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Autophagy, cancer stem cells and drug resistance

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Abstract

Autophagy is a cellular survival mechanism that is induced by cancer therapy, among other stresses, and frequently contributes to cancer cell survival during long periods of dormancy and the eventual outgrowth of metastatic disease. Autophagy degrades large cellular structures that, once broken down, contribute to cellular survival through the recycling of their constituent metabolites. However, the extent to which this fuel function of autophagy is key to its role in promoting stemness, dormancy and drug resistance remains to be determined. Other roles for autophagy in determining cell fate more directly through targeted degradation of key transcription factors, such as p53 and FoxO3A, or by enforcing a reversible quiescent growth arrest, are discussed in this review. This review also highlights the need to parse out the roles of different forms of selective autophagy in stemness, CD44 expression and dormancy that, for example, are increasingly being attributed explicitly to mitophagy. The clinical relevance of this work and how an increased understanding of functions of autophagy in stemness, dormancy and drug resistance could be manipulated for increased therapeutic benefit, including eliminating minimal residual disease and preventing metastasis, are discussed.

Keywords

autophagy; mitochondria; stem cells; drug resistance; CD44; FoxO3A; dormancy; quiescence

Introduction

Macro-autophagy (generally referred to as autophagy) is a highly conserved catabolic process in which double-membraned vesicles called autophagosomes form around cellular cargo, including organelles, protein aggregates and intracellular pathogens, leading to their degradation following fusion of the autophagosome with the lysosome [1–3]. Although much of the regulation of autophagy occurs at the post-translational level, ensuring a rapid

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response to nutrient stress, autophagy-related genes (ATG) genes are also transcriptionally regulated in response to amino acid deprivation and ER stress via the ATF4 and MIT/TFE transcription factors [4,5]. ATG-encoded gene products promote autophagosome formation in three major steps involving the serine kinase activity of the pre-initiation complex, the lipid kinase activity of the initiation complex and the ligase activity of the ATG5/ATG12/ATG16 complex that pulls in processed LC3/ATG8 to nascent phagophores [1].

The pre-initiation complex (containing ATG13, FIP200, ATG101 and the ULK1/ULK2 serine/threonine kinases) is negatively regulated by mammalian target of rapamycin (mTOR) and positively regulated by AMPK, rendering autophagy highly sensitive to both amino acid deprivation and cellular energy deficits [6,7]. ULK1/ATG1 (or ULK2) phosphorylates Beclin1/ATG6 to activate the lipid kinase activity of VPS34 (a class III PI3K), the catalytic component of the initiation complex (also containing ATG14L, VPS15 and other regulatory factors, in addition to Beclin1), increasing phosphoinositol-3-phosphate (PIP3) production. PIP3 promotes recruitment of additional components of the autophagy machinery to the growing phagophore [1], including the ATG5-ATG12/ATG16L-containing conjugation complex that transfers processed LC3-II from ATG3 to phosphatidylethanolamine to permit its integration into the lipid membranes of burgeoning phagophores [1,7].

Processed LC3 at expanding phagophores plays a central role in selecting cargo for degradation through direct interaction with the cargo itself, or indirectly through cargo adaptor molecules that contain specific motifs called LC3-interacting region motifs [8,9]. Selective autophagy includes (but is not limited to) mitophagy [10,11], ribophagy [12,13] and xenophagy [14] in which mitochondria, ribosomes and pathogens, respectively, are selectively targeted for autophagic degradation [3]. Maturation of the autophagosome also requires LC3-related proteins, and this leads to fusion with the lysosome, acid pH-dependent degradation of autophagosomal cargo and recycling of cargo constituents, including nucleotides, fatty acids and amino acids, to the cytosol, where they are now available for various biosynthetic processes that fuel tumor cell growth [1,2].

The role of autophagy in cancer is multifaceted [15], with known functions for autophagy in promoting tumor cell survival by supplying recycled metabolites for growth, modulating mitochondrial function via mitophagy [11,16,17] or interesting new functions in tumor cell migration and invasion via control of focal adhesion turnover and secretion of pro-migratory cytokines [18–21]. Autophagy also plays a central role in the tumor microenvironment [22,23] where, for example, autophagy is induced in cancer-associated fibroblasts by their association with tumor cells, resulting in increased fibroblast production of amino acids provided in a paracrine manner to tumor cells to sustain their growth [24–26]. Autophagy was also recently shown to be required in the liver to prevent the production of arginase-1, which degrades circulating arginine, such that loss of autophagy results in reduced circulating arginine, which limits growth of tumors implanted in autophagy-deficient mice [27]. Intriguingly, components of both the innate and adaptive immune systems also rely on autophagy to either sustain tumor growth or inhibit it depending on context [26,28–30] (Figure 1). Various recent reviews have focused on the role of autophagy in the tumor microenvironment, including anti-tumor immunity [23,26,29,30], in addition to comprehensive reviews on other aspects of the function of autophagy in cancer, such as

tumor metabolism [16,17], cancer therapy [28,31] and cancer metastasis [21]. The goal of this review is to examine more recently reported roles for autophagy in cancer stem cells (CSCs), tumor cell dormancy and related mechanisms of cancer drug resistance.

