

Review Article

Glycogen synthase kinase-3 β is a pivotal mediator of cancer invasion and resistance to therapy

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Tumor cell invasion and resistance to therapy are the most intractable biological characteristics of cancer and, therefore, the most challenging for current cancer research and treatment paradigms. Refractory cancers, including pancreatic cancer and glioblastoma, show an inextricable association between the highly invasive behavior of tumor cells and their resistance to chemotherapy, radiotherapy and targeted therapies. These aggressive properties of cancer share distinct cellular pathways that are connected to each other by several molecular hubs. There is increasing evidence to show that glycogen synthase kinase (GSK)-3 β is aberrantly activated in various cancer types and this has emerged as a potential therapeutic target. In many but not all cancer types, aberrant GSK3 β sustains the survival, immortalization, proliferation and invasion of tumor cells, while also rendering them insensitive or resistant to chemotherapeutic agents and radiation. Here we review studies that describe associations between therapeutic stimuli/resistance and the induction of pro-invasive phenotypes in various cancer types. Such cancers are largely responsive to treatment that targets GSK3 β . This review focuses on the role of GSK3 β as a molecular hub that connects pathways responsible for tumor invasion and resistance to therapy, thus highlighting its potential as a major cancer therapeutic target. We also discuss the putative involvement of GSK3 β in determining tumor cell stemness that underpins both tumor invasion and therapy resistance, leading to intractable and refractory cancer with dismal patient outcomes.

One of the well-recognized but still poorly understood characteristics of cancer is its ability to adapt and survive in a harsh microenvironment. Tumor cells survive the hypoxic and starved conditions by changing their morphological and biological properties and by interacting with tumor-associated stromal components.⁽¹⁾ Another striking characteristic of cancers is their ability to invade host organs and to resist therapeutic insults, thus limiting the success of curative tumor resection and leading to tumor metastasis and therapy failure. These pathological behaviors of cancer are associated with acquisition of the hallmark biological traits of cancer.^(2–4) Despite recent advances in cancer treatments, the existence of unresectable and recurrent cancers that share the persistent capacity for invasion, metastasis and therapy resistance remains a challenge for current medical therapies.^(5,6) Although our knowledge of the molecular and biological mechanisms by which cancer evolves to the refractory stage is increasing,⁽²⁾ few strategies have been established to attenuate the ability of tumors to invade or to prevent the failure of therapy.

Modern options for cancer treatment consist of surgery, cytotoxic or cytostatic chemotherapeutics, radiation, molecular-

targeted and immunomodulating agents, and their multidisciplinary combination.^(7,8) These treatments aim primarily to reduce tumor cell survival and proliferation, but not to eliminate invasive ability or resistance to therapy. Therefore, understanding the pathways by which cancer cells acquire both invasive and therapy-resistant phenotypes is critical for the development of more efficient therapeutic strategies against refractory cancers and, therefore, improvements in patient survival. Apart from the known mediators of invasion and therapy resistance [reviewed in Alexander and Friedl⁽⁹⁾], one emerging candidate is glycogen synthase kinase (GSK)-3 β . This molecule has been extensively implicated in critical cell biology processes and has causal roles in common diseases including glucose intolerance, neurodegenerative disorders and cancer.^(10–12) Here we review previous studies that have reported on the association between therapeutic stimuli/resistance and induction of pro-invasive phenotypes in various cancer types. Many of these cancers have proven to be responsive to experimental treatment which targets GSK3 β . This review focuses on the role of GSK3 β as a molecular hub that connects pathways responsible for tumor invasion and resistance to

therapy, thus highlighting this kinase as a promising multipurpose cancer therapeutic target. It also discusses a putative role for GSK3 β in sustaining the tumor cell “stemness” that is central to both tumor invasion and therapy resistance, thereby leading to intractable cancer and resulting in treatment failure.^(13–15)

Invasion and therapy resistance co-segregate in refractory cancer

High invasiveness and resistance to therapy are common biological and clinical characteristics of refractory cancer that represent major challenges for research and treatment. The mechanisms involved in tumor invasion include cell motility and migration, degradation of the extracellular matrix and interaction with stromal and inflammatory cells. These are orchestrated in a way that enables the tumor to invade the host organ and often beyond.^(3,4) An early morphological and functional change for tumor cells of epithelial origin to acquire a proinvasive phenotype is epithelial to mesenchymal transition (EMT), altering cell behavior to resemble a mesenchymal type.^(16,17) The major modes by which cancers evade the effect of anti-cancer drugs include intrinsic (or constitutive) resistance and acquired resistance. Constitutive resistance to therapy may be due to mutational activation of signaling for cell survival, cytoprotective alterations in the cell cycle and in DNA repair ability, differences in the efficiency of drug uptake and efflux by cancer cells, and insufficient drug delivery [reviewed in Alexander and Friedl⁽⁹⁾]. Acquired resistance to chemotherapeutic and molecular-targeted agents involves distinct mechanisms, including genetic alterations to the drug targets, activation of surrogate prosurvival pathways, and interactions between tumor cells and components of the tumor environment [reviewed in Holohan *et al.*⁽⁵⁾, Ramos and Bentires-Alj⁽⁶⁾ and Alexander and Friedl⁽⁹⁾]. Intra-tumor heterogeneity emerges in many tumors and often underlies their resistance to therapeutic agents.^(18,19)

