Current Topics in Biochemical Research
Vol. 16, 2014

Review

Diversified roles of p67/MetAP2 as a regulator of cell growth and differentiation, in tumor suppression, and in obesity

Bansidhar Datta

Department of Chemistry and Biochemistry, Kent State University, Kent, OH 44242, USA.

ABSTRACT

Communication among different cellular processes such as protein synthesis initiation and cell cycle inside mammalian cells are absolutely required to maintain viability of cells. Any miscommunication within these important cellular events may result in overload or lack thereof of un-translated messages in the cytoplasm and this may lead to abnormal phenotype such as cell death or apoptosis. Eukaryotic initiation factor 2 (eIF2)-associated glycoprotein, p67, whose human homolog is known as MetAP2 (Methionine aminopeptidase 2), regulates protein synthesis initiation by blocking the phosphorylation of the smallest α-subunit of eIF2 and cell cycle progression by inhibiting the activation and activity of extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein (MAP) kinases. Both ERK1/2 MAP kinases are highly activated in tumor cells and p67 inhibits this activation via direct binding to these kinases and thus shows tumor suppression activity. p67 has endopeptidase activity and it is involved in the regulation of phosphorylation of several kinases including eIF2α-specific kinases, ERK1/2 MAP kinases, and possibly several others. p67 uses its N-terminal 1-107 amino-acid-segment to block the phosphorylation site(s) of the targets or cleaves this segment auto-proteolytically that allows phosphorylation of the targets. Due to p67's multifaceted activity in modulating functions of several kinases and possibly other proteins, it is involved in tumor suppression, differentiation of myoblasts, differentiation of cardiomyocytes, and in obesity.

KEYWORDS: p67/MetAP2, tumor suppression, differentiation, obesity, protein synthesis initiation, ERK1/2 MAP kinases

INTRODUCTION

Precise balance among growth, proliferation, and differentiation of mammalian cells, plays important roles in maintaining a normal healthy organism. Any imbalance within these cellular processes leads to disease conditions like tumor formation and possibly other diseases. Several proto-oncogene products and tumor suppressors that control normal cell growth and proliferation have been identified. Among these, eukaryotic initiation factor 2 (eIF2)-associated glycoprotein p67, whose human counterpart known as MetAP2, is unique because it connects the protein synthesis machinery and cell cycle by communicating with the key proteins that control these two most important cellular processes [1]. For example, eIF2 is the key protein that is absolutely required for the initiation of mRNA translation. Phosphorylation at its smallest α-subunit by its specific kinases makes it inactive in translation initiation. However, p67 blocks this inactivation by associating with eIF2 molecules and masking the phosphorylation site of eIF2α [1]. Post-translational modification like O-GlcNAcylation of p67 is essential for it's binding to eIF2 and blocking eIF2α phosphorylation [2-3]. p67 dissociates from eIF2, possibly due to the removal of its O-GlcNAc moieties by specific O-GlcNAcase, and this leads to either auto-proteolysis of p67 or association of the later protein with extracellular signal-regulated kinases 1 and 2 (ERK1/2) [4-8]. The later kinases are a class of mitogen-activated protein (MAP) kinases, which are activated by MEK (MAP/ERK kinase) kinases 1 and 2 [9]. Activation and activity of ERK1/2 MAP kinases regulate cell cycle [9]. Association of p67 with ERK1/2 MAP kinases inhibits their activation and activity *in vitro*,

ex-vivo and *in vivo* [4-8], and thus p67 is directly involved in cell cycle regulation.

Biochemical characterization and gene organization of p67 from mammals

During purification of eIF2 from rabbit reticulocytes or other mammalian cells, p67 co-purifies and glycerol gradient centrifugation can separate p67 from eIF2, which has α-, β-, and γ-subunits. Reconstitution of these two molecules blocks eIF2α phosphorylation mediated by heme-controlled repressor, HCR or HRI [10]. Later, purified rabbit p67 was digested with proteases, peptide fragments were isolated, sequenced, and degenerate oligonucleotides were synthesized. These degenerate oligonucleotides were radiolabeled and used as probes to screen a cDNA library made from KRC-7 tumor hepatoma cells. Subsequently, p67 cDNA from rat cells was cloned and characterized [11]. Further analyses of the amino acid sequences of p67 homologs and orthologs from various species show significant sequence identity among mammals like mouse, rat, and human and even with plants and yeast. It also shows extensive sequence similarities with prokaryotes like *E. coli* and parasites [1, 12]. In addition, there is a sequence context $\mathbf{D}(X)_{10}\mathbf{D}(X)_{62\text{-}68}\mathbf{H}(X)_{32}\mathbf{E}(X)_{31\text{-}93}\mathbf{E}$, where 'X' is any amino acid - uniquely conserved in all species examined so far [12]. These five-conserved amino acid residues (**D**..**D**..**H**..**E**..**E**) seem to coordinate with divalent metal ions when its N-terminal 1-107 amino acid segment is absent, and in full-length molecule these conserved amino acid residues coordinate with its N-terminal lysine-residue rich sequences (mentioned below), and are involved in the auto-proteolysis of p67 in mammals [13]. In *E. coli*, these conserved amino acid residues are involved in the removal of N-terminal methionine and thus, mammalian ortholog of *E. coli* p67 was named as a methionine aminopeptidase, EcMAP or EcMetAP [14-15]. The N-terminus of mammalian p67 contains several unique domains including a lysine-rich sequence I, an acidic acid-rich sequence, and then another lysine-rich sequence II. At physiological pH, these domains form salt-bridges between lysine-rich domain I and acidic residue-rich domain, and acidic residue-rich domain and lysine-rich sequence II *in vitro* [7]. When bound with eIF2, the second lysine-rich sequence II and the down-stream 310-430 amino-acid segment are essential for binding to the eIF2γ subunit [16]. There is also an O-GlcNAcylation domain next to lysine-rich sequence I and together they control the phosphorylation of eIF2α through protein-protein interactions [3]. Another conserved amino acid residue, H231 is involved in the auto-proteolysis of p67 [13] and this residue covalently binds to fumagillin, an anticancer drug isolated from fungi [17]. The H231 residue opens the highly sterically constrained epoxide ring in fumagillin (Fig. 1, [17]) and acts as a suicide inhibitor to p67's auto-proteolysis [13]. Treatment of mammalian cells with fumagillin causes increased levels of p67 due to the inhibition of its auto-proteolysis and it binds to ERK1/2 MAP kinases to inhibit their activation and activity [5]. Since ERK1/2 MAP kinases control cell cycle, this inhibition causes the suppression of cell growth and proliferation of normal cells as well as K-RasV12-transformed mouse fibroblasts both *ex-vivo* and in athymic nude mice [8].