Autophagy in CSCs

Tumor heterogeneity and progression to therapy-resistant disease in numerous different human cancers has been attributed to the properties of so-called CSCs, which have the capacity to self-renew, to regenerate all aspects of tumor heterogeneity and to evade cell killing due in part to their quiescent state, in addition to increased expression of drug transporters and other resistance genes [32–35]. Autophagy has emerged over the past several years as a requirement for the maintenance of stemness in both normal tissue stem cells [36–41] and CSCs [42–48]. The mechanisms by which autophagy contributes to stemness and why stem cells are more dependent on autophagy than non-stem cells are ongoing research interests of many laboratories (Figure 2A). In normal tissue stem cells, autophagy has been shown to promote neurogenesis through the management of oxidative stress responses and supply of metabolites to neural stem cells [39,49] and to be required for hematopoietic stem cell (HSC) maintenance through a FOXO3A-induced autophagy survival program [40]. Autophagy also promotes the survival of mesenchymal stem cells and human embryonic stem cells [37,38] and is required for the quiescent state of muscle stem cells [41]. Autophagy induces pluripotency with pluripotency factor SOX2 repressing mTOR expression, resulting in the increased autophagy necessary to reprogram somatic cells into induced pluripotent stem cells [50,51]. The requirement for autophagy during somatic cell reprogramming to induced pluripotent stem cells is complex, however, with autophagy-dependent degradation of p62 conversely shown to limit reprogramming [52].

Like tissue stem cells, CSCs also show autophagy dependence, with CSCs from primary human ductal carcinoma *in situ* of the breast reliant on autophagy for mammosphere formation, invasive properties and survival both *in vitro* and *in vivo* [53]. Beclin1 expression and autophagic flux are elevated in mammospheres and ALDH+ CSCs derived from mammospheres, compared with tumor cells in the bulk population or grown in 2D culture conditions [54]. Beclin1 and autophagy were also essential for CSC maintenance and tumorigenesis *in vivo* [54]. Similarly, $CD44^{+}CD24^{-/low}$ breast CSCs were dependent on autophagic flux for survival and stem-like properties, including reduced expression of CD24, increased CD44 expression, vimentin expression and a mesenchymal phenotype induced by TGF-β [42]. Two different shRNA screens identified a key role for autophagy in maintaining breast CSCs with Beclin-1/ATG6 emerging from a shRNA screen for genes that modulate breast CSC plasticity [55] and ATG4A emerging from a screen for genes required for mammosphere formation [56]. Indeed, mammospheres showed increased expression of several autophagy and lysosomal genes and ATG4A was shown to promote CSC numbers and *in vivo* tumorigenicity [56]. Other genes that came out of this screen for stemness included components of JAK–STAT signaling pathways [56], which is significant given that STAT3 phosphorylation/activation has also been identified as a molecular readout of autophagy dependency in triple-negative breast cancer [57]; and $CD44^{+}CD24^{-/low}$ CSC secretion of IL-6 (which signals through gp130 to JAK–STAT) is autophagy-dependent and required for CSC maintenance [44].

In mouse models of mammary tumorigenesis, autophagy was required to maintain two distinct pools of CSCs, both the highly invasive, mesenchymal $CD29^{hi}CD61^{+}$ CSCs from MMTV-PyMT and MMTV-Wnt1 transgenic mice and the more luminal ALDH+ CSCs from MMTV-PyMT mice [45,58]. This work showed that autophagy inhibition, through targeted deletion of the FIP200 component of the pre-initiation complex, disrupted both TGF-β/SMAD signaling required for $CD29^{hi}CD61^{+}$ CSCs and the activation of STAT3 required for ALDH+ CSCs [45]. The authors suggested that autophagy regulates turnover of CREB-related transcription factors known to modulate expression of TGF-β2 and TGF-β3 but did not explain how autophagy was required for STAT3-induced stemness, although it should be noted that IL-6, which is dependent on autophagy for its secretion [18,44], acts via gp130 and JAK2 to activate STAT3 [59]. Interestingly, STAT3 has also been reported to regulate expression of several autophagy genes, including Beclin1 and BNIP3 [18].

Stresses prevalent in the unique tumor microenvironment in which CSCs frequently reside, such as hypoxia and TGF-β, promote epithelial to mesenchymal transition (EMT), leading to increased self-renewal and upregulation of CD44 [60–65]. Induction of EMT promotes a CSC phenotype through transcription factors, including Slug and Twist, that activate self-renewal gene expression programs and tumor-propagating properties [60–65]. Significantly, stresses such as hypoxia and TGF-β also induce autophagy, alongside EMT and stemness [66,67], and transcription factors known to promote EMT, such as MITF in melanomagenesis, activate autophagy gene expression [5,68]. Other transcription factors, including the core stemness factors SOX2 and NANOG, have also been linked to autophagy induction [47]. For example, NANOG was recently shown to bind to the BNIP3L promoter, to induce autophagy under hypoxia and promote tumor cell resistance to immune-mediated killing by cytotoxic T cells [69].

