectively stimulate the upregulation of csGRP78 in the tumor cells. As full-length Par-4 has been shown to sensitize normal cells to the action of chemotherapeutic agents, the use of targeted therapy is a safer option in conjunction with Par-4 produced by AVs or AAVs.

3 Effective Utilization of Recombinant Par-4

Recombinant Par-4 produced and purified from bacteria or baculoviral constructs in insect cells is effective in inhibition of metastatic tumor growth [12, 49]. Dr. Zhan and colleagues [53] have further increased the efficacy of recombinant Par-4 by stabilizing the protein in vivo using an Fc-tag. Recombinant Par-4 has been found to be safe in mouse models over extended periods of injection, implying that similar to the observations with Par-4/SAC transgenic mice, extracellular Par-4 does not have any obvious harmful effects in mice. Further genetic engineering of the protein expression constructs and the use of Par-4 mutants that may be more efficient than full-length Par-4 are likely to provide excellent metastasis inhibitor effects. Moreover, as most normal cells lack csGRP78, recombinant Par-4 may be suitable for sensitization of tumors to the action of either radiation or targeted drugs such as enzalutamide or abiraterone in prostate cancer.

4 Par-4 Secretagogues

A number of new compounds such as Nutlin-3a, Arylquin-1 and other arylquin/3-arylquinoline family members, natural products such as withaferin A and curcumin, and FDA-approved drugs such as chloroquine and hydroxychloroquine have been shown to induce the secretion of Par-4 from normal cells [2, 40, 54, 55]. Cancer cells do not secrete Par-4 in response to these secretagogues as the secretory pathway appears to be subdued in most, albeit not all cancer cells, as a defense mechanism against autocrine apoptosis by secreted Par-4. Most normal cells, on the other hand, do not express csGRP78 and are resistant to the action of secreted Par-4.

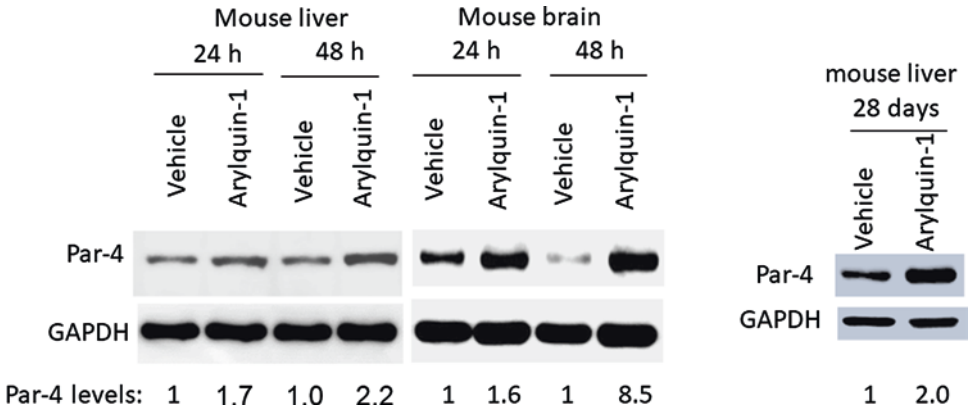

Fig. 1 Arylquin-1 is safely tolerated and induces Par-4 in mouse tissues. C57BL/6 mice were treated with Arylquin-1 (10 mg/kg body weight) for the indicated time periods and Par-4 expression was examined in whole-tissue lysates of the liver and brain by Western blot analysis. Par-4 levels normalized to GAPDH levels are indicated. Note induction of Par-4 in both short-term and long-term treatments

These features ensure the differential effects of Par-4 secretagogues in normal and cancer cells- Par-4 secretion is induced from normal cells by Par-4 secretagogues and this results in paracrine apoptosis of cancer cells. Our mouse studies [34] indicated that metastatic tumors arising in vascular tissues, such as the lungs, are more vulnerable to the apoptotic effects of secreted Par-4. Moreover, Par-4 secreted by normal cells of the host is expected to have the posttranslational modifications that are not otherwise apparent in recombinant Par-4 produced in bacteria. In fact, our clinical trials have indicated that Par-4 secretion from normal cells is induced by HCQ, and that secreted Par-4 is associated with remarkably high levels of apoptosis in diverse primary tumors in cancer patients [39]. These results offer optimism for combining Par-4 secretagogues with standard-of-care treatments to utilize secreted Par-4 as either a radiosensitizer or sensitizer to hormonal therapy (such as with enzalutamide) that is specifically targeted against the tumor. It is important to note that HCQ also functions as an inhibitor of autophagy in tumors and may generate synergistic growth inhibitory effects with secreted Par-4 and radiation treatment of tumor.