The processes of tumor invasion and therapy resistance and their underlying mechanisms are often investigated as independent pathological events in cancer. However, recent studies (shown in Table 1) have demonstrated that tumor cells acquire morphological and functional proinvasive phenotypes with the ability to migrate and invade following the development of resistance to anti-cancer therapeutics (Suppl. References [SR] 1–24) (Data S1).^(SR1–24) The therapeutic modalities included conventional chemotherapeutic agents^(SR1–15), different types of radiation therapy^(SR16–20) as well as bioactive compounds targeting epidermal growth factor (EGF) receptor and vascular endothelial growth factor (VEGF).^(SR21–24) These studies indicate the therapeutic insult and/or resistance elicits proinvasive phenotypes and evokes the invasive capability of tumor cells in a broad spectrum of cancer types, including breast, colorectal, pancreatic, ovarian and prostate cancers, as well as rare intractable tumors (e.g. glioblastoma and osteosarcoma). A large number of preclinical studies have demonstrated that all available cancer treatments, including surgery, facilitate metastatic tumor spread. Such treatment often results in therapeutic benefit, but occasionally also results in resistance, leading to the paradoxical concept of “treatment-induced metastasis” [reviewed in Ebos⁽²⁰⁾]. These experimental and preclinical studies suggest that the invasive behavior of cancer cells and their acquired resistance to therapy may not be separate pathological properties and could, instead, represent interconnected processes [reviewed in Alexander and Friedl⁽⁹⁾].

Regardless of the tumor type and therapeutic agent, the proposed mechanisms for invasion and resistance are attributable to molecular pathways that participate in tumor cell survival, transition to mesenchymal (EMT) and cancer stem cell (CSC) phenotypes, migration and invasion with extracellular matrix degradation, and drug efflux (Table 1). The reported molecules that mediate and interconnect these pathways include growth and transcription factors, chemokines, RAS and Rho family members (e.g. Ras, Rac1) and cell-matrix adhesion molecules (e.g. integrin family and focal adhesion kinase [FAK]) (Table 1).⁽⁸⁾ Bevacizumab is a humanized monoclonal antibody to VEGF used clinically as an anti-angiogenic agent. Acquired resistance to bevacizumab leads to enhanced tumor cell invasion due to the metabolic shift to glycolysis and degradation and remodeling of tumor stromal tissues.^(SR21–24) A better understanding of the biological mechanisms underlying the tight association between tumor invasion and therapy resistance should provide a solid rationale for the development of innovative cancer treatments.⁽⁹⁾

Aberrant GSK3 β in cancer

GSK3 is a family of serine/threonine protein kinases comprising two highly conserved isoforms, GSK3 α and GSK3 β , that show approximately 85% overall homology and 98% homology in their kinase domains. GSK3 is constitutively active in normal cells and its activity is regulated by the differential phosphorylation at serine (S) residues 21 (pGSK3 α ^{S21}) and 9 (pGSK3 β ^{S9}) (both inactive forms), and tyrosine (Y) residues 279 (pGSK3 α ^{Y279}) and 216 (pGSK3 β ^{Y216}) (both active forms) (Fig. 1a,b). GSK3 regulates a diverse array of physiological cellular functions via the phosphorylation of and interaction with various proteins (Fig. 1c). Although the two GSK3 isoforms share many substrates, they are not functionally identical and show some differences in their substrate specificity [reviewed in Beurel *et al.*⁽¹⁰⁾]. Negative regulation of GSK3 activity is desirable to maintain physiological cellular functions. A growing number of studies have demonstrated that deregulated GSK3 activity contributes to the pathogenesis and progression of various diseases, including glucose intolerance, neurodegenerative disorders, and chronic inflammatory and immunological diseases.^(10,21) This points to GSK3 being an attractive and druggable target for a broad spectrum of diseases.⁽²²⁾ Consequently, many GSK3 inhibitor compounds have been developed in academic and pharmaceutical institutes over recent years. Some are reported to inhibit both the α and β isoforms with different affinities,^(SR25–28) while others are specific for GSK3 β (reviewed in SR25,26). Among the latter compounds, it was reported that AR-A014418 does not inhibit the activity of 26 closely related kinases and is, therefore, considered to be highly specific for GSK3 β .^(SR29)

Of the two GSK3 isoforms, most studies in the field of oncology have focused on GSK3 β . This is partly because of the functional redundancy of the two isoforms in regulating the canonical Wnt/ β -catenin pathway, responsible for generating the most prevalent oncogenic signaling.^(23,24) Based on its known effects on several proto-oncoproteins (e.g. β -catenin, cyclin D1 and c-Myc), cell cycle regulators (e.g. p27^{Kip1}) and mediators of EMT (e.g. snail) (Fig. 1c), it has long been recognized that GSK3 β suppresses tumor development [reviewed in Clevers and Nusse,⁽²⁴⁾ Jope *et al.*,⁽²⁵⁾ Luo⁽²⁶⁾ and McCubrey *et al.*⁽²⁷⁾], as discussed later (Table S1).^(SR30–58) Paradoxically, our earlier studies found increased expression and deregulated activity of GSK3 β due to changes in the differential

Table 1. Representative previous studies showing interrelationship between therapeutic stimuli/resistance and pro-invasive phenotype in cancer