When rat p67 cDNA sequence was used to search the genomes from mouse, rat, and human, we found that the true copies of p67 gene from these species that are located in chromosome 10 for mouse, chromosome 7 for rat, and chromosome 12a and 12b for human [18-19]. This human p67 gene in chromosome 12 may influence normal brain functioning [19]. Although, several genes containing some exons and introns from these species are also found in other chromosomes, they are pseudogenes and their percentage mutations vary significantly from species to species [18]. In addition, some chromosomes contain p67 cDNA sequence and no introns; some chromosomes contain a few p67 exons with different lengths as compared to the true copy of the gene, indicating that these are pseudogenes [18]. Mouse pseudogenes are located in chromosomes X, 7, and 11; rat pseudogenes are located in chromosomes X, 1a, 1b, 2, and 18; and human pseudogenes are located in chromosomes 2a, 2b, and 7. Comparing the sequences of exons, their length, and percentage of mutations acquired by these pseudogenes, it is clear that they have originated either by deletion and insertion of exons or due to insertion of jumping genes [18]. Further analyses of the promoter regions of the true copies of p67 genes from mouse, rat, and human revealed the presence of several *cis*-elements – many of them are common to each other and several of them are either tissue-specific or species-specific [18].

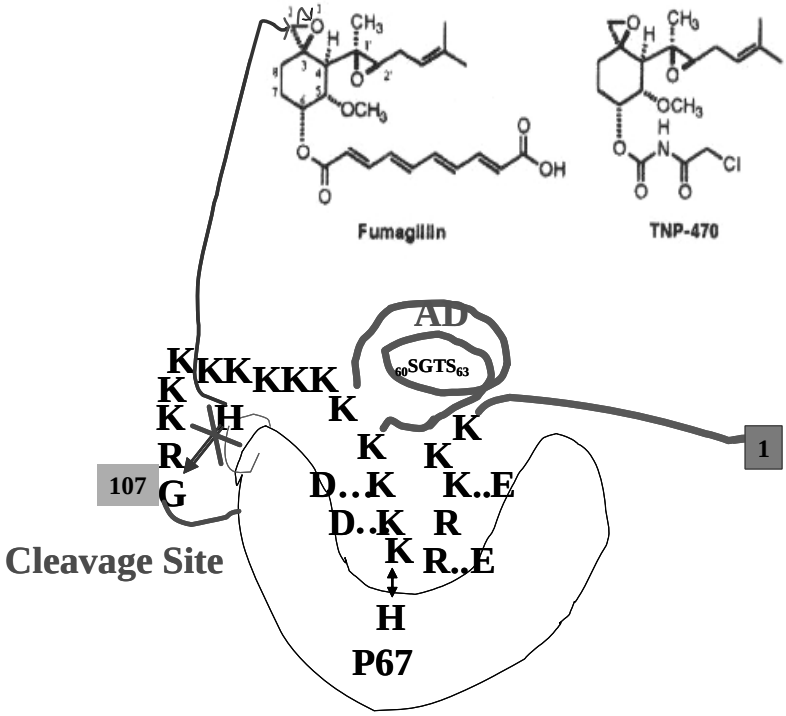

Fig. 1. A schematic view of p67's covalent binding with anti-angiogenic drug fumagillin or TNP-470. The full-length p67 showing its N-terminal p26 segment wraps around its p52 segment that forms the shallow groove juxtaposing the H231 catalytic residue with the cleavage site at R-107, which is located at the end of KI domain. Either lysine-residues or the arginine residues are coordinating with the conserved D251, D262, E364, and E459 residues via positive and negative charges. The chemical structures of fumagillin and its derivative TNP-470 (Adapted from Ref. [17]) are also shown. An arrow shows the N-C bond formation between H231 and the sterically constrained epoxy group of either fumagillin or TNP-470.

Biochemical characterization of MetAP2

In late 80's and early 90's, there was a desperate search for an enzyme, methionine-aminopeptidase (MAP or MetAP) that can remove terminal methionine from proteins. Extensive protein purification schemes were applied, several enzymes were purified, but no unique enzyme showed MAP or MetAP activity. In most cases it was contaminated with other enzymes (for a detailed review see ref. [12] and references therein). An enzyme, named PcMAP was purified to homogeneity from porcine and its amino acid composition was determined [20]. Nearly 95% of the proteins synthesized by the ribosomes contain N-terminal methionine. In some cases, the MAP removes this N-terminal methionine while the nascent peptide is 15-20 amino acids long and is still bound to ribosomes [21-22]. In other words, MAP must be ribosome-bound. Using the cDNA sequence from rat p67, human p67 was cloned and characterized [23]. Using GST (glutathione-S-transferase)-fusion of human p67, an *in vitro* assay was developed to test for the MAP or MetAP activity. In this assay, a tetra-peptide substrate, Met-Gly-Met-Met was incubated with the GST-fusion of recombinant human p67; released amino acid residues were separated by HPLC and identified as methionine. Subsequently, human p67 was named MetAP2 [23]. Later, both MetAP2 and another totally unrelated protein named MetAP1 were also isolated from yeast and showed their involvement in sulfur metabolic pathway in yeast [24-27]. Although, there is > 95% amino acid sequence identity between rat and human p67, comparison of amino acids' compositions from porcine, rat, and human revealed no sequence identity or similarity [12]. In addition, analyses of the MetAP2 activity of highly purified p67 from rabbit reticulocytes and GST-fusion of rat recombinant p67 using the above tetra-peptide revealed no detectable activity that removes N-terminal methionine [12]. In the N-terminus of p67 from mammals like

mouse, rat, and human, there are several uniquely conserved sequences such as lysine residue-rich sequences I (KI domain) and II (KII domain), and acidic residue-rich sequence [1, 12]. A mono-specific antibody that can recognize only the lysine residue-rich sequence I, can identify this sequence in rat, rabbit, and human p67 but not from yeast MetAP1, which is also known as MAP1 [28], indicating that MetAP1 is a unique protein that may have methionine aminopeptidase activity and it does not have any link with p67 or so called MetAP2 (see below for more argument regarding this). In addition, p67 shows dimerization activity with its homologs from rat, rabbit, and human but not with MetAP1 [28]. Altogether, these evidence support the conclusion that p67 from mammals has no MetAP activity.

