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A PINK1-mediated mitophagy pathway decides the fate of tumors—to be benign or malignant?

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ABSTRACT

Macroautophagy/autophagy plays a dual role in cancer depending on the stage of tumorigenesis. Autophagy prevents tumor initiation by suppressing chronic tissue damage, inflammation, accumulation of damaged organelles and genome instability. Autophagy can also sustain tumor metabolism and provide nutrients for tumor growth and survival via nutrient recycling. Moreover, autophagy is required for benign tumors to progress to malignant tumors. Emerging evidence indicates that autophagy or mitophagy can inactivate tumor suppressors such as TP53/TRP53/p53 to promote tumor progression once carcinogenesis has been initiated.

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Autophagy is an evolutionarily conserved lysosomal degradation pathway that plays a critical role in regulating cellular homeostasis under both basal and stress conditions by degrading and recycling proteins and organelles. Autophagy generates nutrients (amino acids and fatty acids), nucleotides, glucose and ATP to support the metabolic needs for cell survival under nutrient-deprivation conditions. Therefore, it is not surprising that autophagy plays an essential role in tumorigenesis. It is now generally thought that autophagy plays a dual role in cancer in a context-dependent manner. During the tumor initiation stage, autophagy acts as a tumor suppressor to prevent chronic tissue damage, inflammation, accumulation of damaged organelles (in particular mitochondria) and genome instability. However, once the tumor is formed, autophagy promotes tumor growth by alleviating metabolic stress, inhibiting cell death (both apoptosis and necrosis) and supplying nutrients [1].

The role of autophagy against tumor initiation has been supported by many studies using genetically engineered mouse models. Mice with allelic loss of the essential autophagy gene *Becn1* have an increased frequency of spontaneous malignancies including lymphomas, liver, lung and breast tumors [2,3]. Moreover, *sh3glb1/bif-1* (SH3-domain GRB2-like B1 [endophilin]) knockout (KO) mice show an increased frequency of spontaneous lymphomas and solid tumors, and *atg4c* KO mice are also susceptible to chemical-induced fibrosarcomas [4,5]. Mice with mosaic deletion of *Atg5* or liver-specific deletion of *Atg5* or *Atg7* develop spontaneous benign adenoma in the livers, which is mediated by the accumulation of hepatic SQSTM1/p62 and subsequent NFE2L2/Nrf2 activation [6,7]. Furthermore, most tumor suppressor genes such as *PTEN* (phosphatase and tensin homolog), *STK11/LKB1* (serine/threonine kinase 11) and *TSC1* (tuberous sclerosis 1)-*TSC2* activate autophagy and inhibit tumorigenesis likely via their inhibition of phosphoinositide 3-kinase-MTOR (mechanistic target of rapamycin) signaling. In contrast, most oncogenes such as *AKT, EGFR* (epidermal growth factor receptor) and *BCL2* (BCL2, apoptosis regulator) inhibit autophagy and promote tumorigenesis [8]. Therefore, all the above evidence collectively supports a tumor suppressor role of autophagy in these contexts.

However, controversial data also reported an opposite role of autophagy in cancer by promoting cancer cell survival via maintaining oxidative metabolism, removing damaged mitochondria and facilitating glycolysis [1,9]. For example, deletion of *Rb1cc1/Fip200* (RB1-inducible coiled-coil 1) in mice, an essential autophagy protein in the ULK1 (unc-51 like kinase 1) complex, suppresses the development of mammary tumors in a *PyMT* (polyoma Middle T) oncogene-driven breast cancer mouse model [9]. Deletion of *Atg7* in mice suppresses the progression of KRAS- and $BRAF^{V600E}$-induced lung cancers and KRAS-driven pancreatic carcinoma [10,11]. Moreover, mice lacking pancreatic ATG5 or ATG7 only present low-grade and pre-malignant pancreatic neoplastic lesions without progression to high-grade malignant pancreatic ductal adenocarcinoma [11]. These findings are very similar to the liver-specific *atg5* or *atg7* KO mice that only develop spontaneous benign adenoma but not malignant hepatocellular carcinoma (HCC). Liver-specific *atg5* KO mice fail to develop HCC even after they are treated with the known hepatic carcinogen diethylnitrosamine (DEN), although control wild-type mice develop HCC after DEN treatment [12]. These data support the notion that autophagy is required to allow benign tumors to progress to malignant tumors, at least in the pancreas and liver. Interestingly, further deletion of *Trp53* in mice with loss of pancreatic *Atg7* or *Atg5* and containing oncogenic *Kras*, accelerates the

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formation of malignant pancreatic ductal adenocarcinoma [11]. Moreover, the expression of several tumor suppressor genes including *Trp53, Rb1* (RB transcriptional corepressor 1*)* and *PTEN* are increased in the liver adenoma tissues of liver-specific *atg5* KO mice [12]. These findings suggest that tumor suppressor gene, and in particular *Trp53*, may decide the role of autophagy in promoting benign tumors to malignant tumors. However, the mechanisms by which autophagy regulates TRP53 activation and promotes the progression of benign tumors to malignant tumors remain largely unknown.

