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SNF1/AMP-Activated Protein Kinases: Genes, Expression and Biological Role

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1. Introduction

Most of the physiological and metabolic processes in any organism are controlled by a regulatory factor network, which includes a lot of protein kinases, protein phosphatases and transcription factors. Protein kinases and phosphatases are key regulators of the majority of transcription factors which control metabolism both in normal and in different pathological conditions; it is a circadian type of regulation [1–5]. AMPK-related kinases SNARK and NUAK1 as well as many others AMPK-related kinases represent molecular components of signalling cascades that control metabolism, gene expression and perhaps cell proliferation in response to cellular, metabolic and environmental stresses [6–8].

The sucrose-non-fermenting protein kinase (SNF1) from *Saccharomyces cerevisiae* and its mammalian counterpart, AMP-activated protein kinase (AMPK), form a family of serine/threonine kinases that acts as a master sensor and regulator of the energy balance at the cellular level as well as the stress response systems, has been critical to our understanding of the whole body energy homeostasis [9]. This family of protein kinases is highly conserved between animals, fungi and plants and is commonly activated in response to cellular and environmental stresses such as nutrient deprivation. Yeast SNF1 responds to glucose deprivation by derepressing genes implicated in carbon source utilization and by modulating the transcription of glucose-regulated genes involved in gluconeogenesis, respiration, sporulation, thermotolerance, peroxisome biogenesis and cell cycle regulation. Activated by environmental stresses AMPK switches off anabolic pathways (e.g. fatty acid and cholesterol synthesis) and induces ATP generating catabolic pathways [9]. Twelve protein kinases (NUAK1, NUAK2, BRSK1, BRSK2, SIK, QIK, QSK, MARK1, MARK2, MARK3, MARK4 and MELK) in the human kinome are closely related to AMPKα_1 and AMPKα_2, thus forming a 14 kinase phylogenetic tree known as "AMPK-related kinases" which represent components of signalling cascades that control metabolism, gene expression and perhaps cell proliferation in response to cellular, metabolic and environmental stresses [9].

The AMP-activated protein kinase system acts as a sensor of cellular energy status that is conserved in all eukaryotic cells. It is activated by a large variety of cellular stresses that increase cellular AMP and decrease ATP levels and also by physiological stimuli, such as muscle contraction, or by hormones such as leptin and cellular adiponectin as well as by

metabolic stresses that either interferes with ATP production or that accelerate ATP consumption [10]. AMPK modulates multiple metabolic pathways. Activation in response to an increase in AMP involves phosphorylation by an upstream kinase, the tumour suppressor LKB1. Once activated, AMPK switches on catabolic pathways that generate ATP, while switching off ATP-consuming processes such as biosynthesis and cell growth and proliferation. Thus, it is a key player in the development of new treatments for obesity, the metabolic syndrome, type 2 diabetes or even cancer. In fact, it has been recently reported that drugs used in the treatment of diabetes, such as metformin and thiazolidinediones, exert their beneficial effects through the activation of AMPK.

The sucrose-non-fermenting protein kinase (SNF1)/AMP-activated protein kinase-related kinase (SNF1/AMP-activated protein kinase; SNARK) is a member of AMPK kinases (NUAK family SNF1-like kinase 2) which is related to serine/threonine protein kinases [7, 9]. SNARK activity is regulated by glucose- or glutamine-deprivation, induction of endoplasmic reticulum stress by dithyothreitol or homocysteine, elevation of cellular AMP and/or depletion of ATP, hyperosmotic stress, salt stress and oxidative stress caused by hydrogen peroxide. However, the regulation of SNARK activity in response to cellular stresses depends greatly upon cell type. It was also shown that SNARK is also regulated by metabolic stress and diabetes [11]. Nuclear localization of SNARK has shown its impact on gene expression [12].

Tsuchihara et al. [13] demonstrated that SNARK(+/-) mice exhibit mature-onset obesity and related metabolic disorders. Moreover, the incidence of both adenomas and aberrant crypt foci were significantly higher in SNARK(+/-) mice than in their wild-type counterparts, suggesting that SNARK deficiency contributed to the early phase of tumourigenesis via obesity-dependent and obesity-independent mechanisms [14]. Recently, Namiki et al. [14] have shown that AMP kinase-related kinase SNARK affects tumour growth, migration, and clinical outcome of human melanoma, further supporting the importance of this protein kinase in cancer development and tumour progression, while AMPK has antioncogenic properties. We have also shown that the SNF1/AMP-activated protein kinase-related kinase is a sensitive marker for the action of ecotoxicant methyl tert-butyl ether (MTBE) as well as silver nanoparticles [15, 16]. These observations support a role for SNARK as a molecular component of the cellular stress response.

SNF1-like kinase 1 (NUAK1) is an AMP-activated protein kinase family member 5, ARK5, which regulates ploidy and senescence, tumour cell survival, malignancy and invasion downstream of Akt signaling, acts as an ATM kinase under the conditions of nutrient starvation [17–20]. Moreover, NUAK1 suppresses the apoptosis, induced by nutrient starvation, and death receptors via inhibition of caspase-8 and caspase-6 activation [21, 22]. Importantly, AMPK-related kinase NUAK1 as well as many others AMPK kinases (including MARK/PAR-1) is regulated by protein kinase LKB1 and USP9X [23, 24].

2. NUAK family SNF1-like kinase 2 (NUAK2), Sucrose nonfermenting AMPK-related kinase (SNARK)

Human NUAK family SNF1-like kinase 2 (NUAK2; EC_number "2.7.11.1") also known as sucrose nonfermenting AMPK-related kinase or skeletal muscle sucrose, nonfermenting 1/adenosine monophosphate activated protein kinase-related kinase (SNARK) is an AMP-

activated protein kinase family member 4, which was identified 10 years ago as an potential mediator of cellular response to metabolic stress [9].

2.1 NUAK2 gene, transcripts and encoded proteins

The human NUAK2 (SNARK) gene (geneID: 81788) is localized on chromosome 1 (1q32,1). The SNARK gene encodes mRNA (GenBank accession number NM_030952) of seven exons. Northern blotting demonstrated that mRNA transcripts (at least two variants) for the SNARK were widely expressed in rodent tissues, but most abundant in rat kidney. Reverse-transcriptase-mediated PCR detected two SNARK cDNA products in RNA from rat heart, skin, spleen, lung, uterus, liver and a neonatal rat keratinocyte cell line, NRKC. The two different SNARK PCR products were cloned, sequenced and found to encode either authentic SNARK (1437 bp) or an internally deleted SNARK transcript (1247 bp) [9]. Whereas rat kidney contained predominantly the intact SNARK transcript and testes expressed only the 1247 bp SNARK transcript, both intact and internally deleted SNARK transcripts were detected in other tested tissues. The ORF encodes a putative protein of 630 amino acid residues with a predicted molecular mass of 70 kDa and a theoretical pI of 9.35. Translation of the SNARK-deleted transcript is predicted to give rise to a prematurely terminated protein of approximately 415 amino acid residues [9].