To improve the MetAP assay, Met-Pro-pNA is used as the substrate and surprisingly proline aminopeptidase is used along with methionine aminopeptidases to release the terminal methionine [29]. In another study, Met-Gly-Pro-AMC is used as a substrate and porcine dipeptidyl peptidase along with the methionine aminopeptidases is used to release the amino acids from AMC (7-amino-4-methyl-courmarin) [30]. In spite of all the published evidence, which suggest that p67 does not have methionine aminopeptidase activity, human p67 is coined as a MetAP2 just to take advantage of the easy, but highly questionable assay for MetAP2. Using this assay hundreds of drugs have been identified as MetAP2 inhibitors (for a detailed review see ref. [1]). None of these drugs except fumagillin and its derivatives show specificity to mammalian p67, although many of them inhibit cell growth in *ex-vivo* conditions [1]. Moreover, MetAP1 does not bind to fumagillin or its derivatives [17]. Although, there is no sequence similarity or functional complementarity between MetAP1 and MetAP2, in many cases the research has been done in MetAP1 and conclusions have been drawn with both MetAP1 and MetAP2 [31]. Only similarity between these two proteins is their pita-bread fold [32], which is also present in several proteins and enzymes like many metalloenzymes, including the dioxygen-carrier protein, hemerythrin; the dinuclear non-heme ironprotein, ribonucleotide reductase; leucine aminopeptidase; urease; arginase; several phosphatases and phosphoesterases [33]. It is not clear why the MetAP2 name for human p67 was chosen. Since human p67 does not have methionine aminopeptidase activity, using the MetAP2 terminology for this protein will divert the scientific knowledge far away from the true biological activity of p67.

Involvement of p67 in proteolysis

In fact, p67 is an endopeptidase rather than a N-terminal methionine aminopeptidase. This is based upon the following facts: Methionine aminopeptidase, EcMAP from *E. coli* was purified, sequenced, cloned, and characterized [14-15]. It shows near 40% overall sequence similarity and five highly conserved amino acid residues – D, D, H, E, and E are positioned almost at near fixed distances as seen in other organisms like yeast, plants, drosophila, mouse, rat, and human [1, 12]. These conserved amino acid residues can bind to divalent metal ions and form the appropriate substrate-binding pocket to facilitate the proteolytic activity. As this molecule (EcMAP) has evolved with time, more exons have added at the N-terminus of its orthologs that provide several unique domains and motifs, which play important roles in cellular activities in higher organisms [1]. Especially in mammals, there are two lysine-rich domains (KI and KII) separated by an acidic-rich sequence, an O-GlcNAcylation site, and a conserved histidine residue (H231) that acts as the catalytic site, whereas its downstream segment maintains divalent metal binding to provide the substrate-binding pocket [1]. This protease also has significant sequence similarities at its substrate-binding pocket with other enzymes and proteins [33]. Surprisingly, most of the MetAP2 inhibitors are chemicals that show structural and functional similarities with typical protease inhibitors [1]. X-ray crystallographic studies show that the downstream 108-480 amino acid segment of p67 (also known as p52 based on its migration on 15% SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) can form a shallow groove where four out of the five conserved amino acid residues D251, D262, E364, and E459 can coordinate with the divalent metal ions [17]. However, in the full-length molecule, the highly positively charged KI domain in the N-terminal 1-107 amino acid segment (also known as p26 based on its mobility in 15% SDS-PAGE) can substitute the action of divalent metal ions and coordinates with the conserved

D251, D262, E364, and E459 amino acid residues (Fig. 1). Indeed, our extensive mutational studies show that the full-length molecule cleaves the p26 segment using H231 during its autocatalysis and the remaining p52 segment also has some auto-cleavage activity [13]. Substitution of D251, D262, E364, and E459 residues with alanine causes increased stability of p67, whereas substituting H231 residue with glutamic acid, increased p67's auto-proteolysis activity significantly while alanine decreased this activity [13]. In addition, substitution of H331, another conserved amino acid residue with alanine increased p67's auto-proteolysis significantly that degraded not only endogenous and exogenous p67, it also degraded several other cellular proteins [13]. This indicates that H331 residue may inhibit the coordination between the N-terminal KI domain or KII domain and the conserved acidic residues present at the shallow groove of p52 segment (Fig. 1). Like rat and mouse p67, human p67 or MetAP2 also has auto-proteolytic activity [34] and its auto-proteolysis is further increased both *in vitro* and *in vivo* by calpains, calcium dependent proteases that are involved in neuronal injury [34].

p67 binds to fumagillin covalently at its catalytic H231 residue [17] and this binding leads to the inhibition of its auto-proteolysis, decreasing its turn over rate, and increasing its cellular concentration [5]. This increased p67 level in cells leads to the association with ERK1/2 MAP kinases and inhibits their activation and activity both *ex-vivo* and *in vitro* [5]. When ERK1/2 MAP kinases bind at the shallow groove of p52 segment of p67, its p26 segment wraps around the $T_{183/202}EY_{185/204}$ phosphorylation sites (Fig. 2) and thus, inhibits their activation and activity.

The N-terminal p26 segment, which is usually generated due to p67's auto-proteolysis, can localize almost everywhere in the cell, although the localization of the full-length molecule is restricted into the cytoplasm (Fig. 3, Part I). Further mutational analyses on the KI, KII, and acidic domain show that the KI domain may be responsible for the generation of multi-nucleated giant cells (Fig. 3, Part II). This may be due to the fact that KI and KII domains either alone or together have the ability to suppress the phosphorylation of several kinases including eIFα-specific kinases [35], cell cycle-specific kinases [36], and possibly others.

Why does a protease like p67 bind to eIF2, the key protein for mRNA translation?