Tumor type	Therapeutic insults	Biological mechanism (Suppl. Reference number)	Therapeutic effect of GSK3 β inhibition and underlying mechanism (Reference number)
Breast cancer	Tamoxifen	EGFR pathway, enhanced tumor cell motility and invasion ^(SR1)	Suppression of invasion through dysregulation of actin-reorganization via down-regulation of WAVE2 ⁽⁶⁸⁾
	Tamoxifen	EMT induction with EGFR pathway-dependent β -catenin activation ^(SR2)	
	Adriamycin	Twist 1-mediated EMT induction and P-gp up-regulation ^(SR3)	
	Doxorubicin and cyclophosphamide alone or in combination	TNF- α /NF- κ B-mediated amplification of CXCL1 paracrine network between carcinoma, myeloid, and endothelial cells ^(SR4)	
Colorectal cancer	Oxaliplatin	NF- κ B-mediated EMT induction with enhanced cell migration and invasion ^(SR5)	Suppression of tumor cell survival and proliferation by inhibition of hTERT/telomerase and promoting p53-dependent apoptosis ^(28–30,32,33) and invasion by down-regulation of WAVE2, ⁽⁶⁸⁾ and enhancing 5-FU effect via PARP1-dependent and AIF-mediated necroptosis ⁽⁷³⁾
	Erlotinib	EMT induction ^(SR21)	
Pancreatic cancer	Gemcitabine	EMT induction with activation of β -catenin and c-Met and acquisition of CSC phenotype ^(SR6)	Suppression of tumor cell survival and proliferation ⁽³⁰⁾ by inhibition of NF- κ B transcriptional activity, ^(34,35) synergistic with gemcitabine by restoration of Rb function ⁽⁶⁰⁾ and inhibition of TP53INP1-mediated DNA repair, ⁽⁷⁰⁾ increase in radiosensitivity and suppression of invasion via FAK/Rac1/MMP-2 and CXCR4/MMP-2 pathways ^(60,67)
	Gemcitabine	Acquisition of EMT and CSC phenotypes with activation of Notch pathway ^(SR7)	
	Gemcitabine, 5-FU, cisplatin	Gene expression profile responsible for EMT phenotype ^(SR8)	
	Gemcitabine	NF- κ B-mediated acquisition of EMT and CSC phenotypes ^(SR9)	
	γ -irradiation	Tumor cell migration and invasion with enhanced MMP-2 activity ^(SR16)	
Ovarian cancer	Erlotinib	EMT induction ^(SR21)	Suppression of tumor cell proliferation by decrease in cyclin D1 expression ⁽³⁹⁾
	Taxol, vincristine Paclitaxel	Increased expression of twist ^(SR10) Acquisition of EMT and metastatic potential ^(SR11)	
	Cisplatin, taxol	Acquisition of EMT via endothelin A receptor-mediated pathway ^(SR12)	
Prostate cancer	Taxol, vincristine Docetaxel	Increased expression of twist ^(SR10) Gene expression profile responsible for EMT phenotype ^(SR13)	Suppression of tumor cell survival and proliferation by eliminating TRAIL resistance, repressing AR activity, ^(41–44) and attenuation of metastasis by depleting CSC population ⁽⁷⁸⁾
Other epithelial cancer			
Endometrial	Ionizing radiation	EMT induction with enhanced cell migration ^(SR17)	Suppression of tumor cell survival by interrupting ERK-mediated prosurvival pathway ⁽⁴⁰⁾
Bladder	Taxol, vincristine	Increased expression of twist ^(SR10)	Suppression of tumor cell survival and proliferation by inhibition of NF- κ B transcriptional activity ⁽⁴⁵⁾
Tongue	Cisplatin	EMT induction and enhanced cell migration and invasion in association with increased BMI1 by down-regulation of miR-200b and miR-15b ^(SR14)	Not shown
Nasopharyngeal	Taxol, vincristine	Increased expression of twist ^(SR10)	Not shown

Table 1 (Continued)

Tumor type	Therapeutic insults	Biological mechanism (Suppl. Reference number)	Therapeutic effect of GSK3 β inhibition and underlying mechanism (Reference number)
Glioblastoma	Doxorubicin	Therapeutic effect and enhancement of doxorubicin effect by a new anti-invasive small molecule (IB) ^(SR15)	Suppression of tumor cell survival and proliferation by restoring p53/p21 pathway and inhibition of c-Myc, NF- κ B and abnormal glycolysis, ^(31,36) synergistic with temozolomide
	Sublethal irradiation	Enhanced tumor cell migration and invasion involving α v β 3 integrin, MMP-2, MMP-9, MT1-MMP, TIMP-2 and BCL-2/BAX rheostat ^(SR18)	and increase in radiosensitivity via Rb-mediated and c-Myc/DMNT3A/MGMT pathways, ^(31,69) suppression of invasion ^(58,59,61) by inhibition of FAK/Rac1/JNK pathway ⁽⁶¹⁾ and induction of CSC differentiation ^(37,77)
	Ionizing radiation	Enhanced tumor cell migration with increased expression of β 3 and β 1 integrins ^(SR19)	
	Bevacizumab	Resistance to the therapy is associated with up-regulation of MMP-2, MMP-9, MMP-12, TIMP1, SPARC and HIF-2 α , and with activation of bFGF-mediated alternate angiogenesis pathway ^(SR22)	
	Bevacizumab	Treatment of tumor xenograft is associated with decrease of mitochondria, induction of glycolytic metabolites (lactate and alanine) and HIF-1 α , and activation of PI3K pathway ^(SR23)	
	Bevacizumab	Resistance to the therapy induced genes associated with a mesenchymal origin, cellular migration/invasion, and inflammation. ^(SR24)	
Osteosarcoma	Low-dose photon irradiation	Enhanced cell migration and invasion concomitant with up-regulation of α v β 3 integrin ^(SR20)	Suppression of tumor cell survival and proliferation by inhibition of NF- κ B transcriptional activity ⁽³⁸⁾ and restoring β -catenin osteosarcoma suppressor (S. Shimozaki <i>et al.</i>) [†]

[†]S. Shimozaki *et al.*, unpublished observation. AIF, apoptosis-inducing factor; AR, androgen receptor; BAX, Bcl-2-associated X protein; bFGF, basic fibroblast growth factor; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; CSC(s), cancer stem-like cell(s); CXCL1, chemokine (C-X-C motif) ligand 1; CXCR4, CXC receptor type 4; DNMT3A, DNA (cytosine-5)-methyltransferase 3A; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; 5-FU, 5-fluorouracil; HIF, hypoxia inducible factor; hTERT, human telomerase reverse transcriptase; JNK, c-Jun N-terminal kinase; MGMT, O⁶-methylguanine DNA methyltransferase; miR, micro-RNA; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; NF- κ B, nuclear factor- κ B; PARP1, poly [ADP-ribose] polymerase 1; P-gp, P-glycoprotein (multidrug resistance); PI3K, phosphatidylinositol-3-kinase; Rb, retinoblastoma; SPARC, secreted protein, acidic, cysteine rich; SR, supplementary reference No.; TIMP, tissue inhibitor of MMP; TNF- α , tumor necrosis factor- α ; TP53INP1, tumor protein p53-inducible nuclear protein 1; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; WAVE2, WAS (Wiskott-Aldrich syndrome) protein family member 2.