Although no autophosphorylated products were detected in samples of immunoprecipitated endogenous SNARK from wild type BHK cells, one major phosphorylated band, possibly a protein doublet, was detected in the immunoprecipitates from SNARK-transfected BHK cells [9]. The size of the phosphorylated band(s) corresponds to the size of SNARK detected in these cell lines by Western blot analysis. Thus, these results demonstrate that SNARK is a protein kinase capable of autophosphorylation *in vitro*. Besides that, immunoprecipitated SNARK protein exhibits phosphotransferase activity with the synthetic peptide substrate HMRSAMSGLHLVKRR as a kinase substrate [9]. SNARK was translated in vitro to yield a single protein band of approximately 76 kDa, possibly a protein doublet, however, Western analysis of transfected BHK (baby hamster kidney) cells detected two SNARK-immunoreactive bands of approximately 76 – 80 kDa.

The NUAK family SNF1-like kinase 2 (NUAK2 or SNARK) is a member 4 of AMPK kinases which are related to serine/threonine protein kinases and contains all 11 catalytic subdomains conserved in these protein kinases. Analysis of the catalytic domain of SNARK with the Prosite program revealed a protein kinase ATP-binding region signature (residues 63 – 89) and a serine/threonine protein kinase active-site signature (residues 175 – 187). The sequences at the C-terminus of SNARK were distinct and not well conserved with C-terminal sequences of other SNF1/AMPK family members. The instability index is computed to be 58.40 with the Protparam Tool program, classifying protein kinase SNARK as an unstable protein [9].

Comparison of the SNARK catalytic subdomains I – XI to other SNF-1/AMPK family members demonstrates that protein kinase SNARK originated very early in eukaryotic evolution, diverging before the divergence of yeast and humans [9]. On the basis of the phylogeny of the catalytic subdomains, SNARK is no more closely related to SNF1 than it is to AMPK and represents a new branch of the SNF1/AMPK family of protein kinases.

2.2 Protein kinase SNARK, its activity and regulation

The NUAK family SNF1-like kinase 2 is a member of AMPK kinases, it is commonly activated in response to cellular and environmental stresses, and it is a molecular component of the cellular stress response, but its precise mechanisms remain unclear [7, 9, 11, 25]. Its activity is regulated by glucose- or glutamine-deprivation, induction of endoplasmic reticulum stress by homocysteine or dithiothreitol, hyperosmotic stress, salt stress, elevation of cellular AMP and/or depletion of ATP, ultraviolet B radiation and oxidative stress caused by hydrogen peroxide. However, the regulation of protein kinase SNARK activity in response to cellular stresses depends greatly upon cell type. Several aspects of SNARK activation and regulation are broadly similar to AMPK [8]. For example, SNARK and AMPK are both AMP-responsive and activated by treatments known to increase the AMP:ATP ratio, including glucose deprivation and chemical ATP production [9, 12]. Nevertheless, the metabolic role of SNARK at the cellular level, particularly in humans, especially in skeletal muscle, is incompletely resolved.

Kuga et al. [12] identified the subcellular localization of SNARK protein. Unlike cytoplasmic localizing AMPKα, SNARK was predominantly localized in the nucleus. This protein kinase is constitutively distributed in the nucleus; even when SNARK is activated by metabolic stimuli such as the AMP-mimetic agent, 5-aminoimidazole-4-carboxamide riboside (AICAR) or glucose-deprivation. Conserved nuclear localization signal was identified at the N-terminal portion (^{68}KKAR^{71}) of protein kinase SNARK. Deletion and point mutation of this part resulted in the cytoplasmic translocation of mutant proteins. Furthermore, GFP fused with the SNARK fragment containing ^{68}KKAR^{71} translocated to the nucleus.

A microarray analysis revealed that nuclear localized SNARK alteres transcriptome profiles and a considerable part of these alterations were canceled by the mutation of nuclear localization signal (first two core lysine residues of ^{68}KKAR^{71} were altered to alanine (^{68}AAAR^{71})), suggesting the ability of SNARK to modulate gene expression is dependent on its nuclear localization. It has been shown that overexpression of protein kinase SNARK in human liver hepatoma cells results in the upregulation (more than 2.0-fold) of 76 mRNA targets and in the downregulation (more than 2.0-fold) of 32 mRNA targets, suggesting that this protein kinase can work as a stress-responsive transcriptional modulator in the nucleus [12].

Moreover, transcriptome profiles of wild-type and nuclear localized signal-mutant SNARK expressing cells were compared to identify the impact of the nuclear localization of SNARK on the regulation of mRNA levels of potential downstream genes. Among the 76 up-regulated probe sets by overexpressed SNARK, only eight probe sets increased more than 2.0-fold in ^{68}AAAR^{71}-overexpressing cells compared with vector-transfected cells. On the other hand, among the 32 down-regulated probe sets by overexpressed SNARK, only 13 probe sets decreased more than 2.0-fold in ^{68}AAAR^{71}-overexpressing cells compared with vector-transfected cells. Thus, overexpressed SNARK altered the gene expression profiles more than nuclear localization signal-mutant SNARK. This result implied that protein kinase SNARK in the nucleus, but not the cytoplasm, has a remarkable impact on gene expression and can work in the nucleus as a transcriptional modulator in response to stress. This data may become a platform to elucidate the molecular mechanism and the physiological signification of protein kinase SNARK.

AMPK and AMPK-related kinases are believed to be activated by increased AMP:ATP ratio through a direct activation mechanism of the allosteric effect and/or indirectly activated by phosphorylation at threonine residue in the activation loop by upstream kinases, LKB1 (serine/threonine protein kinase 11, STK11), CaMKK (calcium/calmodulin-dependent protein kinase kinase 1, alpha), and TAK1 (mitogen-activated protein kinase kinase kinase 7; MAP3K7) [25]. CaMKK and TAK1 are localized in the cytoplasm, but LKB1 is localized in both nucleus and cytoplasm. Therefore SNARK might be phosphorylated in the nucleus by protein kinase LKB1 [12].

Rune et al. [11] showed that skeletal muscle SNARK expression is also regulated by metabolic stress and increases in human obesity, and in response to metabolic stressors. This increase in SNARK mRNA expression may occur as a consequence of systemic factors associated with metabolic impairments in obesity, since exposure of myotubes to elevated levels of TNF-α or palmitate acutely increased SNARK mRNA expression. siRNA against SNARK failed to rescue TNFα- or palmitate-induced insulin resistance, indicating that changes in SNARK expression occur as a consequence, rather than a cause of insulin resistance. Based on this data in human skeletal muscle, in the insulin-resistant and obesity phenotype in whole-body SNARK-haploinsufficient mice [13], SNARK expression in metabolically active tissues beyond skeletal muscle may play a role in whole body energy and glucose homeostasis.