In cellular system, p67 is tightly bound with eIF2 possibly in equimolar amount [10]. The KII domain

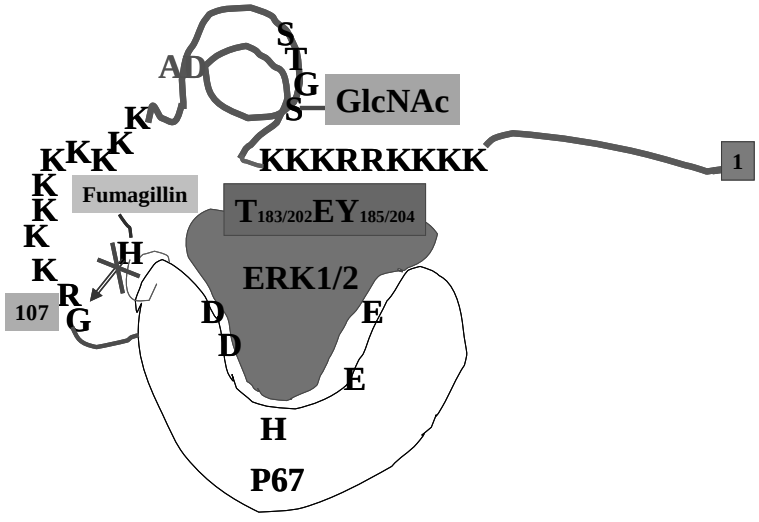

Fig. 2. A schematic view of association between p67 and ERK1/2 MAP kinases. The binding region of ERK1/2 MAP kinases is located in the shallow groove of p52 segment of p67. The ERK1/2 MAP kinases' higher affinity for this site displaces the p26 segment of the molecule that wraps around the phosphorylation sites of these kinases to inhibit its activation by MEK1/2 kinases. Fumagillin binds to H231 residue irreversibly and this inhibits its auto-proteolysis shown by 'X'.

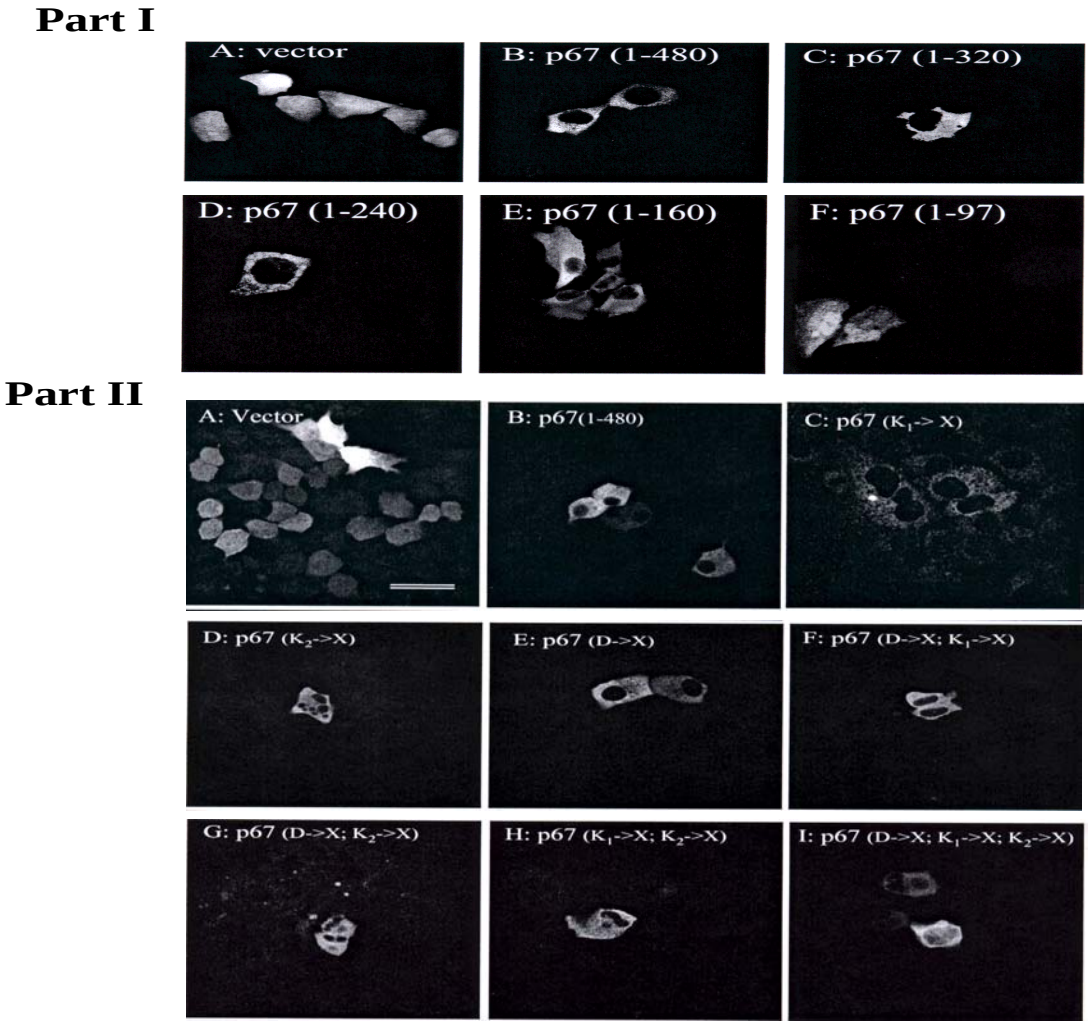

Fig. 3. Intracellular localization of p67, and its deletion and block mutants. The EGFP-fusions of p67 and its various deletion mutants (upper panel, Part I) and block mutants (lower panel, Part II) were transiently transfected into KRC-7 cells. 36 hrs after transfection cells were examined under a microscope. P67($K_1 \rightarrow X$) = K1/2/3, p67($K_2 \rightarrow X$) = K2/9/1, p67($D \rightarrow X$) = D6/2, p67($D \rightarrow X$, $K_1 \rightarrow X$) = D6K1/7, p67($D \rightarrow X$,$K_2 \rightarrow X$) = D6K2/5, p67($K_1 \rightarrow X$,$K_2 \rightarrow X$) = K1K2, and p67($D \rightarrow X$,$K_1 \rightarrow X$,$K_2 \rightarrow X$) = D6K1K2. For detailed description of these mutants see the Ref. [28].

and 310-430 amino acid segment of p67 bind tightly with the γ-subunit of eIF2, covering almost half of the substrate binding pocket [16], thus inhibiting p67's auto-proteolysis. On the other hand, the N-terminal KI domain and the O-GlcNAcylation site ($_{60}SGTS_{63}$) are masking the serine-51 site of the eIFα, thus blocking its phosphorylation from the kinases [1]. Phosphorylation of eIF2α is lethal to eukaryotic cells [1]. Since the serine-51 residue of eIF2α is the site for phosphorylation by several kinases including its four well-characterized kinases [1] and p67's N-terminal lysine residue-rich domains have the ability to suppress the phosphorylation of several kinases [35], it is a perfect natural selection to form a p67/eIF2 complex in eukaryotic cells to sustain their viability. When the active O-GlcNAase removes the O-GlcNAc moieties from the N-terminus and other unidentified sites located at the downstream 108-480 amino acid residues of p67, it dissociates from eIF2. Unprotected eIF2 is phosphorylated at its α-subunit by the responsible kinases and thus, protein synthesis initiation is inhibited [1]. On the other hand, unbound p67 has several options depending

upon the cellular conditions. Its N-terminus can fold around its shallow groove and this will lead to the activation of its auto-proteolytic activity. Subsequently, it will generate the N-terminal p26 segment and an active protease – the 108-480 amino acid segment of the molecule, also known as p52 [13]. The p52 segment may have several cellular targets including cell cycle-regulatory cyclins and cyclin-dependent kinases [36], other cellular kinases [35], and possibly eIFα-specific kinases. It remains to be seen whether the p52 fragment of p67 can act as a general metalloproteinase or not.