phosphorylation of S9 and Y216 residues in gastrointestinal cancers, glioblastoma and osteosarcoma compared to respective normal cells and tissues.^(28–31) These observations suggested that GSK3 β could participate in cancer development and progression, despite the general belief that it has tumor-suppressive functions.^(25–27) We and other research groups have shown that inhibition of GSK3 β activity with specific pharmacological inhibitors, or inhibition of its expression by RNA interference, can preferentially attenuate the survival and proliferation of tumor cells and induce them to undergo apoptosis. This effect has been observed not only in gastrointestinal and pancreatic cancer cells^(28–30,32–35) but also in glioblastoma,^(31,36,37) osteosarcoma (S. Shimozaki, N. Yamamoto, T. Domoto, H. Nishida, K. Hayashi, H. Kimura, A. Takeuchi, S. Miwa, K. Igarashi, T. Kato, Y. Aoki, T. Higuchi, M. Hirose,

R.M. Hoffman, T. Minamoto, H. Tsuchiya, unpublished data, 2016)⁽³⁸⁾ and other malignant neoplasms such as gynecological and urogenital cancers,^(39–47) soft tissue sarcomas,^(48,49) hematological malignancies^(50,51) and lung cancers.^(52,53) This accumulating evidence firmly establishes GSK3 β as a valuable target in cancer treatment.^(11,12)

There has been substantial interest in the molecular mechanisms by which GSK3 β favors tumor progression and by which inhibition of its activity or expression attenuates tumor cell survival, immortalization and proliferation. The reported mechanisms for tumor cell survival include the nuclear factor (NF)- κ B-mediated prosurvival pathway,^(34–36,38) tumor cell resistance to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL),⁽⁴¹⁾ and failure of the p53-mediated tumor suppressor pathway or of the Rb-mediated cell

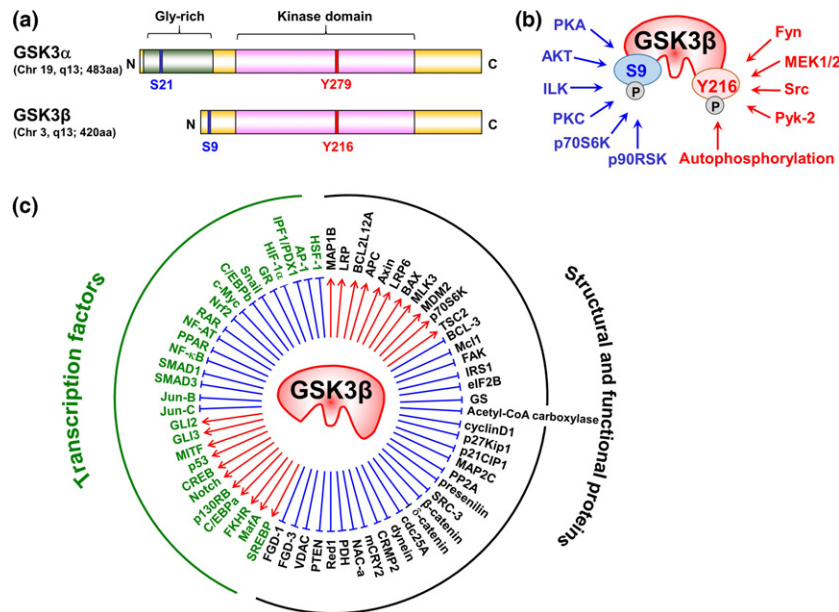


Fig. 1. (a) Comparison of the structural and functional domains of the two GSK3 isoforms, (b) the sites (S9 and Y216) of phosphorylation of GSK3 β by different kinases regulating GSK3 β activity, and (c) the substrates of GSK3 β and proteins that interact with it. (a) GSK3 α (51 kD) and GSK3 β (47 kD) are products of their respective genes located in chromosomes 19q13 and 3q13. The isoforms share high (98%) homology of the catalytic domains, and GSK3 α has a glycine-rich extension at the N-terminal side. Blue and red narrow columns indicate the sites of serine (S) and tyrosine (Y) phosphorylation, respectively. (b) The kinases indicated in blue phosphorylate GSK3 β -S9 resulting in its inactivation, while those indicated in red phosphorylate GSK3 β -Y216 resulting in its activation. (c) GSK3 β stabilizes/activates (red arrows) and destabilizes/inactivates (blue lines) various transcription factors as well as structural and functional proteins. AP-1, activator protein 1; APC, adenomatous polyposis coli; BAX, BCL2-associated X protein; BCL, B-cell lymphoma; C, C-terminal of protein; C/EBP, CCAAT (cytosine-cytosine-adenosine-adenosine-thymidine)-enhancer-binding protein; cdc25A, cell division cycle 25 homolog A; CREB, cAMP (cyclic adenosine monophosphate) response element binding protein; CRMP2, collapsin response mediator protein 2; eIF2B, eukaryotic initiation factor 2B; FAK, focal adhesion kinase; FGD-1/3, FYVE RhoGEF (guanine nucleotide exchange factor) and PH domain-containing protein 1/3; FKHR, forkhead in rhabdomyosarcoma; Gly, glycine; GR, glucocorticoid receptor; GS, glycogen synthase; GSK3 β , glycogen synthase kinase 3 β ; HIF-1 α , hypoxia inducible factor-1 α ; HSF-1, heat shock transcription factor-1; ILK, integrin-linked kinase; IPF1/PDX1, insulin promoter factor 1/pancreatic and duodenal homeobox 1; IRS1, insulin receptor substrate 1; LRP5/6, lipoprotein receptor-related protein 5/6; MafA, musculoaponeurotic fibrosarcoma oncogene homolog A; MAP1B/2C, microtubule associated protein 1B/2C; Mcl1, myeloid cell leukemia 1; mCRY2, mouse cryptochrom 2; MDM2, mouse double minute 2 homolog; MEK, MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) kinase; MITF, microphthalmia-associated transcription factor; MLK3, mixed lineage kinase 3; N, N-terminal of protein; NAC- α , nascent polypeptide-associated complex subunit- α ; NF-AT, nuclear factor of activated T-cells; NF- κ B, nuclear factor- κ B; Nrf2, nuclear factor erythroid 2-related factor 2; p130RB, p130 retinoblastoma; p21CIP1, p21 CDK (cyclin-dependent kinase)-interacting protein 1; p90RSK, p90 ribosomal protein S6 kinase; PDH, pyruvate dehydrogenase; PKA, protein kinase A; PKC, protein kinase C; PP2A, protein phosphatase 2A; PPAR, peroxisome proliferator-activated receptor; PTEN, phosphatase and tensin homolog; Pyk-2, proline-rich tyrosine kinase 2; RAR, retinoic acid receptor; Red1, RNA-editing deaminase 1; S, serine; SRC-3, steroid receptor coactivator-3; SREBP, sterol regulatory element-binding protein; TSC2, tuberous sclerosis complex 2; VDAC, voltage-dependent anion channel; Y, tyrosine