Unlike other Par-4 secretagogues, Arylquin-1 induces robust intracellular Par-4 levels in normal tissues (Fig. 1). Daily treatment of mice with Arylquin-1 for 28 days indicated that Arylquin-1 induces Par-4, yet neither does it reduce mouse weight, nor does it harm normal cells or vital organ functions. Food consumption in mice is also unaffected by Arylquin-1 treatment. Importantly, Arylquin-1 treatment inhibits the growth of metastatic tumors in the mouse lungs (Fig. 2). Our studies have indicated that Arylquin-1 works in a three-pronged fashion, by secretion of Par-4 from normal cells, by induction of apoptosis in tumor cells, and by inhibition of metastasis by binding to vimentin, and is therefore a good candidate for drug development as an inhibitor of cancer metastasis. Moreover, molecules such as Arylquin-1 that induce Par-4 expression in normal cells deserve more attention for prevention of primary or metastatic tumors.

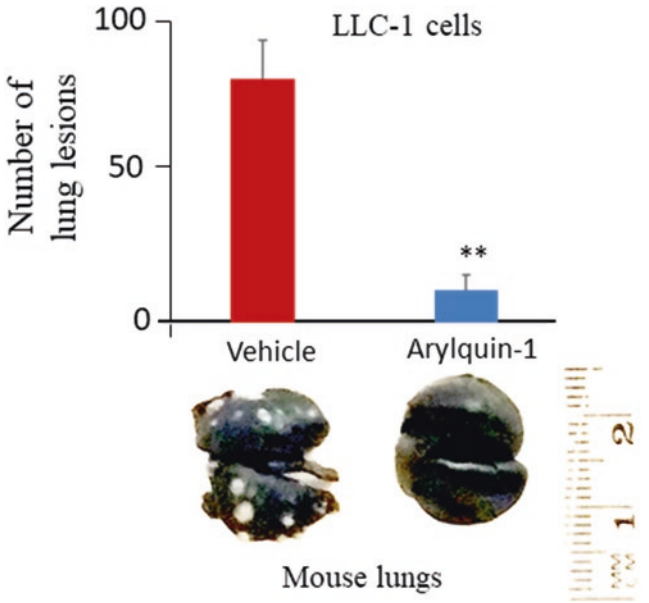

Fig. 2 Arylquin-1 inhibits the growth of lung metastasis. LLC-1 cells (300,000 cells) were injected into C57BL/6 mice through the tail vein, and beginning at 24 h thereafter, the mice were injected i.p. with vehicle or Arylquin-1 (10 mg/kg body weight), once daily for 15 days ($n = 6$ mice in each group). Lungs were resected, perfused, stained with India ink and tumor nodules were scored. Mean ± SD are shown in upper panel and India ink stained lungs are shown in lower panel. $^{**}P < 0.01$ by Student's t-test

5 Inducers of Par-4 Receptor GRP78 on the Cell Surface

The Par-4 receptor csGRP78 is expressed on cancer cells but not on most normal cells. Several lines of evidence indicate that csGRP78 is a targetable receptor [56–58]. It is obvious from our Par-4 transgenic mice that overexpress Par-4 ubiquitously, and from mouse experiments with recombinant Par-4, as well as with Par-4 secretagogue HCQ in cancer patients that normal cells are not harmed by secreted Par-4. This implies that even if some of the normal cells may express csGRP78, secreted Par-4 either does not harm those cells or

those cells are dispensable. In addition to using secretagogues of Par-4, it is important to elevate csGRP78 specifically on cancer cells. Our initial studies have identified a family of FDA-approved drugs that induce csGRP78 in cancer cells but not in normal cells. Further studies to confirm the selective action of these drugs in cancer cells may allow their use to selectively kill cancer cells in conjunction with secreted Par-4 induced by secretagogues. Besides secreted Par-4, csGRP78 may be targeted by antibodies against its carboxyl-terminus. Previous studies have suggested that such antibodies serve as agonists to inhibit the growth of tumor cells [57, 58]. Alternately, genetically engineered CAR-T cells that express csGRP78 agonist antibody, that overexpress Par-4 for secretion, or that display Par-4 at their cell membrane may serve as effective cell therapy against tumors.