Involvement of p67 in cell cycle regulation

Once p67 is dissociated from eIF2 or its cellular concentration is increased due to the inhibition of its auto-proteolytic activity [5] or overexpression of this protein from exogenous expression plasmid [8], it can bind to other high-affinity cellular targets such as ERK1/2 MAP kinases [4-8]. These MAP kinases regulate cell cycle progression in response to signals mediated by growth factors like, EGF (epidermal growth factor) or PDGF (platelet derived growth factor) [9]. When these growth factors bind to their receptors at the cell surface, the receptor tyrosine kinases (RTKs) are activated through trans-phosphorylation and this recruits an adopter protein Grb2, which then binds to a GDP/GTP (guanidine diphosphate/guanidine triphosphate) exchange protein, SOS (son-of-seven less) displaces the GDP from Ras-GDP, a proto-oncogene, with GTP. The GTP-bound Ras then phosphorylates its downstream target, Raf, a serine/threonine kinase. The phosphorylated form of active Raf subsequently phosphorylates the downstream targets, MEK1/2, which are the upstream activators of ERK1/2 MAP kinases [9]. The phosphorylated form of ERK1 modulates the protein translational machinery [37], whereas the phosphorylated form of ERK2 migrates to the nucleus to induce the gene expression of several growth promoting genes, whose protein products drive the cell cycle in different phases [38].

The ERK1/2 MAP kinases bind at the 211-480 amino acid segment of p67, almost the same region where γ-subunit of eIF2 binds [8, 16], masking the whole substrate-binding pocket region as well as the catalytic histidine-231 residue, leaving p67's N-terminal sequences that can wrap around the phosphorylation sites of ERK1/2 MAP kinases to inhibit their phosphorylation by MEK1/2 kinases (Fig. 2). This inhibition of ERK1/2 activation by p67 not only inhibits cell cycle progression, it also modulates the protein synthesis machinery mediated by mTOR signaling pathway [39]. Overall consequence of this inhibition of activation of ERK1/2 MAP kinases leads to the cell growth inhibition [39] and in tumor cells, inhibition of angiogenesis [8].

Involvement of p67 in differentiation

If mammalian cells are growth arrested for an extended period of time, they will undergo apoptosis, a programmed cell death or lineage-specific differentiation. In response to environmental signals, precursor cells differentiate into specific lineages.

(a) Skeletal muscle differentiation

Myoblasts, the satellite cells around the myofibers, multiply and then differentiate into myotubes to fulfill the cellular requirements such as wound healing or muscle regeneration [40]. To study the molecular details of this differentiation pathway, C2C12 mouse myoblasts are routinely used as an excellent model system [41]. When C2C12 myoblasts differentiate into myotubes in differentiation medium, the levels of p67 increase due to its low turn over rate and this increased level associates with the ERK1/2 MAP kinases to inhibit their activation and activity [6]. This then leads to the inhibition of signaling into the myotubes via ERK1/2 MAP-mediated pathways. During these differentiation conditions, rates of global protein synthesis were decreased due to the dissociation of p67 from eIF2α that causes the increased levels of eIF2α phosphorylation [42]. This phosphorylation of eIF2α is however not due to one of its specific kinases, PKR, because its protein level does not correlate with the level of eIF2α phosphorylation [42]. In addition, our recent study shows that the real substrate of PKR could be eIF5 rather than eIF2α [43]. Nonetheless, these results suggest that there is a mutual communication between the cell cycle progression and protein synthesis machinery where p67 acts as a molecular switch at least during differentiation of myoblasts into myotubes.

(b) Cardiac muscle differentiation

Ischemic heart disease, resulting in cardiac muscle loss, is the leading cause of morbidity and mortality

in the human population all over the world. The role of blood vessel formation within diseased blood vessels has become one of the most outstanding puzzles in the biology of cardiovascular disease. The formation of the new blood vessels from existing ones either by splitting or sprouting is known as angiogenesis [44]. The role of angiogenesis in atherosclerosis and other cardiovascular diseases has emerged as a major unresolved issue. Angiogenic cytokine therapy has been widely regarded as an attractive approach for treating ischemic heart disease and for enhancing arterioprotective functions of the endothelium [45]. The study by Moulton *et al.*, shows that two endothelium-specific inhibitors of angiogenesis, endostatin and TNP-470, reduce plaque area in $ApoE^{-/-}$ mice by 85% and 70% respectively [46]. This provides the direct evidence that angiogenesis is involved in the process of plaque formation. Although TNP-470 significantly reduces further growth of atherosclerosis, it does not affect the cholesterol level [46]. TNP-470 is a derivative of fumagillin and is most effective in anti-angiogenesis as compared to fumagillin [17].

Heart development depends upon the levels of A- and B-natriuretic peptides (ANP and BNP) and expression of these peptides is a marker of cardiomyocyte differentiation [47-48]. A family of transcription factors, which include GATA-4, -5, and -6, regulates the expression of ANP and BNP. Genetic, biochemical, and mutational studies both *in vitro* and *in vivo* provide evidence to support that GATA-4 is the upstream activator of transcription factor cascade regulating cardiomyocyte differentiation [49-51]. In cultured cardiomyocytes, phenylephrine (PE)-induced up-regulation of the endothelin-1 promoter was associated with increased phosphorylation of GATA-4, which was sensitive to PD98059, suggesting a role for MEK1/2-ERK1/2 in regulating the expression of GATA-4 [52]. GATA-4 contains a conserved MAPK phosphorylation site at serine 105. This site is phosphorylated in response to agonist stimulation through MEK1-ERK1/2, but only weakly through JNK1/2 or p38 MAPKs [53].