Although the reporting on the role of autophagy in breast cancer CSCs is the most extensive, autophagy has also been implicated in maintaining CSCs in other cancer types, including pancreatic cancer [43,70], bladder cancer [46], colorectal cancer [71], chronic myeloid leukemia [72] and glioblastoma [73]. It remains to be determined to what extent the underlying pathways inducing autophagy in CSCs (Figure 2B), and explaining how autophagy promotes stemness, are conserved from one cancer type to another.

Mitophagy promotes stemness

Autophagy is a broadly acting process operating to degrade numerous different cellular components and until recently the different functions of autophagy in stem cells had not been parsed out [21]. However, the selective degradation of mitochondria, or mitophagy as it is most commonly referred to, has now been directly implicated in stem cell self-renewal [74–77]. Mitophagy was shown to be required for the self-renewal of HSCs by turning over respiring mitochondria to maintain HSCs in a glycolytic state with low levels of oxidative metabolism [78–80]. The balance between glycolysis and oxidative metabolism has been reported in numerous systems to determine rates of stem cell quiescence versus differentiation [75,81–83]. Reducing mitochondrial mass through mitophagy limits oxidative metabolism, making stem cells dependent on glycolysis, which is less efficient in generating ATP than oxidative phosphorylation, thereby contributing to the slow cycling, self-renewing

state that phenotypically defines stem cells [82]. Mitophagy also promotes preferential segregation of younger, more functional mitochondria to daughter CSCs and older mitochondria to daughter non-stem cells [74]. Conversely, suppression of mitophagy (or indeed increased mitochondrial biogenesis) enhances mitochondrial respiration to promote differentiation and loss of stemness. This is associated with mitochondrial remodeling, dispersed cytoplasmic localization of mitochondria and increased expression of enzymes involved in respiration and mitochondrial metabolism (Figure 3) [81,83–86].

Increased mitophagy was detected in esophageal squamous cell carcinoma cells undergoing EMT and inhibition of Parkin-dependent mitophagy in esophageal squamous cell carcinoma cells caused loss of expression of the stem cell marker CD44, leading to cell death [48]. Consistent with these findings, reduced mitochondrial mass distinguishes CSCs from non-CSCs in lung cancer and head and neck cancer [87,88]. In liver cancer, mitophagy has been reported to be required for the maintenance of hepatic CSCs [77]. This was achieved by eliminating p53 localized to mitochondria that was degraded in a mitophagy-dependent manner. When mitophagy was inhibited, PINK1 phosphorylated p53, resulting in its translocation to the nucleus, where it antagonized OCT4 and SOX2 induction of NANOG, a critical transcription factor required for stemness [77]. This intriguing finding suggests that mitophagy may regulate p53 localization and activity more broadly and, given that the stem cell marker CD44 is p53-regulated [89], it will be interesting to determine whether mitophagy-dependent regulation of CD44 levels [48] is p53-dependent. This work also prompts consideration of whether other key transcription factors that localize to the mitochondria, for example STAT3 and FOXO3A, also have their subcellular localization and activity modulated by mitophagy.

Autophagy and tumor dormancy

Disseminated tumor cells (DTCs) at secondary sites can remain dormant for decades, as is apparent from their outgrowth as overt metastatic lesions in breast cancer and prostate cancer patients who were treated effectively years before for their primary disease [90–93]. The insidious nature of dormant cancer cells has lent urgency to efforts to understand the mechanistic basis of dormancy. Autophagy is activated by nutrient deprivation and other stressful conditions that DTCs are probably exposed to when seeding new metastatic sites, leading investigators to test whether autophagy sustains tumor cell viability during dormancy [93]. Indeed, autophagy has been shown to promote the survival of dormant disseminated breast cancer cells and to be required for metastasis following dormancy in preclinical models of breast cancer [94]. Autophagy inhibition effectively reduced the metastatic burden in the lungs of transplanted mice and it was proposed that autophagy is required for the switch from dormancy to tumor cell growth, as autophagy inhibition specifically depleted dormant cells from tumors, leaving the proliferative tumor cells intact [94]. Also, inhibition of autophagy prevented dormant ovarian tumors expressing the ARHI (aplasia Ras homolog member I) tumor suppressor from growing out [95]. ARHI expression is lost in a significant proportion of ovarian cancers [96] and re-expression of ARHI in ARHI-deficient SKOv3 ovarian cancer cells induced autophagy and blocked tumor growth in transplanted mice [95]. Knocking down ARHI in these tumors allowed them to then grow out in an autophagy-dependent manner, indicating that the dormancy enforced by ARHI

expression was autophagy-dependent [95]. Similarly, autophagy inhibition in the Eμ-Myc mouse model of B-cell lymphoma following treatment with alkylating agents blocked tumor recurrence consistent with a role for autophagy in both tumor dormancy and drug resistance [97]. These various studies provide powerful justification for the use of autophagy inhibitors in combination with conventional therapies to eliminate DTCs, minimal residual disease and prevent metastasis.

