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Contributions of PTEN to PML-IV-Mediated Cellular Senescence and Free Fatty Acid-Induced Oxidative Stress

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ABSTRACT OF THE DISSERTATION

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Tumor suppressor PTEN functions differently in the cytoplasm and nucleus. Posttranslational modifications play important roles in regulating activities, functions and subcellular localizations of PTEN. Previously, our laboratory discovered that nuclear PTEN promotes acetylation of tumor suppressor p53. In the present study, we focus on contribution of nuclear PTEN to PML-IV-mediated cellular senescence and to high levels of free fatty acid (FFA)-induced oxidative stress.

PML-IV is a tumor suppressor and the major regulator of cellular senescence. We show

that the C-terminal domain of PML-IV is required for induction of cellular senescence. Nuclear PTEN regulates cellular senescence via interaction with the C-terminus of PML-IV, which is essential for recruiting PTEN into PML-Nuclear Bodies (PML-NBs), enhancing p53 acetylation and inducing cellular senescence.

We also show that high FFA induces nuclear export of PTEN and down-regulates p53 acetylation and protein levels, which leads to inhibition of p53 downstream target GPx-1 and accumulation of ROS in endothelial cells. Furthermore, mTOR/S6K signaling induces phosphorylation of PTEN at Ser380, which decreases PTEN monoubiquitination, promotes PTEN nuclear export and leads to p53/GPx-1 inhibition. This study suggests that alterations of the two posttranslational modifications of PTEN caused by mTOR/S6K are responsible for oxidative stress induced by high FFA.

In summary, we show the regulation of cellular senescence by nuclear PTEN and provide a new mechanism of tumor suppression function of PTEN. We also demonstrate a novel pathway by which high levels of FFA induce oxidative stress through PTEN nuclear export and thus provide novel insight into the role of nuclear PTEN in metabolism.

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Chapter 1

General Introduction

p53

p53 gene is one of the most important tumor suppressor genes. It is mutated or inactivated in more than 50% of human cancers (Nigro et al., 1989). Germ line mutations in TP53 are associated with Li-Fraumeni syndrome, a rare familiar disorder predisposing to several different types of cancers, including sarcomas, brain tumors, breast cancers, and adrenal cortical carcinomas (Malkin et al., 1990).

Somatic mutations of the p53 gene are very frequent in human cancers (Hollstein et al., 1991). Most of them are missense mutations with single nucleoid substitution. Wild type p53 functions as a transcription factor, which can either upregulate or downregulate target gene transcription. Importantly, the majority of p53 mutations found in cancers are located within its DNA binding domain, thus impairing its binding to target DNA and transcriptional activity. Germ line mutations distribute in a similar pattern to somatic mutations. Mutant p53 can abolish wild-type p53 function. Alternatively, they also exert dominant negative activity by tetramer formation with wild-type p53. In addition, mutant p53 have oncogenic function through a gain of function (GOF) mechanism (Muller and Vousden, 2013; Freed-Pastor and Prives, 2012; Rivlin et al., 2011; Magali et al., 2010).

p53 protein has a very short half-life. In a classic model, the level of p53 protein in the unstressed cell is very low due to its ubiquitylation by E3 ligase MDM2 and subsequent degradation by the 26S proteasome. Protein kinases and acetyltransferases activated by cellular stress modify p53 by phosphorylation and acetylation, respectively. As a result, p53 is stabilized and activated in the nucleus. Therefore, p53 phosphorylation and

acetylation are essential for its stability and transcription activation (Bode and Dong, 2004).

The structure of p53

Human p53 protein contains 393 amino acids. It is divided into five functional domains: transactivation domain (residue 1-42) and SH3 domain (residue 63-97) in the N-terminal region, DNA-binding domain (residue 98-292) in central core region, tetramerization domain (residue 300-356) and regulatory domain (residue 363-393) in the C-terminal region(Figure 1.1 A). p53 transactivation domain interacts with transcription factors, acetyltransferases and MDM2 ubiquitin ligase. It forms a tetramer via the tetramerization domain. The p53 tetramer binds to target DNA through DNA-binding domain that is the most frequently mutated region of p53. Nuclear localization and export signals are within the C-terminal regulatory domain (Ferreon, et al., 2009; Bode and Dong, 2004).

The functions of p53

p53 is identified as a "guardian of the genome" (Lane, 1992), due to its role in maintenance of genomic stability through responses to cellular stress signals. It is a sequence-specific DNA-binding protein (Bargonetti et al., 1991; Kern et al., 1991). The DNA consensus sequence of p53 response elements contains two inverted pentameric sequences with the pattern 5'-RRRC (A/ T)|(A/T)GYYY-3'(Wei et al., 2006). Responding to various cellular stresses (including DNA damage, abnormal oncogenic events, hypoxia), p53 regulates its target genes that induce DNA damage repair, cell-cycle arrest, apoptosis, senescence, et al (Horn and Vousden, 2007; Vousden and Lane, 2007; Kruse and Gu 2009). Apoptosis and senescence are powerful tumor suppressive

pathways against uncontrolled proliferation of transformed cells. In addition, cytosolic p53 promotes apoptosis and inhibits autophagy in transcription-independent ways. But the mechanisms are still unclear (Green and Kroemer 2009; Tasdemir et al., 2008; Marchenko and Moll, 2007).

Induction of cell cycle arrest is one of the most important functions of p53. At early stage of cellular stress, activation of p53 can promote cell cycle arrest by transactivation of three genes. Induction of p21/ CDKN1A (cyclin-dependent kinase inhibitor 1A) leads to G1 cell cycle arrest by inhibition of G1 cyclin-dependent kinases (cyclinA/CDK2, cyclinE/CDK2 and cyclinD/CDK4 complexes) (El-Deiry et al., 1993; Harper et al., 1993). Activation of 14-3-3s and GADD45A (growth arrest and DNA damage-inducible gene alpha) triggers G2/M cell cycle arrest (Hermeking et al., 1997; Kastan et al., 1992; Wang et al., 1999). Under extensive stresses, activated p53 can induce transcription of various proapoptotic genes, including p53 upregulated modulator of apoptosis (PUMA) and genes encoding the BH-3–only proteins such as Bax (Bcl-2-associated protein X) and BAK (Bcl-2 antagonist/killer) (Vogelstein et al., 2000; Yu et al., 2001). Moreover, p53 can also promote apoptosis by repression of the transcription of antiapoptotic gene survivin (Hoffman et al., 2002).

Sustained cellular stresses can lead to cellular senescence, defined as a permanent cell cycle arrest. Senescence is regulated by a complex network, in which the tumor suppressors p53 and pRb (retinoblastoma protein) play important roles. It results from many sources of cellular stress, including dysfunctional telomere, DNA damage, oxidative stress, oncogene activation. Cellular senescence mediated by the p53/p21

pathways can be activated by different stimuli in human cells. Telomere shortening activates ATM/ATR and Chk1/Chk2 which in turn phosphorylate p53 (Zhang, et al., 2007; Dimri, 2005). Oncogene Ras activates the RAF-MEK-ERK pathway. As a result, PML-IV is induced. p53 and p300/CBP are recruited into PML-NBs resulting in acetylation of p53 at Lys 382 (Bischof, et al, 2002; Pearson, et al, 2000). Phosphorylation and acetylation increase p53 transcriptional activation, resulting in p21 activation and cells entering permanent cell cycle arrest-cellular senescence.

New p53 target genes are being identified. Some of them are related to oxidative stress, metabolism, development, cell adhesion and so on, indicating that the significance of p53 is far beyond as a tumor suppressor (Vousden and Lane, 2007; Goldstein and Rotter, 2012). Recent evidence suggests that p53 also plays a crucial role in cardiovascular health. Atherosclerotic cardiovascular diseases are the leading death cause in developed countries (Beaglehole and Bonita, 2008). Atherosclerosis is the thickening of the innermost layer of the artery wall. The major cell types in atherosclerotic plaques are vascular smooth muscle cells (VSMCs) and inflammatory cells (macrophages, T lymphocytes, et al). Due to DNA damages, p53 is activated in advanced plagues (Iacopetta et al., 1995; Ihiling et al., 1997; Martinet et al., 2002) and regulates the proliferation of VSMCs (Mercer and Bennett, 2006). Deficiency of p53 promotes the formation of atherosclerosis. Reactive oxygen species (ROS) is the major cause of DNA damage in atherosclerosis. p53 can protect the genome from oxidation by ROS. As a transcription factor, physical levels of p53 maintain a normal basal transcription of antioxidant genes SESN1 (sestrin 1), SESN2 (sestrin 2), and glutathione peroxidase-1

(GPX-1). However, hyper-physical levels of p53 increase transcription of pro-oxidant genes, such as BSX, NQO1 and PUMA, which increases cellular ROS levels and subsequently leads to oxidative damage of DNA (Sablina, et al., 2005). Suppression of p53 results in a significant decrease in the basal transcriptions of the antioxidant genes without affecting the expression of pro-oxidant genes. Our studies suggest that decrease in p53 is responsible for high FFA-induced GPX-1 inhibition and leads to endothelial oxidative stress. These studies will be described in chapter 3.

Regulation of p53

Recent studies show that p53 protein level and activity are tightly regulated by its inhibitors MDM2 and MDM4. Meanwhile, post-translational modifications of p53, such as phosphorylation, acetylation, ubiquitylation, methylation and neddylation, play significant roles in its stabilization, transactivation, subcellular distribution and functions.

Regulation of p53 by MDM2/MDM4

MDM4 is a structurally related protein to MDM2. Compared with the classic model described above, a new model of p53 regulation by MDM2/MDM4 is proposed. In unstressed cells, MDM2 binds to the N terminus of p53 and induces polyubiquitylation on its C-terminal domain, thus inhibiting p53 activity and promoting its degradation. MDM4 binds to and masks p53 transactivation domain, leading to inhibition of the interaction between p53 and transcription cofactors. MDM2 and MDM4 physically associate with each other and form heterodimers, which regulate p53/MDM2 interactions. Therefore, MDM4 stabilize both p53 and MDM2. Under stress, MDM2 degrades itself by autoubiquitination and ubiquitinates MDM4 to target it for proteasomal degradation.

On the other hand, as described above, phosphorylation and acetylation are induced in stressed cells. As a result, p53 is stabilized and transactivated in the nucleus (Pei et al., 2012; Toledo and Wahl, 2006). Interestingly, MDM2 is a downstream target of p53. So, p53 can induce the expression of its own negative regulator, which is an autoregulatory feedback loop (Barak et al., 1993; Picksley and Lane, 1993). MDM2 can also bind to HDAC1 (Histone Deacetylases 1), inducing deacetylation and degradation of p53 (Ito et al., 2002).

Regulation of p53 by phosphorylation

The majority of serine (S)/threonine (T) phosphorylation sites on p53 protein are concentrated within the N-terminal transactivation domain and the C-terminal regulatory domain (Figure 1.1B). Most of these sites are phosphorylated with cellular stress (Dai and Gu, 2010). Our laboratory discovered that Thr55 is a p53 phosphorylation site and showed that Thr55 is phosphorylated in unstressed cells and is dephosphorylated with stress (Li et al., 2007). Phosphorylation of p53 shows redundancy: a specific site can be phosphorylated by several kinases and a specific kinase can phosphorylate several sites (Kruse and Gu, 2009). ATM, ATR, CK1, HIPK2 and ChK1/Chk2 are the major protein kinases responsible for phosphorylation of p53. Several phosphorylation sites have been extensively studied. For instance, in the N-terminal domain, the phosphorylation at Ser6 and Ser9 mediated by protein kinase CK1 family members may play significant roles in development, tumorigenesis and metastatic progression promoted by TGF-β (Cordenonsi et al., 2007; Adorno et al., 2009). Ser46 phosphorylation mediated by HIPK2 is important in p53-mediated apoptosis (Taira et al., 2007; Olsson et al., 2007). Phosphorylation at

Ser15 mediated by ATM/ATR increases p53 stability by inhibiting the interaction between p53 and MDM2 and favors the recruitment of transcriptional coactivators (Toledo and Wahl, 2006). Followed with ionizing radiation, Ser 20 phosphorylation is mediated by CHK1/CHK2. Furthermore, Ser15/Ser20 phosphorylation can lead to or promote consequent phosphorylation of other sites, such as Thr18 phosphorylation (Sakaguchi et al., 2000), Ser 9 phosphorylation, and Ser46 phosphorylation (Saito et al., 2002; Saito et al., 2003). In the C terminus, phosphorylation of Ser392 is induced by ultraviolet (UV) light and enhances specific DNA binding (Matsumoto et al., 2006). ATM-mediated phosphorylation stimulates the recruitment of histone acetyltransferases (HAT) such as p300 and CBP (Lambert et al. 1998; Dumaz and Meek 1999; Feng et al. 2009; Jenkins et al. 2009; Lee et al. 2009), resulting in acetylation of multiple lysine residues on p53.

Regulation of p53 by acetylation

The acetylation of p53 is another important posttranslational modification for p53 activity. Notably, by opposing ubiquitylation on the same sites, acetylation enhances p53 stabilization. Acetylation in tissue culture systems promotes sequence specific binding of p53 to DNA and enhances its transcriptional activity. Histone acetyltranferases (HATs), responsible for acetylation induction, include p300/CBP (CREB-binding protein), p300/PCAF (CBP-associated factor) and MYST family HATs (named for MOZ, Ybf2/Sas3, Sas2 and Tip60/hMOF). Mutations in p300/CBP have been found in several types of human tumors. Up to now, nine lysine residues on p53 protein have been identified as acetylation sites (Figure 1.1B). K120 and K164 are localized within the

DNA binding domain. K120 acetylation mediated by Tip60/hMOF is necessary for activation of proapoptotic genes such as Puma and Bax. K164 acetylation induced by p300/CBP contributes to the activation of most of the p53 target genes. K320 localized in the tetramerization domain is acetylated by p300/PCAF and ubiquitylated by E4F1. Interestingly, both acetylation and ubiquitylation of K320 are important for cell cycle arrest. Six carboxyl-terminal lysines (K370, K372, K373, K381, K382 and K386) are acetylated by p300/CBP and ubiquitinated by MDM2 (Dai and Gu, 2010). Among them, acetylation of p53 at lysine 373/382 is the important for p53 activity (Bode and Dong, 2004). Our laboratory found that in response to DNA damage, nuclear PTEN forms a complex with p300 to maintain high levels of p53 acetylation at 373/382 (Li, et al., 2006). Tumor suppressor PML-IV recruits p53, p300/CBP and nuclear PTEN into PML-nuclear bodies (PML-NBs) and induces p53 acetylation (unpublished data). Conversely, p53 can be deacetylated by Histone Deacetylases (HDACs) such as HDACI and Sir-1 (Sirtuin-1, referred as HDACIII). Deacetylation represses p53-dependent transcriptional activation, cell cycle arrest, apoptosis and cellular senescence (Luo et al., 2000; Luo et al., 2001).

Regulation of p53 by other modifications

p53 can be modified by ubiquitylation. Ubiquitylation is defined as the covalent conjugation of one or more ubiquitin molecules to a protein substrate. It is a series of enzymatic reactions catalyzed by enzymes known as E1 (ubiquitinactivating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin protein ligase). MDM2 is the most important E3 ligase for p53. Distinct from polyubiquitination, which induces degradation, mono-ubiquitination mediated by MDM2 promotes p53 cytoplasmic localization.

Independent of transcriptional activity, cytosolic p53 triggers apoptosis and inhibits autophagy (Green et al., 2009; Tasdemir et al., 2008; Marchenko and Moll 2007). Acetylation sites K370, K372 and K382 on p53 can also be methylated. K372 methylation by Set9 stabilizes p53 (Chuikov et al., 2004). K370 methylation by Smyd2 decreases p53 stability. Nevertheless, methylation of K382 by Set8 represses p53 transcriptional activity.

p53 can be targeted by two other ubiquitin-like proteins, Small Ubiquitin-like Modifier (SUMO) and Neural precursor cell Expressed Developmentally Downregulated protein 8 (NEDD8). Sumoylation at K386 enhances p53 transcriptional activity, while neddylation inhibits p53 transactivation (Toledo and Wahl, 2006).

p53 and cancer therapy

Because of its importance in tumor suppression, p53 is a therapeutic target for cancer treatment. In tumors with p53 inactivation or mutation, reactivation or restoration of functional p53 may be a way to suppress cancer progression. Adenoviral re-induction of p53 into tumor cells has been successful in clinical practice (Senzer and Nemunaitis, 2009). MDM2 and Sirt-1 are negative regulators of p53, and their inhibitors have been developed and used in early clinical trials (Lain et al., 2008; Shangary and Wang, 2009). However, the potential toxicity of these small molecules is debatable. Furthermore, p53 induces survival responses such as DNA repair and cell cycle arrest. So, retention of p53 could protect cancer cells during chemotherapy (Bertheau et al., 2008). Due to the complexity of p53 networks, we need to further investigate the outcomes of its reactivation in cancer treatment.

PTEN

PTEN (phosphatase and tensin homolog deleted on chromosome 10), located on 10q23.3, is one of the most frequently mutated genes in human cancer. It was identified as a tumor suppressor in 1997 (Li and Sun 1997; Li et al., 1998; Steck et al., 1997). Somatic mutations/deletions of PTEN are very common in early and late-stage tumors (Tamguney and Stokoe, 2007; Wang and Jiang, 2008). Intriguingly, germline mutations of PTEN cause a group of autosomal dominant diseases called PTEN hamartoma tumor syndromes (PHTSs), including Cowden syndrome, Lhermitte-Duclos disease, Bannayan-Riley-Ruvalcaba syndrome, and Proteus and Proteus-like syndrome. Aside from multiple harmartomas and developmental disorders, patients with PHTSs have higher risk of other benign tumors. Nevertheless, Cowden syndrome is related to malignant tumors (Baker, 2007; Suils and Parsons, 2003; Salmena et al., 2008; Song et al., 2012). Encoding 403 amino acids, PTEN functions as a multifunctional biological regulator. It plays important roles in G1 cell cycle arrest (Simpson and Parsons, 2001; Di Cristofano and Pandolfi, 2000), apoptosis (Li et al., 1998; Stamboli et al., 1998), development, cell migration inhibition and chromosomal integrity (Wang and Jiang, 2008), et al.