P19 embryonal carcinoma cells have been used for many years to study the cardiac gene expression program during differentiation of cardiomyocytes in *ex-vivo* conditions [54]. To study the cardiac differentiation, P19 cells are usually seeded at a low density in tissue culture dishes containing alpha medium and differentiation is initiated by growing these cells in bacterial grade plastic dishes in the presence of 1% DMSO. Within a few hours, cells started to aggregate and these aggregates are transferred to cell culture petri dishes and allowed to differentiate into cardiomyocytes for different time intervals in growth medium (Fig. 4). We found that P19 aggregates differentiate into cardiomyocytes as evidenced by the expression of β-MHC (Fig. 4A) and during this time, the p67 level gradually decreases (Fig. 4C). At 8 days of differentiation, p67 level decreases to ~7-fold as compared to P19 cells aggregated in the presence of DMSO for 4 days (compare lane 5 with lane 1 in Fig. 4C). During these time intervals of differentiation, the phosphorylated forms of ERK1/2 MAP kinases are undetectable in P19 aggregates and their levels increase gradually to a maximum level at 8 h of differentiation (Fig. 4F, lane 5). These results further confirmed the reciprocal relationship between levels of p67 and activation and activity of ERK1/2 MAP kinases during cardiomyocyte differentiation. In addition, since GATA-4 activation via phosphorylation by ERK1/2 MAP kinases is essential for cardiomyocyte differentiation [52-53, 55] and p67 inhibits the phosphorylation of these MAP kinases [4-8], it is therefore essential to inhibit the expression of p67 while cardiomyocytes are differentiating into cardiac tissues.

Involvement of p67 in tumor suppression

P67's unique affinity for ERK1/2 MAP kinases, which are involved in growth promoting signals to the nucleus and cell cycle regulation, prompted us to examine whether it can suppress tumor growth both in *ex-vivo* and *in vivo* conditions. A potent mutant of K-Ras, K-RasV12, which is found in more than 65% of human cancer cells, is constitutively expressed in mouse NIH3T3 fibroblasts to transform cells that can form *in situ*-tumor in petri dishes. These tumor-forming transformed cells were transfected with a plasmid carrying the exogenous p67 cDNA and both transfected and non-transfected K-RasV12 transformed NIH3T3 cells injected in both dorsal flanks of the athymic nude mice on either side of the midline. After thirteen days of post-injections, tumors were removed from the mice, weighed, and analyzed for the new blood

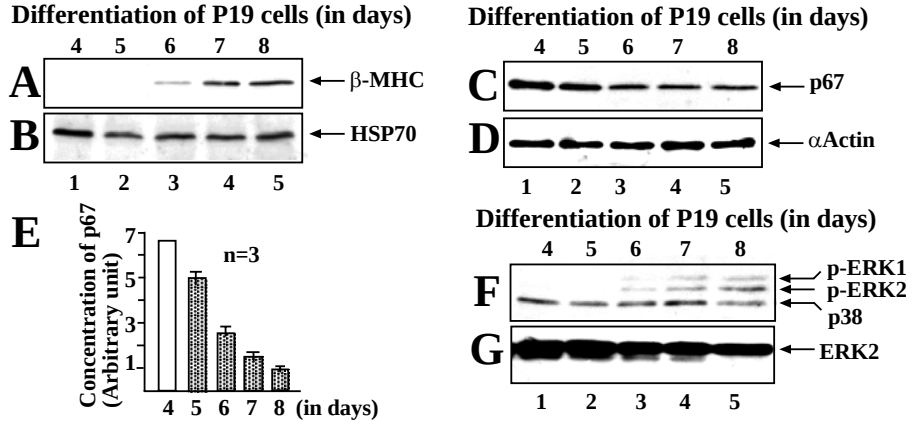

Fig. 4. Expression of p67 and activation of ERK1/2 MAP kinases during differentiation of P19 cells into cardiomyocytes. Around 10^5 cells/ml of P19 cells (ATCC) were seeded on bacterial dishes in the presence of 1% DMSO and kept for 4 days at 37 °C incubator containing 5% CO_2 and 95% air. Cells aggregated rapidly and these aggregates were collected from the cell culture suspension and plated on tissue culture plates. This point is taken as 4 days of differentiation (lane 1). These cell aggregates settled down quickly and were allowed to differentiate into cardiomyocytes in growth medium for indicated times. Cells were harvested, extracts were prepared, and equal amounts of total protein samples were analyzed on Western blots for β-myosin heavy chain (MHC) (A) to test for the progression of differentiation; HSP70, for the loading control (B); p67 (C); αActin, for loading control (D); p-ERK –1 and –2 (F); p38, as a loading control (F); and total ERK2 (G). These experiments were repeated three times and band intensities for p67 were scanned and plotted on a graph (E).

vessel formation in thin slices of the tumors. The results show that indeed overexpression of p67 in transformed cells can suppress tumor growth by inhibiting angiogenesis and this involves the inhibition of the signaling pathways mediated by ERK1/2 MAP kinases [8]. In other reports, it has been shown that there is an increased level of MetAP2 in some tumor cells [56]. Since these researchers always perform analysis with MetAP1 and extend the data to MetAP2 without performing any experiments with MetAP2 [31], it is not clear whether MetAP1 or MetAP2 is overexpressed in those cancer cells. MetAP1 and MetAP2 are two different proteins with different cellular functions.

Involvement of p67 in obesity

Obesity is one of the top health problems along with cancer and heart disease. There are several factors contributing to obesity [57] and a series of drugs are either approved by FDA or are in different phases of clinical trials (see ref. [58] for a detailed review). A few such drugs, beloranib or CKD-732, a TNP-470 derivative, fumagillin itself and its derivative, TNP-470, have anti-obesity effects and the molecular mechanism of their actions is through the suppression of signal regulated by ERK1/2 MAP kinases [59]. It has been shown that beloranib binds to human p67 reversibly whereas fumagillin or TNP-470 binds to this protein irreversibly [1, 17]. Molecular details of beloranib binding to human p67 are not known, although, it has higher affinity for p67 and higher efficacy for suppressing tumor growth as compared to TNP-470 [60]. When mammalian cells were treated with fumagillin that binds at H231 residue of p67, the level of p67 increases due to its low turn over rate [5]. Similarly, when obese C57BL/6J mice were treated with fumagillin, their inguinal subcutaneous fat pads show significantly smaller adipocyte size and higher density [61]. Expression levels of p67 along with TIE-2, angiopoietin-1 and angiopoietin-2 mRNA in gonadal adipose tissues were significantly up-regulated in fumagillin-treated obese mice [61]. Detailed studies with obese mice and phase II trials with obese patients using TNP-470 and beloranib showed significance promise for these drugs in the treatment of obesity (for a detailed review, see ref. [58]). Both TNP-470 and beloranib show significant structural

similarity, especially the identical locations of the two epoxy groups. The most sterically constrained epoxy group of TNP-470 binds to p67 irreversibly via covalent bond with the imidazole ring of histidine 231 [17]. It is therefore puzzling as to how beloranib binds to p67 reversibly. Since TNP-470 binds to p67 irreversibly, long-term treatment of obese patients with this drug will have adverse side effects including severe neurological impairments. This is due to the fact that higher cellular concentrations of p67 have the ability to shut-down or down-regulate the functions of several key molecules that are required to sustain viability of mammalian cells apart from the adipose cells.