The relatively quiescent and motile state of CSCs that, like dormant tumor cells are dependent on autophagy for survival, has linked CSCs to dormancy and indeed CSC markers are upregulated on DTCs in the bone marrow of breast cancer patients [98], leading to the suggestion that dormant tumor cells are in fact CSCs [93,95]. In a switchable mouse model of pancreatic ductal adenocarcinoma (PDAC), dormant tumor cells that survived K-Ras inactivation to promote tumor regrowth upregulated autophagy and showed features of CSCs, including the ability to form tumor spheroids, high CD44 expression and increased tumor initiation properties *in vivo* [70]. Interestingly, these dormant PDAC CSCs had an increased dependence on autophagy and mitochondrial function, including β-oxidation of fatty acids, than non-CSCs for spheroid formation and survival, with dormant PDAC CSCs being more sensitive to inhibition of either autophagy or oxygen consumption than non-CSCs [70].

Autophagy may promote the dormancy of DTCs by supplying key metabolites or, as discussed above for CSCs, autophagy may play a more instructive role in dormancy by turning over key transcription factors that modulate the dormant stem-like state [93,99,100]. Alternatively, autophagy may promote tumor cell dormancy by ensuring a reversible quiescent state and preventing irreversible senescence, as was previously reported in muscle stem cells and HSCs [40,41,79]. The LKB1-AMPK axis is a major modulator of HSC homeostasis, acting to promote stem cell quiescence, mitochondrial function, lipid metabolism and survival [101–103] and, as previously mentioned, LKB1-AMPK signaling is also a potent activator of autophagy [6]. Quiescence and cell survival are coordinated downstream of LKB1-AMPK activation via a $p27^{Kip1}$-dependent growth arrest in G1 of the cell cycle and the aforementioned AMPK-induced activation of the pre-initiation complex and AMPK-dependent phosphorylation of ULK1 [6,104]. Deletion of $p27^{Kip1}$ results in rapid apoptotic cell death under metabolic stress and LKB1-AMPK signaling [104], suggesting a mechanism by which autophagy induction is linked to growth arrest to promote survival. Interestingly, loss of LKB1 was also associated with survival of aneuploid HSCs [102], with autophagy inhibition shown in separate studies to preferentially kill aneuploid cells due to accumulation of autophagic cargo in the lysosome [105,106]. Given the role of CSCs in therapy resistance and disease recurrence [35,107], and that dormant DTCs contribute to the metastatic outgrowth of cancers over time [91], understanding how autophagy can be effectively inhibited to suppress both of these phenotypes is a major challenge for translational cancer research.

Autophagy mediates cancer drug resistance

A wide range of cancer therapies has been shown to induce autophagy and in most cases, although not all, autophagy has been shown to promote tumor cell survival and contribute to

therapy resistance (Figure 4A) [108–110]. For example, in estrogen receptor-positive breast cancer, inhibition of autophagy sensitized resistant tumors to tamoxifen-induced killing [111,112]. Similarly, in prostate cancer, autophagy inhibition overcame resistance to enzalutamide [113]. Autophagy is also induced in response to treatment of gastrointestinal stromal tumor (GIST) cells with Imatinib™ and inhibition of autophagy, including with the lysosomotropic agent chloroquine (CQ), caused tumor cell apoptosis [114]. Numerous studies in other cancer types and in response to other cancer therapies confirm that autophagy is both induced by treatments used and confers resistance to the treatment [28,110].

The therapeutic induction of autophagy is frequently attributed to reduced mTOR activity leading to autophagy derepression, and this is most obvious with therapies targeted at inhibiting PI3K, AKT or indeed mTOR itself [109]. However, the induction of autophagy by other conventional and non-conventional treatments is varied and not completely understood. DNA damage-induced p53 activity may explain how autophagy is induced by conventional genotoxic agents, such as radiation or cisplatin, as a result of p53-mediated induction of autophagy regulators, such as DRAM1 [115]. However, the role of p53 in these responses is complicated by the fact that p53 is also pro-apoptotic and, depending on context, can also inhibit autophagy [116]. Other aspects of cancer therapy, including increased production of reactive oxygen species due to mitochondrial damage and an ER stress response due to protein aggregation may explain activation of autophagy via induction of the activity of the FOXO and ATF4 transcription factors, respectively, that are known to induce autophagy genes, such as ATG5, LC3 and others [4,40,117].