Interestingly, SNARK has anti-apoptotic properties, acting through a TNF-α-sensitive nuclear NF-κB-mediated mechanism. Thus, the SNF1/AMP kinase-related kinase 2, which is induced in response to various forms of metabolic stress, was identified as an NF-κB-regulated anti-apoptotic kinase that contributes to the tumour-promoting activity of death receptor CD95 (APO-1/Fas) in apoptosis-resistant tumour cells [26]. The death receptor CD95 induces apoptosis in many tissues. However, in apoptosis-resistant tumour cells, stimulation of CD95 induces up-regulation of a defined number of mostly anti-apoptotic genes, resulting in increased motility and invasiveness of tumour cells. The majority of these genes are known NF-κB target genes. One of the CD95-regulated genes is the serine/threonine kinase (SNARK). It was shown that up-regulation of SNARK in response to CD95 ligand and tumour necrosis factor α depends on activation of NF-κB. Overexpression of SNARK rendered tumour cells more resistant, whereas a kinase-inactive mutant of SNARK sensitizes cells to CD95-mediated apoptosis. Furthermore, small interfering RNA-mediated knockdown of SNARK increases the sensitivity of tumour cells to death receptor CD95 ligand- and TRAIL-induced apoptosis. Importantly, cells with reduced expression of SNARK also showed reduced motility and invasiveness in response to CD95 engagement. SNARK therefore represents an NF-κB-regulated anti-apoptotic gene that contributes to the tumour-promoting activity of CD95 in apoptosis-resistant tumour cells [26].

Kim et al. [27] have investigated the effect of Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) on human cancer cells for identification of potential target genes. It was found that LMP1 upregulated the expression of protein kinase SNARK compared with the empty vector transfected control cells. Moreover, SNARK expression increased drug resistance in response to doxorubicin, whereas knockdown of SNARK by siRNA effectively inhibited LMP-1-mediated increase of cell survival. SNARK stimulates the expression of anti-apoptotic genes BCL6 and BIRC2; knockdown of these genes decreased the SNARK-

mediated increase of cell survival. These results suggest that SNARK is a downstream cellular target of LMP1 in malignant cells [27].

2.3 Protein kinase SNARK and tumourigenesis

Members of the AMP kinase family play an important role in tumourigenesis [6]. This activity is believed to be due to their activation by various forms of metabolic stress such as glucose deprivation, a condition to be expected within solid tumours [28]. Recently, Namiki et al. [14] showed that AMP kinase-related kinase NUAK2 affects tumour growth, migration, and clinical outcome of human melanoma. This study further supports the importance of NUAK2 in cancer development and tumour progression, while AMPK has antioncogenic properties.

Although several *in vitro* studies have suggested that metabolic stress as well as genotoxic or osmotic stresses induce SNARK activation, the physiological roles of protein kinase SNARK remain uncertain. Using SNARK-deficient mice helps to clarify the *in vivo* function of this kinase. Interestingly, SNARK(+/-) mice exhibited mature-onset obesity and related metabolic disorders [13]. Thus, an increased bodyweight in these mice is accompanied by fat deposition, fatty changes of the liver, and increased serum triglyceride concentration. These mice also exhibited hyperinsulinemia, hyperglycemia, and glucose intolerance, symptoms which are similar to those of human type II diabetes mellitus accompanied with obesity. Obesity is regarded as a risk factor for colorectal cancer. To investigate whether SNARK deficiency is involved in tumorigenesis in the large intestine, obese SNARK(+/-) mice were treated with a chemical carcinogen, azoxymethane, a chemical carcinogen that induces aberrant crypt foci, colorectal adenoma, and adenocarcinoma. The incidences of both adenomas and aberrant crypt foci were significantly higher in SNARK(+/-) mice than in their wild-type counterparts, suggesting that SNARK deficiency contributed to the early phase of tumourigenesis via obesity-dependent and -independent mechanisms [13].

2.4 Activation of protein kinase SNARK during muscle contraction

Sakamoto et al. [29] have shown that deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. In LKB1-lacking muscle, the basal activity of the AMPKalpha2 isoform was greatly reduced and was not increased by the AMP-mimetic agent, 5-aminoimidazole-4-carboxamide riboside (AICAR), by the antidiabetic drug phenformin, or by muscle contraction. Moreover, phosphorylation of acetyl CoA carboxylase-2, a downstream target of AMPK, was profoundly reduced. Glucose uptake stimulated by AICAR or muscle contraction, but not by insulin, was inhibited in the absence of LKB1. Contraction increased the AMP:ATP ratio to a greater extent in LKB1-deficient muscles than in LKB1-expressing muscles. These studies establish the importance of LKB1 in regulating AMPK activity and cellular energy levels in response to contraction and phenformin.

Recently, Koh et al. [30] showed that muscle contraction also increases protein kinase SNARK activity and that this effect blunted in the muscle-specific LKB1 knockout mice. It is known that the signaling mechanisms that mediate the important effects of contraction, to increase glucose transport in skeletal muscle, occur through an insulin-independent mechanism. Moreover, muscle-specific knockout of protein kinase LKB1, an upstream kinase for AMPK and AMPK-related protein kinases, significantly inhibited contraction-

stimulated glucose transport, suggests that one or more AMPK-related protein kinases are important for this process. It has been shown that expression of a mutant SNARK in mouse tibialis anterior muscle impaired contraction-stimulated, but not insulin-stimulated, glucose transport. The impaired contraction-stimulated glucose transport was also observed in skeletal muscle of whole-body SNARK heterozygotic knockout mice [30]. Thus, SNARK, the fourth member of the AMP-activated protein kinase catalytic subunit family, is activated by muscle contraction and is a unique mediator of contraction-stimulated glucose transport in skeletal muscle.

There is data that NUAK2 is a TNFalpha-induced kinase which regulates myosin phosphatase target subunit 1 (MYPT1) activity by phosphorylation at a site other than known Rho-kinase phosphorylation sites (Thr696 or Thr853) responsible for inhibition of myosin phosphatase activity [31]. Moreover, Suzuki et al. [32] observed the induction of cell-cell detachment during glucose starvation through F-actin conversion by protein kinase SNARK. Recently, Vallenius et al. [33] have shown that an association between AMP kinase-related kinase SNARK and myosin phosphatase Rho-interacting protein (MRIP) reveals a novel mechanism for regulation of actin stress fibers via activation of MLCP (myosin light chain phosphatase). Moreover, new roles for the LKB1-NUAK pathway in controlling myosin phosphatase complexes and cell adhesion have been shown [34].