PTEN protein is composed of two major domains: the phosphatase (residue 1-185) and C-terminal domains (residue 186-403). Within the C terminus, there is a lipid-binding C2 domain (residues 186-351), two PEST sequences involved in protein stability (residues 350-375 and 379-396) and a PDZ domain responsible for protein-protein interactions (Planchon et al., 2008). PTEN has been found in both nucleus and cytosol. In

general, it is predominantly localized in nucleus in quiescent and normal cell. But in active and tumor cells, PTEN primarily localizes in cytoplasm (Whiteman et al., 2002).

PTEN in cytoplasm and nucleus

Cytoplasmic PTEN has both lipid and protein phosphatase activity. As a lipid phosphatase, PTEN dephosphorylates phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) to phosphatidylinositol (4, 5)-biphosphate (PIP2) at the plasma membrane, thereby directly antagonizing the activity of PI3 kinase (PI3K) (Salmena, et al., 2008). Since PI3K/AKT has a variety of downstream targets, PTEN can exert diverse effects through its negative regulation of PI3K/AKT pathway, including cellular growth and survival, metabolism, cardiovascular health, tumor suppression and so on. For instance, downregulation of AKT by PTEN results in increase of p27, leading to apoptosis. Therefore, cytosolic PTEN is pro-apoptotic. Glycogen synthase kinase-3 (GSK3) is a serine/threonine kinase which phosphorylates and inactivates glycogen synthase (Doble and Woodgett, 2003; Dent et al., 1989; Dent et al., 1990; Fiol et al., 1988). AKT inhibits GSK-3 by phosphorylation of its serine residues (Oudit and Penninger, 2009). Correspondingly, PTEN inhibits glycogen synthase activity and plays an important role in glycogen metabolism. Importantly, insulin binding to its receptor causes activation of PIP3/AKT pathway. Therefore, upregulation of PTEN impairs insulin pathway and contributes to insulin resistance, which is a characteristic of type 2 diabetes (Cohen and Leroith 2012; Pal et al., 2012). Apart from its contribution to metabolism, PTEN is involved in biological regulation of cardiovascular health medicated by PI3K/AKT signaling (Oudit and Penninger, 2009). Loss of PTEN reduces apoptosis of

cardiomyocytes (Schwartzbauer and Robbins, 2001), enhances physiological myocardial hypertrophy (Oudit et al., 2004), attenuates pathological hypertrophy and increases resistance to heart failure (Oudit et al., 2008). On the other hand, PTEN is a protein phosphatase. PTEN dephosphoryaltes p85β, a regulatory subunit of PI3K, providing another mechanism to inhibit PI3K signaling (He et al., 2010). Focal adhesion kinase (FAK) serves as a protein substrate of PTEN. Thus, PTEN functions to inhibit cell adhesion and migration (Tamura et al., 1998) and suppress invasion and metastasis of tumors (Wang and Jiang, 2008). Activation of non-receptor Tyr kinase c-SRC confers resistance of breast cancer cells to human epidermal growth factor receptor 2 (HER2; also known as ERBB2)-targeted therapy (Zhang et al., 2011). Tyrosine residue 416 on SRC is a direct substrate for PTEN protein phosphatase activity. Loss of PTEN leads to activation of SRC and contributes to the ERBB2-targeted therapy in breast cancers (Song, et al 2012).

Nuclear localization of PTEN is crucial for its tumor suppressor function (Baker, 2007). In nucleus, PTEN plays important roles in regulation of cell cycle progression and genomic stability. Although major components in PI3K/AKT pathways also exist in nucleus, there is limited evidence to support that PTEN works as a nuclear PIP3 phosphatase (Deleris et al 2003; Deleris et al 2006). In reality, there has been a study showing that nuclear pool of PIP3 is insensitive to PTEN catalysis (Lindsay et al., 2006). Apparently, the lipid phosphatase function of PTEN in nucleus is still unclear (Salmena et al, 2008; Baker, 2007; Lian and Cristofano, 2005). However, nuclear PTEN is able to function as a protein phosphatase and dephosphorylate MAPK, leading to decrease in

cyclin D1 level and induction of G0-G1 cell cycle arrest (Chung and Eng, 2005; Chung et al., 2006, Salmena et al., 2008; Gil et al., 2007; Planchon et al., 2007; Lian and Cristofano, 2005). In nucleus, PTEN promotes centromere integrity by interacting with centromere protein C (CENPC), a protein required for kinetochore assembly. RAD51 is a key protein involved in double-strand break (DSB) repair. Nuclear PTEN induces DNA damage responses by upregulation of RAD51 transcription. Independent of phosphatase activity of PTEN, the two above functions contribute to maintenance of chromosome stability (Salmena et al., 2008; Song et al., 2012; Shen et al., 2007; Baker, 2007; Planchon et al., 2007).

Interestingly, cytoplasmic and nuclear PTEN stabilize p53 through different pathways. In cytosol, AKT phosphorylates MDM2, the E3 ligase of p53. As a result, more MDM2 molecules accumulate in the nucleus, target, and degrade p53. Therefore, cytoplasmic PTEN can promote p53 stability by inhibiting AKT in a phosphatase dependent manner (Mayo and Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). In nucleus, independent of phosphatase activity, PTEN interacts with p53 directly and prevents its degradation by MDM2 (Tang and Eng, 2006). Further, our laboratory found that in response to DNA damage, nuclear PTEN forms a complex with p300/CBP to maintain high p53 acetylation level and enhance its transcriptional activity (Li et al., 2006). Conversely, p53 functions as a transcriptional activator of PTEN, binds to its promoter and upregulates PTEN mRNA levels (Stambolic et al., 2001). However, physical interaction of p53 and PTEN promotes caspase-mediated PTEN degradation (Tang et al, 2006).

Nuclear-cytoplasmic trafficking

Nuclear Localization of PTEN is a dynamic process, associated with cell cycle status, hormone stimulation and cellular proliferation (Lian and Di Cristofano, 2005). For instance, in MCF-7 cells, nuclear PTEN reaches the highest level in G1-phase and lowest in S-phase (Ginn-Pease and Eng, 2003). Endometrial expression of PTEN is very sensitive to hormones (Mutter et al., 2000; Guzeloglu-Kayisli et al., 2003). However, PTEN lacks obvious typical nuclear localization signal sequences (NLS) or nuclear export sequences (NES), which make it difficult to explore the molecular mechanisms of PTEN nuclear/cytoplasmic trafficking (Lian and Di Cristofano, 2005; Gil et al., 2007). Four NLS-like sequences localized in PTP (residue 159-164) and C2 domains and a cytoplasmic localization signal (CLS) within the N terminus were identified in PTEN. Combined mutations of NLS-like sequences lead to nuclear exclusion. Accordingly, mutations of CLS cause increase of nuclear accumulation (Chung et al., 2005; Denning et al., 2007). Several other mechanisms of nuclear/cytoplasmic shuttling have been proposed. PTEN can enter the nucleus by passive diffusion (Liu et al., 2005). In U87MG human glioblastoma cells, PTEN was reported to be transported into nucleus in an active way dependent on RAN GTPase (Gil et al., 2006). PTEN physically interacts with MVP (major vault protein), which functions as a molecular carrier for nuclear/cytoplasmic trafficking (Yu et al., 2002; Mossink et al., 2003). Importantly, posttranslational modifications are related to PTEN subcellular distribution. In U87MG cells, phosphorylation dead mutants that target the C-terminal tail, such as S380A, prefer nuclear localization (Gil et al., 2006).). PTEN can be ubiquitinated by E3 ubiquitin ligase

NEDD4-1 (Neural-precursor-cell-Expressed Developmentally Downregulated4-1) (Trotman, et al., 2007). Monoubiquitination is correlated to PTEN nuclear import. A mono-ubiquitylation site is present at K289 and regulates its nuclear localization. K289E mutant identified in Cowden syndrome predominantly localizes in cytoplasm. On the contrary, deubiquitation of PTEN by deubiquitylation enzyme herpesvirus-associated ubiquitin-specific protease (HAUSP, also known as USP7) leads to cytoplasmic accumulation (Song et al., 2008). However, the molecular mechanisms of PTEN nuclear export are still unclear. Our studies show that palmitic acid suppresses PTEN monoubiquitination via induction of PTEN-Ser380 phosphorylation, leading to PTEN nuclear export and inhibition of p53 acetylation and GPX-1 expression. Those results will be described in Chapter 3.

Regulations of PTEN

Expression and function of PTEN can be regulated by several mechanisms, including epigenetic silencing, transcriptional modulations, downregulations by non-coding RNAs, post-translational modifications and protein-protein interactions (Salmena et al., 2008; Tamguney and Stokoe, 2007).

In many types of cancers, epigenetic silencing through aberrant PTEN promoter methylation suppresses expressions and impairs tumor suppressor functions of PTEN (Garcia et al., 2004; Goel et al., 2004; Kang et al., 2002; Mirmohammadsadegh et al., 2006; Hollander et al., 2011). Stem cell factor sal-like protein 4 (SALL4) was shown to repress *PTEN* transcription by interacting with the epigenetic repressor Mi-2/NuRD complex, which has chromatin-remodelling ATPase and histone deacetylase activities

(Lu et al., 2009). Transforming growth factor β (TGFβ) is the first discovered transcriptional factor of PTEN (LI and Sun, 1997). Aside from p53, two other transcription activators of PTEN have been identified, including the peroxisome proliferation-activated receptor γ (PPARγ) (Patel et al., 2001) and the early growth-regulated transcription factor-1 (EGR-1) (Virolle et al., 2001). In addition, resistin, a cytokine involved in inflammation and insulin resistance, (Shen et al., 2006) and phytoestrogens such as genistein (in soy), resveratrol (in red wine) and quercetin (in fruit and vegetables) (Waite et al., 2005) can also upregulate PTEN transcription levels through different mechanisms. On the other hand, several transcription factors and signaling pathways have been shown to regulate PTEN transcription negatively. For instance, in the haematopoietic system, the leukaemia-associated factor EVI1 (ecotropic virus integration site 1 protein; also known as MECOM) represses *PTEN* transcription directly (Yoshimi et al., 2011). SNAIL and the oncogenic factor inhibitor of DNA-binding 1 (ID1) can compete with p53 for binding on the *PTEN* promoter, thus inhibiting *PTEN* transcription (Escriva et al., 2008; Lee et a., 2009). NFκB is a transcription suppressor of PTEN. Signaling pathways including MKK4 (Mitogen activated protein kinase kinase-4) and JNK promote cell survival by repressing PTEN transcription through activation of NFκB (Xia et al., 2007). Interestingly, pathways occurring downstreams of active NOTCH1 can promote or repress PTEN transcription through the CBF-1 transcription factor (Chappell et al., 2005; Whelan et al., 2007) and the HES-1 transcription factor (Palomero et al., 2007), respectively.

MicroRNAs (miRNAs) are a group of endogenous non-coding single-stranded RNAs (about 22-25 nucleotides in length) that regulate gene expression through imperfect base-pairing to sequences in the 3′ untranslated region (UTR) of target mRNAs (Bartel et al., 2009). A number of miRNAs have been documented to target PTEN in a variety of human diseases (Song et al., 2012). In mice models, high expressions of miR17-92 stimulate the development of benign lymphoproliferative diseases, Burkitt's lymphoma and autoimmunity by downregulating PTEN (Xiao et al., 2008; He et al., 2005). Within miR 17-92, miR-19 alone is sufficient to promote lymphomagenesis in c-myc-induced B cell lymphoma (Olive et al., 2009) and cooperate with Notch1 in T-cell acute lymphoblastic leukaemia (Mavrakis et al., 2010). The contributions of miR-19 to haematopoietic cancers are partially due to its suppression of PTEN expression. miR-21 is also shown to negatively regulate PTEN in several different human tumors (Meng et al., 2006; Meng et al., 2007; Ma et al., 2011).

Importantly, posttranslational modifications, such as phosphorylation, ubiquitination, acetylation and oxidation tightly regulate PTEN stability, activity, function and subcellular localization. Phosphorylations of serine/threonine sites at PTEN C terminus (Ser370, Ser380, Thr382, Thr383, and Ser385) stabilize it and decrease its lipid phosphatase activity (Georgescu et al., 1999; Torres and Pulido, 2001; Vazquez et al., 2000; Salmena et al., 2008). In contrast, phosphorylation at Thr366 decreases its stability (Maccario et al., 2007). It is proposed that phosphorylation of the C-terminal tail set PTEN in a "close" and stable conformation, which attenuate its attachment to plasma membrane and PIP3 phosphatase activity. Ser380, Th382 and Thr383 are referred to as

STT cluster, which plays important roles in PTEN function. Accordingly, alanine substitutions at STT cluster open the closed and stable conformation, which enhance PTEN phosphatase activity (Vazquez et al., 2000; Leslie and Downes, 2004). Casein kinase 2 (CK2) shows partial contribution to phosphorylation of the STT clusters *in vitro* (Torres and Pulido, 2001). However, it does not phosphorylate Ser380 directly (Al-Khouri et al., 2005). Glioma tumor suppressor candidate region 2 (GLTSCR2, also known as PICT-1) binds to PTEN, induces its phosphorylation at Ser380 and increases its stability (Okahara et al., 2006; Yim et al., 2007). Our study identifies that another protein kinase S6K is able to phosphorylate PTEN at Ser380 directly. Those results will be discussed in Chapter 3. As mentioned above, NEDD4-1 is a well-known E3 ubiquitin ligase of PTEN. By binding to PTEN, NEDD4-1 induces both monoubiquitination and polyubiquitination of PTEN (Wang et al., 2007). Lys13 and Lys289 are recognized as monoubiquitylation sites of PTEN (Trotman et al., 2007). Monoubiquitylation by NEDD4-1 and deubiquitation by HAUSP modulate cytoplasmic/nuclear traffic of PTEN (Song et al., 2008). In contrast, polyubiquitination leads to proteasome-mediated degradation of PTEN in cytoplasm (Wang et al., 2007; Yim et al., 2009). In addition, WWP2 (also known as atrophin-1-interacting protein 2, AIP-2) is a NEDD4-like protein. It can function as E3 ubiquitin ligase of PTEN and mediate its ubiquitylation-dependent degradation (Maddika et al., 2011). It has been proposed that phosphorylation of the C-terminal tail of PTEN increases its stability, inhibits polyubiquitination and plasma membrane targeting (Tolkacheva et al., 2001; Torres and Pulido 2001; Vazquez et al., 2000). It was documented that the phosphorylated the C-terminal of PTEN inhibits

ubiquitination induced by NEDD4-1 (Wang et al., 2008). And the phosphorylated C terminus inhibits membrane localization of PTEN (Vazquez et al., 2006). Maccario et al showed that plasma membrane attachment of PTEN promotes both mono- and polyubiquitination of PTEN, which indicates that C-terminal phosphorylation downregulates ubiquitination by suppression of membrane localization (Maccario et al., 2010). This conclusion is consistent with previous findings. Our study discloses a novel signaling pathway by which phosphorylation of Ser380 of PTEN inhibits its monoubiquitination in nucleus and promotes nuclear export. Those results will be discussed in Chapter 3. In addition, PTEN can be regulated by acetylation and oxidation. Lys 125 and Lys 128 located within the catalytic cleft of PTEN can be acetylated by p300/CREB-binding protein (CBP)-associated factor (PCAF). Therefore, PCAF functions a negative regulator of the catalytic activity of PTEN (Okumura et al., 2006). Conversely, PTEN is deacetylated by Sirtuin 1 (SIRT1) (Chae and Broxmeyer, 2011). Catalytic activity of PTEN has been reported to be abolished by ROS (Lee et al., 2002; Leslie et al., 2003; Kwon et al., 2004; Seo et al., 2005), which induce the formation of an intramolecular disulfide bond between Cys71 and Cys124 through oxidation(Lee et al., 2002).

Numerous proteins have been identified to interact with PTEN and modulate its stability, subcellular distribution, phosphatase activity, tumor suppressor function, and so on. Because of the existence of the PDZ-domain-binding motif on the C-terminal tail, PTEN is able to interact with members of the membrane guanylate-kinase inverted (MAGI) family, such as MAGI2 and MAGI-3, which contain PDZ motifs. These interactions

increase its stability and membrane targeting (Wu, X. et al., 2000; Wu, Y. et al., 2000; Vazquez et al., 2001). Direct interaction of the motor protein myosin V and PTEN promote the transport of PTEN to the membrane, which is required for antagonizing PtdIns (3,4,5)P3 signaling (van Diepen et al., 2009). PTEN-mediated inhibition of cellular transformation induced by oncogenic protein MSP58 requires the interaction of the C terminus of PTEN with MSP58 (Okumura et al., 2005).

PML

Promyeolocytic leukemia nuclear bodies (PML-NBs) are protein complexes predominantly localized in the nucleus. PML-NBs are also known as nuclear domins-10 (ND10) or PML oncogenic domains (PODs) (Everett and Chelbi-Alix, 2007). Diameter of PML-NBs is 0.3-10 μm. There are 10-30 bodies per nucleus. The expression of PML-NBs depends on cell cycle and differentiation in normal tissues. In response to cellular stress and senescence, the number and size of PML-NBs are increased (Zimber, 2004). Close to 80 proteins localize in PML-NBs, including transcription factors, such as p53, pRb, DNA damage responsive proteins such as ATM, ATR, apoptosis regulators, such as Daxxx, et al (Zimber, 2004; Krieghoff-Henning, and Hofmann, 2008). Therefore, PML-NBs play important roles in transcription regulation, DNA-damage responses, regulation of apoptosis and regulation of cellular senescence, et al (Zimber, 2004; Krieghoff-Henning and Hofmann, 2008; Bermardi and Pandolfi, 2007).