Such direct molecular mechanisms may explain how autophagy is upregulated in response to different therapies, but an alternative or parallel explanation is that therapy is selecting for those cancer cells that already have high levels of autophagy, namely therapy refractory CSCs. For example, different tumor types showed increased autophagy in response to irradiation treatment, and inhibition of autophagy reduced clonogenic survival of breast, lung and cervical cancer cell lines following irradiation [118]. Significantly, autophagy inhibition specifically reduced clonogenic survival of radioresistant tumor cells but not radiosensitive subclones, consistent with the radioresistant cells being more autophagy dependent [118]. Autophagic flux was also selectively higher in cisplatin-resistant bladder cancer cells and autophagy inhibition specifically depleted drug-resistant bladder CSCs [46]. In primary human glioblastoma (GBM), MST4 kinase (encoded by *STK26*) was upregulated due to promoter hypomethylation in glioblastoma stem cells (GSCs) in response to irradiation [119]. Elevated MST4 activity induced phosphorylation and activation of the ATG4B protease, leading to increased autophagic flux in GSCs, increased self-renewal properties and sphere formation, in addition to increased tumorigenicity *in vivo* [119]. Direct targeting of ATG4B or autophagy inhibition with CQ promoted the therapeutic effects of radiation in a GBM transplant model. This was associated with loss of GSC self-renewal capacity. Consistent with these findings, levels of MST4, phospho-ATG4B and LC3B staining correlated negatively with patient outcome for GBM. These findings suggest that there are indeed specific molecular mechanisms promoting autophagy induction in tumor cells, but also that at least some of these mechanisms are specific to CSCs and could contribute to explaining how CSCs are key mediators of drug resistance.

Recent work has identified a negative feedback loop wherein FoxO3A, a key transcriptional inducer of ATG genes in response to nutrient stress and reactive oxygen species, particularly in stem cells [40], is itself turned over by autophagy [120]. When autophagy was inhibited using CQ, FoxO3a accumulated in tumor cells, leading to increased expression of its pro-apoptotic target gene *Puma*, resulting in programmed cell death [120]. Indeed, this study showed an essential role for FoxO3A in binding to the gene regulatory region of *Puma* to promote synergistic tumor cell killing by genotoxic agents, including doxorubicin and etoposide, in combination with CQ [120]. Interestingly, CQ and autophagy inhibition also synergized with Nutlin-1 to activate p53 to super-induce *Puma* and cause cell death [120]. Together, this ever-growing body of research showing autophagy playing a cytoprotective effect provides a strong rationale to combine cancer therapeutic approaches with agents that inhibit autophagy [110,121].

Targeting autophagy for improved cancer treatment

As already alluded to, treatment with CQ or hydroxychloroquine (HCQ) is one of the most commonly used approaches to inhibit autophagy in the clinic to promote tumor cell killing by conventional chemotherapeutics (Figure 4B) [110,121]. CQ is a FDA-approved drug initially derived from the bark of the cinchona tree and used to treat malaria, arthritis and lupus, making it relatively cheap and accessible. Although CQ and HCQ inhibit autophagy, their mode of action is at the lysosome where they are trapped by protonation, leading to alkalinization of the lysosomes and inhibition of lysosomal acid protease activity [121]. Interestingly, metastatic tumor cells appear to be preferentially sensitive to CQ, and this was attributed to greater dependence on lysosomal function than non-metastatic tumor cells, but whether these metastatic cancer cells also exhibited increased stem-like properties was not examined [122]. Multiple clinical trials have now reported on the efficacy of combining CQ with conventional chemotherapies [110], such as in GBM treatment, where CQ in combination with temozolomide more than doubled patient survival times compared with temozolomide alone [121,123,124]. Similarly, CQ in combination with doxorubicin for the treatment of non-Hodgkin lymphoma in dogs showed improved overall drug response and progression-free survival compared with animals receiving doxorubicin alone [125]. CQ has now been tested in a range of different human cancers, for example in combination with gemcitabine for the treatment of PDAC [126], in combination with radiation for the treatment of metastatic breast cancer [127] and in combination with rapamycin analogs for different types of solid tumor and melanoma [128,129], in addition to ongoing trials comparing HCQ to CQ in adjuvant therapies [110].