2.5 Protein kinase SNARK as a regulator of whole-body metabolism

Ichinoseki-Sekine et al. [35] provide evidence for a robust effect on whole body metabolism by hemiallelic SNARK deficiency, suggesting that this AMPK-related kinase is a previously unrecognized important regulator of whole-body metabolism. They have investigated the *in vivo* effects of altering expression of SNARK by using hemiallelic loss of SNARK on whole body metabolic homeostasis and physical activity behaviour. Homozygous SNARK-deficient mice have a high incidence of embryonic lethality, whereas the heterozygous *S*NARK-deficient mice have an obvious metabolic phenotype with mature-onset obesity and increased white adipose tissue mass evident after the age of 4 month.

Activation of SNARK by upstream kinase LKB1 occurs by phosphorylation of Thr^{208}, a conserved threonine residue equivalent in position to Thr^{172} within the activation loop of $AMPK\alpha_2$ [36]. LKB1 is attractive as a regulator of SNARK activity by virtue of its nuclear localization, which is coincident with the predominant nuclear localization of SNARK [12], but SNARK may also directly mediate some physiological effects of LKB1. Several aspects of SNARK regulation and activity are broadly similar to those of AMPK, which can be summarized as follows. First, SNARK possesses AMPK-like phosphotransferase activity; second, activation of SNARK is AMP responsive; third, SNARK activity is increased by AICAR, albeit in a cell-specific manner; and fourth, SNARK is activated by treatments known to increase AMP/ATP ratio or disrupt ATP production, including glucose deprivation and chemical ATP depletion among others [36].

Possible AMPK activation of SNARK, secondary to the activation of AMPK by these treatments, has not been investigated but raises the possibility that one or more activities previously attributed to the AMPK-signaling cascade may be attributable, in part, to SNARK activation. In addition, similarities between AMPK and SNARK regulation do not necessarily infer that SNARK activity directly mirrors AMPK activity in the context of

cellular metabolism. Cell-specific differences are reported between SNARK and AMPK activity and pharmacological activation as well as in the relative rates of phosphorylation and peptide substrates phosphorylated [36].

Examining metabolic and anthropometric effects of SNARK deficiency, the core finding in Ichinoseki-Sekine et al. [35] investigation is that the provision of voluntary exercise opportunities to SNARK(+/-) mice results in habitually increased daily physical activity (~2-fold) compared with SNARK(+/+) mice, commensurate with the prevention of mature-onset obesity to which these animals are genetically predisposed. Physical activity resulted in a reduction in total body mass, liver mass, and white and brown adipose tissue mass in both exercise groups compared with sedentary controls. At termination of the study, body mass was similar between genotypes in the physically active mice. The prevention of weight gain in the active SNARK(+/-) mice occurred despite a 10% increase in food intake. Differences in physical activity were not attributable to sex, age, or disrupted circadian rhythm, nor were they attributable to any intrinsic deficit in forced exercise capacity/muscle energetic associated with SNARK deficiency. Direct SNARK-dependent modulation of whole body metabolism, similar to AMPK effects in the context of carbohydrate and lipid metabolism has not been demonstrated yet. However, SNARK is predominantly and constitutively localized in the nucleus, where it is likely to be regulated by protein kinase LKB1 or other unidentified kinases [12].

Interestingly, the SNARK gene expression and kinase activity is tissue specific, and its activity profile differs significantly from the AMPKα_2 activity profile. Therefore, targeting SNARK could potentially affect whole body metabolic homeostasis, and a more thorough examination of the physiological role of SNARK is warranted. Thus, protein kinase SNARK is a novel regulator of whole body metabolic homeostasis and highlights yet another protein kinase as an exciting new addition to the already extensive paradigm of homeostatic regulation by cellular energy sensors.

2.6 Protein kinase SNARK and PFKFB-3 alternative splicing

The new aspect of the biological role of protein kinase SNARK was demonstrated in SNARK-deficient mice by investigation of the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB-3) mRNA and its alternative splice variants in the liver, lungs, testes, heart, and skeletal muscle [37]. Bifunctional enzyme PFKFB is a key regulatory enzyme of glycolysis which also participates in glucose phosphorylation [38, 39]. The PFKFB-3 expression significantly increased due to hypoxia in different normal and cancer cell lines via hypoxia inducible transcription factor (HIF)-dependent mechanism [40, 41]. Hypoxia also induces expression of PFKFB-3 in different mouse organs in vivo, except skeletal muscle [42]. High expression level and phosphorylation status of PFKFB-3 as an important glycolytic regulator was determined in different malignant tumours [43–47]. Because SNARK deficiency contributed to the early phase of tumourigenesis and is important in cancer development and tumour progression [13, 14], investigation of the expression of PFKFB-3 and its alternative splice variants, which have different proliferative properties [48], is necessary for understanding the role of SNARK deficiency in tumourigenesis.

As shown in Fig. 1, the expression levels of PFKFB-3 mRNA significantly increases in the liver and lung of SNARK(-/-) knockout mice as compared to corresponding tissues of

control C57BL/6 mice [37]. At the same time, PFKFB-3 mRNA expression level in skeletal muscle of SNARK knockout mice decreases without significant changes in the heart as compared to control animals. Thus, SNARK deficiency leads to variable changes of PFKFB-3 mRNA expression in different mouse tissues.

Reverse-transcriptase-mediated PCR of the carboxyl-terminus of PFKFB-3 mRNA detected three – four cDNA products in RNA from the liver, lung, testis, heart, and skeletal muscle of control C57BL/6 and SNARK knockout mice (Fig. 2). This heterogeneity is a result of alternative splicing of the PFKFB-3 mRNA in tissue specific manner. Alternative splice variants of PFKFB-3 mRNA were identified by sequence analysis of cloned fragments. The major difference among the members of these bifunctional enzyme alternative splice variants is the length and composition of the carboxyl-terminal region (Fig. 3 and 4), supporting the idea that this terminus of the various enzyme isoforms serve to adapt the kinetic properties of the catalytic core to metabolic exigencies of a particular tissue. Alternative splice variants of PFKFB-3 also have different amounts and sequence positions of serine residues which are very important in the regulation of isozyme activity via phosphorylation [39, 46]. It was shown that the pattern of alternative splice variants of the PFKFB-3 mRNA differs in different mouse organs (Fig. 2).

Results of this study strongly support the SNARK dependent regulation of PFKFB alternative splicing. Thus, the level of smallest alternative splice variant increases in the heart and liver of SNARK knockout mice compared with control mice. However, the level of longest alternative splice variant decreases in the skeletal muscle of SNARK knockout mice compared with control mice (Fig, 2). Therefore, investigation of different alternative splice variants of PFBFB-3 isozymes is important for comprehension of tissue-specific regulation mechanisms of glycolysis. The precise molecular mechanisms, whereby SNARK participates in the splicing of PFKFB as well as a role of different isoenzymes in the regulation of glycolysis, await further study.