CONCLUSIONS

Eukaryotic initiation-factor 2-associated glycoprotein, p67 plays important roles in the cellular systems via communicating with key proteins that are involved in the regulation of several cellular pathways. For example, it controls the rate of global protein synthesis by modulating the function of eIF2. p67 also regulates cell cycle by modulating the functions of growth promoting ERK1/2 MAP kinases that control the proto-oncogene, Ras-mediated cell signaling pathways. Most of these key proteins bind to p67 at its highly conserved p52 segment that forms a shallow groove and uses the N-terminal p26 segment of p67 to regulate their activities. Therefore, in various stress conditions such as viral infection, heme-deficiency, amino acid starvation, ER stress, growth factor-deficiency, differentiation, tumorigenesis, and others, this p26 segment has been subjected to various modifications such as O-GlcNAcylation/deglycosylation [1], possibly phosphorylation [35], and in worse case scenario it has been cleaved from the molecule due to the activation of p67's auto-proteolysis [13]. This activation of auto-proteolysis may be the key mechanism by which tumor cells inactivate p67's action towards eIF2 and ERK1/2 MAP kinases such that cancer cells can grow. Higher levels of eIF2α phosphorylation could be seen in several cancer cells [62-65]. However, the question remains as to how cancer cells tolerate high levels of eIF2α phosphorylation. In mammals, the p26 segment of p67 has the most degenerate sequences as compared to its other p52 segment, whose sequence is highly conserved [1]. In addition, there is an extensive cleavage of p67 that generates p26 segment in most cancer cells. This cleavage of p67 is due to its intrinsic proteolytic activity that has evolved from *E. coli* [1]. Auto-proteolysis of p67 generates the N-terminal p26 segment that can localize to any part of the cells and may cause giant cell morphology (Fig. 3). The downstream p52 segment of p67 may act as a general protease that may have several cellular targets including cell cycle regulators, signaling molecules, eIF2α-specific kinases, and possibly others. Based on the cellular targets for degradation, there will be different cellular outcomes. Due to the multifaceted roles of p67 in cellular system, different classes of organic molecules have been used to inhibit its activity, but in all cases a different name such as MetAP2 is used instead of p67. Using a simplistic and controversial assay, hundreds of drugs have been synthesized and these drugs showed cell growth inhibition due to their reversible or irreversible binding to MetAP2. MetAP2 shares > 95% sequence identity with p67 [1]. Since many of these chemicals or so called MetAP2 inhibitors are identified based upon a wrong function of p67, their true effects or side effect will be apparent only after these drugs go through the different phases of clinical trials.

ACKNOWLEDGEMENTS

The author would like to thank the past and present lab members for their valuable discussions. The author would also like to thank the editors at Research Trends for valuable comments and suggestions to improve the manuscript.

CONFLICT OF INTEREST STATEMENT

The author confirms that there are no conflicts of interest.

REFERENCES

1. Datta, B. 2009, Biochem. Biophys. Acta, 1796, 281.
2. Datta, B., Ray, M. K., Chakrabarti, D., Wylie, D. E. and Gupta, N. K. 1989, J. Biol. Chem., 264, 20620.
3. Datta, R., Choudhury, P., Ghosh, A. and Datta, B. 2003, Biochemistry, 42, 5453.
4. Datta, B., Datta, R., Ghosh, A. and Majumdar, A. 2004, Arch. Biochem. Biophys., 427, 68.