A central challenge in assessing the efficacy of autophagy inhibitors in cancer therapy is to develop better, more reliable markers of autophagic flux *in vivo* to determine whether drug combinations are indeed effectively inhibiting autophagy as part of the treatment modality and outcome response [110]. This is particularly important as CQ has autophagy-independent effects, including inhibition of the ATM kinase [130] and effects on vasculature [121]. For example, recent studies showed that the growth suppressive effect of CQ was independent of autophagic flux in metastatic cancer cells and attributed to lysosomal dysfunction more specifically [122]. Added to this, CQ is currently used at micromolar doses, and such doses have been linked to toxicities, including fatal blood loss

[110,121,131]. Furthermore, as autophagy is required systemically for tissue homeostasis in response to stress [132], one of the major concerns with autophagy inhibition as a therapeutic approach is whether the adverse consequences of systemic autophagy inhibition can be tolerated. This has spurred investigators to develop more potent analogs of CQ that are active at lower doses and have fewer side-effects, including Lys05, a dimeric version of CQ [131]. Interestingly, as autophagy and mTOR are both critically regulated at the lysosome [133,134] and the potent pro-growth activity of mTOR can dampen autophagy induction, Amaravadi and colleagues [135] recently took the innovative approach of screening for novel drugs that target the lysosome to both block autophagy and simultaneously inhibit mTOR activity. The lead compound they developed is a dimeric quinacrine (DQ661), which is derived from Lys05, has improved lysosomal targeting capability and impairs the activity of palmitoyl-protein thioesterase (PPT1), which is required for mTOR interaction with Rheb at the lysosome [135]; inhibition of PPT1 also prevents mTORC1 from associating with the lysosomal membrane, causing mTOR inhibition [136]. DQ661 appears to function much more effectively than existing mTOR inhibitors to suppress tumor growth and concomitantly inhibits autophagy by inhibiting the lysosome in a mouse melanoma model, a human colon cancer model and an orthotopic PDAC model [135]. Interestingly, PPT1 appears to be the common molecular target at the lysosome of CQ, HCQ and Lys05, in addition to DQ661, and deletion of PPT1 inactivated the ability of CQ or CQ derivatives to block autophagy [136]. Increased PPT1 expression in human cancers was linked to poor prognosis and, conversely, knockout of PPT1 in tumor cells inhibited tumor growth, tumor spheroid formation and tumorigenesis *in vivo*, suggesting that PPT1 may be a good therapeutic target [136].

As scientific understanding of autophagy has increased, the development of targeted small molecule inhibitors to key regulatory nodes in autophagy pathways has emerged as an alternative therapeutic approach, beyond CQ and related antimalarials (Figure 4B). For example, development of targeted inhibitors of ULK1 [137,138], VPS34 [139] and other enzymes required for autophagy [140] is a work in progress and Vescor Therapeutics LLC has been set up by leaders in the field to specifically develop new drugs along these lines (https://vescortx.com/pipeline/). Beyond the development of autophagy inhibitors, the development of specific mitophagy inhibitors also seems that it could prove productive, given the concerns about global autophagy inhibition for tissue homeostasis and that mitophagy appears to underlie many of the relevant functions previously attributed to general autophagy. Use of specific inhibitors will also be powerful moving forward to explore how autophagy determines stemness, whether dormant tumor cells are autophagy-dependent CSCs and which autophagy functions are key to understanding how autophagy promotes drug resistance and cancer recurrence.

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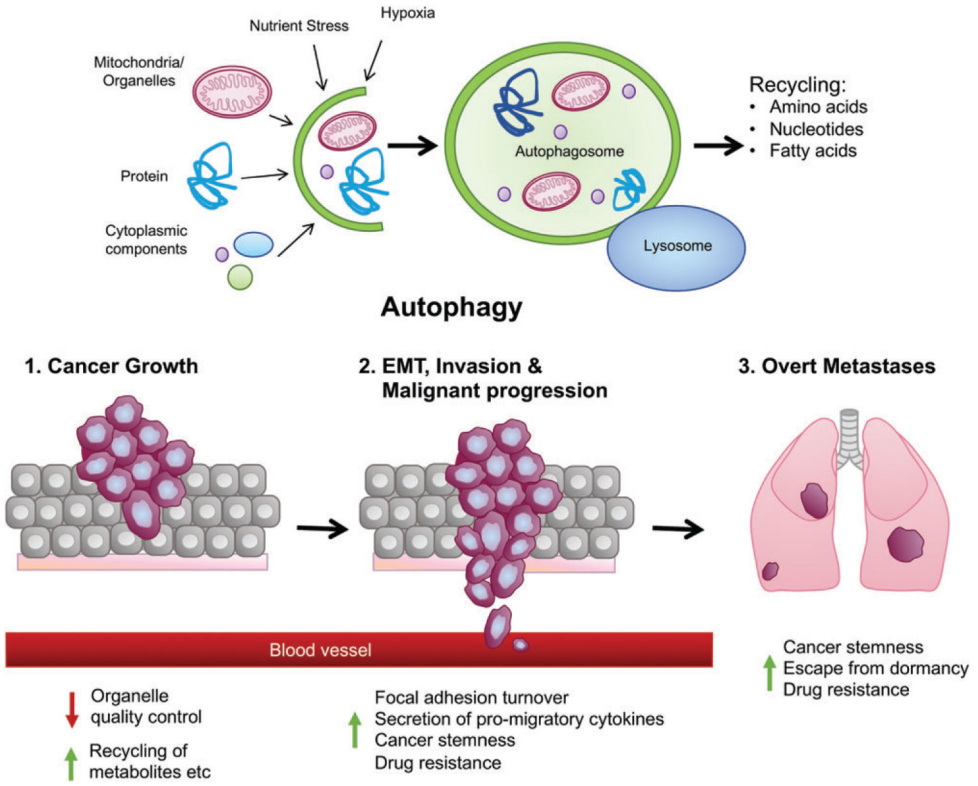

Figure 1.