cycle regulation.^(31,32) Preservation of telomere length by maintaining activity of human telomerase reverse transcriptase (hTERT) and telomerase also immortalizes tumor cells in response to the aberrant activation of GSK3 β .⁽³⁰⁾ Cell proliferation pathways mediated by c-Myc and cyclin D1 sustain tumor cell proliferation that is dependent on GSK3 β .^(36,39) In osteosarcoma and rhabdomyosarcoma, the induction of β -catenin signaling, a well-known tumor suppressive mechanism in these cancers, has been linked to decreased cell proliferation following GSK3 β inhibition (S. Shimozaki *et al.*, unpublished data).⁽⁴⁹⁾ Another recent study reported that mitotic catastrophe caused by disruption of centrosome biodynamics was associated with attenuated tumor cell survival and proliferation following GSK3 β inhibition.⁽⁵⁴⁾ Importantly, GSK3 β has little impact on the cell survival, immortalization and growth of normal cells, where the differential phosphorylation of S9 and Y216 residues functions to fine tune GSK3 β activity.^(28–31,34) The growing body of evidence on the role of aberrant GSK3 β in sustaining and promoting cancer cell survival, immortalization and proliferation, together with the differential effects of GSK3 β inhibition on cancer and normal cells, underpins the targeting of GSK3 β as a novel cancer treatment.^(11,12)

GSK3 β mediates both the invasive phenotype and therapy resistance in refractory cancer

The dependency of cancer cells on GSK3 β for their survival and proliferation has encouraged further studies on whether aberrant GSK3 β participates in tumor cell invasion and therapy resistance, the two major determinants of patient outcome.^(3–6) As shown in Table 1, most tumors that acquire pro-invasive phenotypes as they evade therapy are susceptible to experimental treatment involving inhibition of GSK3 β . Here we review what is known about the involvement of GSK3 β in tumor invasion and resistance to therapy based on our previous studies in pancreatic cancer and glioblastoma. These are representative of lethal tumors and are characterized by high invasive capacity and resistance to available therapies.^(55,56)

GSK3 β and cancer invasion. While GSK3 β plays pivotal roles in cytoskeletal organization, cell polarity and migration in the physiological processes of organogenesis and wound healing,⁽⁵⁷⁾ little is known about its role in cancer cell motility, migration and invasion. Earlier studies showed that lithium chloride and some indirubins, both classical GSK3-inhibiting agents, inhibited the migration and invasion of glioblastoma

cells, but the mechanism underlying this effect was not clear.^(58,59) Subsequently, we demonstrated that inhibition of GSK3 β by a specific pharmacological inhibitor and by RNA interference decreases the capacity for migration and invasion of pancreatic cancer and glioblastoma cells and of their tumors in animal models.^(60,61) Morphologically, these effects were associated with reduced formation of lamellipodia and invadopodia, the horizontal and vertical cellular surface microarchitectures, respectively, that drive cell migration, stromal degradation and invasion.^(62,63) Inhibition of GSK3 β disrupted the functional subcellular colocalization of Rac1⁽⁶⁴⁾ and cortactin⁽⁶³⁾ with the cytoskeletal molecule F-actin in lamellipodia and invadopodia, respectively. These changes in the pro-invasive phenotype of tumor cells following GSK3 β inhibition coincide with the suppression of molecular pathways mediated by focal adhesion kinase (FAK),⁽⁶⁵⁾ guanine nucleotide exchange factors (GEF)/Rac1^(64,66) and c-Jun N-terminal kinase (JNK). This results in decreased expression of matrix metalloproteinase (MMP)-2 and membrane type (MT) 1-MMP. Our observations therefore suggest that GSK3 β sustains tumor invasion via the promotion of morphological and functional pro-invasive cellular phenotypes and that the molecular axis involves FAK, GEFs/Rac1 and JNK (Fig. 2).^(60,61)

Subsequent to our studies,^(60,61) other workers have reported the proinvasive effect of GSK3 β in pancreatic, colorectal and breast cancer cells in association with modulation of cytoskeletal organization and cytokine-mediated matrix degradation.^(67,68) These studies highlight the pivotal role of GSK3 β in tumor invasion and probably also in metastatic spread.