The PML protein, first identified in acute promyelocytic leukemia (APL), is a tumor suppressor and essential for the proper assembly of PML-NBs (Bernardi and Pandolfi, 2007). In most of the APL patients, PML gene is fused to the retinoic acid receptor α

(RAR α) as a result of the t(15; 17) chromosomal translocation(de The, et al., 1991; Kakizuka, et al., 1991). PML-RARα fusion protein compromises the integrity of PML-NBs and contributes to the pathogenesis of APL (Fogal, et al., 2000; Stanchina, et al., 2004; Yoshida, et al., 2007). PML protein expression is decreased or abolished in a variety of human cancers, such as prostate adenocarcinomas, colon adenocarcinomas, lung carcinomas, breast carcinomas, lymphomas, et al (Gurrieir, et al, 2004). PML protein can be phosphorylated by extracellular regulated kinase (ERK), checkpoint kinase-2(CHK2), and casein kniase-2(CK2), et al. It contains three covalent sumoylation sites. Most of proteins in PML-NBs can also be sumolyated by SUMO. Therefore, sumoylation of PML protein is essential for the proper assemble of PML-NBs and recruitment of component proteins (Zimber, 2004; Krieghoff-Henning and Hofmann, 2008; Bermardi and Pandolfi, 2007).

The PML gene is located on chromosome 15q22 and has nine exons. PML isoforms are illustrated in Figure 1.2. All of PML isoforms have the same N terminus containing a-RING finger, two B-boxes, and a coiled-coil domain. And they together form the RBCC/TRIM motif, encoded by exons 1 to 3. This motif is necessary for PML-NBs formation, homo-multimerization, as well as apoptotic, tumor suppressor and anti-viral functions. Because of the existence of NLS (nuclear localization signal) in exon 6, most of PML isoforms are predominantly localized in nucleus. Alternative splicing within exons 4 through 9 give rises to a variety of PML proteins with different central regions or the C-terminal domains, which may give rise to various functions and different binding regions to other molecules(Jensen, et al., 2001; Bermardi and Pandolfi, 2007; Everett and

Chelbi-Alix, 2007; Krieghoff-Henning and Hofmann, 2008). For instance, AML1 and PML are the two leukemia-associated factors. PML-I interacts with AML1 through its C-terminal region and recruits the AML-1 and p300 in PML-NBs. Consequently, PML-I enhances AML1-mediated transcription and promotes myeloid cell differentiation (Nguyen, et al., 2005). Only PML-I and PML-VI are able to increase transactivation of NFAT (nuclear factor of activated T cell) (Lo, et al., 2008). PML-III associates with centrosome and controls its duplication. Thus, PML-III plays a role in genome stability (Xu, et al., 2005). PML protein mediates anti-viral function. PML-II is the only one that can interact with adenovirus type 5 E4 Orf3 protein (Hoppe, et al., 2006). HPV (human papillomaviruse) E6 proteins localize in PML-NBs. However, E6 proteins colocalize with PML isoforms I-IV, but not V and VI (Guccione, et al., 2004).

Among all of the PML isoforms, the most extensive research is focused on PML-IV. It plays important roles in transcription regulation, cell differentiation, tumor suppression, apoptosis and cellular senescence, et al (Guo, et al., 2000; Zhong, Salomoni, et al., 2000; Zhong, Salomoni and Pandolfi, 2000). c-Myb is a transcription factor and regulates cell growth and differentiation of hemapoietic cells. PML-IV associates with c-Myb and enhances c-Myb mediated transcription (Dahle, et al., 2004). c-Myc inhibits differentiation of hematopoietic precursor cells. PML-IV is able to induce differentiation of hemapoietic cells by interacting with c-Myc and destabilizing it (Buschbeck, et al., 2007). PML-IV induces apoptosis by repression of transactivation of survivin (Xu, et al., 2004).

More importantly, PML-IV is a p53 downstream target and contributes to p53-mediated apoptosis and senescence (Guo, et al., 2000; Pearson, et al., 2000; Pearson and Pelicci, 2001; Bischo, et al., 2002; de Stanchina, et al., 2004). Among, all the isoforms, only PML-IV interacts with p53 and regulates p53 activities (Fogal, et al., 2000). It binds to residues 120-290 of p53, which is located in DNA-binding domain. p53 interacts with the C terminus(amino acids 361-633) of PML-IV(Guo, et al., 2000). Together, all of the above suggest that the C terminus of PML-IV is responsible for its unique properties. The role of the C terminus of PML-IV in senescence will be described in chapter 2.

PML-IV regulates the two main senescence pathways. In the p53/p21 pathway, the N terminus of PML binds to MDM2, sequesters it to nucleolus and protects p53 from proteosome-mediated degradation, thereby resulting in p53 stabilization (Bernardi, et al., 2004). In Ras-induced senescence, upregulated PML recruits p53 and p300/CBP to PML-NBs, followed by acetylation of p53 at Lys382 and transcription activation by p53 (Pearson, et al., 2000; Salmoni and Pandolfi, et al., 2002; Bernardi, et al., 2004). Human SIRT2 deacetylates p53 and inhibits PML/p53-induced cellular senescence (Langley, et al., 2002).

In the p16/pRb pathway, PML-IV interacts with pRb and associates with a macromolecular complex that contains corepressors such as NCoR/SMART, Ski/Sno and histone deacetylases (HDAC1) in the PML-NBs. This complex is essential for the transcriptional repression mediated by pRb (Alcalay, et al., 1998; Bischof, et al., 2005; Caino, et al., 2009). PML interacts with pRb through its N terminus that is shared with all isoforms. However, while PML-II and PML-IV can both interact with pRb, only PML-IV

is capable of inducing cellular senescence via p16/pRb pathway, suggesting that the C terminus of PML-IV is important in this process. Overall, PML overexpression is sufficient to cause senescence, correlating with the induction of p21 and repression of Rb/E2F-dependent genes. In PML-induced senescence, the cell type determines which one of the two pathways is predominant (Mallette, et al., 2004; Bischof, et al; 2005). Interestingly, PML-IV can regulate nuclear trafficking of PTEN (Song, et al., 2008).

Free Fatty Acids

Overweight and obesity, recently increased in worldwide prevalence, are linked to increased incidence of type II diabetes mellitus (DM2) and cardiovascular diseases. A sedentary lifestyle (Zelber-Sagi et al., 2011) and unhealthy diet (Pietinen et al., 1996) are considered the major causes of these disorders. Saturated fatty acids (SFAs) and hydrogenated or *trans* fatty acids (TFAs) in the diet are the most important risk factors contributing to disease.

Free fatty acids (FFAs), unbranched aliphatic monocarboxylic acids, are derived from triacylglycerols and phospholipids. Fatty acids are categorized into 3 main groups on the basis of the existence of double bonds: i) SFAs, which do not contain double bonds; ii) monounsaturated fatty acids (MUFAs), containing only 1 double bond; and iii) polyunsaturated fatty acids (PUFAs), containing at least 2 double bonds. The 2 PUFAs, linoleic acid (LA) and alpha-linolenic acid (ALA), which are fundamental for organisms and must be gained from diet, are also identified as essential fatty acids (EFAs). Most double bonds in unsaturated fatty acids are in the *cis* configuration; *trans*-fatty acids (TFAs) are uncommon in organisms. The source of SFAs is primarily animals: red meat

and dairy products. MUFAs are rich in sunflower, corn, soybean, peanut, and olive oils. They can also originate from desaturation of SFAs in the body. PUFAs are found in nuts and vegetable oils. TFAs are mainly industrially produced: partially hydrogenated vegetable oils (PHVOs). Through β-oxidation, long-chain FFAs can be metabolized to acetyl-CoA and enter the citric acid cycle (Cascio et al., 2012).

Functions of FFAs

FFAs can be used in the synthesis of polar lipids, such as phospholipids and sphingolipids, which are fundamental components of cell membranes. FFAs affect the dynamics and plasticity of membranes where they are embedded (Kien et al., 2009). For instance, palmitic acid, an SFA, can decrease membrane fluidity (Leekumjorn et al., 2008). UFAs stabilize membranes and maintain or even increase their fluidity (Langner et al., 2000; Rogerson et al., 2006).

FFAs can covalently modify the cytoplasmic proteins that are involved in signal transduction and affect protein activities and the transduction of downstream signals. For example, palmitic acid can modify Src family kinases and G proteins. Palmitoylation is necessary for their localization in lipid rafts, which protects them from low temperature extraction by non-ionic detergent. Src family kinases, Fyn and Lck, and the linker for activation of T cells (LAT) are important signaling molecules in T cell receptor-mediated signaling. Palmitoylated LAT, Fyn and LcK are localized in rafts. Mutations of the palmitoylation sites within LAT inhibit its trafficking to rafts and suppress recruitment of LAT-binding proteins Vav to rafts, thus affecting the following signal transduction (Liang et al., 2001). While PUFAs, such as arachidonic acid and eicosapentaenoic acid,

inhibit Fyn palmitoylation and preclude Fyn localization to rafts, which may contribute to immunosuppressive effects of PUFAs (Webb et al., 2000). Eicosapentaenoic acid displaces palmitated Lck and LAT from lipid rafts by changing raft compositions (Stulnig et al., 2001).

FFAs can also function as signaling molecules. For example, through the oxidative pathway, arachidonic acid (AA) produces eicosanoids, such as prostaglandins (PGs), leukotriens (LTs) and thromboxanes (TBXs), which have autocrine and/or paracrine effects on surrounding cells. In addition, FFAs can activate a family of G protein-coupled receptors (GPR/FFARs). Short-chain FFAs, such as formate and acetate, activate GPR41 and induce the expression of leptin in both a mouse adipocyte cell line and mouse adipose tissue in culture (Xiong et al., 2004). GPR40 can be activated by medium- and long-chain FFAs, expediting glucose-stimulated insulin secretion from pancreatic β-cells (Itoh et al., 2003). FFAs can also bind to nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs), which is important for maintenance of lipid homeostasis in the body.

FFAs in metabolic and cardiac health

The complex roles of FFAs in metabolic and cardiac health have been studied for several decades. In general, different groups of FFAs exert different impacts on LDL/HDL levels, insulin sensitivity, and cardiovascular health.

It is widely accepted that intake of MUFAs and PUFAs can increase HDL level and decrease LDL level. Furthermore, a UFA-rich diet can improve insulin sensitivity in DM2 patients, inhibit fat abdominal accumulation, and reduce the incidence of

cardiovascular diseases (Ascherio et al., 2002), stroke (Iso et al., 2002) and heart arrhythmia (Charnock et al., 1991). These favorable effects may arise from their interaction, directly or after production of eicosanoids, with nuclear receptors such as PPARs. The ligand–receptor binding induces the expression of lipolytic genes and suppression of lipogenic ones.

Conversely, excessive intake of TFA increases LDL level and decreases HDL level (Mensink and Katan, 1992), which is associated with weight gain, insulin resistance, DM2 and cardiovascular disorders. However, the precise mechanisms are not clear. Notably, SFAs with 12 to 16 carbon atoms increase plasma LDL level (Hu et al., 2001). Long-chain SFAs (12-18 carbon atoms) are related to insulin resistance, glucose intolerance, inflammation, and metabolic disorders. SFAs can induce the gene expression of lipogenic enzymes, thus leading to triglyceridemia, abdominal fat accumulation and insulin resistance. Intriguingly, SFAs are an independent risk factor for cardiovascular disorders (Micha and Mozaffarian, 2008). With their involvement in signaling pathways, they cause endothelial dysfunction. SFA-induced endothelial apoptosis involves p38 mitogen-activated protein kinase (MAPK) signaling (Chai and Liu, 2007), NF-ĸB activation (Staiger et al., 2006) and GSK-3β/Wnt/beta-catenin signaling (Zhu et al., 2010). SFAs reduce nitric oxide availability (Kim et al., 2005) and promote inflammatory responses (Umpierrez et al., 2009). More importantly, SFAs increase oxidative stress in endothelial cells (Inoguchi et al., 2000). However, the underlying mechanisms of how SFAs induce endothelial oxidative stress are still unclear. Moreover, more understanding is needed of the differences in individual SFAs. Palmitic acid (C16) is the most common

polysaturated acid in the human body. Chapter 3 demonstrates that high PA levels cause oxidative stress by mTOR/S6K signaling.

mTOR/S6K

Mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol 3-kinase (PI3K)-related kinase family (Laplante and Sabatini DM, 2012). It is a sensor of nutrient supply, energy metabolism and cellular stress. Integrated with other components, it forms two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), modulated by the regulatory proteins raptor and rictor, respectively. The activity of mTORC1 is regulated by nutrients, growth factors, DNA damage signals, and hypoxia. mTORC1 can be directly activated by the small GTPase protein Rheb, which is negatively regulated by the TSC1/2 (Figure 1.3). For instance, AKT can phosphorylate TSC2, which leads to the relief of the suppression of Rheb, resulting in the ultimate activation of mTORC1 (Huang and Manning, 2008; Sun et al., 2008). In addition, growth factors can activate the Rheb-mTORC1 pathway by inactivating TSC1/2 through insulin signaling pathways. Interestingly, independent of TSC1/2, amino acids activate mTORC1 through Rag GTPases. However, the underneath meachnism is not clear. In contrast, energy restriction, DNA damage and hypoxia inhibit mTORC1 activity. A decrease of ATP levels activates the AMP-dependent protein kinase (AMPK), which inhibits the activity of mTORC1 by inducting phosphorylation of TSC2. LKB-1 can phosphorylate AMPK, resulting in suppression of mTORC1 signaling (Corradetti et al., 2004; Inoki et al., 2003; Shaw et al., 2004). mTORC2 is regulated by growth factors. However, the mechanism is unclear (Yang and Ming, 2012).

As a sensor of nutrient availability, mTORC1 is associated with metabolic disorders. Sustained high levels of amino acids constantly activate mTORC1 (Um et al., 2004; Khamzina et al., 2005). In animal models of obesity and metabolic disorders, basal mTORC1 activity is increased in metabolically active organs and tissues, such as live and skeletal muscles (Khamzina et al., 2005; Drake et al., 2010), as well as vasculature (Wang et al., 2009) and heart (Sung et al., 2011; Turdi et al., 2011). In mouse muscle cell lines, high FFAs can activate mTOR/S6K (Castaneda et al., 2012). However, the mechanism by which mTOR/S6K is activated is still unclear. In addition, mTOR is closely related to cardiovascular health. In the cardiovascular system, mTOR activation promotes cellular survival by suppressing apoptosis and autophagy. Inhibition of mTOR reduces endothelial angiogenesis and impairs the protection against ischemic injury in cardiomyocytes (Chong et al., 2011).

mTORC1 is the upstream kinase of the serine/threonine kinase ribosomal protein S6K and the eukaryotic initiation factor 4E-binding protein 1 (4EBP1). Phosphorylation of S6K by mTORC1 promotes translation of ribosomal proteins, mRNA biogenesis and cell growth. Phosphorylation of 4EBP1 by mTORC1 leads to mRNA translation by releasing 4EBP1 from eIF4E. mTORC2 regulates cytoskeleton organization, cell size, cell migration, and cell cycle progression. Interestingly, an mTORC2 downstream target is Akt, which is the positive regulator of mTORC1 (Yang and Ming, 2012).

S6K (ribosomal S6 kinase) which is a direst downstream target of mTORC1, was initially identified as the protein kinase for phosphorylation of the ribosomal S6 protein (rpS6), a component of the 40S ribosomal subunit. Because of the variety of downstream effectors,

S6K is associated with protein synthesis, cytoskeletal rearrangement, proliferation, cell survival and aging. Importantly, *in vitro* experiments and studies using mouse models indicate the involvement of S6K in insulin resistance, obesity and diabetes. However, the underlying mechanism is still unclear.

Our studies show that high levels of FFAs can activate mTOR/S6K in EC nuclei, induce phosphorylation of nuclear PTEN, lead to PTEN nuclear export and ultimately cause endothelium damage. These studies will be discussed in chapter 3.