5. Datta, B., Datta, R., Majumdar, A. and Balusu, R. 2004, Biochemistry, 43, 14821.
6. Datta, B., Datta, R., Ghosh, A. and Majumdar, A. 2005, Exp. Cell Res., 303, 174.
7. Datta, B., Datta, R., Ghosh, A. and Majumdar, A. 2006, Arch. Biochem. Biophys., 452, 138.
8. Majumdar, A., Ghosh, A., Datta, S., Prudner, B. C. and Datta, B. 2010, Biochemistry, 49, 10146.
9. Raman, R., Chen, W. and Cobb, M. H. 2007, Oncogene, 26, 3100.
10. Datta, B., Chakrabarti, D., Roy, A. L. and Gupta, N. K. 1988, Proc. Natl. Acad. Sci., USA, 85, 3324.
11. Wu, S., Gupta, S., Chatterjee, N., Hileman, R. E., Kinzy, T. G., Denslow, N. D., Merrick, W. C., Chakrabarti, D., Osterman, J. C. and Gupta, N. K. 1993, J. Biol. Chem., 268, 10796.
12. Datta, B. 2000, Biochimie, 82, 95.
13. Datta, B., Ghosh, A., Majumdar, A. and Datta, R. 2007, Biochemistry, 46, 3465.
14. Ben-Bassat, A., Bauer, K., Chang, S. Y., Myambo, K., Boosman, A. and Chang, S. 1987, J. Bacteriol., 169, 751.
15. Liang, S. M., Allet, B., Rose, K., Hirschi, M., Liang, C. M. and Thatcher, D. R. 1985, Biochem. J., 229, 429.
16. Ghosh, A., Datta, R., Majumdar, A., Bhattacharya, M. and Datta, B. 2006, Exp. Cell Res., 312, 3184.
17. Liu, S., Widom, J., Kemp, C. W., Crews, C. M. and Clardy, J. 1998, Science, 282, 1324.
18. Datta, B., Earl, D., Rood, M. and Datta, S. K. 2014, Int. J. Mol. Genet., 5, 1.
19. Borg, K., Stankiewicz, P., Bocian, E., Kruczek, A., Obersztyn, E., Lupski, J. R. and Mazurczak, T. 2005, Hum. Genet., 118, 267.
20. Kendall, R. L. and Bradshaw, R. A. 1992, J. Biol. Chem., 267, 20667.
21. Jackson, R. and Hunter, T. 1970, Nature, 227, 672.
22. Wilson, T. B. and Dintzis, D. B. 1970, Proc. Natl. Acad. Sci. USA, 66, 1282.
23. Li, X. and Chang, Y. H. 1995, Biochem. Biophys. Acta, 1260, 333.
24. Li, X. and Chang, Y. H. 1995, Proc. Natl. Acad. Sci. USA, 92, 12357.
25. Chen, S., Vetro, J. A. and Chang, Y. H. 2002, Arch. Biochem. Biophys., 398, 87.
26. Vetro, J. A. and Chang, Y. H. 2002, J. Cell Biochem., 85, 678.
27. Dummitt, B., Micka, W. S. and Chang, Y. H. 2003, J. Cell Biochem., 89, 964.
28. Ghosh, A., Tammali, R. Balusu, R., Datta, R., Chattopadhyay, A., Bhattacharya, M. and Datta, B. 2014, Int. J. App. Biotech. Biochem., 4, 25.
29. Hu, X. V., Chen, X., Han, K. C., Mildvan, A. S. and Liu, J. O. 2007, Biochemistry, 46, 12833.
30. Garrabrant, T., Tuman, R. W., Ludovici, D., Tominovich, R., Simoneaux, R. L., Galemmo, Jr. R. A. and Johnson, D. L. 2004, Angiogenesis, 7, 91.
31. Zheng, F., Bhat, S., Gabelli, S. B., Chen, X., Miller, M. S., Nacev, B. A., Cheng, Y. L., Meyers, D. J., Tenney, K., Shim, J. S., Crews, P., Amzel, L. M., Ma, D. and Liu, J. O. 2013, J. Med. Chem., 56, 3996.
32. Arfin, S. M., Kendall, R. L., Hall, L., Weaver, L. H., Strewart, A. E., Matthews, B. W. and Bradshaw, R. A. 1995, Proc. Natl. Acad. Sci. USA, 92, 7714.
33. Zhang, P., Nicholson, D. E., Bujnicki, J. M. Su, X., Brendle, J. J., Ferdig, M., Kyle, D. E., Milhous, W. K. and Chiang, P. K. 2002, J. Biomed. Sci., 9, 34.
34. Clinkinbeard, T., Ghoshal, S., Craddock, S., Pettigrew, L. C. and Guttmann, R. P. 2013, Brain Res., 1499, 129.
35. Datta, B., Datta, R. and Tammali, R. 2014, Int. J. Biotech. Biochem., 10, 141.
36. Datta, B. and Datta, R. 2014, Int. J. Biotech. Biochem., 10, 113.
37. Anjum, R. and Blenis, J. 2008, Nat. Rev. Mol. Cell. Biol., 9, 747.
38. Deschenes-Simard, X., Kottakis, F., Meloche, S. and Ferbeyre, G. 2014, Cancer Res., 74, 412.
39. Chappell, W. H., Steelman, L. S., Long, J. M., Kempf, R. C., Abrams, S. L., Franklin, R. A. Bäsecke, J., Stivala, F., Donia, M., Fagone, P., Malaponte, G., Mazzarino, M. C., Nicoletti, F., Libra, M., Maksimovic-Ivanic, D., Mijatovic, S., Montalto, G., Cervello, M., Laidler, P., Milella, M., Tafuri, A., Bonati, A., Evangelisti, C., Cocco, L., Martelli, A. M. and McCubrey, J. A. 2011, Oncotarget, 2, 135.

40. Buckingham, M. and Rigby, P. W. J. 2014, Dev. Cell, 28, 225.
41. Yaffe, D. and Saxe, O. 1977, Nature, 270, 725.
42. Datta, B. and Datta, R. 2014, Int. J. Adv. Res. Chem. Sci., 1, 1.
43. Tammali, R., Datta, R. and Datta, B. 2014, Intl. J. Biotech. Biochem., 10, 127.
44. Folkman, J. 1995, Nat. Med., 1, 27.
45. Simons, M. and Ware, J. A. 2003, Nat. Rev. Drug Discov., 2, 863.
46. Moulton, K. S., Heller, E., Konerding, M. A., Flynn, E., Palinski, W. and Folkman, J. 1999, Circulation, 99, 1726.
47. de Bold, A. J. 1985, Science, 230, 767.
48. Levin, E. R., Gardner, D. G. and Samson, W. K. 1998, New Engl. J. Med., 339, 321.
49. Charron, F. and Nemer, M. 1999, Sem. Cell & Dev. Biol., 10, 85.
50. Molkentin, J. D. 2000, J. Biol. Chem., 275, 38949.
51. Zheng, B., Wen, J. K. and Han, M. 2003, Biochemistry (Moscow), 68, 795.
52. Morimoto, T., Hasegawa, K., Kaburagi, S., Kakita, T., Wada, H., Yanezume, T. and Sasayama, S. 2000, J. Biol. Chem., 275, 13721.
53. Liang, Q., Wiese, R. J., Bueno, O. F., Dai, Y. S., Mazkham, B. E. and Molkentin, J. D. 2001, Mol. Cell Biol., 21, 7460.
54. Marcel, A. G., Heyden, V. D. and Defize, L. H. K. 2003, Cardiovascular Res., 58, 292.
55. Eriksson, M. and Lippa, S. 2002, J. Biol. Chem., 277, 15992.
56. Selvakumar, P., Lakshmikuttyamma, A., Das, U., Pati, H. N., Dimmock, J. R. and Sharma, R. K. 2009, Mol. Cancer, 8, 65.
57. Nguyen, D. M. and El-Serag, H. B. 2010, Gastroenterol. Clin. North Am., 39, 1.
58. Joharapurkar, A. A., Dhanesha, N. A. and Jain, M. R. 2014, Diabetes, Meta. Synd. Ob: Targets and Therapy, 7, 73.
59. Hughes, T. E., Kim, D. D., Marjason, J., Proietto, J., Whitehead, J. P. and Vath, J. E. 2013, Obesity, 21, 1782.
60. Chun, E., Han, C. K., Yoon, J. H., Tim, T. B., Kim, Y. K. and Lee, K. Y. 2005, Int. J. Cancer, 114, 124.
61. Lijnen, H. R., Frederix, L. and Van Hoef, B. 2010, Obesity, 18, 2241.
62. Kim, S. H., Forman, A. P., Mathews, M. B. and Gunnery, S. 2000, Oncogene, 19, 3086.
63. Zykova, T. A., Zhu, F., Zhang, Y., Bode, A. M. and Dong, Z. 2007, Carcinogenesis, 28, 1543.
64. Kim, S. H., Gunnery, S., Choi, J. K. and Mathews, M. B. 2002, Oncogene, 21, 8741.
65. Wang, S., Raven, J. F., Durbin, J. E. and Koromilas, A. E. 2008, PLoS One, 3, e3476.