GSK3 β and cancer therapy resistance. The combination of multiple agents having different targets and mechanisms of actions is important in the treatment of cancer in order to optimize the therapeutic effects and minimize the adverse effects. This is particularly relevant for the treatment of refractory cancer, and, hence, molecular-targeted therapy is often combined with conventional chemotherapeutics and/or radiation therapy, as well as with other targeted agents.^(7,8) As discussed above, accumulating evidence on the effects of GSK3 β inhibition against cancer cell survival and proliferation has led us to

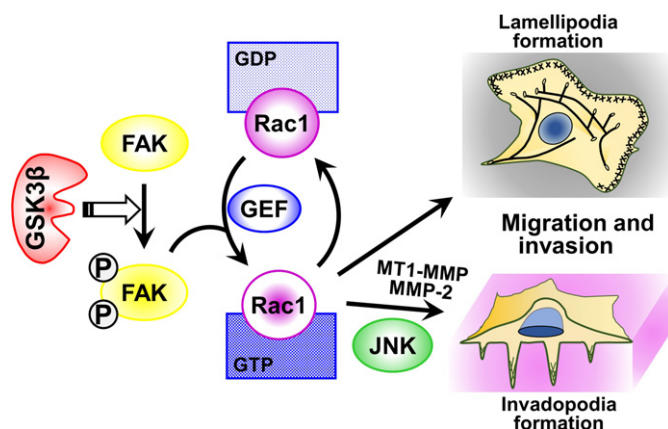


Fig. 2. Putative molecular pathway through which deregulated GSK3 β promotes tumor cell migration and invasion. The exact molecular pathway by which GSK3 β mediates the activation of FAK (open arrow) remains to be determined. FAK, focal adhesion kinase; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GSK3 β , glycogen synthase kinase 3 β ; GTP, guanosine triphosphate; JNK, c-Jun N-terminal kinase; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; circled P, phosphorylation.

address whether this could be used in combination with chemotherapy and irradiation.

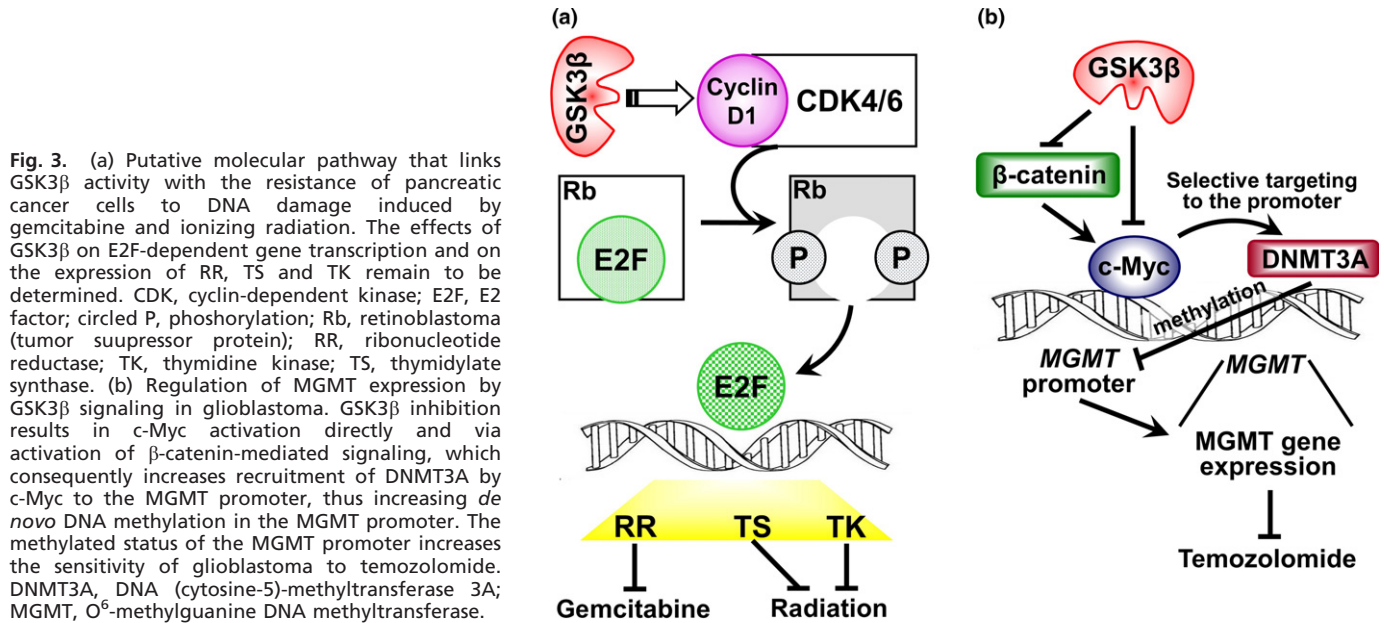
The standard treatment modalities prescribed are often ineffective in patients with refractory tumors such as pancreatic cancer and glioblastoma.^(55,56) We have shown that administration of a GSK3 β -specific pharmacological inhibitor (e.g. AR-A014418)^(SR29) at a dose below that which results in a therapeutic effect when given on its own can significantly enhance the cytotoxic effects of temozolomide and gemcitabine.^(31,60,69,70) These are the standard chemotherapeutic agents prescribed for patients with glioblastoma and pancreatic cancer, respectively.^(55,56) Low-dose GSK3 β inhibitor given in combination with chemotherapeutic agents had additive and synergistic effects. Investigation of the underlying molecular mechanism is crucial to justify treatment combinations of GSK3 β inhibitor with other anti-cancer therapeutics (Fig. 3). In glioblastoma, the combined effect was attributed to modulation of the mouse double minute (Mdm)2/p53 and c-Myc-mediated pathways resulting in epigenetic silencing of O⁶-methylguanine DNA methyltransferase (MGMT), a clinically proven biomarker of temozolomide efficacy⁽⁵⁶⁾ in tumor cells (Fig. 3b).^(31,69) In pancreatic cancer, inhibition of tumor GSK3 β activity decreased cyclin D1/cyclin-dependent kinase (CDK)4/6 complex-dependent phosphorylation of Rb tumor suppressor protein.⁽⁶⁰⁾ One of the important functions of Rb is to bind the oncogenic transcription factor E2F1 and inhibit its transcriptional activity.^(71,72) GSK3 β may, therefore, sensitize pancreatic cancer to gemcitabine by downregulating the expression of ribonucleotide reductase, a well-known pharmacological biomarker of gemcitabine⁽⁵⁵⁾ that is regulated by the Rb/E2F pathway (Fig. 3a).^(71,72) In addition to modulation of this pathway, we found that downregulation of the *TP53INP1* gene (encoding tumor p53-induced-nuclear-protein 1) involved in DNA repair is associated with the chemosensitizing effect of GSK3 β inhibitor.⁽⁷⁰⁾ The GSK3 β inhibitor used in these studies also sensitized both pancreatic cancer and glioblastoma to ionizing radiation.^(31,60) This radio-sensitizing effect may depend on the restoration of Rb function following GSK3 β inhibition, resulting in the inability of E2F1 to induce the transcription of thymidylate synthase and thymidine kinase (Fig. 3a).