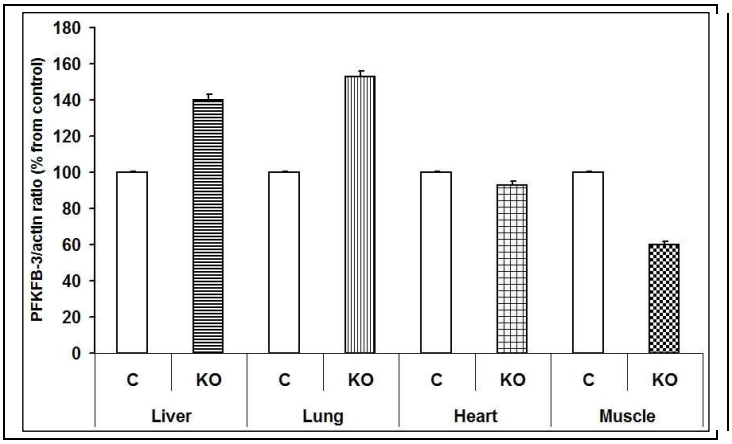

Fig. 1. Real time PCR analysis of PFKFB-3 mRNA expression in liver, lung, heart and skeletal muscle of control C57BL/6 male mice (C) and SNARK(-/-) knockout mice (KO). Amplification of PFKFB-3 mRNA was carried out using M4 forward and M5 reverse primers. Intensities of PFKFB-3 mRNA expression were normalized to β-actin mRNA [37].

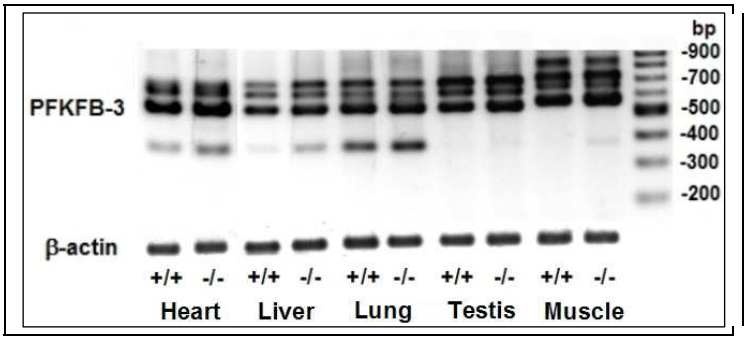

Fig. 2. RT-PCR analysis of PFKFB-3 mRNA expression in the heart, liver, lung, testis and skeletal muscle of control C57BL/6 (**+/+**) and SNARK knockout mice (**-/-**). Amplification of PFKFB-3 mRNA was carried out using M3 forward and M6 reverse primers. The amplified PCR products were run on an agarose gel. Intensities of PFKFB-3 mRNA bands were normalized to β-actin mRNA [37].

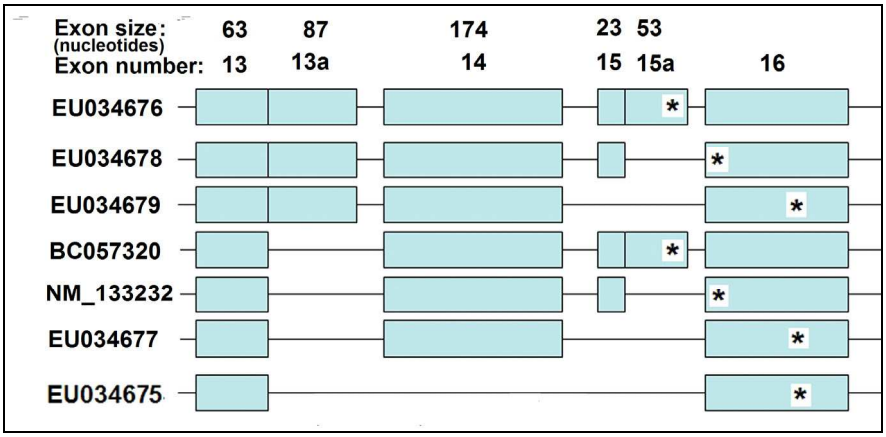

Fig. 3. Schematic representation of exon structure of mouse PFKFB-3 mRNA alternative splice variants. Most of these splice variants do not have exon 13th. Some of splice variants have 15th and 15tha exons which alters the reading frame, amino acid sequence and length of C-terminus. One splice variant is shortest because it does not have exon 14th. Position of three possible stop codons for the different alternative splice variants of PFKFB-3 mRNA are shown by asterisk. The GenBank accession number of alternative splice variants of mouse PFKFB-3 is noted on the left [38].

2.7 SNARK kinase as a stress sensor and sensitive marker of silver nanoparticles and methyl tert-butyl ether action

Recently we have shown that the expression of SNF1/AMP-activated protein kinase (SNARK) increases in different organs of male Wistar rats intratracheally instilled by 30% silver nanoparticles (28-30 nm) in sodium chloride matrix aerosol in dose 50 μg/kg (or 0.05 mg/kg) body weight (=15 μg of silver) [15]. Silver nanoparticles were prepared in the

Laboratory No. 84 of the Paton Electric Welding Institute of The National Academy of Sciences of Ukraine. The expression levels of the SNARK mRNA were analyzed in the lung, liver, brain, heart, kidney and testis using quantitative polymerase chain reaction on the 1st, 3rd or 14th day after one-time intratracheally treated rats with silver nanoparticles.

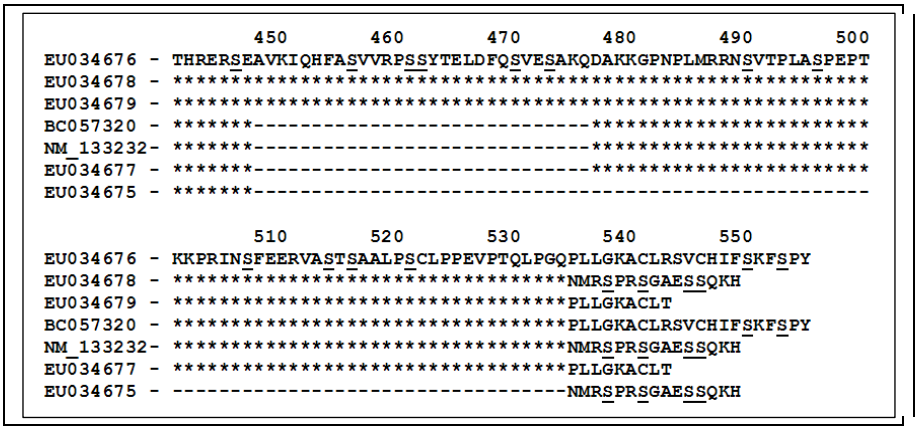

Fig. 4. Amino acid sequence of alternative splice variants of mouse PFKFB-3. Differences in amino acid sequences and length of C-terminus of different alternative splice variants of mouse PFKFB-3 are shown. Serine residues are underlined. The GenBank accession number of alternative splice variants of mouse PFKFB-3 is noted on the left [38].