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Figure 1.1

p53 function domains and the major phosphorylation /acetylation sites

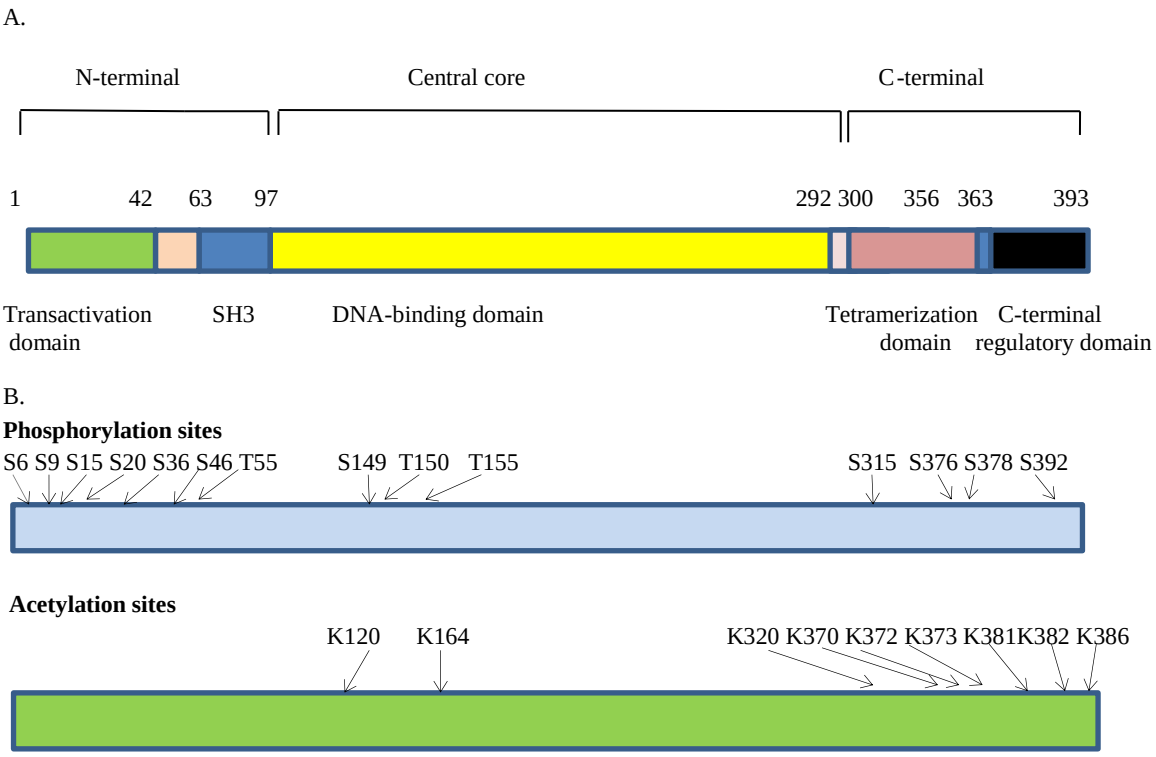


Figure 1.2 PML isoforms

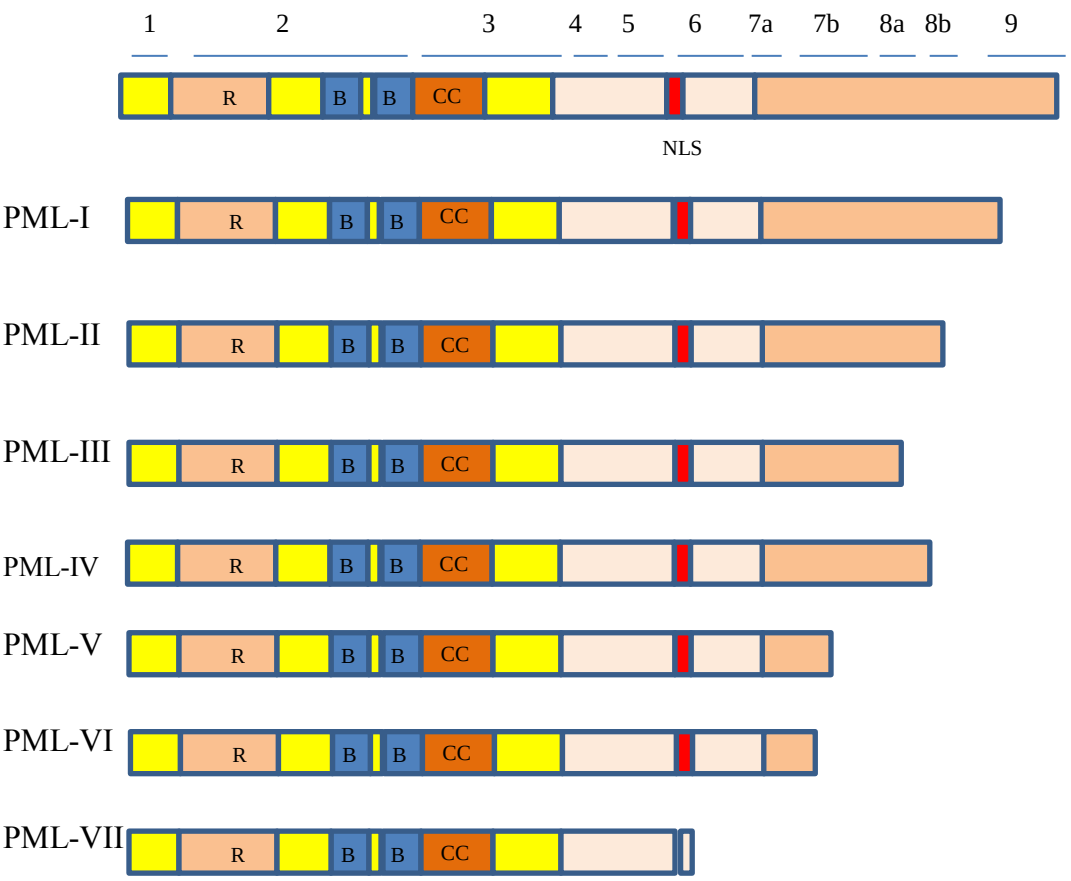
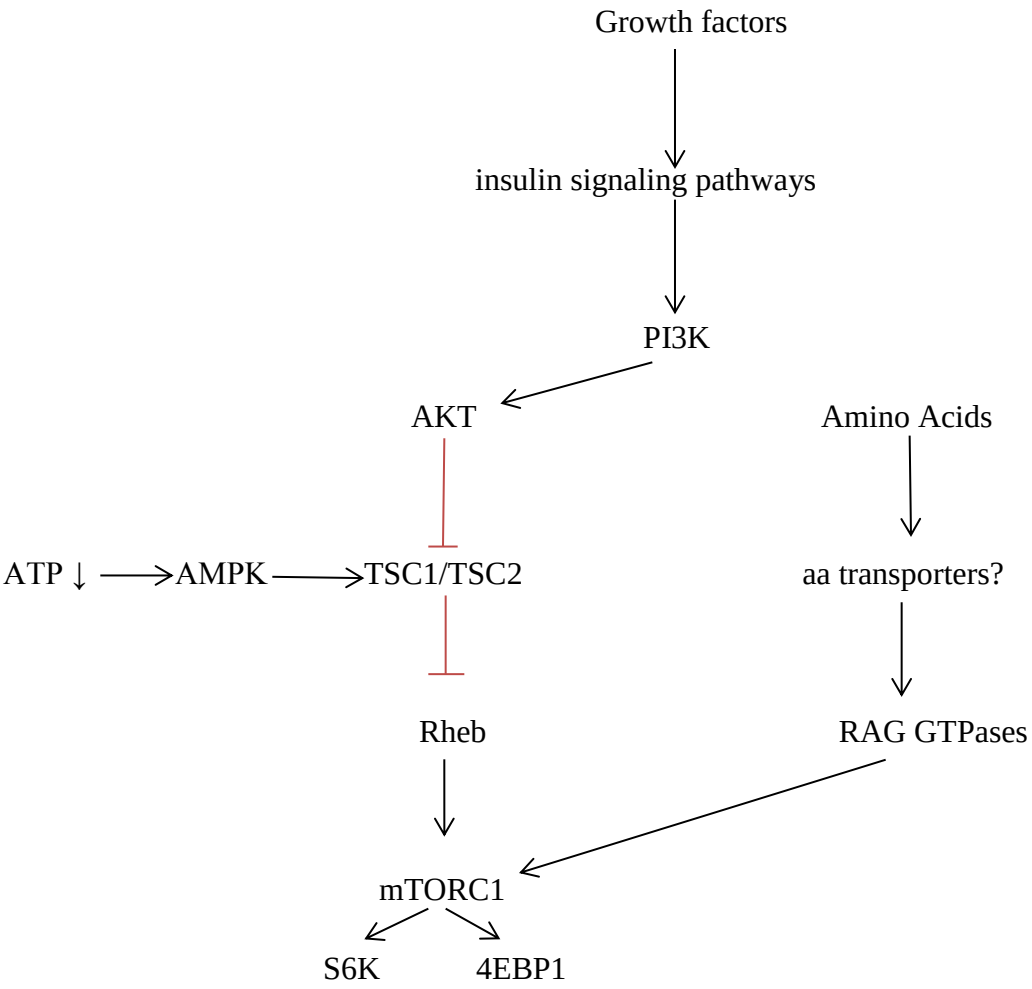


Figure 1.3 The regulators and effectors of mTORC1.



Note: activate: ⟶ ; inhibit: ⊣

Chapter 2

The role of the C-terminal domain of PML-IV in p53/p21 mediated cellular senescence

Abstract

Promyeolocytic leukemia protein isoform IV (PML-IV) is a key regulator of cellular senescence (Dimri, 2005; Caino, et al., 2009). Under cell stress, PML-IV recruits p53 and acetyltransferase CBP/p300 to Promyeolocytic leukemia nuclear bodies (PML-NBs) where p53 is acetylated and activated, thus activating p21 and inducing cell senescence. Despite its significance, the mechanism is not thoroughly understood. Our laboratory found that nuclear PTEN is required for cellular senescence via the p53/p21 pathway. Here, we showed that the C-terminal domain of PML-IV is involved in binding to nuclear PTEN and inducing p53 acetylation. Also, the C-terminal domain of PML-IV is crucial for inducing cellular senescence. Thus the interaction of PML-IV-PTEN plays an important role in recruiting PTEN into PML-NBs, enhancing p53 acetylation and inducing cellular senescence.

Introduction

After a limited number of divisions in vitro, normal cells enter an irreversible cell cycle arrest called “replicative senescence”, which is mainly caused by telomere shortening in human cells, and oxidative stress in mouse cells. Non-telomeric signals, such as DNA-damaging agents, oncogenic activation and inadequate culture conditions can induce “premature senescence”. Replicative and premature senescence are collectively called cellular senescence. Cellular senescence is a complicated process and is different in human and mouse cells. In human cells, senescence is induced by both telomeric and non-telomeric signals and regulated by both the p53/p21 and p16/pRb (retinoblastoma protein) pathways (Dimri, 2005). In response to cell stresses, p53 can activate p21 and induce senescence. p16, induced by various stimuli, inhibits CyclinD/cyclin-dependent kinase 4 (Cdk) 4 and 6, thus resulting in hypophosphorylation of pRb, loss of the transcription of E2F target genes, and cellular senescence. The two pathways feature some crosstalks. For example, pRb can also be activated by p21 (Ben-Porath and Weinberg, 2005). Nevertheless, in mouse cells, cellular senescence is triggered by non-telomeric signals alone and is regulated predominantly via the p53/p21 pathway. However, similar to the wild-type counterparts, $p21^{-/-}$ mouse embryonic fibroblasts (MEFs) can still enter cellular senescence, which suggests that p21 is not the only conduit of p53 during the induction of senescence in mouse cells. Therefore, in addition to p21, other p53 targets may also contribute to senescence induction in mouse cells (Pantoja and Serrano 1999).

Morphologically, senescent cells are flat and enlarged, with positive staining for senescence-associated ß-galactosidase (SA- ß-gal) at pH 6. These cells form a heterochromatin structure called senescence-associated heterochromatin foci (SAHFs), whose formation requires repression of E2F target genes mediated by hypophosphorylated pRb. Senescent cells may contribute to age-related decline in tissue function, structure and other abnormalities. Senescence is considered an anti-tumor mechanism. Oncogene-induced senescence keeps benign hyperproliferative lesions without additional cooperating mutations in check and limits their development. Progression to malignant tumor involves bypassing senescence pathways. In cancer treatment, radiation and chemotherapeutic drugs can induce cellular senescence in tumor cells, thereby functioning as a backup plan for apoptosis to prevent tumor progression (Dimria, 2005; Narita and Lowe, 2005; Zhang, 2007). However, senescent cells secret cytokines, chemokines, growth factors and other factors that may promote cancer development (Collado and Serrano, 2010). Our current understanding of the role of senescence in cancer development is still limited. For more effective cancer treatment, further studies on pathways and players involved in cellular senescence are needed. Overexpression of the tumor suppressor PML-IV induces senescence by engaging both p53 and Rb pathways (Figure 2.1) (Dimri, 2005; Caino, et al., 2009). In Ras-induced senescence, activated PML-IV recruits p53 and acetyltransferase CBP/p300 into PML-NBs (Pearson, et al., 2000), then p53 is acetylated and activated, thus leading to p21 transcription. PML can also bind to MDM2 and protect p53 against degradation, resulting in p53 stabilization (Figure 2.1 A). In the p16/pRb pathway, PML-IV interacts with pRb

in association with a macromolecular complex containing transcription corepressors such as N-CoR and HDAC. This complex is essential for the transcriptional repression of E2F-dependent genes (Figure 2.1 B). However, a thorough understanding of the mechanism by which PML-IV induces cellular senescence is still lacking.

PTEN is a tumor suppressor and localized in both cytoplasm and nucleus. Nuclear PTEN is crucial for its tumor suppressor function (Baker, 2007). Our laboratory found that in response to DNA damage, nuclear PTEN forms a complex with p300 to enhance p53 acetylation (Li, et al., 2006). As mentioned previously, acetylation of p53 plays an important role in p53 transcriptional activation and cellular senescence mediated via the p53/p21 pathway, so nuclear PTEN may be involved in cellular senescence. However, few studies have focused on the role of nuclear PTEN in cell senescence. PML-IV is the main regulator in cellular senescence. However, how it functions together with PTEN in senescence was unknown. Our laboratory found that nuclear PTEN is required for cellular senescence via the p53/p21 pathway. Here, we showed that the interaction of the C-terminal domain of PML-IV to PTEN plays a role in recruiting PTEN into PML-NBs, enhancing p53 acetylation and inducing cellular senescence.

Results

Generation and characterization of the PML deletion mutants

The full-length PML gene contains 9 exons. Alternative splicing within exons 4 through 9 produces different PML isoforms with different central regions or C-terminal domains, which may result in various functions and diverse binding regions to other molecules. Among all the PML isoforms, only PML-IV can interact with p53 and PTEN (unpublished data) in the nucleus and induce p53 acetylation and cellular senescence, suggesting that the C terminus of PML-IV is essential for its association with p53 and PTEN, induction of p53 acetylation and cellular senescence.

Distinct from the C-terminal regions of other isoforms, the C terminus of PML-IV is composed of 64 amino acids: a fragment of 50 amino acids encoded by exon 8 and a unique 14-amino acid tail. To further characterize the C-terminal domain of PML-IV, deletion mutants within the C-terminal domain of PML-IV were generated (Figure 2.2). Flag-tagged full-length (Flag-FL) and Δ14 and Δ64 deletion mutants were overexpressed in U2OS cells and immunostained with Flag antibody. Like FL, Δ14 and Δ64 mutants are localized in the nucleus (Figure 2.3), suggesting PML-NBs integrity is intact in cells overexpressing the mutants.

PML deletion mutants interact with p53 but not PTEN

To investigate interaction between PML deletion mutants and p53, flag-tagged FL PML-IV, Δ14 and Δ64 mutants were overexpressed in U2OS cells. Lysates were immunoprecipited with p53 antibody (Santa Cruz, sc-6243) and the immunocomplex were immunoblotted with Flag antibody. This coimmunoprecipitation (CoIP) experiment

shows that deletion mutants bind to p53 with the same strength as full length PML-IV (Figure 2.4A). To examine PTEN-PML-IV interaction, endogenous PTEN was pulled down by immunoprecipitation with anti-PTEN antibody (Santa Cruz, sc-7974) and IP products were immunoblotted with Flag antibody. In contrast to p53 interaction, interactions of Δ14 and Δ64 with PTEN are much weaker than that of full length PML-IV with PTEN, suggesting that the C-terminal region of PML-IV is involved in binding to PTEN but not p53 (Figure 2.4B).

PML deletion mutants fail to induce p53 acetylation

Our laboratory has shown that full length PML-IV induces p53 acetylation at Lys373 and Lys382 in U2OS cells (unpublished data). To test the abilities of PML deletion mutants to induce p53 acetylation, U2OS cells were transfected with FL PML, Δ14, and Δ64 and the lystates were subsequently assayed for p53 acetylation using acetyl-p53 specific antibodies.

As shown in Figure 2.5, the two C-terminal deletion mutants reduce their ability to enhance p53 acetyaltion, suggesting that the C-terminal domain of PML-IV is required for induction of p53 acetylation at Lys373 and 382. The inability of induction of p53 acetylation might be due to reduced interaction of the mutants with PTEN.

PML-IV deletion mutants are unable to induce cellular senescence

Because p53 acetylation is important for PML-IV induced senescence (Langley, et al., 2002), we examined whether PML-IV deletion mutants can induce cell senescence. In those experiments, MCF-7 cells were transfected with empty vector, FL, Δ14, and Δ 64 PML. After G418 selection for 11 days, cells were stained with Senescence β-

Galactosidase Staining Kit (Cell Signaling, 9860). Images were obtained using microscope. SA- ß-gal positive cells were also counted and shown in Figure 2.6 B. The assay shows overexpression of full length PML-IV indeed induce cell senescence in MCF-7 cells, while overexpression of the C-terminal deletion mutants are not able to induce cellular senescence. Immunoblotting confirms equal levels of overexpressed proteins and vinculin (Figure 2.6 C). Together, these results suggest the C-terminal domain of PML-IV is required for induction of cellular senescence by PML-IV.

Discussion

Previous studies showed that all of the PML isoforms can recruit p53 and p300/CBP to PML-NBs, yet only PML-IV enhanced p53 acetylation and induced premature senescence (Bischof, et al., 2002). PML isoforms contain the same N terminus but differ in central regions or the C-terminal domains, which may give rise to various functions and different protein-protein interactions (Bernardi and Pandolfi, 2007). Although PML-IV is responsible for inducing p53 acetylation and cellular senescence, little is known about specificity of its C terminus. Our study defines the essential role of the C terminus of PML-IV in p53 acetylation and cellular senescence. In our experiments, the C-terminal deletion mutants of PML are localized in nucleus and form intact nuclear bodies (Figure 2.3), but fail to induce p53 acetylation at Lys 373 (Figure 2.5 A) and Lys 382 (Figure 2.5 B). They can not induce cellular senescence (Figure 2.6). Deacetylase Sir-1 was found to counteract p53 acetylation by CBP/p300 and antagonize the induction of cellular senescence by PML-IV overexpression (Langley, et al., 2002). Therefore, failure to induce cellular senescence is concomitant with lack of p53 acetylation, which agrees with our results.

Like PML, nuclear PTEN is important in maintaining p53 acetylation (Li et al., 2006). Previous studies showed some links between PTEN and PML. PTEN cooperates with PML to inactivate the nuclear AKT pathway (Trotman et al., 2006). PML favors PTEN nuclear localization (Trotman et al., 2007; Song et al., 2008). Importantly, previous experiments of our laboratory showed that PTEN forms a complex with PML-IV in the nucleus. The association increases with DNA damage (unpublished data), which suggests

that nuclear PTEN is localized in PML-NBs and the interactions of PML and PTEN play an important role in regulating p53 acetylation with DNA damage. In the present study, PML-IV deletion mutants interact with p53 but not PTEN, yet fail to induce p53 acetylation. Thus, PML-IV interacts with PTEN through its C terminus, and the interaction is required for p53 acetylation induced by PML-IV overexpression. The current study is the first to locate the binding region of PML-IV to PTEN and define the essential role of this interaction in regulating p53 acetylation.

Recent evidence has indicated the importance of PTEN in cellular senescence. PTEN induces senescence dose-dependently through the AKT pathway (Alimonti, et al., 2010). In prostate cancer, loss of a single PTEN allele promotes cell growth and loss of both alleles induces senescence, called PTEN-loss-induced cellular senescence (PICS). If cells have a normal expression level of PTEN, overexpression of PTEN causes senescence (Chen, et al., 2005; Peeper, 2010). In glioma, PTEN –deficient cells entered senescence after IR treatment. PTEN-proficient cells showed apoptosis after IR exposure. Therefore, the status of PTEN determined cell fates in glioma (Lee et al., 2011). Previous results from our laboratory showed that nuclear PTEN is required for PML-IV-induced cellular senescence (unpublished results). This present study shows that PML-IV C-terminal deletion mutants lose their binding to PTEN, thus failing to induce p53 acetylation and cellular senescence. The interaction of the C-terminal region of PML-IV and nuclear PTEN is essential for inducing cellular senescence by PML-IV by upregulating p53 acetylation. Therefore, nuclear PTEN and p53 acetylation are required for cellular senescence induced by PML-IV via the p53/p21 pathway.