6 Par-4 Expression as an Indicator of Tumor Relapse

A number of reports in literature assert that Par-4 loss contributes to tumor recurrence following therapy and affects patient survival [2, 59]. The presence of cytoplasmic versus nuclear Par-4 may also be a harbinger of response to therapy and tumor relapse. Moreover, as Par-4 is secreted and full-length Par-4 protein is detectable in the plasma or serum samples, it is important to determine whether Par-4 levels in circulation truly reflect the ability of the individual to prevent malignant growth of pre-clinical tumors or relapse of primary or metastatic tumors. Such studies will require large cohorts of normal individuals and cancer patients at various stages of cancer development. As Par-4 levels may be influenced by glucose and fat levels in diet, smoking, alcohol, genetic factors, comorbidities, and associated treatments, a thorough record of these variables will be essential in assessing the significance of circulating Par-4 as a gauge of treatment response and outcome.

7 Approaches to Modulate Par-4 in Neuronal and Other Diseases

Several lines of evidence suggest that Par-4 plays a critical role in promoting cell death in neurodegenerative diseases. Elsherbini and Bieberich [60] and Guo et al. [61] have described mechanisms by which Par-4 sensitizes neurons to apoptosis in various experimental models of Alzheimer's disease. The apoptosis antagonizing transcription factor (AATF) binds to Par-4 via its leucine zipper domain and confers neuroprotection by preventing the binding of Par-4 to the amyloid β and β-amyloid precursor protein (APP) intracellular domain (AICD). Prevention of this interaction of Par-4 with AICD using either an LZ domain peptide of Par-4 or AATF, or a small core peptide from TRL-4 binding region of AATF may block the effects of Par-4.

Par-4 levels are increased in midbrain dopaminergic neurons of monkeys and mice exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine prior to loss of tyrosine hydroxylase immunoreactivity and dopaminergic neuronal death [61]. As blockade of Par-4 induction prevents neuronal cell death in these model systems, approaches using small molecules, peptides, or RNA-interference that prevent Par-4 levels from increasing in response to local cues need further investigation for long-term safety and effectiveness.

Interestingly, Par-4 has been suggested to constitute a molecular link between impaired dopamine signaling and depression, as Par-4 directly interacts with the dopamine D2 receptor [62]. Lack of interaction between the LZ domain of Par-4 and the dopamine D2 receptor showed an enhanced dopamine-cAMP-CREB signaling pathway in neurons with impairment of dopamine signaling and increased depression [62]. These results suggest that Par-4 functions to link dopamine signaling and depression. Par-4, dopamine D2 receptor, and calmodulin protein levels are critical in schizophrenia, major depression, and bipolar disorder. Par-4 levels are lower in schizophrenia and bipolar disorder relative to normal controls, and Par-4 decreased by 67% in individuals with major depression relative to normal controls. These observations need careful evaluation in the context of the apoptotic function of Par-4 and its link with dopamine signaling pathways in schizophrenia, major depression, and bipolar disorder [63]. A better understanding of the nuanced relationship between Par-4 and these pathways will enable the design of strategies to precisely modulate Par-4 levels to thwart these disorders.

Apoptosis in cardiomyocytes contributes to cardiac remodeling after myocardial infarction (MI). A recent study has suggested that circular RNA circ_0068655 soaks up endogenous miR-498 to inhibit its activity and increase Par-4/PAWR expression [64]. Experiments at the University of Toronto suggest that the microRNA miR-17-3p exerts a fundamental role in heart development and functions by targeting Par-4 to inhibit mouse cardiac fibroblast senescence [65]. Repression of Par-4 by miR-17-3p elevates the transcription of CEBPB and FAK to downregulate E-cadherin and promote upregulation of N-cadherin, vimentin, Oct4, and Sca-1, for self-renewal. Similarly, an unbiased assessment of circular RNAs, miRNAs, or peptides that regulate Par-4 expression in pancreatic β-cells may provide insights for targeting Par-4 and inhibition of apoptosis associated with type 2 diabetes.

8 Conclusions and Future Perspectives

In conclusion, numerous studies have reiterated that Par-4 is a modular, multi-faceted protein. Par-4 functions as a transcriptional co-regulator to either repress or activate gene