In a recent study, Liu et al. [13] (see the related punctum in this issue of the journal) investigated the mechanism of how autophagy is required for benign hepatic adenoma to progress into malignant HCC. Cancer stem cells (CSCs) are a subset of tumor cells that express stem cell markers and play a critical role in the tumorigenesis of HCC. Using cultured HepG2 cells, a human hepatoma cell line that is comprised of approximately 5% PROM1/CD133$^+$ CSCs of the total HepG2 population under normal conditions, Liu et al. first demonstrated that pharmacological or genetic inhibition of autophagy decreases the PROM1$^+$ CSC population. Moreover, inhibition of autophagy also decreases the sphere formation of HepG2 cells, indicating impaired self-renewal ability of CSCs caused by inhibiting autophagy. In contrast, activation of autophagy by rapamycin or serum starvation increases the number of PROM1$^+$ CSCs. In addition to HepG2 cells, the percentage of PROM1$^+$ cells is also significantly higher in liver tumor cells isolated from DEN-treated wild-type mice than the tumor cells isolated from the liver-specific *atg5* KO mice. Together, these data demonstrate that autophagy positively regulates hepatic CSCs. By further dissecting the mechanisms, Liu et al. found that activation of autophagy increases the protein levels of NANOG but not POU5F1/OCT4 (POU class 5 homeobox 1) and SOX2 (SRY [sex determining region Y]-box 2), the key transcription factors that are involved in the self-renewal of embryonic stem cells and CSCs.

TP53 is the most commonly mutated gene in human cancers including HCC. As discussed above, the expression levels of TRP53 increase in both normal and tumor tissues of liver-specific *atg5* KO mice [12]. TRP53 is also associated with more malignant grades of pancreatic tumors with ATG7 or ATG5 deficiency [11]. Interestingly, unlike HepG2 cells that express wild-type TP53, Liu et al. found that modulation of autophagy has no effects on the levels of PROM1$^+$ CSCs in Hep3B and Huh7 cells, another 2 human hepatoma cell lines that either do not express TP53 (Hep3B) or express a mutant TP53 that lacks DNA binding ability (Huh7). However, inhibition of autophagy decreases the levels of PROM1$^+$ CSCs when wild-type TP53 is overexpressed in Hep3B and Huh7 cells. Conversely, knockdown of *TP53* using siRNA or inhibition of nuclear transport of TP53 by pifithrin-α increases the protein levels of PROM1 and NANOG in HepG2 cells. Liu et al. further identified 2 possible TP53-binding motifs, which are adjacent to the POU5F1-SOX2 binding site in the promoter region of *NANOG*. Wild-type TP53 and a phosphomimetic form, TP53^{S392D}, but not the nonphosphorylated form, TP53^{S392A}, prevent the binding of POU5F1-SOX2 to this promoter region and suppress the expression of NANOG. Collectively, these data indicate that autophagy deficiency leads to the accumulation and activation of TP53, which negatively regulates NANOG and PROM1$^+$ CSCs in cultured hepatoma cells.

How does lack of autophagy lead to the accumulation of hepatic TP53 protein? The authors found that either pharmacological inhibition of autophagy (via the phosphatidylinositol 3-kinase inhibitor 3-methyladenine or the lysosomal inhibitor bafilomycin A_1) or genetic knockdown of *ATG5* with an *ATG5*-specific shRNA increase TP53 and its phosphorylated form TP53 (p-S392) but do not affect the *TP53* mRNA level. These data indicate that the regulation of TP53 is at the post-translational level.

Mitophagy is a selective form of autophagy for removing damaged or excess mitochondria. Liu et al. further found that activation of mitophagy by treating HepG2 cells with carbonyl cyanide m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, increases the levels of PROM1, NANOG and the number of spheres. Conversely, inhibition of mitophagy by treatment with Mdivi-1, a DNM1L/Drp-1 inhibitor that inhibits mitochondrial fission, decreases the levels of PROM1, NANOG and the number of spheres. Further analysis from the subcellular fractionation and immunostaining experiments revealed that TP53 (p-S392) is present in mitochondrial, cytoplasmic and nuclear fractions. CCCP treatment increases the levels of mitochondrial TP53 (p-S392) but markedly decreases the nuclear TP53 (p-S392) in HepG2 cells. In contrast, Mdivi-1 significantly increases nuclear levels of TP53 (p-S392) but decreases mitochondrial TP53 (p-S392). These data suggest that mitophagy may suppress TP53 activation and in turn trigger the expression of NANOG to positively regulate CSCs.

Recent research has revealed an important PINK1 (PTEN induced putative kinase 1)-PRKN/PARK2/parkin pathway that regulates mitophagy in mammalian cells [14,15]. PRKN is an E3-ubiquitin ligase. PINK1 is a mitochondrial resident serine/threonine protein kinase, which is required for recruiting PRKN to depolarized mitochondria and activating its E3 ligase activity. Once PRKN is recruited and activated at mitochondria, it ubiquitinates a subset of mitochondrial outer membrane proteins and further recruits mitophagy receptor proteins such as SQSTM1, NBR1 (NBR1, autophagy cargo receptor), CALCOCO2/NDP52 (calcium binding and coiled-coil domain 2) and OPTN (optineurin) to trigger mitophagy [15].