Acetylation of p53 is important for its stability, transcriptional activity and functions. In tumors with p53 inactivation or mutation, reactivation or restoration of functional p53 could be important for cancer treatment. Senescence of precancerous and cancer cells is a tumor suppressive mechanism. As discussed above, the interaction of the C terminus of PML-IV and nuclear PTEN plays important roles in p53 acetylation and cellular senescence. Investigating the clinical significance of these findings would be of interest. A peptide molecule composed of the last 64 amino acids of the PML-IV C terminus and an NLS signal can be constructed and overexpressed in cancer cell lines. Without a physical association with p53, this peptide alone may be able to interact with nuclear PTEN, enhance p53 acetylation and induce cellular senescence. With overexpression of this peptide, p53 tumor suppression functions, such as inhibition of cancer cell proliferation and suppression of tumor invasion, need to be tested both *in vitro* and *in vivo*. If this peptide can enhance p53 tumor suppression functions, it may be considered a new drug to treat cancer. However, this peptide may compete with endogenous PML-IV for interaction with PTEN. Also, it may fail to enhance p53 acetylation and not induce cellular senescence. In this scenario, the peptide would inhibit p53 tumor suppression function and promote cancer progression. Therefore, further studies are needed to characterize this peptide.

Materials and Methods

Cell culture and transfection

Cell lines were originally purchased from ATCC. U2OS cells were cultured in McCoy's 5A medium (Mediatech, InC. Cat # 10-050-CV) supplemented with 10% fetal bovine serum (Omega Scientific, InC. Cat# FB-01). MCF-7 cells were cultured in DMEM/F-12 50/50 medium (Mediatech, InC. Cat# 10-092-CV) supplemented with 10% fetal bovine serum and 4μg/ml insulin (Invitrogen, Cat#12585-014). Cell transfection was performed with FuGENE 6 (Roche, Cat#11988387001).

The C-terminal deletion mutant construction

The original pCIFlagPML-IV plasmid was a gift from Dr. Keith N. Leppard. The last 14 or 64 amino acids at the C-terminal region were deleted. The primer information to make these two deletion mutants is listed below.

Construct	Primer sequence
Δ 14	Forward: CCGCTCGAGATGGATTACAAGGATGACGACGATAAGATGGAGCCTGCACCCG XhoI Reverse : CCGGAATTCTCATTCATTGTCAATCTTGAG EcoR I
Δ 64	Forward:

	CCGCTCGAGATGGATTACAAGGATGACGACGATAAGATGGAGCCT GCACCCG XhoI Reverse : CCGGAATTCTCATTCATTGTCAATCTTGAG EcoR I

Western blot analysis

Cells were lysed with lysis buffer (50 mM Tris-HCL, pH=8.0, 120 mM NaCl, 0.5% NP-40, 1 mM DTT, 2 mg/ml aprotinin and 2 mg/ml leupeptin). Cell lysates were boiled with SDS loading buffer for 5 min, placed on ice for 10 min and spinned down to recover all fluid. The protein samples were subjected to SDS-PAGE (10%), followed by Western blot with specific antibodies. The information of all primary and secondary antibodies used is listed below. The membranes were developed with chemiluminescent substrate (Pierce, 34080) for 5 min at room temperature prior to film exposure.

Primary antibody preparation

Name	Company and Cat.#	Dilution
p53	Santa Cruz, sc-126	1:4000
Vinculin	Sigma, V9131	1:4000
Flag	Sigma, 3165	1:10000
Ace-373	Millipore, 06-916	1:5000

Ace-382	Cell signaling, 2525	1:1000

Secondary antibody preparation

Goat anti-rabbit HRP	Bio-Rad, 170-6515	1:5000
Goat anti-mouse HRP	Bio-Rad, 170-6516	1:4000

Immunoprecipitation

To detect interaction of full-length and deletion mutants of PML-IV with p53, about 1×10^7 U2OS cells were transfected with indicated plasmids with FuGENE 6 (Roche) and harvested 30 hours after transfection. Cell lysates were incubated with 1μg of anti-p53 polyclonal antibody (FL-393, Santa Cruz, sc-6243) and 15 μl of Protein A agarose beads (Pierce, #20334) for 6 hours at 4℃. The amounts of PML-IV in the immunoprecipitates were determined by Western Blot with anti-Flag antibody. To detect interaction of full-length and deletion mutants of PML-IV with PTEN, about 1×10^7 U2OS cells were transfected with indicated plasmids with FuGENE 6 (Roche) and harvested 30 hours after transfection. Cell lysates were incubated with 0.5 μl of anti-PTEN polyclonal antibody (Cell Signaling, 9552) and 15μl of Protein A agarose beads for 6 hours at 4℃. The amounts of PML-IV in the immunoprecipitates were determined by Western Blot with anti-Flag antibody.

Immunostaining

U2OS cells were seeded on coverslips and transfected with plasmids expressing 0.5 μg Flag-tagged PML cDNAs as indicated individually. Immunostaing was performed as

described previously (Li et al., 2006). The primary antibody used was anti-Flag (Sigma, 3165).

Senescence assay

MCF7 cells were transfected with indicated plasmids for 24 hours and selected with 500 μg/ml Geneticin (Life technologies, Cat# 10131-035) for 5 days. Then, they were kept in medium containing 5% FBS for 6 days. Thereafter, cells were fixed and stained with β-galactosidase staining kit (Cell Signaling, Cat# 9860). Images were taken and analyzed with stereo scope 1 (Color/grayscale).

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Figure 2.1 Regulatory events in PML-mediated cell senescence pathways

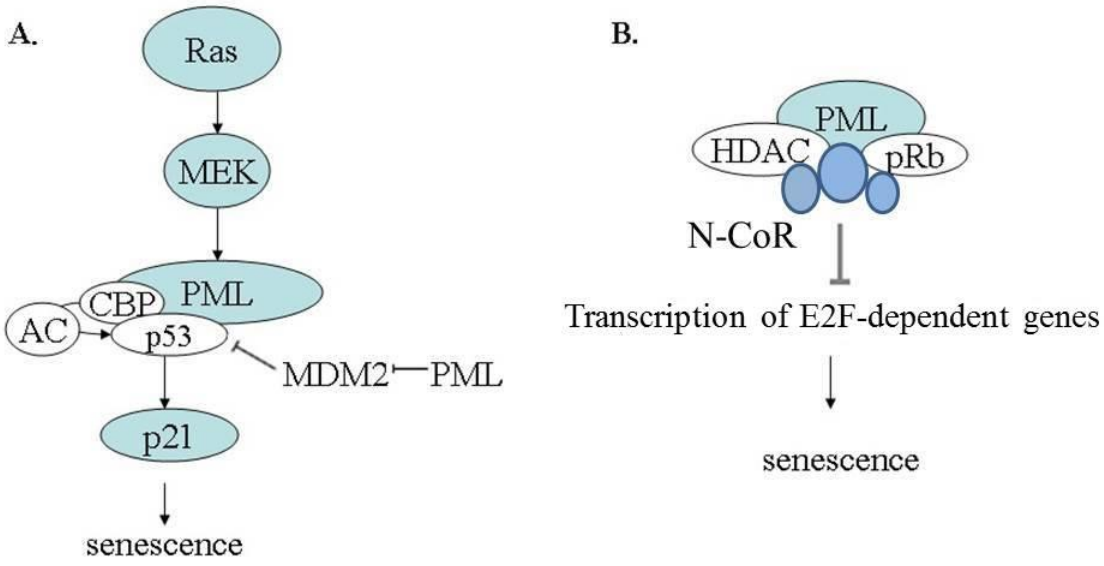


Figure 2.2 Construction of the C-terminal deletion mutants of PML-IV

In Δ14 mutant, last 14 amino acids were deleted; in Δ64, last 14 amino acids and exon 8 were deleted, so 64 amino acids were deleted in total.

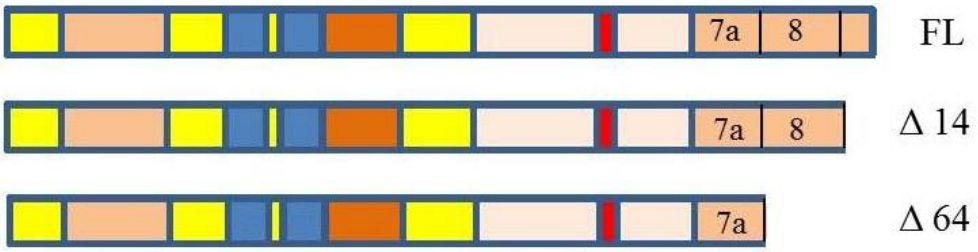


Figure 2.3 Nuclear localization of PML-IV deletion mutants

U2OS cells were transfected with plasmids expressing individual Flag-tagged PML cDNAs as indicated, fixed, and stained for Flag-PML. Stained cells were mounted with DAPI and viewed by immunofluorescence microscope. Localizations of indicated PMLs were shown.

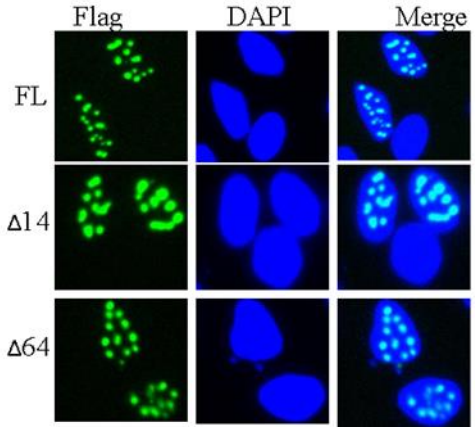


Figure 2.4

PML deletion mutants fail to interact with PTEN

A. The C-terminal deletion mutants of PML-IV bind to endogenous p53. U2OS cells were transfected with FL, Δ14 and Δ64 PML expression plasmids. The interactions of PML and p53 were assayed with CoIP. **B**. **PML-IV deletion mutants fail to interact with PTEN**. U2OS cells were transfected with FL, Δ14 and Δ64 PML expression plasmids. The interactions of PML and PTEN were assayed with CoIP.

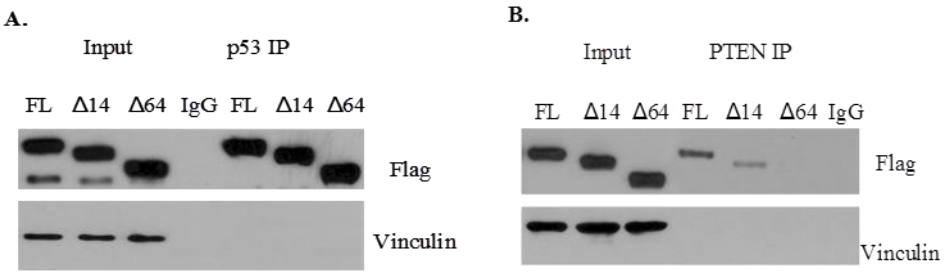


Figure 2.5

PML deletion mutants fail to induce p53 acetylation.

U2OS cells were transfected with full length and deletion mutants of PML-IV. Acetylation levels of p53 on Lys 373(A) and Lys 382(B) were tested using specific antibodies.

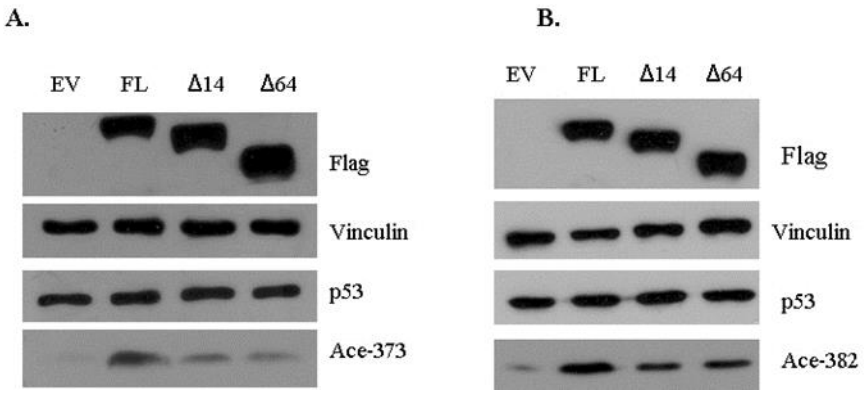
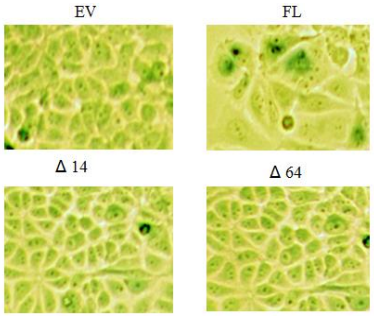


Figure 2.6

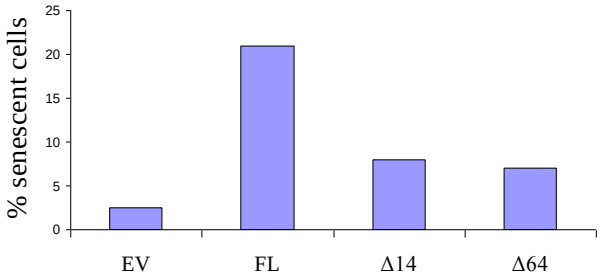
The C-terminal deletion mutants of PML-IV fail to induce cellular senescence.

A. Empty vector, FL, Δ14, and Δ 64 PML were transfected in MCF-7 cells. 24 hours after transfection, transfected cells were selected by antibiotics G418 (final concentration: 500ug/ml).11 days after drug selection, cells transfected with different PML forms were stained with SA-ß-gal. B. The numbers of SA-ß-gal positive cells per 500 cells were counted and percentages of senescent cells were calculated. C. To detect levels of proteins in stained cells, the same transefections were performed in parallel. Cell lysates were immunoblotted to detect overexpressed and endogenous proteins

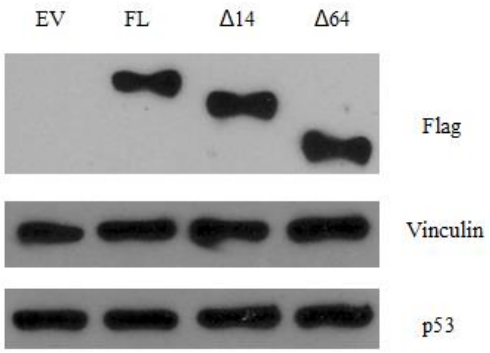
A.


EV
FL
Δ 14
Δ 64

B.


% senescent cells
25
20
15
10
5
0
EV
FL
Δ14
Δ64

C.


EV
FL
Δ14
Δ64
Flag
Vinculin
p53

Chapter 3

PTEN nuclear export mediates free fatty acid-induced endothelial oxidative stress

Abstract

High levels of free fatty acids (FFAs) in circulation lead to oxidative stress in endothelial cells and contribute to the development of cardiovascular diseases. However, the underlying mechanisms remain unclear. Here, we treated human umbilical vein endothelial cells (HUVECs) with palmitic acid (PA) and found that high PA treatment decreases the levels of p53 protein and its acetylation, thus inhibiting the p53 downstream target GPX-1 and increasing the production of intracellular ROS. Furthermore, activation of mammalian target of rapamycin (mTOR)/S6K by high PA treatment induces phosphorylation of PTEN at Ser 380, which enhances the interactions between PTEN and its deubiquitylating enzyme USP7, resulting in decreases of monoubiquitination and PTEN nuclear localization, followed by p53/GPX-1 inhibition. Pharmacologic inhibition of mTOR or S6K blocks high PA-mediated PTEN phosphorylation at Ser380 and the consequent downstream events, suggesting that mTOR/S6K is indispensable for PA-induced oxidative stress through PTEN nuclear export and p53/GPX-1 inhibition. Our research discloses a novel pathway of high levels of FFA-mediated oxidative stress in endothelial cells and provides new insight into an important role of nuclear PTEN in metabolism.

Introduction

Metabolic syndrome is a group of medical disorders including central obesity, insulin resistance, hypertension and dyslipidemia that increase the risk of cardiovascular diseases when occurring together. The prevalence of metabolic syndrome in adults is about 20%-25% in the world (Kereiakes and Willerson, 2003; Haffner and Taegtmeyer, 2003). Obesity and insulin resistance are the two major risk factors. Obesity is considered to almost always precede insulin resistance. One of the characteristic abnormalities in obesity is high levels of circulating FFAs (Colberg et al., 1995; Laws et al., 1997). High FFA levels increase ROS formation in endothelial cells and result in cardiovascular dysfunctions (Inoguchi et al., 2000). Increased levels of polysaturated fatty acids induce insulin resistance and cardiovascular complications (Steinberg et al., 1996; Wang et al., 2006; Ginsberg and MacCallum, 2009; Quehenberger and Dennis, 2011). However, the mechanism of high FFA level increasing ROS level and causing cardiovascular dysfunctions is not well defined.

Oxidative stress, resulting from excess of production or inadequate clearance of ROS, or both, is believed to be one of the major players in the endothelium damage and pathogenesis of metabolic syndrome (Roberts and Sindhu, 2009; Bashan et al., 2009). Antioxidants can correct or even reverse cardiovascular dysfunctions caused by oxidative stress (Keaney et al., 1995; Ohara et al., 1995; Vita et al., 1998). Especially, antioxidants could reduce or suppress the progression of atherosclerosis in animal models (Pratico et al., 1998; Kita et al., 1987). ROS such as hydroxyl radical (·OH), superoxide (O_2 ·-) and hydrogen peroxide (H_2O_2) are very active but short-lived oxygen derivatives. They are

mainly generated by mitochondrial respiration and enzyme resources such as cytochrome P-450 (CYP) isoforms and NADPH oxidase. H_2O_2 has a longer biological half- life than O_2 ˙⁻ and can pass through the lipid biolayer. Thus, H_2O_2 is more atherogenic than O_2 ˙⁻ (Khatri et al., 2004; Cai, 2004; Paravicini and Touyz, 2006; Bienert et al., 2007). As expected, cleansing of H_2O_2 is more effective in suppressing the occurrence and development of atherosclerosis.