Other recent studies have also reported that GSK3 β inhibition allows therapy-resistant colon and pancreatic cancer cells to become susceptible to 5-fluorouracil (5-FU),⁽⁷³⁾ and renal cell carcinoma cells to become susceptible to a synthetic multikinase inhibitor (sorafenib) that targets growth signaling and angiogenesis.⁽⁷⁴⁾ Together, these studies suggest that GSK3 β participates in multiple molecular pathways used by various cancer types to evade chemotherapy, radiotherapy and targeted therapies.

Collectively, a growing body of experimental evidence supports the notion that GSK3 β is a molecular “hub” that mediates and connects various pathways responsible for tumor invasion and resistance to therapy. Together with the role of GSK3 β in promoting tumor cell survival and proliferation and its differential functions between tumor and normal cells [reviewed in Miyashita *et al.*⁽¹¹⁾ and McCubrey *et al.*⁽¹²⁾], this evidence reinforces the promise of novel cancer therapeutic strategies that target GSK3 β .

Future perspectives

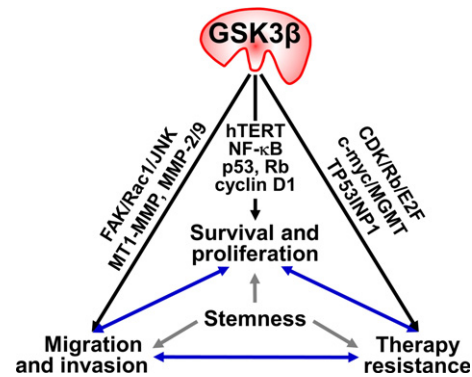
Targeting of the biological pathways that link tumor invasion and therapy resistance is clearly an attractive prospect for the



development of innovative cancer treatments. Another important challenge is to understand the biology of cancer initiating or stem-like cells (CSC) so that “stemness” phenotypes can also be targeted. Based on the clonal evolution model of tumorigenesis and on the normal stem cell concept, CSC are conceptually defined as a population of tumor cells with self-renewing and pluripotent capacity.⁽¹³⁾ CSC have been identified in a wide range of hematopoietic malignancies and solid tumors and are assumed to be progenitors of the entire neoplastic cell population. One of the most striking characteristics of CSC is their influence on tumor cell plasticity, intra-tumor heterogeneity, invasion and metastasis, therapy resistance and tumor relapse after surgery and adjuvant treatment.^(14,75) Eradication of CSC is, therefore, of paramount clinical importance for combatting refractory cancer.^(15,76)

GSK3 β has pivotal functions in normal cell biology^(10,21,22) and is also central to the processes of cancer cell survival, proliferation, invasion and therapy resistance, as discussed above. A number of studies have reported that CSC phenotypes share unique molecular pathways and tumor–environment interactions that are also associated with tumor invasion and therapy resistance.^(14,75) This leads us to propose a working hypothesis whereby GSK3 β underlies the basal mechanism for sustaining the CSC phenotype (Fig. 4). Recent studies have shown that GSK3 β negatively controls the differentiation of malignant glioma cells,⁽⁷⁷⁾ and that GSK3 β inhibition results in depletion of cancer stem-like or progenitor-like cells and attenuation of metastatic spread in prostate cancer.⁽⁷⁸⁾ Consistent with the physiological roles of GSK3 β in negatively regulating canonical Wnt/ β -catenin and hedgehog signaling,^(10,23,24) inhibition of GSK3 β is a prerequisite for the maintenance of “stemness” phenotypes in embryonic and hematopoietic stem cells.^(79,80) Therefore, future work should aim to understand the influence of GSK3 β on both tumor and normal stem cell phenotypes. This knowledge can be applied for the development of novel cancer treatments that target GSK3 β .

One of the concerns about targeting GSK3 β for cancer treatment is that its inhibition may promote the progression of existing tumors [reviewed in Takahashi-Yanaga⁽²²⁾, Jope *et al.*,⁽²⁵⁾ Luo⁽²⁶⁾ and McCubrey *et al.*⁽²⁷⁾]. A number of



studies have focused on the putative tumor suppressive role of GSK3 β (Table S1).^(SR30–58) Many of these report inactivation or activation of GSK3 β as a mediating event in pathways leading to tumor progression or suppression, respectively. An inverse association between tumor expression of GSK3 β (either total or active form pGSK3 β ^{Y216}) and the survival of cancer patients has been reported.^(SR33,36,37) Other studies found causal links between pharmacological GSK3 β inhibition and/or depletion of GSK3 β expression (e.g. gene knockout and RNA interference) or activity (e.g. recombinant kinase-dead or constitutively active mutant form) and tumor cell survival, proliferation^(SR40,52) and susceptibility to cancer therapies.^(SR32,34,58) GSK3 β inhibition strategies and the development of GSK3 β -targeted agents therefore require careful evaluation to determine whether the tumor promoting function of GSK3 β is counteracted by its putative tumor suppressor function in different cancer types.