It was shown that the expression of protein kinase SNARK mRNA increases in the liver, lung and brain on the 1st, 3rd and 14th day after one-time treatment of rats with silver nanoparticles, being more intense (more than 2 fold) on the 3rd and 14th day in the brain and liver and on the 1st day in the lung (Fig. 5 and 6). Results of Fig. 6 and 7 also indicated that SNARK mRNA expression does not change significantly in the heart and testes on the 1st

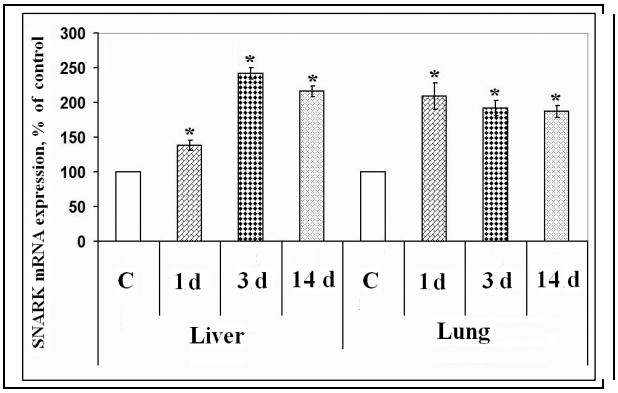

Fig. 5. The effect of silver nanoparticles on the expression of protein kinase SNARK mRNA in the liver and lung in 1, 3 or 14 days (d) after treatment. Values of SNARK mRNA expressions were normalized to β-actin mRNA expression; C – control; $n = 3$; * - $P < 0.05$ [15].

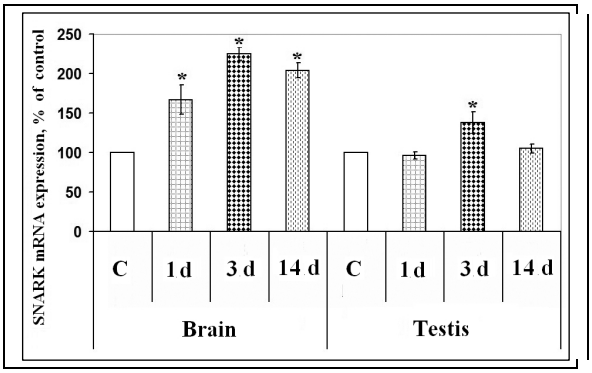

Fig. 6. The effect of silver nanoparticles on the expression of protein kinase SNARK mRNA in the brain and testis in 1, 3 or 14 days (d) after treatment. Values of SNARK mRNA expressions were normalized to β-actin mRNA expression; C – control; n = 3; * - $P < 0.05$ [15].

and 14th days after treatment of rats with silver nanoparticles, but there is a clear increase of SNARK expression on the 3rd day as compared to control animals. Results of Fig. 7 show that SNARK mRNA expression also increases in the kidney on the 1st, 3rd and 14th day after treatment of rats with silver nanoparticles, being more intense on the 3rd and 14th days; however, this induction is significantly less when compared to the liver, lung or brain.

Thus, one-time intratracheally instilled silver nanoparticles change the expression of the protein kinase SNARK in different rat organs, not only in the lung tissue. Moreover, this effect of silver nanoparticles on the expression of the protein kinase SNARK strongly depends on time after the treatment of rats with these nanoparticles in a tissue-specific manner. These results correlate to data from Lefebvre and Rosen [7] whom have shown that the regulation of protein kinase SNARK activity in response to cellular stresses greatly depends upon cell type.

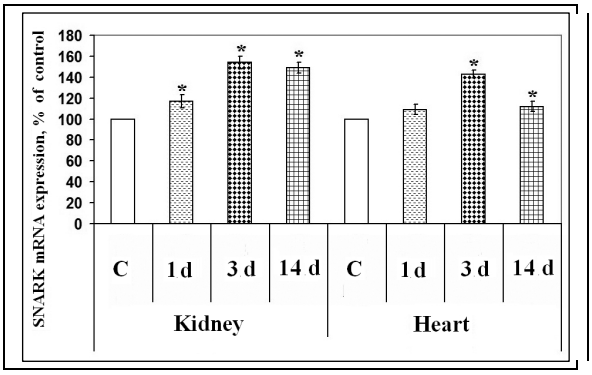

Fig. 7. The effect of silver nanoparticles on the expression of protein kinase SNARK mRNA in the kidney and heart in 1, 3 or 14 days (d) after treatment. Values of SNARK mRNA expressions were normalized to β-actin mRNA expression; C – control; n = 3; * - $P < 0.05$ [15].

Shimada et al. [49] demonstrated that the intratracheally instilled ultrafine nanoparticles are able to translocate from the mouse lung into systemic circulation. Precise mechanisms of the anatomical translocation (crossing the air-blood barrier) of inhaled nanoparticles at the alveolar wall are not fully understood. Silver nanoparticles are widely used in the field of biomedicine, but a comprehensive understanding of how silver nanoparticles distribute in the body and the induced toxicity remains largely unknown. Tang et al. [50] investigated the distribution and accumulation of silver nanoparticles in rats with subcutaneous injection. Rats were injected with either silver nanoparticles SNPs or silver microparticles (SMPs) at 62.8 mg/kg, and then sacrificed at predetermined time points. Silver content analysis by Inductively coupled plasma mass spectrometry was used for determination of silver content in different organs. Results indicated that silver nanoparticles translocated into the blood circulation and distributed throughout the main organs, especially in the kidney, liver, spleen, brain and lung in the form of particles. Ultrastructural observations indicate that those silver nanoparticles that had accumulated in organs could enter different kinds of cells. Moreover, silver nanoparticles also induced blood-brain barrier (BBB) destruction and astrocyte swelling, and caused neuronal degeneration [50].

There is data that silver nanoparticles are more toxic than silver microparticles or ions [51–53]. Powers et al. [53] have shown that silver nanoparticles have the potential to evoke developmental neurotoxicity even more potently than known neurotoxicants. Silver ions inhibited replication and increased cell death in undifferentiated cells, and selectively impaired neurite formation. Silver nanoparticles in D. melanogaster induce heat shock stress, oxidative stress, DNA damage and apoptosis [54]. Thus, silver nanoparticles up-regulate the expression of heat shock protein 70, the cell cycle checkpoint p53 and cell signaling protein p38 all of which are involved in the DNA damage repair pathway. Moreover, the activity of caspase-3 and caspase-9, markers of apoptosis was significantly higher in silver nanoparticles exposed organisms.

It is possible that silver nanoparticles create stress conditions which affect the expression of protein kinase SNARK mRNA via unfolded protein response signals through activation of inositol requiring enzyme-1 (endoplasmic reticulum–nuclei-1) and alternative splicing of XBP-1 [55 – 57]. Endoplasmic reticulum stress signalling activates inositol requiring enzyme-1 and XBP-1 which control diverse cell type- and condition-specific transcriptional regulatory networks. However, the cellular mechanism for survival under stress conditions is complex and further investigation of the mechanism by which silver nanoparticles affects protein kinase SNARK expression as well as biologic significance of silver nanoparticles induced alteration in the expression of these genes is needed. The stage is now set for the elucidation of the molecular mechanisms responsible for these important SNARK responses to silver nanoparticles action.