To protect against damage caused by oxidative stress, human cells acquire a very advanced antioxidant enzyme system, including superoxide dismutases (SODs), glutathione peroxidases (GPXs), and catalase. SODs convert O_2 ˙⁻ into H_2O_2. Both GPXs and catalase are able to covert H_2O_2 into H_2O and O_2 (Li et al., 2000). GPXs are a selenocysteine-containing enzyme family consisting of GPX-1, GPX-2, GPX-3 and GPX-4. GPX-1 is the most abundant member in human cells (Lubos et al., 2011). GPXs are more effective than catalase in reducing H_2O_2 level in erythrocytes and heart mitochondria (Cohen and Hochstein 1963, Antunes et al., 2002). Because the expression of catalase is very low in the vascular endothelium system (Antunes et al., 2002; Lin et al., 2004; Zhang et al., 2005), GPX-1 plays a major role in H_2O_2 detoxification in cardiovascular cells. It is the most dominant antioxidant enzyme in the cardiovascular system. GPX-1 level is decreased in cardiovascular diseases (Lubos et al, 2011). It protects against coronary artery diseases (Flohe 1988).

The antioxidant gene GPX-1 is a downstream target of tumor suppressor protein p53. Due to its role as a transcription factor, p53 induces GPX-1 expression (Hussain et al., 2004; Tan et al., 1999). Under physical conditions, p53 maintains a basal transcription

level of GPX-1. Downregulation of p53 leads to an obvious decrease in transcription of GPX-1, thus resulting in elevated level of intracellular ROS and excessive DNA oxidation. Accordingly, restoration of p53 to its physical level promotes the expression of GPX-1 and suppresses ROS production (Sablina et al., 2005). Interestingly, p53 plays a fundamental role in regulating cells in atherosclerotic plaques (Mercer and Bennett, 2006). Vascular smooth muscle cells (VSMCs) and inflammatory cells (macrophages, T lymphocytes, dendritic cells and mass cells) are the major cell types in plaques. Bone marrow stromal cells can trans-differentiate into VSMCs (Sata et al., 2002; Calpice et al., 2003). In atherosclerosis, endogenous p53 limits cell proliferation and protects VSMCs against apoptosis. Therefore, deficiency of p53 promotes atherosclerosis formation. $ApoE^{-/-}$mice are atherosclerosis-prone. As compared with $p53^{+/+}$ $ApoE^{-/-}$ mice, $p53^{-/-}$ $ApoE^{-/-}$ mice showe accelerated atherosclerosis and more advanced plaques. In cell culture, p53 deficiency is protective for VSCMs. Under cellular stress, such as serum deprivation or UV irritation, $p53^{-/-}$VSMCs undergo enhanced apoptosis with DNA damage responses. The introduction of wild type p53 with a conditional allele into $p53^{-/-}$ VSMCs rescued apoptosis and inhibited activation of DNA damage pathways. Endogenous p53 can also inhibit the trans-differentiation of stromal cells into VSMCs (Mercer and Bennett, 2006).

Posttranslational modifications such as phosphorylation and acetylation are required for p53 stabilization and transcriptional activation (Bode and Dong, 2004). Our lab showed that nuclear PTEN maintains high p53 acetylation, which is independent of its phosphatase activity (Freeman et al., 2003; Li et al., 2006). As mentioned above, p53

activity is associated with cellular ROS levels, because of its role as a transcription factor of antioxidant genes such as GPX-1. The mechanism by which high FFA levels increase ROS production and cause cardiovascular dysfunction is unclear. We hypothesize that high FFA levels induce oxidative stress by inhibiting p53/GPX1 signaling through nuclear PTEN.

To test this hypothesis, we treated cultured human endothelial cells with high FFAs. High FFA treatment inhibits p53 expression and transcriptional activation, which decreases GPX-1 level. PTEN posttranslational modifications and nuclear export are responsible for these downregulations. This current study provides a mechanism of the contribution of hyperlipidemia to the induction of endothelial oxidative stress and development of cardiovascular diseases in metabolic syndrome.

Results

PA treatment decreases GPX-1 protein and mRNA levels and increases ROS accumulation

Palmitic acid (PA) is the most common saturated fatty acid found in body. In healthy adults, the concentration of PA is about 0.15 mM (Fraser et al., 1999). Therefoer, The concentration of PA at 0.4 mM mimics the pathological condition (Wu et al., 2007). To investigate the effects of high levels of FFAs on GPX-1 in endothelial cells (ECs), we treated HUVECs with PA under different conditions and observed the changes of GPX-1 protein and mRNA levels. p53 is a transcription factor of GPX-1, so the protein level of p53 was also detected. At first, HUVECs were exposed to 0.4 mM PA for different time durations. As shown, high PA treatment significantly decreases both p53 and GPX-1 protein and mRNA levels in HUVECs after 8-16 hour incubation (Figure 3.1 A, C). Then, HUVECs were treated with PA at various concentrations for 16 hours. As expected, 0.4 mM and higher concentration of PA cause drops in both p53 and GPX-1 protein and GPX-1 mRNA levels in a dose-dependent manner (Figure 3.1B, D).

GPX-1 is the major antioxidant enzyme for clearance of H_2O_2 in ECs. To test its activity after PA treatment in ECs, we measured the intracellular DCF which exhibits H_2O_2 levels specifically. The increases of intracellular H_2O_2 levels are correlated with the decreases of GPX-1 protein levels (Figure 3.1E, F. $p<0.01$), indicating that GPX-1 inhibition by PA treatment leads to the accumulation of ROS in ECs. In the following experiments, we treated ECs with 0.4 mM PA for 16 hours.

PA treatment inhibits p53 acetylation via inducing PTEN nuclear export

p53 is a transcription factor of GPX-1. As shown above, GPX-1 protein and mRNA levels decrease in PA-treated cells. In the meantime, p53 protein level drops in ECs after PA application. It suggests that both p53 stability and transcriptional activity are impaired by PA. Posttranslational modifications, such as phosphorylation and acetylation are required for p53 stabilization and transcriptional activation (Bode and Dong, 2004). To further elucidate the mechanism for the inhibition of p53, we tested the levels of several modifications of p53 in PA-treated ECs. Without affecting phosphorylation sites (Ser15, 20, 46, 392 and Thr55), PA causes the significant decreases of acetyl-373 and acetyl-382 of p53 in a time-dependent manner (Figure 3.2A).

It is well known that p53 acetylation is tightly regulated. p300 functions as acetyltransferases of p53 (Ito et al., 2001). PML (Pearson et al., 2000) and PTEN (Li et al., 2006) induce and maintain p53 acetylation. In contrast, SIR-1 is a deacetylase of p53, which decreases acetylation of p53 (Solomon et al., 2006; Luo et al., 2001). However, PA does not change expression levels of those proteins (Figure 3.2B). Cytoplasmic and nuclear PTEN regulate p53 activity through different pathways (Freeman et al., 2003; Tang and Eng, 2006). Our lab is the first to show that only nuclear PTEN is responsible for maintenance of high p53 acetylation (Li et al., 2006). Thus we checked if PA affects subcellular distribution of PTEN. Intriguingly, PA treatment induces PTEN translocation from nucleus to cytoplasm. In contrast, subcellular localization of p300 or Sirt-1 is not affected (Figure 3.2C). All of the above findings imply an important role of decreases of nuclear PTEN in PA-mediated p53 acetylation downregulation. To further confirm it, PTEN expression plasmids with either nuclear localization signal (NLS) or nucleus

exclusion signal (NES) were transfected into ECs. As expected, nuclear-localized PTEN, but not cytoplasmic-localized PTEN, rescues PA-mediated degradation of p53 and its acetylation down-regulation (Figure 3.2D). To investigate whether acetylation alteration is required for p53 inhibition induced by PA, ECs were transfected with empty vector (EV), p53 WT, or 2KR (K373R/K382R) mutant. As shown in Figure 3.2E, 2KR abolishes p53 inhibition mediated by PA, demonstrating an essential role of acetylation in PA-induced p53 down regulation. To confirm the indispensable role of PTEN in suppression of p53 mediated by PA, PTEN was knocked downed with siRNAs in ECs. It was illustrated that knock-down of PTEN inhibits p53 degradation and its acetylation alteration induced by PA (Figure 3.2F). In all, our results suggest that downregulation of p53 acetylation caused by nuclear PTEN reduction is responsible for PA-mediated p53 inhibition.

Ser380 phosphorylation by S6K suppresses PTEN mono-ubiquitination and nuclear localization in PA-treated cells

Subsequently, we intended to explore the underneath mechanisms of PTEN nuclear export. As documented, mTOR/S6K signaling plays a fundamental role in regulation of PTEN nuclear export during G1/S transitions (Liu et al., 2007). To figure out if mTOR/S6K is involved in PA-mediated PTEN nuclear export, we treated ECs with sodium salicylate (NaSal), a specific inhibitor of S6K. As illustrated in Figure 3.3A, B, inhibition of S6K by NaSal completely eliminates PTEN nuclear export, p53 inhibition, and GPX-1 down-regulation in PA-treated ECs. Intriguingly, PA treatment activates nuclear S6K by induction of its phosphorylation at Thr389. Phosphorylation of PTEN at

Ser380 is associated with its nuclear distribution (Chang et al., 2008; Planchon et al., 2007). We also checked levels of phosphorylation of PTEN at Ser380. Concomitantly, phosphorylation of nuclear PTEN at Ser380 is increased, which can be abolished by NaSal (data not shown).

To determine whether S6K is a direct upstream kinase inducing PTEN phosphorylation at Ser380, *in vitro* kinase assay was performed with purified GST-PTEN and S6K protein. As a result, S6K phosphorylates Ser380 of PTEN *in vitro* directly. This phosphorylation can be completely abolished by NaSal (Figure 3.3C). *In vivo*, knockdown of S6K by siRNA blocks phosphorylation of PTEN at Ser380 in PA-treated ECs (data not shown). This study for the first time shows that S6K is a direct upstream kinase of PTEN at Ser380 and activated S6K by PA treatment phosphorylates Ser380.

Accompanied with upregulated phosphorylation at Ser380, PTEN monoubiquitination, which promotes its nuclear import (Trotman et al., 2007), is inhibited by PA treatment and rescued by NaSal (data not shown). To explore the relationship between Ser380 phosphorylation and monoubiquitination, we constructed S380A (phosphorylation defect mutant) and S380D (phosphorylation mimic mutant) plasmids, transfected them into ECs and tested their monoubiquitination levels. Compared to wild type, S380A shows higher level of monoubiquitination while S380D shows lower level of monoubiquitination than wild type (Figure 3.3D). Those results suggest that phosphorylation of S380 on PTEN inhibits its monoubiquitination.

The suppression of monoubiquitination may be due to the decrease of ubiquitination or increase of deubiquitination. NEDD4-1, an ubiquitin ligase of PTEN, induces

monoubiquitination by binding to PTEN (Wang et al., 2007). Herpesvirus-associated ubiquitin-specific protease (HAUSP, also known as USP7) is a deubiquitylation enzyme of PTEN. It also interacts with PTEN. In contrast to monoubiquitination, deubiquitination leads to PTEN cytoplasmic accumulation (Song et al., 2008). To search the reason for the downregualtion of monoubiquitination by phosphorylation, we checked whether S380 phosphorylation affects the interactions of PTEN with NEDD4-1 or USP7. As illustrated, S380A displays weaker binding to USP7 than wild type, whereas S380D displays stronger binding to USP7. Nevertheless, interaction of PTEN with NEDD4-1 is not impeded by phosphorylation (Figure 3.3E). Correspondingly, the interaction between USP7 and endogenous PTEN is enhanced by PA treatment and abolished by NaSal, which blocks S380 phosphorylation by inhibition of S6K (Figure 3.3F). In addition, PA treatment itself does not affect the binding of NEDD4-1 to PTEN (Figure 3.3G).

Taken together, PA-treatment activates S6K, which function as a direct upstream kinase and promotes phosphorylation of PTEN at S380. Consecutively, by augmenting integration of PTEN with USP7, phosphorylation inhibits mono-ubiquitination of PTEN, leading to its nuclear export.

mTOR/S6K pathway is involved in PA-induced PTEN S380 phosphorylation and nuclear export

mTOR, a Ser/Thr kinase inhibited by rapamycin, is a sensor of nutrient excess (Carrera, 2004). mTOR has been shown to activate S6K by mediating its direct phosphorylation of T389 (Burnett et al., 1998; Isotani et al., 1999). In the present study, PA treatment increased S6K phosphorylation at T389. To test whether mTOR is involved in PA-

mediated PTEN S380 phosphorylation and nuclear export, we treated EC with RAD001, an mTOR inhibitor. As shown, inhibition of mTOR by RAD001 blocks PA-mediated PTEN nuclear export (Figure 3.4A), p53 degradation, GPX-1 downregulation and inhibition of p53 acetylation (Figure 3.4B). To further confirm the indispensable role of mTOR in those events, another mTOR inhibitor AZD8055 was applied in PA-treated ECs and similar results were obtained (Figure 3.4C). Importantly, RAD001 abolishes PA-induced phosphorylation of S6K on T389, phosphorylation of PTEN on S380 and decrease of PTEN monoubiquitination (data not shown). In addition, the augmented binding of PTEN and USP7 is blocked by RAD001 in PA-treated ECs (Figure 3.4D). Intriguingly, immunostaining results show that RAD001 and Nasal abolished PTEN nuclear export in PA-treated ECs (Figure 3.4E), indicating that mTOR/S6K pathway plays an essential role in PA-mediated PTEN nuclear export.

Taken together, our data suggest that PA treatment activates mTOR/S6K pathway, and upon activation, S6K phosphorylates PTEN at S380. Phosphorylation of S380 induces conformation change of PTEN, which promotes interaction between PTEN and USP7, resulting in decrease of monoubiquitination and nuclear export of PTEN. The decrease of nuclear PTEN leads to decrease of p53 acetylation, stability, and transcriptional activity. Ultimately, GPX-1 expression is inhibited. As a result, ROS formation is increased, which causes endothelium damage (Figure 3.4F).

Discussion

In the current study, we elucidated that high levels of FFAs induces oxidative stress in endothelial cells (ECs) by GPX-1 inhibition. High FFAs can activate mTOR/S6K, and S6K can directly phosphorylate PTEN at S380. Phosphorylation of PTEN at S380 suppresses monoubiquitination and promotes its nuclear export, thus leading to decreased p53 protein level and transcription. As a p53 downstream target, GPX-1 expression is suppressed and its antioxidant function impaired. In brief, this study discloses a novel pathway by which hyperlipidemia causes endothelium oxidative damage.

Hyperlipidemia is one of the characteristics of metabolic syndrome. It causes oxidative stress and is an independent risk factor for the cardiovascular diseases (Zhang et al., 2012), which is the leading death cause of the developed countries. However, the mechanism of how high FFAs increase ROS and cause cardiovascular dysfunctions remains unclear.

mTOR signaling is closely related to cardiovascular health (Chong et al., 2011). In agreement with mTOR being a sensor of nutrient excess (Patti and Kahn, 2004; Carrera, 2004), mTOR activity can be induced by high levels of amino acids. Studies of animal models with high-fat-diet–induced obesity revealed that cytosolic or total mTOR is activated in skeletal muscles (Liu et al., 2012; Khamzina et al., 2005; Drake et al., 2010), vasculature (Wang et al., 2009), and heart (Sung et al., 2011; Turdi et al., 2011), which is associated with insulin resistance (Rosner et al., 2012). In mouse muscle cell lines, FFAs can activate mTOR/S6K (Castaneda et al., 2012). The current study is the first to demonstrate that high levels of FFAs activate mTOR/S6K in human EC nuclei, followed

by endothelium damage caused by oxidative stress. Future studies of the mechanisms by which high FFAs activate mTOR/S6K are needed. In addition, mTOR is an oncogenic protein. Thus, restriction of fatty acids intake is beneficial for prevention of cancer and inhibition of cancer progression.

In the present study, we also disclose the modulation of PTEN subcellular localization by mTOR/S6K signaling. Previous studies demonstrate that monoubiquitylation by NEDD4-1 induces PTEN nuclear import (Trotman et al., 2007), but deubiquitation by HAUSP promotes its cytoplasmic accumulation (Song et al., 2008). Nuclear Localization of PTEN is a dynamic process. Several mechanisms of nuclear/cytoplasmic shuttling have been proposed (Lian and Di Cristofano, 2005). However, the regulation of PTEN nuclear export is still unclear. mTOR/S6K is reported to be involved in PTEN nuclear export during G1/S transition (Liu et al., 2007), but the underlying mechanism is still lacking. This study revealed that mTOR induced phosphorylation of S6K at T389 in PA-treated EC nuclei. Then, S6K directly phosphorylated PTEN at S380. Further, phosphorylation at S380 reduced its monoubiquitination by enhancing the interaction of PTEN and HAUSP, for PTEN nuclear export (Figure 3). Moreover, these results illustrate the relationship between phosphorylation and monoubiquitination, which is consistent with the previous finding that C-terminal phosphorylation downregulates ubiquitination induced by NEDD4-1 by suppressing PTEN membrane localization (Wang et al., 2008; Maccario et al., 2010).

The role of CK2 as a direct protein kinase to target S380 of PTEN is not well defined (Torres and Pulido 2001; Vazquez et al., 2000). Another tumor suppressor, GLTSCR2,

induces phosphorylation of PTEN at Ser380 (Okahara et al., 2006; Yim et al., 2007). The current research is the first to identify S6K as a direct upstream kinase of PTEN at Ser 380. LKB1 can phosphorylate PTEN at S385 in combination either with S380, T382 or T383 (Mehenni et al., 2005). LKB1 negatively regulates mTOR signaling (Shaw et al., 2004), so it can induce phosphorylation at T382 or T383 but not S380. The stronger association of PTEN with HAUSP may be due to a conformational change induced by phosphorylation at S380. Use of X-ray or NMR for structure analysis of PTEN with S380 phosphorylation by PA treatment would be of interest. Compared with the original structure, if the conformation of PTEN is changed by phosphorylation at S380, the new conformation may also promote or inhibit interactions of PTEN with other proteins and affect related functions.