The development and administration of GSK3 β inhibitors for the treatment of chronic diseases, including diabetes

mellitus, neurodegenerative disorders and cancer, requires serious awareness of the safety issues. A major concern is whether and how GSK3 β can be targeted to treat disease, because its systemic inhibition may lead to unwanted effects by disrupting multiple biological pathways (Fig. 1c). Most notably, long-term inhibition of GSK3 β may initiate tumorigenesis due to its role in suppressing proto-oncogenic pathways, in particular that are mediated by β -catenin [reviewed in Takahashi-Yanaga⁽²²⁾, Jope *et al.*,⁽²⁵⁾ Luo⁽²⁶⁾ and McCubrey *et al.*⁽²⁷⁾]. A previous study showed that genetic deletion of GSK3 β in mammary epithelial cells resulted in β -catenin activation and induced intraepithelial neoplasia that progressed to the development of adenocarcinoma.^(SR51) Moreover, overexpression of wild-type and constitutively active mutant GSK3 β in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-mediated, transformation-resistant mouse epidermal cells suppressed EGF-mediated and TPA-mediated anchorage-independent growth in soft agar and tumorigenicity in rodents (Table S1).^(SR56) However, there is currently no direct evidence to support tumor development *in vivo* following treatment with a GSK3 β inhibitor [reviewed in Miyashita *et al.*⁽¹¹⁾ and SR27]. As discussed in previous studies that report cancer therapeutic effects of GSK3 β inhibition (Table 1), none of the available experimental GSK3 β inhibitors induces neoplastic transformation of non-neoplastic (normal) cells or tumor development in experimental animal models [reviewed in Miyashita *et al.*⁽¹¹⁾ and SR27]. Long-term prescription of lithium, the only GSK3 β inhibitor approved for the treatment of bipolar disorder since the 1950s, has not been associated with an increased risk of cancer or death from cancer.^(SR59) Post-translational regulation of GSK3 β activity via the phosphorylation of S9 and Y216 (pGSK3 β ^{S9} versus pGSK3 β ^{Y216}) (Fig. 1b) in response to various stimuli could partly underlie a mechanism that protects normal cells from the detrimental effects of GSK3 β inhibition.

Despite the concerns outlined above, clinical trials for neurodegenerative diseases and cancer have tested some seed pharmacological GSK3(β) inhibitor compounds and also approved medicines with the ability to inhibit GSK3 β activity (Table S2). The former trials include AZD-1080 (AstraZeneca) for the treatment of Alzheimer's disease (phase I), and NP031112 (tideglusiv; Noscira SA) for the treatment of progressive supranuclear palsy (NCT01049399: phase IIb)^(SR60,61) and of Alzheimer's disease (NCT01350362: phase II).^(SR62,63) Clinical trials for cancer treatment have used LY2090314 (Eli Lilly) alone for acute leukemia (NCT01214603: phase II), and the same compound in combination: (i) with gemcitabine, the combined folate, 5-FU and oxaliplatin (FOLFOX) regimen or the combined gemcitabine and nab-paclitaxel regimen for metastatic pancreatic cancer (NCT01632306: phase I/II); and

(ii) with pemetrexed and carboplatin for advanced or metastatic solid cancer (NCT01287520: phase I).^(SR64,65) Our group is undertaking the phase I/II clinical trials by repurposing approved GSK3 β -inhibiting medicines (combined cimetidine, lithium, olanzapine and valproate regimen) in combination with gemcitabine for advanced pancreatic cancer (UMIN000005095) and with temozolomide for recurrent glioblastoma (UMIN000005111) (T. Furuta, H. Sabit, D. Yu, K. Miyashita, M. Kinoshita, M. Kinoshita, Y. Hayashi, Y. Hayashi, T. Minamoto, M. Nakada, unpublished data, 2016).

Currently, information regarding the side effects of GSK3 β inhibitors is limited because the clinical trials have evaluated only a small number of seed compounds and also because lithium chloride is the only currently approved inhibitor for clinical use. It is, therefore, difficult to predict what serious adverse events, if any, will occur in patients treated with GSK3 β inhibitors. The extent of GSK3 β inhibition and the differential effects between normal and diseased cells/tissues (therapeutic window) are critical determining factors for therapeutic efficacy and undesirable side effects. It is clear that GSK3 β activity is deregulated in various pathological conditions, including many, but not all, cancer types (Table 1).^(10–12,22,25–27) Identifying the range of GSK3 β inhibition that is efficient for treatment of disease but which results in minimal detrimental effects to normal tissues and organs will be important for the clinical application of GSK3 β inhibitors. Numerous studies have shown that, in addition to differential phosphorylation at S9 and Y216 residues (Fig. 1a,b), the subcellular localization of GSK3 β together with various upstream pathways can regulate the expression and activity of this kinase [reviewed in Beurel *et al.*⁽¹⁰⁾ and Takahashi-Yanaga⁽²²⁾]. New classes of GSK3 β inhibitors that spatially and temporally control the expression and activity of GSK3 β may therefore be required to reduce the risk of adverse events such as carcinogenesis that may be associated with long-term GSK3 β inhibition.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Previous studies reporting the putative tumor suppressor roles of GSK3 β .

Table S2. Clinical trials of GSK3 β inhibitors for treatment of diseases.

Data S1. Supplementary references.