Liu et al. found that knockdown or overexpression of PINK1 increases or decreases PROM1$^+$ CSCs and the formations spheres, respectively, in HepG2 cells, which correlates with the levels of TP53 (p-S392). Results from the co-immunoprecipitation experiments revealed that mitochondrial TP53 and TP53 (p-S392) but not the cytosolic TP53 directly interact with PINK1. Moreover, immunoprecipitated PINK1 from HeG2, Hep3B and Huh7 cells could directly phosphorylate recombinant GST-TP53 protein in vitro. These data suggest that PINK1 negatively regulates hepatic CSCs likely by phosphorylating TP53 at S392. Intriguingly, suppression of autophagy by 3-methyladenine, sh*ATG5*, or sh*ATG7* increases PINK1 and levels of TP53 (p-S392) but decreases NANOG. As a result, ATG5-knockdown HepG2 cells suppress the tumorigenesis in a xenograft nude mouse model, which is reversed if the expression of PINK1 is also suppressed. These data suggest that inhibition of autophagy and/or mitophagy increases the levels of PINK1-mediated phosphorylated TP53, which can no longer

be removed by mitophagy. Phosphorylated TP53 then translocates to the nucleus where it suppresses the expression of NANOG and in turn prevents the progression of benign to malignant liver tumors.

While this important work by Liu et al. has largely expanded our understanding of how autophagy is required for the progression of benign liver adenoma to malignant HCC, several important questions remain to be answered. It is well known that PINK1 is cleaved and degraded in healthy mitochondria via the N-end rule pathway [16]. The levels of PINK1 are almost undetectable in most cells including cancer cells unless the mitochondria are depolarized. Therefore, under normal conditions, the contribution of PINK1 to the regulation of cellular TP53 levels would be less significant given the very low levels of PINK1 on healthy mitochondria.

In contrast, under impaired autophagy conditions, lack of autophagy (or more specifically mitophagy) can lead to the accumulation of damaged mitochondria, which may have elevated levels of PINK1 that would phosphorylate and activate TP53 resulting in the suppression of NANOG and CSCs. Indeed the same group has previously reported that a large portion of mitochondria in *atg5* KO hepatocytes have lower mitochondrial membrane potential compared with wild-type hepatocytes [12], which may lead to a higher PINK1 level in the autophagy-deficient hepatocytes. However, the PINK1 levels have not been directly compared between wild-type and *atg5* KO hepatocytes in these studies. Intriguingly, it seems that PINK1 only regulates the phosphorylation and activation of TP53 but does not affect the total levels of TP53 because knockdown or overexpression of PINK1 has no effects on the total levels of TP53 in HepG2 cells [13]. In contrast, CCCP decreases, whereas Mdivi-1 increases, total levels of TP53 in HepG2 cells. Because PINK1 is not essential for the changes of total TP53 and the level of PRKN is undetectable in HepG2 cells (in fact PRKN is undetectable in many other cancer cells), one would argue that CCCP- or Mdivi-1-induced changes of total TP53 are independent of the PINK1-PRKN mitophagy pathway.

This finding raises interesting possibilities that CCCP or Mdivi-1 may regulate TP53 levels through other mechanisms independent of mitophagy. For example, depolarization of mitochondria by CCCP will lead to decreased ATP production that may cause activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) and inhibition of MTOR. Because rapamycin and serum starvation also decrease TP53 levels and both inhibit MTOR in HepG2 cells, the possibility that MTOR may regulate TP53 stability should be determined in the future. While Mdivi-1 may inhibit mitophagy, it also blocks mitochondrial fission, resulting in the accumulation of elongated mitochondria. It will also be interesting to determine whether mitochondrial dynamics can regulate TP53 levels. Furthermore, PINK1 may also regulate TP53 activation independent of its role in regulating mitophagy. One well-known mechanism that regulates the stability and levels of TP53 is the mouse MDM2 (transformed mouse 3T3 cell double minute 2)-mediated ubiquitination and proteasomal degradation of TRP53. A previous study from the same group has reported that liver-specific *atg5* KO mice have increased hepatic TRP53 levels due to decreased levels of MDM2 [12]. It will be interesting to investigate whether CCCP, Mdivi-1 or PINK1 would affect MDM2 levels in hepatocytes in the future. In conclusion, despite many unanswered questions, this work from Liu et al. further affirms the dual role of autophagy in tumorigenesis in which autophagy acts as a tumor suppressor at the tumor initiation stage but promotes existing tumors to progress from benign to malignant tumors. They further demonstrated the role of autophagy and/or mitophagy in the suppression of TP53 likely involving a role of PINK1, which decides the fate of a tumor to be benign (lack of autophagy but has TP53 activation) or malignant (with autophagy but lack of TP53).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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