Most of studies that are working on the significance of PTEN in cardiovascular health are focused on its negative regulation of PIP3/AKT in cytoplasm or nucleus (Deleris et al., 2003). Our study provides a new insight into a role of nuclear PTEN in endothelium health through regulation of p53 acetylation. p53 is the most important tumor suppressor. It plays an important role in regulation of the formation and development of atherosclerosis (Iacopetta et al., 1995; Ihiling et al., 1997; Martinet et al., 2002; Mercer and Bennett, 2006). With antioxidant function, physical levels of p53 maintain a normal basal transcription of antioxidant genes SESN1 (sestrin 1), SESN2 (sestrin 2), and GPX-1(Sablina, et al., 2005). Our laboratory is the first to introduce the role of p53/GPX-1 inhibition in ROS accumulation and endothelium damages under hyperlipidemia condition. As indicated in our study, functions of p53 and PTEN which are two important

tumor suppressors are impaired under high FFAs condition. It suggests an intimate link between hyperlipidemia and cancer.

Oxidative stress is one of the major vascular effects of hyperlipidemia. The ROS and reactive nitrogen species (RNS) released by high levels of FFAs can induce oxidative stress, which causes apoptosis of ECs, vascular smooth muscle cells (VSMCs) and cardiomyocytes (Bashan et al., 2009). In addition, ROS decreases the production of bioactive nitric oxide ($NO\cdot$), which is an important vasodilator and plays a significant role in vasopretection. FFA-mediated endothelial dysfunction caused by ROS is improved by vitamin C treatment (Pleiner et al., 2002). Recently, antioxidant vitamin treatment has been beneficial in preventing atherosclerosis in animal experiments and clinical studies (Ozkanlar and Akcay, 2012). Nevertheless, an efficient antioxidant system in the endothelium is needed for self-defense. As discussed above, GPX-1 is a key antioxidant enzyme in vascular ECs and is associated with diabetes (Grankvist et al., 1981; Lei et al., 2007) and cancer development (Lei et al., 2007). Importantly, the activity and bioavailability of GPX-1 are closed related to cardiovascular health (Schnabel et al., 2005). In the current study, high FFA levels induced oxidative stress by repressing GPX-1 expression. Inhibition of p53/GPX-1 promoted H_2O_2 accumulation (Figure 1), which could be abolished by mTOR/S6K inhibitors (data not shown). Intriguingly, GPX-1 is indispensable for maintaining endothelial function and NO bioavailability (data not shown). Suppression of GPX-1 may play an important role in FFA-mediated endothelial oxidative damage.

Materials and Methods

Antibodies used in the present study and their commercial sources were as follows: anti-GPX1, anti-phospho-Ser15, anti-phospho-Ser20, anti-phospho-Ser46, anti-acetyl-p53 antibody (Lys373, Lys382), anti-PTEN, anti-phospho-PTEN-ser-380, anti-S6K, anti-phospho-S6K-T389, anti-HAUSP antibodies were purchased from Cell Signaling Technology; anti-p53 (DO1), anti-phospho-Ser392, anti-PML, anti-p300, anti-Sirt-1, anti-H3, anti-TBP, anti-Nedd4-1, anti-PAPR-1, anti-ubiquitin antibodies were from Santa Cruz Biotechnology; anti-myc (Ab910B) antibody was from Abcam, and anti-vinculin (VIN-11-5) antibody was from Sigma. Anti-TAF1 (Ab1230) and anti-Thr55-Phos (Ab202) antibodies were home generated. S6K inhibitor NaSal (10 mM), mTOR inhibitor RAD001 (20 nM), TSA (0.5 μM), palmitic acid and bovine serum albumin (BSA) were purchased from Sigma. The proteasome inhibitor MG132 was purchased from A.G Scientific, Inc.

Cell culture

Human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) were growth in EGM-2 and EGM-MV (Lonza Walkersville, Md) at 37 ℃ in an atmosphere of 95% air and 5% CO2. HUVECs or HAECs from passages 2 to 5 were used for experiments.

Preparation of free fatty acid-albumin complexes

Palmitic acid (PA) was added into cell culture medium as PA-BSA complex as described previously[37] with a minor modification described by Wang et al.[7] Briefly, a stock solution of palmitic acids (0.2 M) was prepared in 100% ethanol and kept at 4 ℃. The palmitic

acids were coupled with 10% fatty-acid free bovine serum albumin (BSA) to a concentration of 4 mM. These solutions were sterilized by filtration through 25 mm syringe filters (Fisher Scientific, 09-719A) and stored at -20 ℃. Control solution containing ethanol and BSA was similarly prepared. The final concentration of ethanol in the culture medium is lower than 0.1%. The concentration of PA applied in the experiments was 0.4 mM. Mock cells were incubated with FFA-free BSA as presented in PA-treated cells.

Nuclear/cytoplasmic fractionation

Subcellular fractionation was performed as described.[38] Briefly, one 100 mm-plate of HUVECs were washed with PBS and resuspended in 350 μl of 1:5 diluted buffer A [50 mM Hepes (pH 7.4) ,1 mM EDTA, 10 mM mannitol, 1 mM DTT, 2 μg /ml aprotinin, 2 μg/ml leupeptin, and 1 mM PMSF]. After incubation on ice for 10 min, cells were homogenized with 25 G needle for 10 strokes. After brief centrifugation at 6,000×g at 4 ℃, the supernatant (cytoplasmic fraction) was collected. The pellet was washed with buffer A and then resuspended in 350 μl of L-buffer [50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonident P-40, 1 mM DTT, 2 μg/ml aprotinin, 2 μg /ml leupeptin, and 1 mM PMSF]. After centrifugation, the supernatant (nuclear fraction) was collected. Nuclear and cytoplasmic fractions were assessed by immunoblotting of histone H3 or TBP and vinculin, respectively.

PTEN GST-pull down and in vitro kinase assay

In vitro kinase assay was performed using bacterially expressed purified PTEN[39] and baculovirus expressed and purified human S6K protein (1-421, T412E active; Millipore)

in a phosphorylation buffer containing 20 mM Hepes, pH 7.8, 20 mM $MgCl_2$, 20 mM glycerophsphate, 1 mM DTT, 100 μM ATP, 1 mM EDTA, 1 mM PMSF. The phosphorylation reaction was resolved by 10% SDS-PAGE and immunoblotted with anti-PTEN (phospho S380) antibody. The GST-PTEN proteins were detected by immunoblotting with anti-PTEN antibody (cell signaling) after stripping.

Western blot analysis and immunoprecipitation

Whole-cell extract was prepared by lysing cells in lysis buffer containing 20 mM Tris-Cl (pH 7.9), 150 mM NaCl, 0.5% NP-40, 20% glycerol, 2 mM EDTA, 0.5 mM DTT, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 0.5 mM PMSF. For in vitro binding assay, cell lysates were immunoprecipitated with 1 μg anti-PTEN or anti-HA antibody. The amounts of HAUSP (also known as USP7) and NEDD4-1 in the immunoprecipitates were determined by anti-HAUSP and anti-NEDD4-1 antibody, respectively. For detecting p53 Thr55 phosphorylation, the cell lysate was immunoprecipitated with anti-Thr55-Phos antibody Ab202 and immunoblotted with anti-p53 (DO-1) antibody. To normalize the p53 protein levels, MG132 (20 μM, Calbiochem) was applied 6 hr prior to cell harvesting. For the PTEN ubiquitination assay, HUVECs were transfected with Myc-ubiquitin and then treated with palmitic acid for 16 hours in the presence or absence of NaSal or RAD001. The nuclear extract was subjected to immunoprecipitation with anti-myc antibody, followed by immunoblotting with anti-PTEN antibody.

Immunostaining

HUVECs were seeded on coverslips and treated with PA in the presence and absence of RAD001 and NaSal. After incubation, the cells were fixed with 3% formaldehyde for 20

min, permeabilized with 0.5% Triton X-100 for 10 min, and pre-blocked with PBS containing 3% BSA for at least 1 hour. The cells were incubated with anti-PTEN (#9552, Cell signaling) at 4℃ overnight and then incubated with the secondary antibody conjugated with Alexa fluor 568 (1:500 dilution, Invitrogen) for 2 hours. After incubation, the cells were mounted with mounting solution containing 1 μg/ml DAPI. Images were obtained with a Nikon E-800 fluorescence microscope.

Measurement of ROS

To detect the generation of intracellular ROS, the ROS-sensitive fluorescent indicator 2',7'-dihydrodichlorofluorescin diacetate (DCFDA, Molecular Probes) was used in HUVECs or HAECs according to manufacturer's protocol [Or as described previously.[40]] Confluent ECs in 96-well plates were preincubated with the fluorescence probe DCFDA (10 μM) for 30 min. After removal of medium from wells, cells were washed three times in PBS, followed by measurement of fluorescence intensity at 485-nm excitation and 538-nm emission spectra with a fluorescence microplate reader. Data are presented as the fold of increase in DCF fluorescence compared with that in unstimulated cells.

Transient transfection and siRNA gene silencing

Cell transfection was performed using TransPass HUVEC Transfection Reagent (New England Biolabs) with 1 μg PTEN expression vector (NLS-PTEN or NES-PTEN), 1 μg HA-PTEN (HA-WT-PTEN, HA-S380A-PTEN or HA-S380D-PTEN), 1 μg p53 expression vector (pcDNA3-p53-WT or pcDNA3-p53-2KR), or empty vector. For PTEN knock down studies, ECs were transfected with human-specific PTEN siRNA (5'-AAGAUCUUGACCAAUGGCUtt-3') or scrambled siRNA (Cell Signaling, Inc.) for 48h

using BioT transfection reagent (BiolandScientific LLC, Cerritos, CA) according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Sigma), and RT-PCR was performed using SuperScript One-Step RT-PCR kit (Invitrogen) according to manufacturer's protocol. One set of primers was designed to amplify GPX1 mRNA (forwarded: 5'-TCCCTCTGAGGCACCACGGTC-3' and reversed: 5'-TTGGCGTTCTCCTGATGCCCAAAC-3'). Another set of primers for GAPDH (forwarded: 5'-AGGTGAAGGTCGGAGTCAAC-3' and reversed: 5'-GACAAGCTTCCCGTTCTCAG-3') was used as a control.

Statistical analysis

All results were analyzed with unpaired Student *t* test or 1-way ANOVA, except for those obtained from the time-course studies, which were analyzed with repeated-measures ANOVA. Values are expressed as mean ± SD for all assays. Significance was accepted at $P<0.05$.

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Figure 3.1

PA treatment decreases GPX-1 protein and mRNA levels and increases ROS accumulation

HUVECs were treated with 0.4 mM palmitic acid (PA) for different time durations (A). They were treated with different concentrations of PA for 16 hours (B). p53 and GPX-1 protein levels were detected by Western Blot with specific antibodies. Vinculin was an internal control. In those experiments, PA was complexed to BSA. And cultures with FFA-free BSA were used as controls (Mock). The blot is a representative of 3 blots from 3 independent experiments. The bar graphs shown in A and B (the upper panel) shows quantitative data of the optical density in p53 and GPX1 protein levels (n=3; *p<0.01 vs. control). (C) HUVECs were treated with PA for different time durations. The GPX-1 mRNA levels were measured with reverse transcription polymerase chain reaction (RT-PCR) or real-time quantitative RT-PCR (qRT-PCR). (D) Cells were treated with different concentrations of PA for 16 hours. The GPX-1 mRNA levels were detected. (E) and (F), intracellular ROS was assessed by the fluorescence intensity of dichlorofluorescin (DCF) emission. Data are presented as mean fold increases (±SD) in treated groups over basal values from three independent experiments. *p<0.01 vs. control.

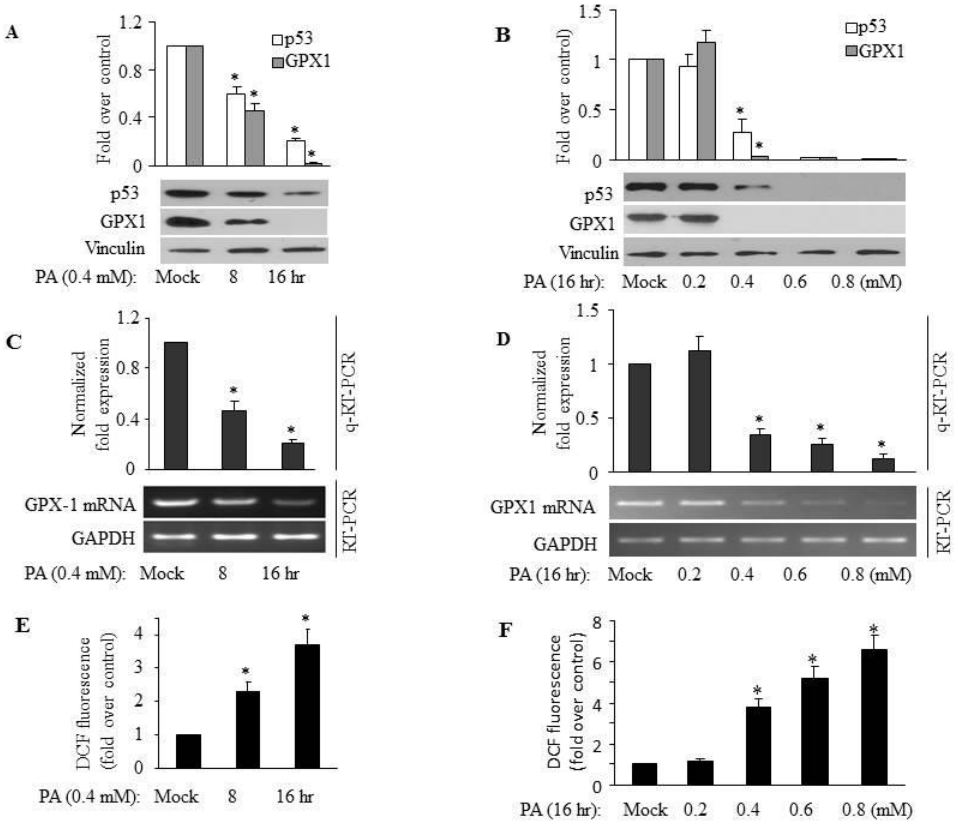

A
Fold over control
p53
GPX1
Vinculin
PA (0.4 mM): Mock 8 16 hr
B
Fold over control
p53
GPX1
Vinculin
PA (16 hr): Mock 0.2 0.4 0.6 0.8 (mM)
C
Normalized fold expression
q-RT-PCR
GPX-1 mRNA
GAPDH
RT-PCR
PA (0.4 mM): Mock 8 16 hr
D
Normalized fold expression
q-RT-PCR
GPX1 mRNA
GAPDH
RT-PCR
PA (16 hr): Mock 0.2 0.4 0.6 0.8 (mM)
E
DCF fluorescence (fold over control)
PA (0.4 mM): Mock 8 16 hr
F
DCF fluorescence (fold over control)
PA (16 hr): Mock 0.2 0.4 0.6 0.8 (mM)

Figure 3.2 PA treatment inhibits p53 acetylation via inducing PTEN nuclear export

(A). HUVECs were treated with 0.4 mM PA for 8 or 16 hours. Major modifications were detected by Western Blot with specific antibodies. When p53 phosphorylation or acetylation was assayed, cells were treated with MG-132 before harvest. (B). HUVECs were treated with 0.4 mM PA for 8 or 16 hours. PML, SIRT1, p300 and PTEN protein levels were measured by Western Blot with specific antibodies. (C). HUVECs were treated with 0.4 mM PA for 16 hours. Subcellular fractionation experiments were performed. The protein levels of PTEN, Vinculin, H3, TAF1 and TBP in the nuclear and cytoplasmic fractions were detected with specific antibodies. Histone H3, TAF1 and TBP were used as nuclear controls. Vinculin was used as the cytoplasmic control. (D). ECs were transfected with Empty Vector (EV) or PTEN expression constructs tagged with nuclear localization signal (NLS-PTEN) or cytoplasmic localization sequence (NES-PTEN). Transfected cells were incubated with PA or its vehicle. The protein levels of PTEN and p53 were detected. Acetylation levels of p53 among different groups were compared. (E). HUVECs were transfected with the EV, p53 wild-type (WT) or 2KR (K373/K382) mutant expression plasmids. Transfected cells were treated with PA or its vehicle. The levels of p53 protein and its acetylation were detected. (F). HUVECs were treated with 0.4 mM PA or its vehicle accompanied with control siRNA or PTEN siRNA transfection. The p53 and PTEN protein levels were assayed by Western Blot. Acetylation levels of p53 among different groups were compared. The blot is a representative of 3 blots obtained from 3 separate experiments.