Results of investigations clearly demonstrate that silver nanoparticles have a significant effect on important regulatory mechanisms which control metabolic processes in different tissues via SNARK gene expression, which can be considered as a sensitive marker for silver nanoparticles action. These results suggest that more caution is needed in biomedical applications of silver nanoparticles as well as higher level of safety in the silver nanoparticles production industry.

We have also studied the effect of different doses of ecotoxicant methyl tertbutyl ether on the expression protein kinase SNARK in the liver, lung and heart [16]. Results of this

investigation demonstrated that methyl tertbutyl ether affects the expression protein kinase SNARK in the liver, lung and heart in dose dependent and tissue specific manner and that very small dose induce the expression of protein kinase SNARK in all tested vital organs in rats (Fig. 8 and 9). There is data that methyl tertbutyl ether can initiate the variety of neurotoxic, allergic and respiratory illnesses, liver hypertrophy and leukaemia in humans as well as the following cancers in rats and mice: kidney, liver, testicular and lymph nodes, initiate development of leukemia [58, 59]. We have recently shown that methyl tertbutyl ether affects the expression of PFKFB-4 mRNA and its alternative splicing [60]. Thus, SNARK gene expression can be considered as sensitive markers for the methyl tertbutyl ether action.

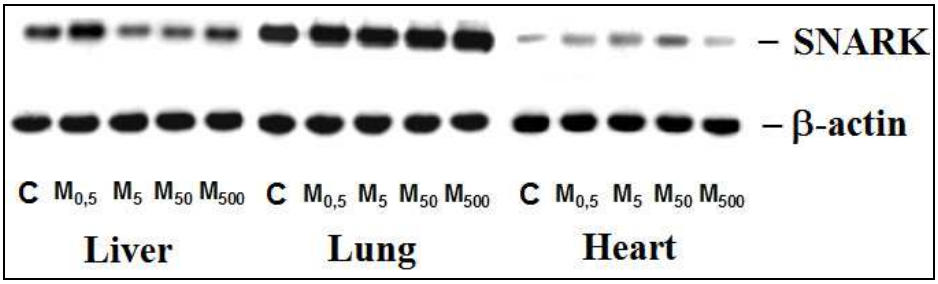

Fig. 8. Effect of methyl tertbutyl ether [0.5 ($M_{0,5}$); 5 (M_5); 50 (M_{50}) та 500 (M_{500}) mg/kg body weight during two months] on SNARK mRNA expression in the liver, lung and heart by reverse-transcriptase-mediated PCR. C – control rats.

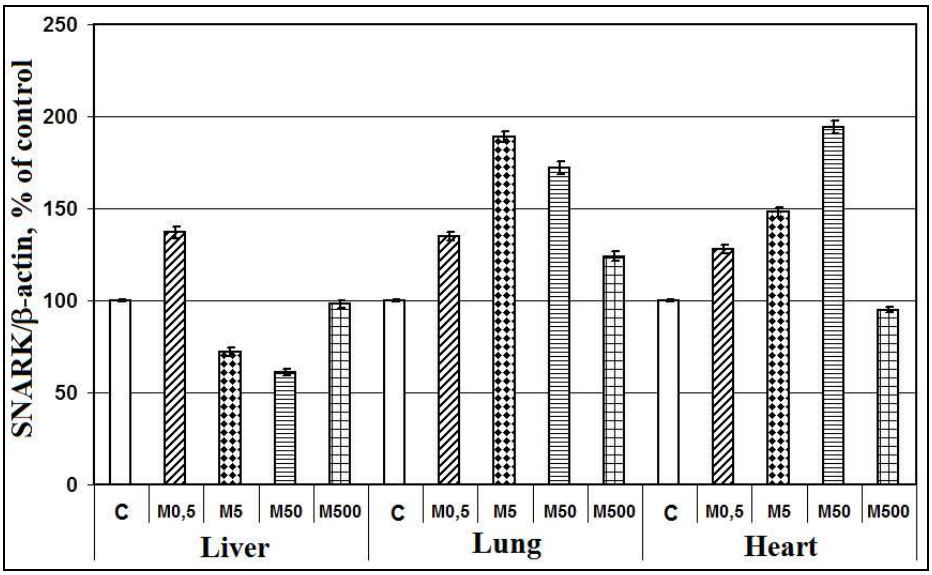

Fig. 9. Effect of methyl tertbutyl ether [0.5 ($M_{0,5}$); 5 (M_5); 50 (M_{50}) та 500 (M_{500}) mg/kg body weight during two months] on the expression levels of SNARK mRNA in the liver, lung and heart by quantitative PCR. C – control rats.

3. SNF1-like kinase, 1 (NUAK1) AMP-activated protein kinase family member 5, ARK5

Human NUAK family SNF1-like kinase 1 (NUAK1; EC_number "2.7.11.1") is an AMP-activated protein kinase family member, ARK5. AMP-activated protein kinases (AMPKs) are a class of serine/threonine protein kinases that are activated by an increase in intracellular AMP concentration. They are a sensitive indicator of cellular energy status and have been found to promote tumor cell survival during nutrient starvation. The human gene encoded protein kinase NUAK1 (ARK5) is located on chromosome 12 ("12q23.3"; GeneID: 9891). This gene encodes a protein of 661 amino acid residues. ARK5, which is the fifth member of the AMPK catalytic subunit family, is a tumor malignancy-associated factor at the downstream of Akt [19]. ARK5 is a tumour cell survival and invasion-associated factor. The activated ARK5 induces cell survival during nutrient starvation and death receptor activation, and tumor cell invasion and metastasis [18–21].

However, the precise mechanisms of how ARK5 activity inhibits caspase dependent cell death remains to be determined. Both cell death and cell survival are important for cellular homeostasis; therefore, an imbalance of their signalling causes several disease states, including tumourogenesis.

ARK5, as an AMP-activated protein kinase family member 5, is a tumour progression-associated factor that is directly phosphorylated by AKT at serine 600 in the regulatory domain, but phosphorylation at the conserved threonine residue on the active T loop has been found to be required for its full activation [18, 19]. Suzuki et al. [61] identified serine/threonine protein kinase NDR2 as a protein kinase that also phosphorylates and activates ARK5 during insulin-like growth factor-1 (IGF-1) signalling. Upon stimulation with IGF-1, protein kinase NDR2 was found to directly phosphorylate the conserved threonine 211 on the active T loop of protein kinase ARK5 and to promote cell survival and invasion of colorectal cancer cell lines through ARK5.