transcription events depending on the cellular context. Alternately, Par-4 regulates downstream events by binding to diverse other proteins through its PAF domain, SAC domain, or LZ domain. These biochemical changes activated by Par-4 result in distinct phenotypic alterations that include apoptosis induction, autophagy induction or inhibition dependent on the co-parallel cues and context, and inhibition of inflammation and EMT. Par-4 expression or function is compromised in most tumors, depression, schizophrenia, and bipolar disorder, but Par-4 activation contributes to neuronal degeneration, cardiac aging, and pancreatic β-cell apoptosis in type 2 diabetes. As Par-4 is downregulated, inactivated or mutated in tumors, reinstating functional levels of Par-4 in tumors and/or in circulation is offered as a key strategy to inhibit the growth of various tumors (Fig. 3). Multiple approaches that are considered for replenishing Par-4 levels include: (a) gene therapy using AVs or AAVs to express various domains of Par-4, (b) recombinant Par-4, (c) secretagogues of Par-4, and (d) retention of Par-4 in the nucleus. Other relatively underexplored approaches, include miRNAs or siRNAs against Par-4 regulators such as UACA [66], or circular RNAs [67], and specific regulators of the Par-4 promoter [68] to induce Par-4 gene expression. Such approaches may also include the use of nanoparticles, such as nanoliposomes that are effective in the delivery of Par-4 plasmid into tumors [69]. Moreover, natural products and dietary supplements such as withaferin A, flavonoids, 3,3′-Diindolylmethane derived from indole-3-carbinol found in cruciferous vegetables, or the AKT1 inhibitor phenylbutyl isoselenocyanate (which was derived through extensive structure-activity studies from naturally occurring phenylalkyl isothiocyanates) that are suggested to induce apoptosis in cancer cells through a Par-4-based mechanism deserve further studies [70–73]. On the flip side, a reverse approach is necessary in neurodegenerative diseases including Alzheimer's and Parkinson's, cardiac aging, and pancreatic islet β-cell degeneration to subdue Par-4 expression or function (Fig. 3). Moreover, careful modulation of Par-4 in schizophrenia and depression is necessary in view of the delicate balance between Par-4 replenishment to overcome these episodes and preventing apoptosis of the neurons (Fig. 3). The prognostic significance of both circulating Par-4 levels and cytoplasmic/nuclear compartmentalization of intra-tumoral Par-4 needs extra attention to recognize the value of Par-4 in cancer prognosis. Thus, Par-4 that was first discovered in prostate cancer cells undergoing apoptosis [74], is ubiquitously expressed in various tissues and cell types, and serves as a therapeutic target in diverse diseases.

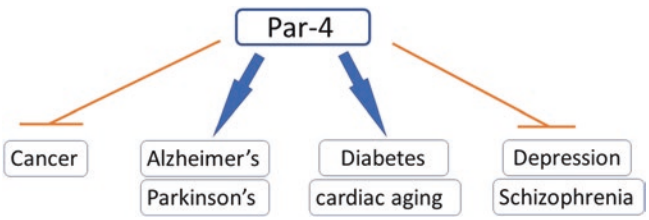

Fig. 3 Par-4 suppresses cancer, depression, and schizophrenia, but promotes neuronal diseases, diabetes, and cardiac aging. Par-4 is downregulated, mutated, or inactivated in cancer and replenishment of functional Par-4 can induce apoptosis and inhibit tumor growth and metastasis. Similarly, loss of Par-4 function is associated with depression and schizophrenia, and restoration of functional Par-4 may counteract these disorders. On the other hand, Par-4 pro-apoptotic function is causally associated with Alzheimer's disease, Parkinson's disease, diabetes and cardiac aging, and strategies to inhibit Par-4 function may alleviate these diseases

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Conflict of Interest VMR is founder of Parcure, LLC, a startup with interest in developing Par-4-based therapies for cancer and other diseases.

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Correction to: Involvement of Par-4 in Breast Cancer

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Correction to:
Chapter 7 in: V. M. Rangnekar (ed.), *Tumor Suppressor Par-4*,
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After initial publication of the book, various errors were identified in chapter 7, *Involvement of Par-4 in Breast Cancer*, that needed correction. The following corrections have been made in the updated version of the chapter.

1. The word "FOXOa3" has been changed to "Foxo3a" throughout the chapter.
2. The word "NFkB" has been changed to "NF-κB" throughout the chapter.
3. In figure 4, the word "NFkB" has been changed to "NF-κB".
4. In figure 6, the text "Golgi to ER" has been changed to "ER to Golgi".
5. In figure 7, the text "Golgi/ER/GRP78" has been changed to "ER/Golgi/GRP78" and word "FOXOa3" has been changed to "Foxo3a". Also, the repetition of protein name CTNNB1 is excluded and has been mentioned only once.
6. In figure 2, the text in Luminal B subtype "ER-/PR-/+/HER2-/+High Ki67 index" has been changed to "ER+/-/PR-/+/HER2-/+High Ki67 index".
7. In figure 5, the repetition of protein name "Ki67" is excluded and has been mentioned only once.

The updated online version of this chapter can be found at:
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Index

V. M. Rangnekar (ed.), *Tumor Suppressor Par-4*, https://doi.org/10.1007/978-3-030-80558-6