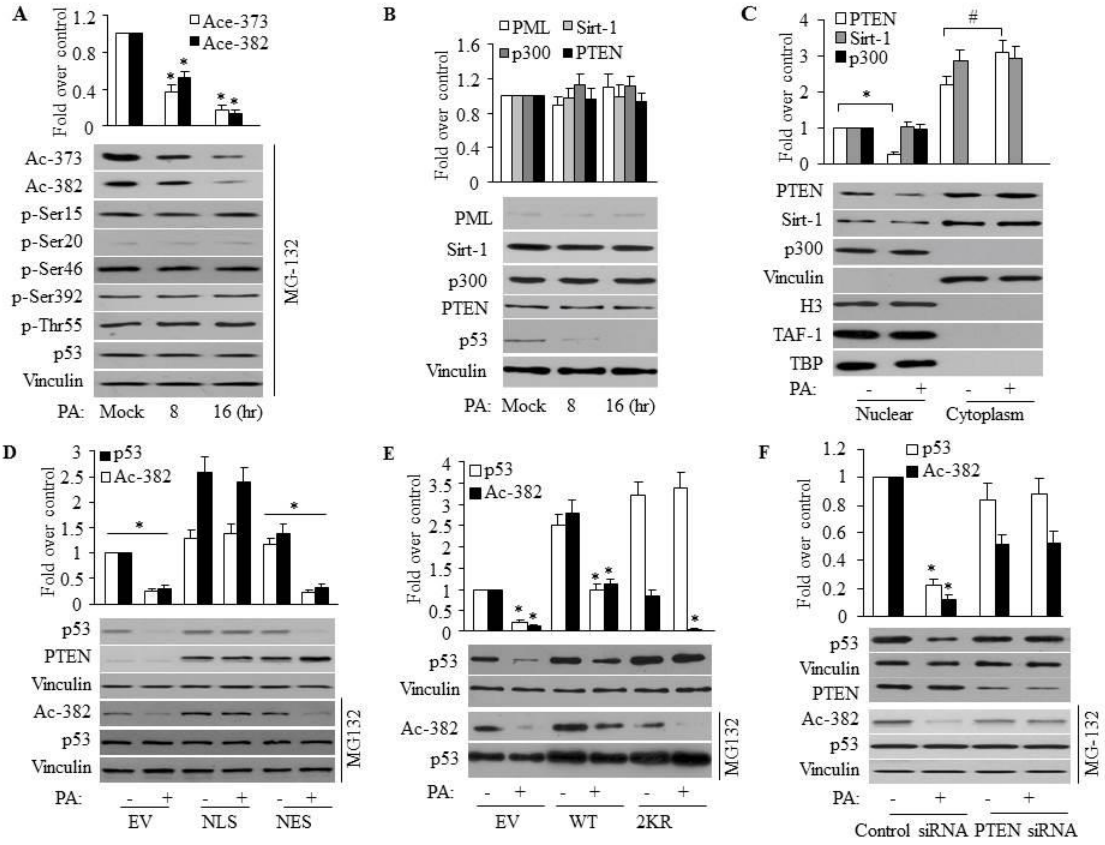

A
Fold over control
Ace-373
Ace-382
Ac-373
Ac-382
p-Ser15
p-Ser20
p-Ser46
p-Ser392
p-Thr55
p53
Vinculin
MG-132
PA: Mock 8 16 (hr)
B
PML
Sirt-1
p300
PTEN
p53
Vinculin
PA: Mock 8 16 (hr)
C
PTEN
Sirt-1
p300
H3
TAF-1
TBP
PA:
Nuclear
Cytoplasm
D
p53
Ac-382
PTEN
Vinculin
MG132
EV
NLS
NES
E
WT
2KR
F
Control siRNA
PTEN siRNA

Figure 3.3

Ser380 phosphorylation by S6K suppresses PTEN mono-ubiquitination and nuclear localization in PA-treated cells

In A, B and F, HUVECs were treated with PA or its vehicle, in presence or absence of S6K inhibitor NaSal (10 mM). (A). Subcellular fractionation experiments were performed. PARP-1 was used as internal control of nuclear fraction. Nuclear/cytoplasmic distributions of PTEN were assayed in different groups. (B). Whole lysate of treated cells were immonoblotted with specific antibodies. The levels of p53 protein and its acetylation and GPX-1 protein were assayed. (C). GST or GST-PTEN plasmids was transformed into E. coli (BL21). The recombinant proteins GST or GST-PTEN was purified and incubated with 0.1 μg of recombinant human p70S6K kinase in phosphorylation kinase assay buffer at 37 ℃ for 30 min. S6K kinase inhibitor NaSal was added as a negative control. Phosphorylation of PTEN at Ser380 and PTEN protein levels were detected by Western Blot with specific antibodies. (D). In indicated groups, ECs were transfected with Myc-ubiquitin and EV, plasmids encoding, HA-WT-PTEN, HA-PTEN-S380A or HA-PTEN-S380D, respectively. Subcellular fractionation experiments were performed. Nuclear lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-HA for Ub-PTEN. Monoubiquitinations in ECs overexpressing different plasmids were detected. (E). ECs were transfected with EV, plasmids encoding HA-WT-PTEN, HA-PTEN-S380A or HA-PTEN-S380D. Nuclear lysates were immunoprecipitated with anti-HA and immunoblotted with anti-USP7. Correspondingly, cytoplasmic lysate were immunoprecipitated with HA and blotted with NEDD4-1.

PTEN-USP7 or PTEN-NEDD4-1 interactions were detected, respectively. (F). As mentioned above, PTEN-USP7 interactions were assayed in treated EC. (G). ECs were treated PA or its vehicles. PTEN-NEDD4-1 interactions were assayed.

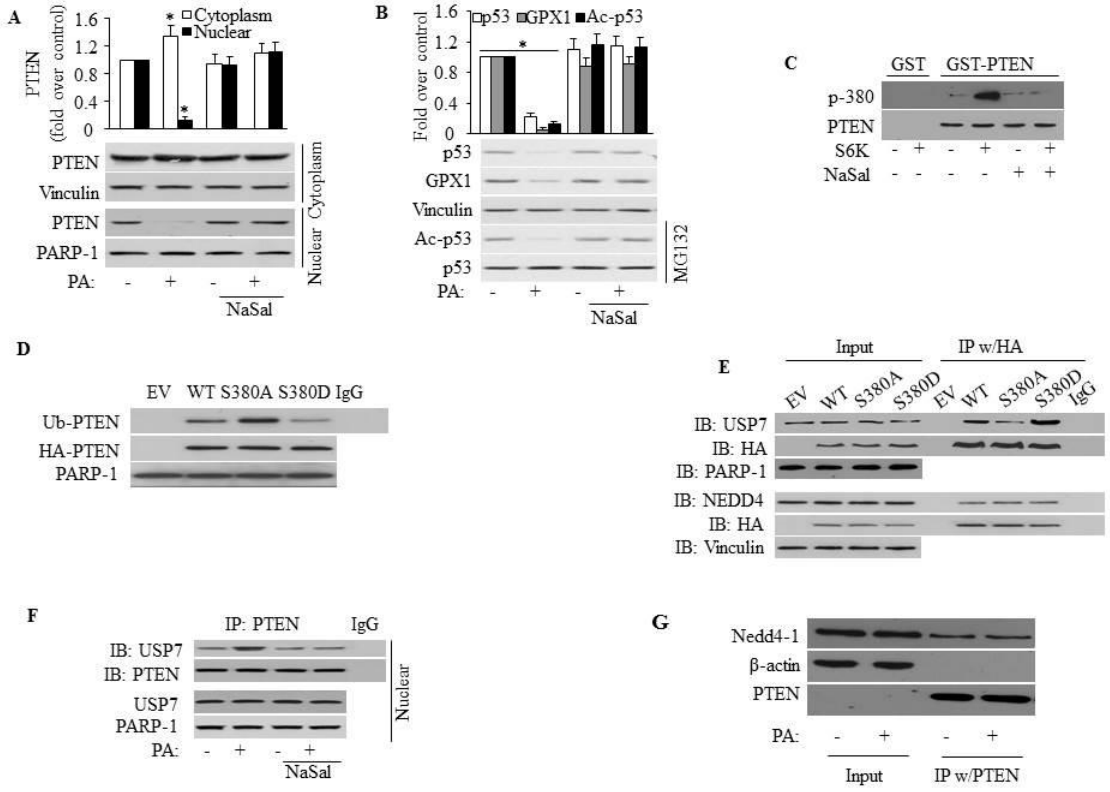
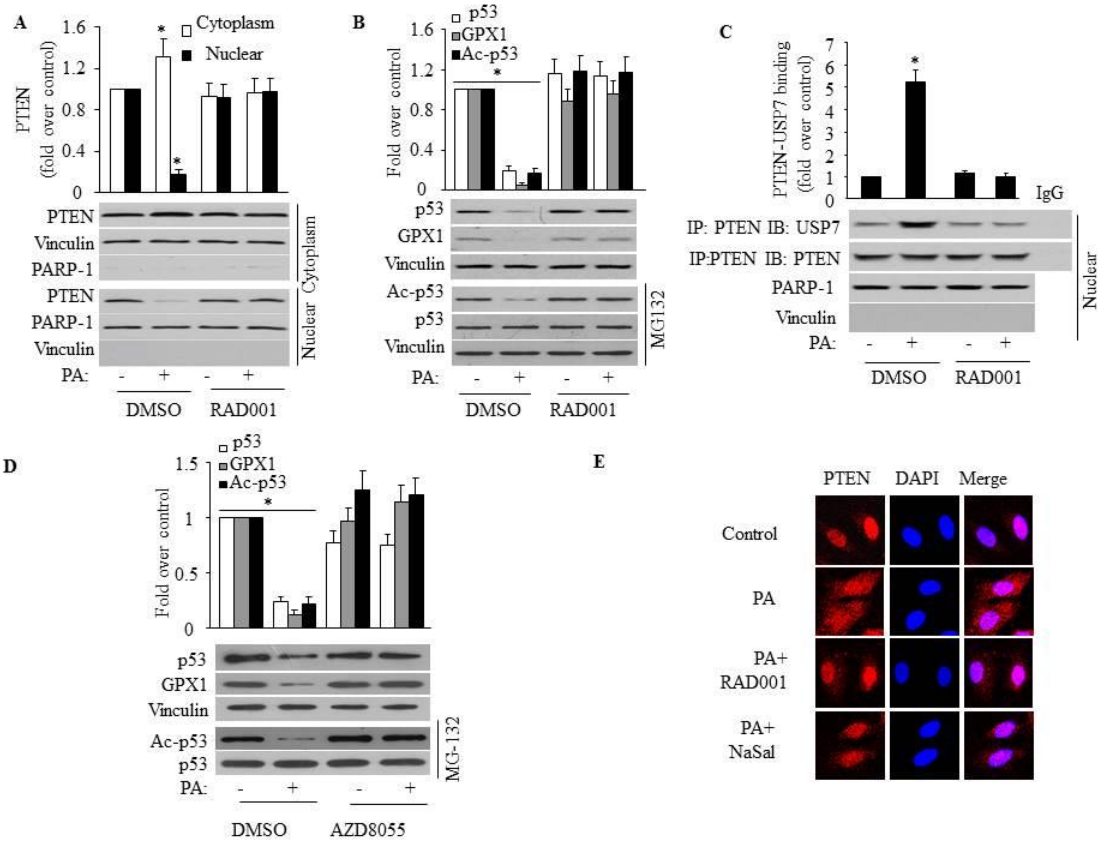


Figure 3.4

mTOR/S6K pathway is involved in PA-induced PTEN S380 phosphorylation and nuclear export

In A, B and C, HUVECs were treated with 20 nM of RAD001 or its vehicle DMSO prior to incubation with PA. (A) Subcellualar fractionation experiments were performed with treated cells. Distributions of PTEN in nuclear and cytoplasmic fractions were assayed with Western Blot with anti-PTEN. (B). The whole lysate of treated ECs were immunoblotted with corresponding antibodies. The levels of p53 protein and its acetylation and GPX-1 proteins were assayed. (C). PTEN-USP7 interaction in nuclear fraction were detected as described in Figure 3.3.E. (D) HUVECs were treated with 150 nM of AZD8055 or its vehicle DMSO prior to treatment with PA. In total lysates, the levels of p53 protein and its acetylation and GPX-1 proteins were detected with specific antibodies. The blot is a representative of 3 blots obtained from 3 separate experiments. *p<0.01 vs. control. (E). HUVECs were treated with vehicle (Control) and PA in the presence or absence of RAD001 and NaSal. Immunostaining experiments were performed with anti-PTEN and anti-actin antibody. DAPI was used to visualize nuclei. Images were merged to observe nuclear export of PTEN under different conditions. (F). A proposed model of increase of oxidative stress by palmitic acid.


A
Cytoplasm
Nuclear
PTEN (fold over control)
PTEN
Vinculin
PARP-1
Nuclear Cytoplasm
PA:
DMSO
RAD001
B
p53
GPX1
Ac-p53
Fold over control
MG132
C
PTEN-USP7 binding (fold over control)
IgG
IP: PTEN IB: USP7
IP:PTEN IB: PTEN
Nuclear
D
AZD8055
MG-132
E
PTEN
DAPI
Merge
Control
PA
PA+ RAD001
PA+ NaSal
F
palmitic acid(0.4 mM)
mTOR
RAD001
Nasal
p-S6K-T389
p-PTEN-S380↑
conformational change
mono-ub of PTEN↓
PTEN-USP7↑
PTEN
Ace-p53↓
p53 protein level and Txn↓
nucleus
GPX-1↓
ROS↑
cytoplasm
cardiovascular endothelium damage

Chapter 4

Conclusion

Tumor suppressor protein PTEN has been found in both nucleus and cytosol. As a lipid phosphatase, cytoplasmic PTEN dephosphorylates PIP3 to PIP2 and down-regulates the activity of PI3K. Through its inhibition of PI3K/AKT pathway, PTEN modulates cellular growth and survival, metabolism, cardiovascular health and tumor suppression in cytoplasm (Salmena, et al., 2008). Nuclear function of PTEN is also essential for its tumor suppressor activity. Nuclear PTEN participates in regulation of cell cycle progression and genomic stability (Baker, 2007). PTEN activities, functions and subcellular distribution are regulated by epigenetic silencing, transcriptional modulations, non-coding RNAs, post-translational modifications and protein-protein interactions (Tamguney and Stokoe, 2007). Here, we investigate the significance of PTEN in PML-IV-mediated cellular senescence and free fatty acids-induced oxidative stress. Independent of its phosphatase activity, PTEN plays an important role in both processes by maintenance of high levels of p53 acetylation.

Contributions of nuclear PTEN in PML-IV mediated cellular senescence

Tumor suppressor PML-IV is the key regulator of cellular senescence, a permanent cell cycle arrest, and it has been shown that overexpression of PML-IV can induce senescence (Dimri, 2005; Caino, et al., 2009). However, underlying mechanism for this senescence is unclear. Previous studies in our lab found that PTEN, p53 and PML-IV interact with each other and form a complex in the nucleus. Nuclear PTEN and p53 acetylation are required for cellular senescence mediated by PML-IV via p53/p21 pathway. My study described in chapter 2 provides additional evidence for this conclusion.

Specifically, I have constructed the C-terminal deletion mutants of PML-IV and identified PTEN binding domain on PML-IV. Intriguingly, I showed that the deletion mutants that fail to bind to PTEN and to induce p53 acetylation were unable to induce cellular senescence. Interactions between nuclear PTEN and the C terminus of PML-IV induce p53 acetylation, which increases transcription activity of p53. As a result, expression of p21 is increased, leading to cell senescence. In all, we demonstrate the indispensable role of nuclear PTEN in cellular senescence mediated by PML-IV via p53/p21 pathway. Those results support the importance of the physical interaction between PML-IV and nuclear PTEN and provide an intimate link between nuclear PTEN and PML-IV medicated cellular senescence. Induction of cellular senescence in cancer cells has been used as a therapy for tumors (Narita and Lowe, 2005). Thus, nuclear PTEN and PML-IV could be targets for cancer treatment.

Roles of PTEN phosphorylation and nuclear export in free fatty acid-induced endothelial oxidative stress

Hyperlipidemia, one of medical disorders of Metabolic syndrome, is correlated to cardiovascular diseases. Several mechanisms of endothelial dysfunction caused by high levels of FFAs have been proposed. Recently, it is shown that oxidative stress induced by abnormal levels FFAs lead to endothelium damages (Zhang et al., 2012). However, the mechanisms of induction of oxidative stress by high FFAs are not well defined. My results described in chapter 3 provide a novel pathway that is through PTEN phosphorylation and nuclear export.

GPX-1, a downstream target of p53, is a key antioxidant enzyme in endothelium cells (ECs). In FFA-treated ECs, decreased p53 protein and acetylation levels lead to the inhibition of GPX-1 and accumulation of ROS. Further, we found that PTEN nuclear export is responsible for the down regulation of p53. Because mTOR /S6K was reported to be responsible for PTEN nuclear export in G1/S transition (Liu et al., 2007), we investigated whether mTOR/S6K is also involves in our research. Our results indicate that high levels of FFAs activate mTOR/S6K and once activated, S6K directly phosphorylates PTEN at Ser380. This phosphorylation suppresses the monoubiquitination of PTEN by enhancement of interaction of PTEN with its deubiquitination enzyme HAUSP. PTEN nuclear export which is caused by the decrease of monoubiquitination results in p53/GPX-1 inhibition and oxidative stress.

Together, PTEN nuclear export is a pivot in the novel pathway, which illustrates a role of nuclear PTEN in metabolism. In EC nuclei, high levels of FFAs activate mTOR/S6K, which induces phosphorylation of PTEN at 380 directly. Sequentially, decrease of monoubiquitination by phosphorylation induces PTEN nuclear export. As a result, p53 acetylation is decreased, leading to reduction of GPX-1 and accumulation of ROS. In the end, oxidative stress is induced in high levels of FFAs treated ECs. Importantly, inhibitors or genetic knockdown of mTOR or S6K blocked FFA-induced PTEN phosphorylation at S380 and all the consequent downstream events, suggesting that mTOR/S6K is required for induction of oxidative stress in high levels of FFAs treated ECs.

In this study, two tumor suppressors, p53 and PTEN are implicated in metabolism in the same pathway, which suggests a new link between cancer and metabolism. The relationship of the two modifications of PTEN, monoubiquitination and phosphorylation is unveiled. By suppression of monoubiquitinaiton, phosphorylation at Ser380 promotes PTEN nuclear export. Our study is the first to explore the mechanism of PTEN nuclear exclusion mediated by mTOR/S6K. Major players in the pathway, such as GPX-1, p53, nuclear PTEN and mTOR/S6K can be used as clinical targets in Metabolism syndrome.

Summary

These 2 studies underline the substantial roles of maintenance of p53 acetylation by nuclear PTEN in both cellular senescence and hyperlipidemia-induced oxidative stress. Induction of senescence in precancerous and cancer cells is an important mechanism for tumor suppression. Oxidative stress plays important roles in endothelium damage and cardiovascular disease. Therefore, p53 acetylation and PTEN nuclear localization are associated with two leading causes of death in developed countries, heart disease and cancer, which indicates their significance in human health. p53 has been targeted in cancer treatment since 1996 (Lane et al., 2010; Suzuki and Matsubara, 2011). However, little is known about its potential for treatment of cardiovascular diseases.

Clearly, future studies on how maintenance of p53 acetylation by nuclear PTEN will shed light on the treatment of tumor and heart disease. PTEN nuclear export is related to endothelium damage, so retaining PTEN in the nucleus could benefit patients with cardiovascular diseases. LKB1 which inhibits mTOR has been shown to promote PTEN nuclear retention (Liu et al., 2011). Positive regulators of LKB1 and inhibitors of

mTOR/S6K could be candidates for effective treatment of cardiovascular disease. Further investigations of these potential drugs are needed.

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