During IGF-1 signaling, phosphorylation at three residues (threonine 75, serine 282 and threonine 442) was also found to be required for NDR2 activation. Among these three residues, phosphorylation of serine 282 seemed to be most important for NDR2 activation (the same as for the mouse homologue) because its aspartic acid-converted mutant (NDR2/S282D) induced ARK5-mediated cell survival and invasion activities even in the absence of IGF-1. Threonine 75 in protein kinase NDR2 was required for interaction with protein S100B, and binding was in a calcium ion-dependent and phospholipase C-gamma-dependent manner [61]. Thus, protein kinase NDR2 is an upstream kinase of ARK5 that plays an essential role in tumour progression through an AMP-activated protein kinase ARK5.

NUAK1 acts as an ATM kinase under the conditions of nutrient starvation and plays a key role in tumour malignancy downstream of Akt signalling [19]. Matrigel invasion assays demonstrated that both overexpressed and endogenous ARK5 showed strong Akt dependent activity. In addition, ARK5 expression induced activation of matrix metalloproteinase 2 (MMP-2) and MMP-9. In nude mice, ARK5 expression was associated

with a significant increase in tumour growth and significant suppression of necrosis in tumour tissue. Interestingly, only the ARK5-overexpressing PANC-1 cell line tumour showed invasion and metastasis in nude mice, although Akt was activated in tumours derived from both PANC-1 and ARK5-overexpressing PANC-1 cell lines.

Suzuki et al. [21] have investigated the mechanisms of induction of cell survival by protein kinase ARK5 and have shown that ARK5 suppresses the apoptosis induced by nutrient starvation and death receptors via inhibition of caspase 8 activation. Thus, human hepatoma HepG2 cells undergo necrotic cell death within 24 h after the start of glucose starvation, and the cell death signaling has been found to be mediated by death-receptor-independent activation of caspase 8. When HepG2 cells were transfected with ARK5 expression vector and subjected to several cell death stimuli, ARK5 was found to suppress cell death by glucose starvation and TNF-alpha, but not by camptothecin or doxorubicin. Western blotting analysis revealed that glucose starvation induced Bid cleavage and FLIP degradation following caspase 8 activation in a time-dependent manner, and ARK5 overexpression clearly delayed Bid cleavage, FLIP degradation, and caspase 8 activation. These results demonstrated that cell survival induced by ARK5 is, at least in part, due to inhibition of caspase 8 activation.

AMP-activated protein kinase family member 5 also negatively regulates procaspase-6 by phosphorylation at serine 257, leading to resistance to the FasL/Fas system the key regulator promoting cell death and cell survival [22]. Fas is a type I transmembrane protein mediating intracellular cell death signalling upon the stimulation of Fas ligand (FasL). When Fas is activated by the ligation of FasL, an intracellular interaction of Fas death domain (Fas-DD), FADD, and caspase-8 (death inducing signalling complex (DISC) recruitment) is initiated for the activation of executioner caspase; and cellular FLIP is well known as the inhibitor of DISC recruitment. The serine/threonine protein kinase Akt induces cell survival as a result of phosphorylation and several cell death-associated factors, such as Bad, caspase-9 and Forkhead, upon the stimulation of growth factor receptor and integrin-induced cell signaling. Although active caspase-6 overexpression induced cell death in SW480 and DLD-1 cell lines, SW480 cells, but not DLD-1 cells, exhibite strong resistance to procaspase-6 overexpression. Moreover, mutant caspase-6, in which the serine 257 was substituted by alanine (caspase-6/SA), induced cell death and FLIP degradation, even in SW480 cells. Active ARK5 was found to phosphorylate wild-type caspase-6 in vitro, but not caspase-6/SA, and the prevented activation of caspase-6 was promoted due to its phosphorylation by active ARK5 in vitro.

AMPK-related kinases NUAK1 and many others (including MARK/PAR-1) are regulated by protein kinase LKB1 and USP9X [23, 24]. Moreover, there is data that the LKB1-NUAK pathway plays important role in controlling myosin phosphatase complexes and cell adhesion [34]. NUAK1 regulates ploidy and senescence; cells that constitutively express NUAK1 suffer gross aneuploidies and show diminished expression of the genomic stability regulator LATS1, whereas depletion of NUAK1 with shRNA exerts opposite effects [17].

AMP-activated protein kinase-related kinase 5 (ARK5/NUAK1) is expressed in rat skeletal muscle and phosphorylated by electrically elicited contractions and 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR). Increased phosphorylation of ARK5 by

muscle contractions or exposure to AICAR, however, is insufficient to activate this protein kinase in skeletal muscle, suggesting that some other modification (e.g., phosphorylation on tyrosine or by Akt) may be necessary for its activity in muscle [62].

4. Conclusions

NUAK family SNF1-like kinase includes two kinases, NUAK1 and NUAK2; both are members of AMP-activated protein kinases which are related to serine/threonine protein kinases. The sucrose-non-fermenting protein kinase (SNF1)/AMP-activated protein kinase-related kinase (SNF1/AMP-activated protein kinase; SNARK) is a member 4 of AMPK kinases (NUAK family SNF1-like kinase 2; NUAK2). SNF1-like kinase 1 (NUAK1) is an AMP-activated protein kinase family member 5, ARK5. Protein kinase NUAK2 (SNARK) is a molecular component of the cellular stress response and an important regulator of whole-body metabolism. Protein kinase SNARK was consistently localized in the nuclei. It has been shown that the nuclear localizing SNARK alters transcriptome profiles. It therefore represents a NF-κB-regulated anti-apoptotic gene that contributes to the tumour-promoting activity of death receptor CD95 in apoptosis-resistant tumour cells and plays an important role in cancer development and tumour progression. SNARK affects tumour growth, migration, and clinical outcome of human melanoma. Protein kinase SNARK is also activated by muscle contraction and is a unique mediator of contraction-stimulated glucose transport in skeletal muscle. Moreover, association between AMP kinase-related kinase SNARK and myosin phosphatase Rho-interacting protein reveals a novel mechanism for regulation of actin stress fibers via activation of myosin light chain phosphatase. Protein kinase NUAK1 (ARK5) regulates ploidy and senescence, tumour cell survival, malignancy and invasion downstream of Akt signalling and suppresses apoptosis induced by nutrient starvation and death receptors via inhibition of caspase-8 and caspase-6 activation. It is interesting to note that the expression of SNARK is a sensitive marker of silver nanoparticles and methyl tert-butyl ether toxic effect.

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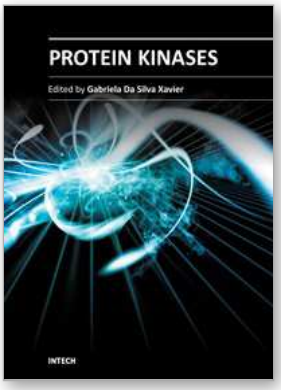

Protein Kinases

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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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