Multifaceted roles of autophagy in cancer. Autophagy is a catabolic process by which cells degrade large cellular cargoes, such as organelles, ribosomes and intracellular pathogens that are captured inside double-membraned autophagosomes before fusing with the lysosome and resultant constituent metabolites (amino acids, nucleotides, fatty acids) released to the cytsosol for reuse in biosynthetic processes and cell growth. This fuel function of autophagy is important in terms of promoting tumor cell survival at many stages in tumorigenesis. Autophagy also performs an organelle quality control function as part of cellular homeostasis that is important in both normal and tumor cells. As cells progress to becoming invasive, autophagy plays a role in promoting cell migration through focal adhesion disassembly and secretion of pro-migratory cytokines, such as IL-6. Autophagy also plays a role in the tumor microenvironment in modulating recruitment and response of T cells to the tumor and providing tumor cells with nutrients via amino acid transfer from cancer-associated fibroblasts to the tumor. Finally, emerging data have identified a role for autophagy in maintaining CSCs and tumor dormancy, both of which may play into drug resistance of cancers, minimal residual disease and metastatic latency.

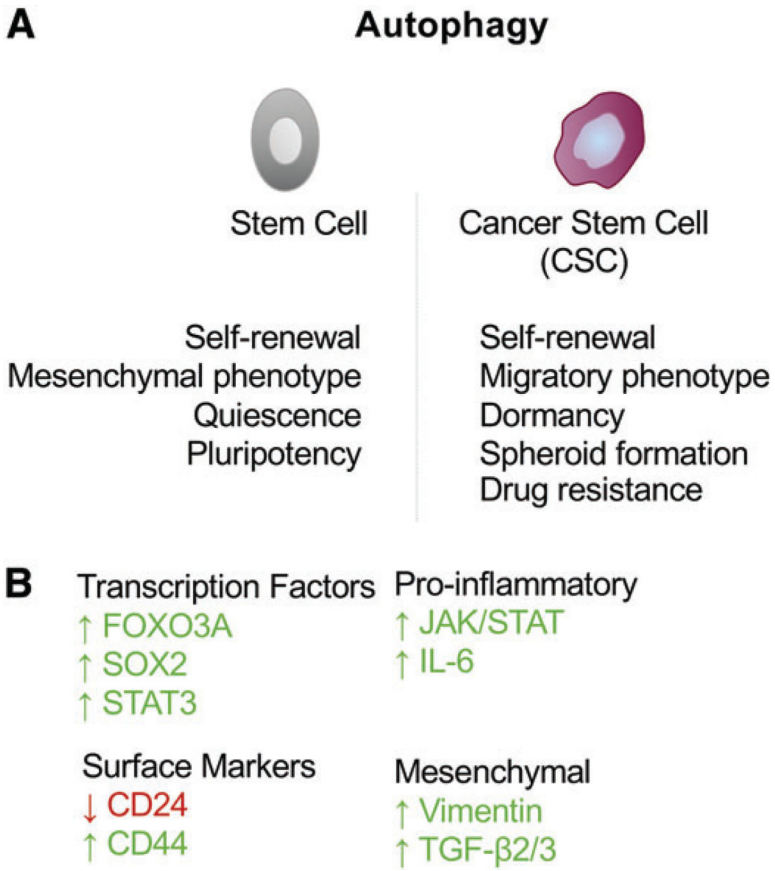

Figure 2.

Autophagy in CSCs. (A) Autophagy plays an important role in maintaining both normal tissue stem cells and CSCs. The survival and quiescence of normal tissue stem cells appears dependent on autophagy and autophagy has also been reported to promote pluripotency. In CSCs, autophagy promotes expression of stem cell markers such as CD44 as well as expression of mesenchymal markers such as vimentin. Autophagy also promotes spheroid formation, *in vivo* tumorigenesis and drug resistance consistent with a critical role in maintaining CSCs. Inhibition of autophagy limits tumor dormancy and promotes outgrowth of metastases. (B) Key transcription factors have been linked to the induction of autophagy and the stem cell state, including FOXO3A, which induces expression of autophagy genes in stem cells and is itself turned over by autophagy. Also, SOX2 and STAT3 have been shown to modulate autophagy genes and to determine the stemness of CSCs.

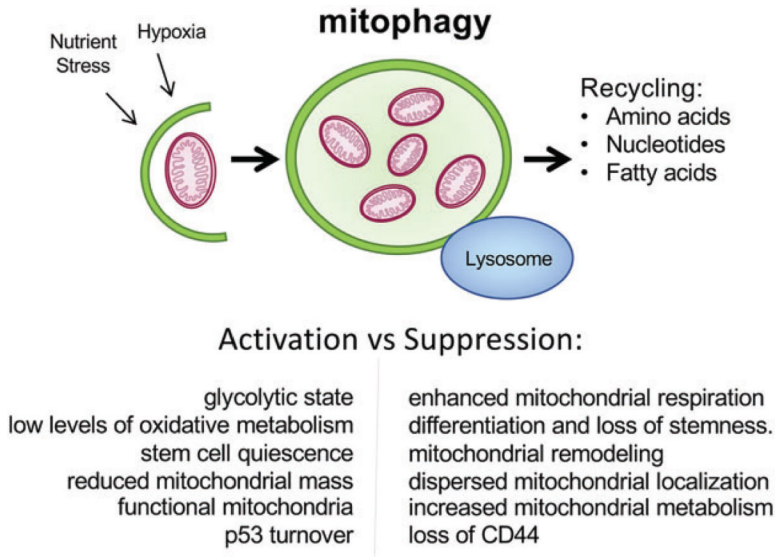

Figure 3.

Mitophagy promotes stemness. Mitophagy is a selective form of autophagy in which mitochondria are specifically targeted for degradation at the autophagosome. Recently, mitophagy has been specifically implicated in maintaining the stem cell state by promoting turnover of mitochondria and limiting the capacity of the stem cell for oxidative phosphorylation and making stem cells more dependent on glycolysis for energy demands. This has also been proposed to contribute to the quiescent state of stem cells. Inhibition of mitophagy suppressed CD44 expression and also promoted translocation of p53 to the nucleus, where it has been reported to antagonize expression of stem cell genes.

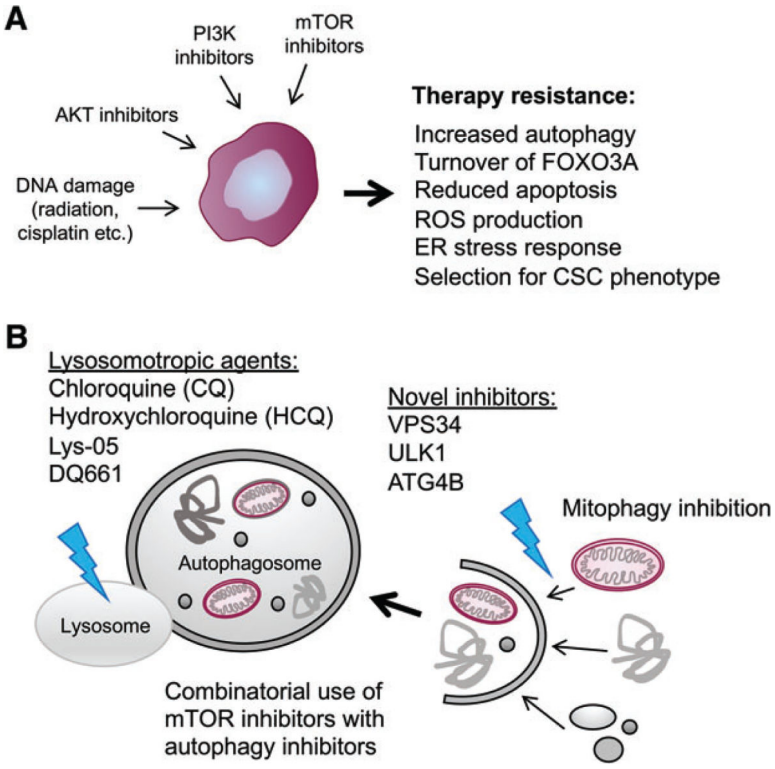

Figure 4.

Autophagy promotes cancer drug resistance. (A) Autophagy is induced in tumors by many different cancer therapeutic approaches, including irradiation, inhibition of PI3K, AKT or mTOR, as well as other conventional and targeted therapies. As a result of autophagy induction, tumor cells are more resistant to apoptosis, with an interesting mechanism revealed recently showing targeted turnover of FOXO3A by autophagy to prevent FOXO3A-dependent induction of *Puma*, a BH3-only pro-apoptotic protein [119]. Autophagy may also promote drug resistance by promoting selection for a CSC phenotype, as has been suggested by work in breast cancer [117] and glioblastoma [118]. (B) CQ and its derivatives are the mainstay of efforts to inhibit autophagy in a clinical setting and this has seen some efficacy in combination with conventional therapies for some cancers and drug combinations. Clinical trials with the combination of CQ and mTOR inhibitors are ongoing and there is particular interest in testing DQ661, which has the dual activity of inhibiting autophagy and mTOR at the lysosome. New generation autophagy inhibitors include small molecules targeted at catalytic components of autophagosome biogenesis, including ULK1, VPS34 and ATG4B.